# The *necrotic* Gene in Drosophila Corresponds to One of a Cluster of Three Serpin Transcripts Mapping at 43A1.2

**Clare Green,* Elena Levashina,† Carol McKimmie,* Tim Dafforn,‡ Jean-Marc Reichhart† and David Gubb***

**Department of Genetics, University of Cambridge, Cambridge CB2 3EH, England, ‡Department of Haematology, University of Cambridge, CIMR, Cambridge CB2 2XY, England and †UPR 9022 C.N.R.S., Institut de Biologie Moléculaire et Cellulaire, 67084 Strasbourg, France*

Manuscript received February 24, 2000
Accepted for publication July 3, 2000

ABSTRACT

Mutants of the *necrotic (nec)* gene in *Drosophila melanogaster* die in the late pupal stage as pharate adults, or hatch as weak, but relatively normal-looking, flies. Adults develop black melanized spots on the body and leg joints, the abdomen swells with hemolymph, and flies die within 3 or 4 days of eclosion. The TOLL-mediated immune response to fungal infections is constitutively activated in *nec* mutants and pleiotropic phenotypes include melanization and cellular necrosis. These changes are consistent with activation of one or more proteolytic cascades. The *nec* gene corresponds to *Spn43Ac*, one of a cluster of three putative serine proteinase inhibitors at 43A1.2, on the right arm of chromosome 2. Although serpins have been implicated in the activation of many diverse pathways, lack of an individual serpin rarely causes a detectable phenotype. Absence of *Spn43Ac*, however, gives a clear phenotype, which will allow a mutational analysis of critical features of the molecular structure of serpins.

THE serpins (*ser*ine *p*roteinase *in*hibitors) form a divergent group of proteins that are found in plants, animals, and viruses and have been most widely characterized in mammals (Carrell and Travis 1985; Marshall 1993; Potempa *et al.* 1994; Wright 1996). Serpins bind to the active site of their target proteases as competitive substrates that block the protease activity. The binding of serpins to their cognate protease to form a Michaelis complex occurs via a "bait" region on the exposed loop of the serpin. The protease cleaves the serpin between the P1 and P1′ residues, which releases the loop and covalently attaches the serpin to the protease. The serpin then undergoes a large internal rearrangement in which the unconstrained reactive center loop (RCL) inserts into β-sheet A (Loebermann *et al.* 1984; Baumann *et al.* 1991, 1992). These changes trap the protease in an inactive, covalently linked complex with the serpin. Under some conditions, the conformational change within the serpin can occur spontaneously, without cleavage, to give an inactive "latent" conformation (Mottonen *et al.* 1992; Carrell *et al.* 1994; Schreuder *et al.* 1994; Lomas *et al.* 1995). In the absence of serpins, serine proteases may cleave their normal substrate to give an activated form that can initiate a proteolytic cascade. In mammals, a variety of proteolytic cascades, including blood coagulation, fibrinolysis, complement activation, and the inflammatory response, are regulated in this way (Boswell and Carrell 1988; Potempa *et al.* 1994).

Invertebrate serpins are less well characterized. Several serpins have been isolated in *Manduca sexta* (Kanost *et al.* 1989; Jiang *et al.* 1994, 1996) and eight in *Drosophila melanogaster* (Clark *et al.* 1995; Bayer *et al.* 1996; Han *et al.* 2000). No genetic functions have been identified with serpin transcripts although one of them is known to inactivate trypsin-like proteases *in vitro* (Han *et al.* 2000).

In the mouse, genetic knockout of a large number of serpins has failed to identify mutant phenotypes (D. Lomas, personal communication), with the exception of antithrombin, which causes fetal abortion. By this criterion, most serpins are functionally redundant. The target specificity of serpins tends to be toward a general class of proteases and the serpin/protease balance is actively regulated. As a consequence, lack of an individual serpin usually results in upregulation of similar family members and has limited phenotypic consequences. In humans, a number of pathologies are associated with serpin abnormalities (Lomas *et al.* 1992; Aulak *et al.* 1993; Bruce *et al.* 1994; Davis *et al.* 1999) and a few of these result from simple lack-of-function mutations (Erdjument *et al.* 1988). In general, however, loss of an individual serpin activity causes little or no phenotypic change. Pathological changes, however, can be caused by gain-of-function serpin mutations. One class of mutation gives serpin protein, which forms concatenated polymers in which the RCL of one molecule inserts into β-sheet A of another. These polymers have no protease inhibitory activity and their accumulation leads to a

*Corresponding author:* D. Gubb, Department of Genetics, Downing St., University of Cambridge, Cambridge CB2 3EH, England. E-mail: d.gubb@gen.cam.ac.uk

range of disease states. Polymerization of a mutant form of the acute phase serpin, $\alpha_1$-antitrypsin, is associated with liver disease and emphysema in humans (Lomas *et al.* 1992). The polymerized serpin is retained within hepatic inclusions, leading to reduced levels in the plasma. Serpin polymerization has also been linked to dementia (Davis *et al.* 1999), while more unusual serpins may suppress tumor growth (Maspin; Zou *et al.* 1994); inhibit caspases (CrmA; Takahashi *et al.* 1996); and bind DNA, leading to heterochromatinization in avian blood cells (Ment; Grigoryev *et al.* 1999). In all these cases, the ability of serpin molecules to undergo conformational changes underlies their biological activity.

The cloning and sequencing of three Drosophila serpin transcripts is described here. *Spn43Aa* is just proximal to the *prickle* (*pk*) transcript (Gubb *et al.* 1999), while *Spn43Ab* and *Spn43Ac* are within the first 5′ intron of *pk* (Figure 1). Developmental expression profiles and imaginal disc *in situ* hybridization patterns of the *Spn43* transcripts are shown. The deduced amino acid sequences of the serpins are compared and possible reactive centers are identified. The cytogenetic location of *nec* was reported by Heitzler *et al.* (1993) and its role in the TOLL-mediated innate immune response to fungal infection by Levashina *et al.* (1999). The necrotic phenotype is further characterized in this report and its rescue by a genomic DNA fragment containing *Spn43Ac* is demonstrated in transgenic flies. Both the $nec^1$ and $nec^2$ chromosomes have lesions within the coding region of the *Spn43Ac* transcript.

## MATERIALS AND METHODS

**Drosophila stocks:** The *nec* alleles and the *Df(2R)sple-D1* and *Df(2R)sple-D2* chromosomes used in this study are from Heitzler *et al.* (1993). *Df(2R)pk-78k* is from Gubb and Garcia-Bellido (1982), *Df(2R)nap-2* from Ringo *et al.* (1991), and *Df(2R)pk-sple-25* and *Df(2R)pk-30* from Gubb *et al.* (1999). The entire *nec* region is deleted by *Df(2R)pk-78k* (42E3;43C3), while the molecular boundaries of *nec* are defined by the overlapping deletions *Df(2R)sple-D1* (43A1.2;43B2) and *Df(2R)nap-2* (41F4-9;43A1.2). The fluorescent *CyO-GFP* balancer stock *In(2LR)O, Cy* $dp^{lvl}$ *pr* $cn^1$ *P*[$w^{+mC}$ *Act:GFP]* was constructed by Reichhart and Ferrandon (1998). The *white-mottled-4* ($w^{m4}$) mutation (Muller 1930) was used to test whether the *Spn43A* transcripts act as dominant suppressors or enhancers of heterochromatic position effect variegation.

**Cloning and sequencing:** Standard molecular biological techniques were used (Sambrook *et al.* 1989). Genomic inserts were subcloned from phage in the 43A1.2 region isolated from the EMBL3 library of John Tamkun (Gubb *et al.*1999). *Spn43Aa* cDNAs were isolated from the imaginal disc library of Brown and Kafatos (1988) using a 3.25-kb *Eco*RI fragment (Figure 1) from phage FP11/2 (http://www.gen.cam.ac.uk/dept/gubb.html). *Spn43Ab* and *Spn43Ac* cDNAs were isolated from the larval and adult head libraries of Russell and Kaiser (1993) using the 3.2-kb *Sal*I and 2.1 + 6.0-kb *Sal*I fragments, respectively, from the adjacent phage insert (FP10/2, http://www.gen.cam.ac.uk/dept/gubb.html). Putative full-length cDNAs *Spn43Aa-NB3*, *Spn43Ab-SL2*, and *Spn43Ac-SH8* were subcloned into pBluescript SK+ (Stratagene, La Jolla, CA) and sequenced. Subcloned genomic fragments from the phage walk were sequenced to identify the precise intron/exon structure of these transcripts.

The boundaries of the *Df(2R)pk-30* deletion within the *pk* and *Spn43Ab* transcripts were defined by sequencing across the deletion end points. Genomic DNA from homozygous *Df(2R)pk-30* and wild-type (Canton-S) flies was PCR amplified from 5′ and 3′ primers (CATCGGCACTCGGATCACA and GTTCTCGAGAGATGGTGAC, respectively). The amplification products were 2.9 kb for Canton-S and 1.8 kb for *Df(2R)pk-30*. The *Spe*I/*Xho*I fragment (Figure 1) from the *Df(2R)pk-30* PCR product was subcloned into pBluescript SK+ and sequenced from vector primers. The $nec^1$ and $nec^2$ mutations were PCR sequenced using the CTGGCTGCTCAGACCT TCGCC and CATGGGCGTGGGATACTCCAC primers.

**Analysis of sequence data:** The Wisconsin Package Version 9.1 [Genetics Computer Group (GCG), Madison, Wisconsin] was used for sequence alignment and assembly. DNA sequences for each transcript were compared to database sequences using the Blast program (Altschul *et al.* 1990) and protein motifs were compared using the ProSite database (Bairoch *et al.* 1997).

**Northern hybridization:** Total and poly(A)$^+$ RNA extractions and Northern blotting experiments were performed as described in Lemaitre *et al.* (1996). Probes corresponding to the cDNA of *Spn43Aa*, *Spn43Ab*, and *Spn43Ac* were amplified by PCR using internal-specific primers. A total of 5 μg of poly(A)$^+$ RNA was loaded for each track. A ribosomal protein (*Rp49*) probe was used as a loading control (O'Connell and Rosbach 1984).

**Tissue *in situ* hybridization:** Random-primed digoxygenin (DIG) DNA probes were made with the Boehringer kit and developed with DAB. Template DNAs were gel-purified inserts of the 1.3-kb *Eco*RI fragment of *Spn43Aa-NB3*, the 1.3-kb *Eco*RI fragment of *Spn43Ab-SL2*, and the 0.6 + 0.7-kb *Eco*RI fragments of *Spn43Ac-SH8*.

**Transformation of flies:** Genomic constructs of each of the three serpins were made using the *pWhiteRabbit* transformation vector (Dunin-Borkowski and Brown 1995) and genomic fragments of 3.2, 5.2, and 7 kb, respectively (Figure 1). These genomic fragments were cut from phage FP11/3 and FP10/2, in the case of *Spn43Aa* and *Spn43Ac*, while the *Spn43Ab* rescue fragment was cut from a cosmid isolated from the library of J. Tamkun. A total of 1 mg/ml of transformation construct DNA plus 0.25 mg/ml of the helper plasmid *ppi25.7wc* were microinjected into *yellow white* embryos (Spradling 1986).

**Test for RCL activity in SPN43Ac:** To test whether the SPN43Ac protein functions as an active serine protease inhibitor, the putative protease cleavage site (P1 and P1′, Figure 4) was changed from L438-S439 to P438-P439 by PCR-directed mutagenesis. The resulting construct was sequenced, cloned into pUAST, and rescue experiments were made as in Levashina *et al.* (1999).

**Modeling of SPN43Ac structure:** A model of the tertiary structure of SPN43Ac was created using the Modeler program (Sali and Blundell 1993). The template structure to which the SPN43Ac sequence was fitted is based on the X-ray crystal structures of *M. sexta* serpin 1K (Li *et al.* 1999) and human α1-antitrypsin (P. R. Elliott, X. Y. Pei, T. Dafforn, R. J. Read, R. W. Carrell and D. A. Lomas, unpublished data).

## RESULTS

**Identification of transcripts:** Three short transcripts were identified on developmental Northern blots within a 10-kb region in 43A1.2 (Figure 1). Flies heterozygous

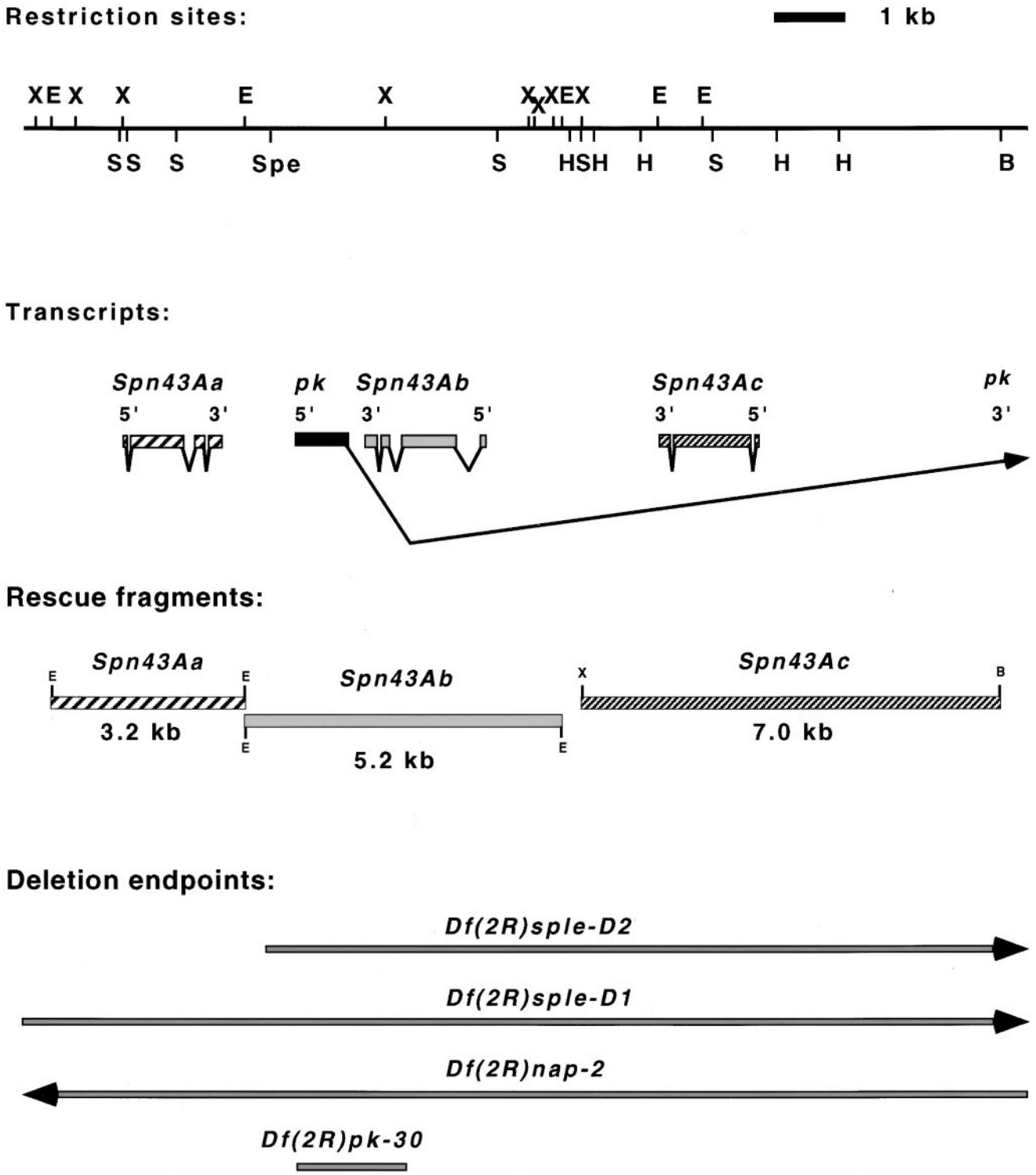


FIGURE 1.—Molecular map of the *Spn43A* cluster. The *Spn43A* transcripts map within ~10 kb, one proximal to *pk*, on the same strand, and two in the opposite orientation, within the 5′ intron of *pk*. The *Df(2R)sple-D2/Df(2R)nap-2 trans*-heterozygotes survive and give a pk nec phenotype. The nec phenotype of *Df(2R)sple-D2/Df(2R)nap-2* flies is rescued by a 7.0-kb *XB* genomic fragment that includes *Spn43Ac*. Restriction sites: *E, S, Spe, B, X,* and *H* (*Eco*RI, *Sal*I, *Spe*I, *Bam*HI, *Xho*I, and *Hin*dIII).

for the overlapping deletions that remove the two distal transcripts (*Df(2R)sple-D2/Df(2R)nap-2*) express amorphic *pk* and *nec* mutant phenotypes, but are otherwise wild type (Figure 1). A fourth transcript, expressed at much lower levels, was later identified, which corresponds to the 5′ exon of the *pk* transcript (GUBB *et al.* 1999). *Spn43Aa* maps just proximal to *pk*, while *Spn43Ab* and *Spn43Ac* map within the 5′ *pk* intron.

**Characterization of the nec mutant phenotype:** Balanced stocks of *nec* alleles tend not to give homozygous flies. This is true of our $nec^1/CyO$ and $nec^2/CyO$ stocks and although *trans*-heterozygous $nec^1/nec^2$ flies survive for several days (see below), the females are sterile. Given that *nec* females are completely sterile and *nec* males are weak, any homozygous flies that might hatch in a balanced stock would not give progeny. Under these conditions, accumulation of additional lethal mutations, or genetic modifiers, would not be selected against. In this study, the nec phenotype was characterized in $nec^1/nec^2$ mutant flies, which show a nec phenotype indistinguishable from that of the overlapping deletion combination *Df(2R)sple-D2/Df(2R)nap-2*.

The survival rate of $nec^1/nec^2$ larvae was compared with that of heterozygous balancer larvae, using the *CyO-GFP* balancer (MATERIALS AND METHODS). In the progeny of $nec^1/CyO\text{-}GFP \times nec^2/CyO\text{-}GFP$, normal and fluorescent first instar larvae were found close to the expected 1:2 ratio ($nec^1/nec^2$):(*nec/CyO-GFP*); 811 of 1850 third instar larvae were scored. These larvae were reared separately until eclosion and both classes were viable. Between 10 and 20% of the *nec* larvae show brown spots around the posterior spiracles. Both nec and Cy larvae pupated normally, but ~10% of nec pupae fail to eclose. These results confirm that the major deleterious effect of the *nec* mutation occurs after the embryonic and larval stages.

Adult $nec^1/nec^2$ flies show a patchy distribution of melanotic spots of variable position and intensity (Figure 2). These spots are restricted to the cuticular surfaces; melanotic masses were never observed within internal tissues. The abdomens of nec adults gradually swell with hemolymph and are extremely distended by 48 hr posteclosion.

Transmission electron microscopy of mutant tissues showed that the epidermal cells undergo necrosis (Figure 3). The sites of necrosis correspond to the sites of extensive melanization of the cuticle, although it is unclear whether the cuticular melanization is caused by the necrosis of the underlying epidermal cells. Interestingly, an additional layer of healthy epidermal cells is seen beneath the necrotic cells. The necrotic mutant phenotype is clearly pleiotropic and it is unclear what causes the lethality of adult *nec* flies.

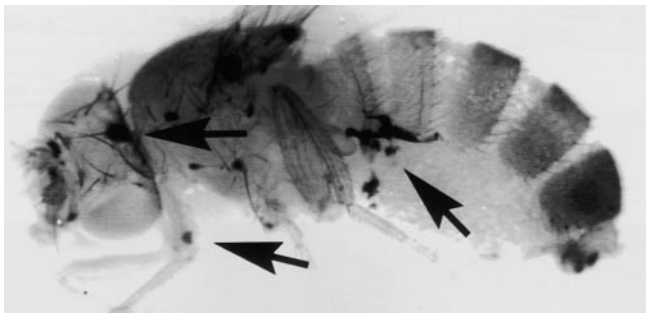

FIGURE 2.—$nec^1/nec^2$ adult male. Melanotic patches (arrows) are distributed over the body surface, but preferentially near cuticular joints and sutures. Patches tend to be concentrated at the leg joints, proboscis, ocelli and eyes, the lateral and dorsal thoracic regions, and near the segment boundaries in the abdomen.

**Nucleotide and deduced amino acid sequences:** Each of the three short transcripts in 43A1.2 (Figure 1) shows homology to the serpin family. The most proximal cDNA, *Spn43Aa-NB3*, is 1300 nucleotides long, *Spn43Ab-SL2* is 1333 nucleotides, and *Spn43Ac-SH8* is 1523 nucleotides. These cDNAs encode putative 370-, 394-, and 477-amino-acid peptides, respectively (Figure 4). *Spn43Ac* has two short introns while *Spn43Aa* and *Spn43Ab* each have three (Figure 1). All three proteins contain putative signal peptides (mnhwlsiillgvwisapeg, SPN43Aa; maviisclllllatvsqs, SPN43Ab; and maskvsillllltvhllaaqtfa, SPN43Ac; NIELSEN *et al.* 1997), suggesting that they encode secreted proteins.

The SPN43A serpins are widely diverged from each other, with similar divergences between these serpins and the *D. melanogaster Acp76A* serpin, *M. sexta*, *Bombyx mori*, and mammalian serpins (Table 1). The reactive center loops of SPN43Aa and SPN43Ac contain hinge regions (AAGAS and ASAAS, respectively) typical of inhibitory serpins. The target protease specificity of serpins is strongly influenced by the sequence at the $P_1$ $P_1'$ site on the reactive center loop. The residues in these positions in SPN43Aa and SPN43A*c* are MS and LS, respectively (Figure 4). These residues suggest that SPN43Aa is in the antitrypsin inhibitor class, while SPN43Ac is in the antichymotrypsin inhibitor class. The SPN43Ab reactive center loop lacks the typical hinge region and instead contains bulky residues, which would suggest that it is noninhibitory.

The structural homology between the Drosophila SPN43A proteins and serpins for which three-dimensional structures have been solved allows putative three-dimensional structures to be assigned. Any insertions or deletions within the peptide sequences with respect to known serpin scaffolds will be apparent. With all three SPN43A serpins, the only changes are in surface loops that are unlikely to contribute to function, with the exception that SPN43Ac contains a long N-terminal extension of 88 amino acids that includes polyglutamine repeats. Among known serpins, the presence of a polyglutamine repeat is unique to SPN43Ac. Polyglutamine repeats have been described in a number of mammalian proteins, where their function remains unclear, and are also found in Drosophila proteins. Other unusual features of the SPN43A serpins are that SPN43Ab is highly basic (with a predicted isoelectric point of 10); while SPN43Aa and SPN43Ac contain a leucine zipper motif.

**Temporal expression patterns:** *Spn43Aa* is expressed predominantly in the early pupae and at much lower levels in the embryo and late larval stages. *Spn43Ab* is

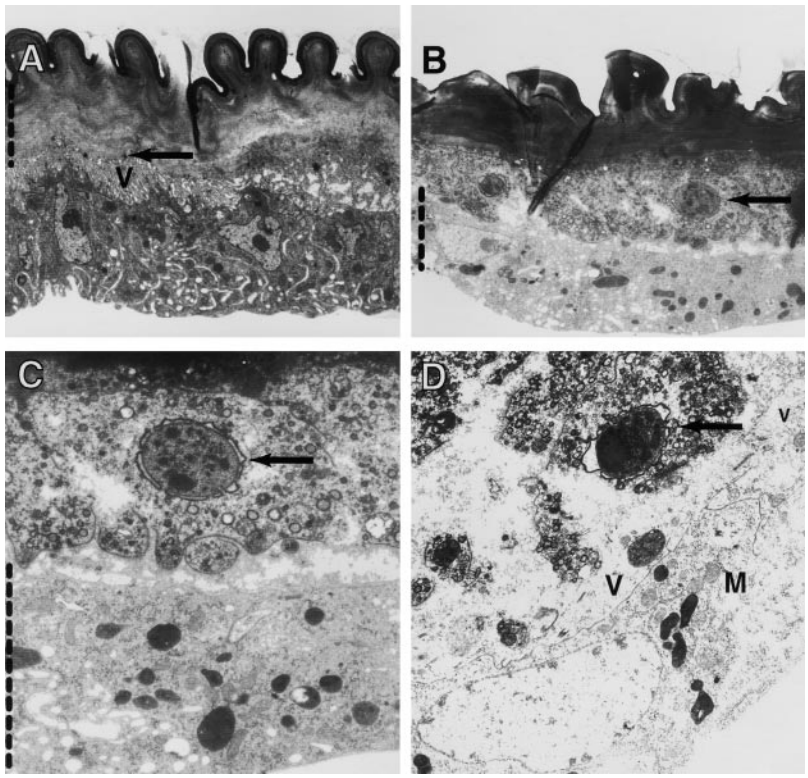


FIGURE 3.—Electron microscope sections of adult cuticle and epithelium. (A) Wild type showing lightly stained cuticle (dashed line). The underlying epithelial cells show irregular light nuclei containing discrete nucleoli and a microvillar layer (V) that is presumably secreting the basal layer of cuticle (arrow). Necrotic cells are shown in B–D with electron-dense cuticle, from a melanized region in an adult $nec^1/nec^2$ fly. This epithelial layer shows no microvilli and the nuclei are rounded (arrows) with irregular inclusions, with the appearance of fragmenting chromatin. An additional layer of healthy proliferating cells (dashed line) underlies the primary epithelium. In D, the necrotic cell nucleus (arrow) shows blebbing of nuclear membrane and is surrounded by vesiculated cellular debris. In contrast, the underlying cells show lightly stained nuclei, normal looking mitochondria (M), and microvilli on the apical surface (V).

expressed from late embryogenesis onward, with the exception of the early pupal stages. *Spn43Ab* and *Spn43Aa* are on opposite DNA strands and their temporal expression patterns are reciprocal. The *pk* and *Spn43Aa* transcripts are in the same 5′ to 3′ orientation (Figure 1) and are expressed at similar stages (GUBB *et al.* 1999). *Spn43Ac* is expressed at very low levels in the larva and early pupae and at moderate levels in late pupae and adults (Figure 5).

**Spatial expression patterns:** In general, the *Spn43A* transcripts are not expressed at high levels in imaginal discs. Localized expression of *Spn43Aa* occurs at the sites of innervated bristles on the notum and wing and both *Spn43Aa* and *Spn43Ab* are expressed weakly in the eye disc. *Spn43Ab* gives concentric rings in the leg disc with a central dot at the position of the presumptive tarsal claw and is expressed after the morphogenetic furrow in the eye (Figure 6). *Spn43Ac* expression was not

```
M S9B     KKDVRYADVPELDAKMIEMSYEGDQASMIIILPNQVDGITALEQKLKDPK.ALSRAEERL
SPN43Aa   DNWYYYADYPELDAKAIELFFENINLTMWFILPNQRSGLQALEQKLKGVDFNLLEDRWQW
SPN43Ab   LQNFNYAEVNSLDAKVVELPYQNPDFSMLLLLPNRKDGLRSLQQSLSGKNL.LAEIGA.L
SPN43Ac   DDVYGLAELPELGATALELAYKDSATSMLILLPNETTGLGKMLQQLSRPEFDLNRVAHRL
SP1       MGTFRANYFRDLDAQVIELPYLNSNLSMTIFLPREVEGLSAL.......EEKIVGFARPL
SP2       FQSFRAAHDSELGAKIIELPYRNSSLSMLIFLPDQVDGLSEL.......EKKIVGFKPKL
SP6       KFNYGFFE..DLGCTALEMPYQDSDLSMFVLLPQERTGIYALAEKLK..TVNLVDLADKL
H α1AC    HLTIPYFRDEELSCTVVELKYTGNA.SALFILPDQDK..MEEVEAMLLPETLKRWRDSLE
B AC2     RGDYKYGESAVLNAQLIEIPYKGDQSSLIVVLPKDKDGITQLQEALKDPK.TLETAQQSM

M S9B     YNTEVEIYLPKFKIETTTDLKEVLSNMNIKKLFTPGAARLENLLKTKESLYVDAAIQKAF
SPN43Aa   QSVSV..YLPKFKFEFDTDLRPTLHKMGISAMFSDAADFSNIFQDSPIGTRITKVQHKTF
SPN43Ab   SQQKVEVLLPKFSVTFGLGLEGPFKKLGVHTMFSRDGDFGNM.YRMFVSHFINAVEHKAN
SPN43Ac   RRQSVAVRLPKFQFEFEQDMTEPLKNLGVHQMFTPNSQVTKLM.DQPV..RVSKILQKAY
SP1       VAKEVYLKLPKFKIEFRDELKETLEKLGIRELFTDKSDLSGLFADKS.GGKVSQVSHKAF
SP2       SKMDVTLRLPKFKIEFFAQLNKILVAMGIQDAFEKSADFKDLVENSN..VHVKKVIHKAF
SP6       TVEEVHVKFPKFKVDYSLELAEKLKQLGITKMFTDQAEFSNLLESPEG.VFVSKVLHKAT
H α1AC    FREIGELYLPKFSISRDYNLNDILLQLGIEEAFTSKADLSGITGARNL..AVSQVVHKAV
B AC2     YSTEVDLYLPKFKIETETNLKDVLSNMNVNKIFNNDAQITRLLKGE..SLSVSEAIQKAF

M S9B     IEVNEEGAEAAAANAFGIVPASLILY....PEVHIDRPFYFELKIDGIPMFNGKVIE
SPN43Aa   IDVNEIGCEAAGASYAAGVPMSLPLDPKT...FVADHPFAFIIRDKHAVYFTGHIVKF
SPN43Ab   VEVTEAGVDQPLETGLLKGLFSRSKK......FEADHPFVFAIKYKDSIAFIGHIANYAYV
SPN43Ac   INVGEAGTEASAASYAKFVPLSLPPKPTE...FVANRPFVFAVRTPSSVLFIGHVEYPTPMSV
SP1       LEVNEEGAEAAGATSVAVTNRAGFSTF.....LMADHPFAFVIRDANTIYFQGRVVSP
SP2       IEVNEEGAEAAAATALLFVRLSVPMPSSQ.MVFNADHPFAYVIRDRETIYFQGHFVKPNE
SP6       IEVNEEGTEAAAATGMIMMTRMMTFPLQFQ....ADRPFLYVIWNKKNILFAGAFVKAA
H α1AC    LDVFEEGTEASAATAVKITLLSALVETRTIV..RFNRPFLMIIVPTDTQNIFFMSKVTNPKQA
B AC2     IEINEEGAEAAAANAFAVVFMSAVVSQPLVF..KANHPFVFFLKGDGVTLFNGVFHP
          --------------------------------
              ^^^^^       **
```

FIGURE 4.—Sequence alignment. The C-terminal region of the SPN43A serpins compared with the *M. sexta* Serpin-1 exon 9B variant (alaserpin SwissProt P14754); human α1-antichymotrypsin (EMBL X68733) and *B. mori* antichymotrypsin II (SwissProt P80034); and *D. melanogaster* serpins SP1, 2, and 6 (EMBL AJ251744, 251745, and 251749). Conserved amino acids are in boldface type and the *L*-zipper motifs in SPN43Aa and SPN43Ac are shown in bold italics. The reactive center loop is underlined (---), the hinge region is indicated (^^^^^), and the hinge sequences of SPN43Aa and SPN43Ac are shown in boldface. The protease cleavage sites (P1 and P1′) are marked with **.

**TABLE 1**

**Percentage amino acid identity (similarity) of serpins**

| | SPN43Ab | SPN43Ac | ACP76A | Mand | Antitrypsin *Bm* | Antielastase pig | Antichymotrypsin *Bm* | Antichymotrypsin Human |
|---|---|---|---|---|---|---|---|---|
| SPN43Aa | 35 (46) | 36 (46) | 22 (30) | 37 (46) | 31 (42) | 34 (46) | 33 (44) | 32 (42) |
| SPN43Ab | | 30 (38) | 22 (34) | 27 (40) | 25 (37) | 31 (42) | 26 (38) | 27 (36) |
| SPN43Ac | | | [a] | 34 (44) | 32 (46) | 34 (45) | 33 (46) | 29 (39) |

Comparison of the SPN43A serpins with the most closely related serpin sequences: Drosophila accessory gland protein ACP76A (EMBL U90947), Manduca (Mand) *Serpin-1* variant 9B (SwissProt P14754), *Bombyx mori* (*Bm*) antitrypsin (SwissProt P22922), pig leucocyte elastase inhibitor (SwissProt P80229), *Bombyx mori* antichymotrypsin (SwissProt P80034), and human α1-antichymotrypsin (EMBL X68733).

[a] The SPN43Ac sequence is too dissimilar to ACP76A for an alignment to be made.

detected in imaginal discs. SPN43Ac protein, however, is present in adult fat body, but not detected in epidermis or blood cells (J.-M. Reichhart, unpublished results).

**Heterochromatic inactivation:** To test whether any of the 43A serpins might stabilize heterochromatin, similar to the avian MENT serpin (Grigoryev *et al.* 1999), deletions in the 43A1.2 region were crossed with $w^{m4}$ to see if they acted as suppressors or enhancers of position effect variegation (for review, see Henikoff 1990). In a $w^{m4}$ background, *Df(2R)pk-sple-51/+*, *Df(2R)nap-2/+*, *Df(2R)sple-D2/+*, *Df(2R)sple-D1/+*, *Df(2R)pk-30/+* and $nec^{1}/+$ all retain the normal level of variegated *w* expression (data not shown). In addition, the homozygous deletion of *Spn43Ab*, in $w^{m4}$; *Df(2R)pk-30/Df(2R)pk-30* flies, does not modify the $w^{m4}$ phenotype.

**Identification of the *nec* transcript:** The genetic limits for the *nec* transcript are within the overlap between *Df(2R)sple-D2* and *Df(2R)nap-2* (Figure 1), an interval that extends for 17–20 kb within the *pk* 5′ intron (Gubb *et al.* 1999). The *Df(2R)sple-D1* breakpoint is proximal to *Spn43Aa* (Figure 1) by at least 8 kb (data not shown), but the phenotype of the *Df(2R)spleD1/Df(2R)nap-2* heterozygote is indistinguishable from that of *Df(2R) spleD2/Df(2R)nap-2*; the nec and pk phenotypes do not become more extreme, implying that *Spn43Aa* cannot be *nec* and that there is no additional phenotype that could be attributed to the *Spn43Aa* transcript. Similarly, the *Spn43Ab* transcript appears to be redundant as the *Df(2R)pk-30* deletion gives only an amorphic pk pheno-

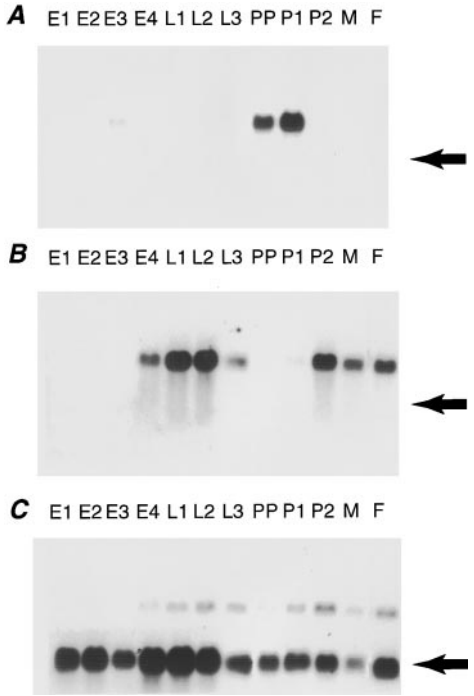


Figure 5.—Developmental Northerns. Repeated probings of a single filter with (A) a *Spn43Aa* probe, (B) a *Spn43Ab* probe, and (C) *Spn43Ac* + *Rp49* (arrow, and at equivalent positions in A and B) probes. The *Spn43Aa* transcript is expressed at high levels during the prepupal and early pupal stages. *Spn43Ab* and *Spn43Ac* are expressed from late embryo to adult stages except that *Spn43Ab* is inactive during the prepupal and early pupal stages, while *Spn43Aa* is being actively transcribed. Developmental stages: embryonic, (E1) 0–3 hr, (E2) 3–6 hr, (E3) 6–12 hr, (E4) 12–24 hr; larval, (L1, 2, and 3) first, second, and third instars; pupal, (PP) white prepupae, (P1) 12–48-hr pupae, (P2) 48–96-hr pupae; adult male (M); and female (F).

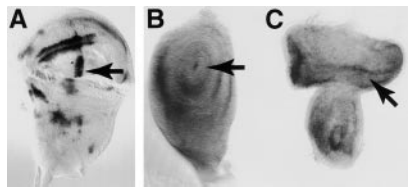


Figure 6.—Imaginal disc tissue *in situ*. (A) *Spn43Aa* hybridizes to presumptive sites of sensory bristles and sensillae in the wing, either side of the anterior D/V boundary and the presumptive vein 3 campaniform sensillae (arrow). *Spn43Aa* does not hybridize to the leg disc or eye disc, except weakly behind the morphogenetic furrow. *Spn43Ab* does not hybridize to the wing disc, but the leg (B) disc shows rings of staining close to segment boundaries and at the center of the disc at the site of the presumptive tarsal claws, and the eye disc (C) shows staining along the lateral boundaries and the morphogenetic furrow (arrow). *nec* transcripts were not detected in imaginal discs.

type (*Df(2R)pk-30* flies can be maintained as a fertile homozygous stock). Sequencing across the *Df(2R)pk-30* deletion endpoints confirms that it corresponds to a microdeletion of 1306 bp, extending from bp 27 of the *pk* 5′ exon to 7 bp 5′ of the second intron of *Spn43Ab*, including the putative active site loop. These results imply that *Spn43Ac* might correspond to *nec*, but do not preclude an unidentified transcript, or a requirement for more than one of the *Spn43A* serpins.

**Rescue of phenotype:** Transgenic rescue constructs containing genomic fragments spanning the three *Spn43A* transcripts (Figure 1) were tested in a $nec^1$ $bw^D$/*Df(2R)pk-78k* background (Table 2). For the *P[Spn43Aa$^+$]* and *P[Spn43Ab$^+$]* crosses, nec flies hatched, although at the reduced frequencies compared to nec$^+$ siblings. These flies developed necrotic patches within 24 hr and died within 3 days of eclosion. In contrast, $nec^1$ $bw^D$/*Df(2R)pk-78k; P[Spn43Ac]/+* transformants eclosed at the expected frequency. When reexamined 10 days later, the $nec^1$ $bw^D$/*Df(2R)pk-78k; P[Spn43Ac]/+* flies remained wild type for nec, indicating that the *P[Spn43Ac]* insert rescues the necrotic phenotype completely. The viability of $nec^1$ $bw^D$/*Df(2R)pk-78k; P[Spn43Ac]/+* flies under these conditions is indistinguishable from their $nec^1$ $bw^D$/*CyO; P[Spn43Ac]/+* siblings, with >98% surviving for 10 days. At 29°, 114/118 adult $nec^1/nec^2$*; UAS-p[Spn43Ac]/Gal4-da* survive for 7 days (Figure 8), with 113 remaining alive after 9 days.

Having established rigorously that the *Spn43Ac* corresponds to *nec*, we henceforth refer to this transcript as *nec*.

**Characterization of the $nec^1$ and $nec^2$ mutations:** PCR sequencing of DNA from $nec^1$/*Df(2R)pk-78k* and $nec^2$/*Df(2R)pk-78k* flies identified a 6-bp deletion in $nec^1$, resulting in deletion of two isoleucine residues at positions 118 and 119. In $nec^2$, Q37 is replaced by a stop codon, giving a 5′ truncation of the peptide within the polyglutamine repeat.

**Suppression of adult lethality:** *nec* has recently been shown to control expression of the antifungal peptide Drosomycin via the TOLL pathway (Levashina *et al.* 1999). To investigate whether the lethal nec phenotype is associated with activation of the immune response, double mutant flies were constructed with a loss-of-function allele of *spaetzle* (*spz*), the gene coding for the *Toll*-ligand. As shown in Figure 7, *nec; spz* flies survive significantly longer than their *nec* siblings (from the cross of $nec^1$/*CyO; spz/TM3* × $nec^2$/*CyO; spz/TM3*). Half of the *nec* flies are dead within 2 days, while the partially rescued *nec; spz* flies only start to die after day 2 and it is 5.5 days before half are dead.

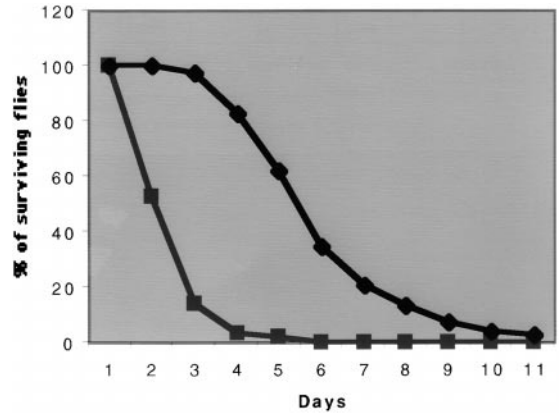


Figure 7.—The survival of adult *nec vs. nec; spz* flies at 25°. A total of 222 $nec^1/nec^2$ (◆) and 81 $nec^1/nec^2$; $spz^{rm7}$ (■) flies were counted and transferred daily. Confidence intervals of 95% are ≤0.1 for each point.

**Activity of NEC reactive center loop:** To test whether the nec phenotype is linked to a serine protease inhibitory function of NEC, the P1 P1′ residues (L438 and S439) of the putative reactive center loop were altered to proline residues (see materials and methods), which are never found at these positions within an active inhibitor. The resulting construct (*P[UAS-nec$^{PP}$]*) was tested for complementation of the nec phenotype in a simple survival test. The ubiquitously expressed

**TABLE 2**

**Test crosses for rescue of nec phenotype**

| Transgene | w | P[w$^+$] bw$^D$ | Cy or If | Cy If |
|---|---|---|---|---|
| *Spn43Aa* | 55/93 = 0.59[a] | 34/93 = 0.37[a] | 383 | 174 |
| *Spn43Ab* | 3/19 = 0.16[a] | 2/19 = 0.11[a] | 59 | 24 |
| *Spn43Ac* | 56/99 = 0.57[a] | 96/99 = 0.97[b] | 417 | 176 |

*w; nec$^1$ bw$^D$/If; P[w$^+$, Spn$^+$]/+* males were crossed to *w; Df(2R)pk-78k/CyO* females. The $F_1$ Cy$^+$ If$^+$ progeny are heterozygous $nec^1/nec^-$ and are phenotypically w; nec or nec P[w$^+$] bw$^D$ (unless they carry a transgene that rescues the nec phenotype). $F_1$ Cy, If, and Cy If progeny will be nec$^+$, with either w or P[w$^+$] bw$^D$ eyes, depending on whether they carry the $w^+$ allele on the transgene. The expected frequency of the w and P[w$^+$] bw$^D$ classes is 1/6 [Σ(Cy + If + Cy If)], *i.e.*, 93 for *Spn43Aa*, 19 for *Spn43Ab*, and 99 for *Spn43Ac*. In the *P[Spn43Aa$^+$]* and *P[Spn43Ab$^+$]* crosses, w nec and P[w$^+$] bw$^D$ nec flies hatched at reduced frequencies compared to their Cy, If, and Cy If siblings. In contrast, the *P[Spn43Ac]* test cross gave survival of one-sixth (96/593) of P[w$^+$] bw$^D$ : Cy, If, and Cy If progeny, while their w siblings hatched at reduced frequency and remained nec.

[a] Developed necrotic patches and died within 72 hr of eclosion.

[b] Survived at least 10 days after eclosion with nec$^+$ phenotype.

**TABLE 3**

**Test crosses for activity of *nec$^{PP}$***

| Transgene | Cy (w Sb) | w nec Sb | w$^+$ Cy$^+$Sb$^+$ |
|---|---|---|---|
| *nec$^{PP}$* | 242 | 8[a] | 2[a] |
| *nec$^+$* | 306 | 13[a] | 37[b] |

To test the activity of the NEC (SPN43Ac) reactive center loop, *w; nec$^2$ bw$^D$/CyO; P[w$^+$, Gal-4$^{da}$]/MKRS, Sb* males were crossed to *w; Df(2R)pk-78k/CyO; P[UAS, w$^+$, nec$^{PP}$]/MKRS, Sb* females. The $F_1$ progeny consisted of 242 Cy flies (with combinations of w and Sb), 8 w nec Sb, and 2 w$^+$ Cy$^+$ Sb$^+$ nec (confirming that the *nec$^{PP}$* transgene does not rescue the nec phenotype). The control cross of *w; nec$^2$ bw$^D$/CyO; P[w$^+$, Gal-4$^{da}$]/MKRS, Sb* males to *w; Df(2R)pk-78k/CyO; P[UAS, w$^+$, nec$^+$]/MKRS, Sb* females gave 306 Cy, 13 w nec Sb, and 37 wild-type flies.

[a] Developed necrotic patches and died within 72 hr of eclosion.

[b] Survived at least 10 days after eclosion with nec$^+$ phenotype.

*Gal4-da* strain was used to drive expression of *P[UAS-nec$^{PP}$]* using the expression system of Brand and Perrimon (1993). The *nec$^1$/nec$^2$; P[UAS-nec$^{PP}$]/Gal4-da* flies expressed a nec phenotype indistinguishable from their *nec$^1$/nec$^2$; P[UAS-nec$^{PP}$]*/MKRS siblings, with an adult half-life of ~1.5 days posteclosion. In contrast, the wild-type *P[UAS-nec]* construct (Levashina *et al.* 1999) rescues the nec phenotype completely to give a healthy fertile stock in *nec$^1$/nec$^2$; Gal4-da/P[UAS-nec$^{PP}$]* flies (Table 3, Figure 8). Western blots of hemolymph proteins from *nec$^2$/Df(2R)pk78k; Spn43Ac$^{PP}$/+* flies show a strong SPN43Ac-PP protein band, confirming that the transgenic protein is expressed and is stable (N. Pelte and J.-M. Reichhart, unpublished data).

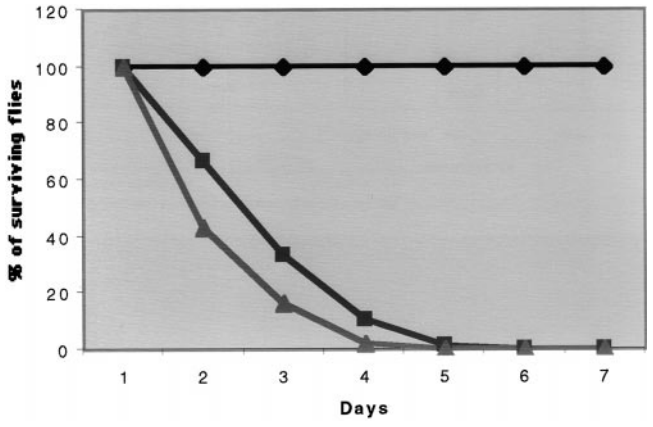


Figure 8.—The survival of adult *nec vs. nec-PP* flies at 29°. A total of 153 *nec$^1$/nec$^2$* (■); 81 *nec$^1$/nec$^2$; UAS-nec$^{PP}$/Gal4-da* (◆); and *237 nec$^1$/nec$^2$; UAS-nec$^{PP}$/Gal4-da* (▲) flies were counted and transferred daily. Confidence intervals of 95% are ≤0.1 for each point.

## DISCUSSION

We show here that the *Spn43Ac* serpin transcript corresponds to the *nec* gene and that it is not functionally redundant. Deletions that include all three serpins of the *Spn43A* cluster do not enhance the necrotic phenotype compared to that of the *nec$^1$/nec$^2$* mutant combination. The other two serpins, *Spn43Aa* and *Spn43Ab*, are more typical of serpins in that a deletion gives no detectable phenotype. The *nec$^1$* and *nec$^2$* mutations both map within the *Spn43Ac* transcript and a genomic fragment that includes this transcript completely rescues the nec phenotype. The *nec$^1$* mutation deletes two isoleucine residues (I118 and I119; Figures 4 and 9) within the putative helix-A of the serpin, leaving the remainder of the nucleotide sequence in frame. This alteration would probably disrupt helix-A and the underlying β-sheet B, which could cause misfolding of the protein. The *nec$^2$* mutation causes a Q37 to stop codon transition within the N-terminal polyglutamine repeat that deletes the entire serpin domain. The nec mutant phenotype is not rescued by a transgenic construct carrying L438P + S439P transitions within the putative RCL of NEC, confirming that the NEC protein acts as an active serine protease inhibitor.

In arthropods, injury initiates proteolytic cascades, leading to rapid blood clotting and melanization at the site of injury. The black patches in *nec* mutants may result from melanization via activation of a phenol oxidase cascade that is under the direct control of NEC. Alternatively, the melanization might be a secondary reaction to cellular damage caused by the activation of a distinct protease cascade in the absence of functional NEC. The additional layer of epidermal cells underlying necrotic patches is reminiscent of the wound healing response seen in mammalian systems.

The demonstration of activation of the TOLL-mediated immune response by NEC (Levashina *et al.* 1999) prompted us to construct the double mutant *nec; spz* strain. In this double mutant strain the nec melanotic patches underlying the cuticle are delayed in appearance and reduced in size. In addition, the survival of adult *nec; spz* flies is extended relative to *nec* flies (Figure 7). Interestingly, constitutive activation of the TOLL pathway in the dominant mutant *Toll$^{10B}$* does not cause a necrotic phenotype or adult lethality. We propose that the NEC target protease(s) is upregulated by activation of the TOLL pathway. This response would be masked in *Toll$^{10B}$* flies, as the serpin activity of the wild-type *nec* allele would be sufficient to block excess protease. In a *nec* mutant, however, activation of the TOLL pathway would lead to expression of active protease(s) without the inhibitory NEC serpin. In this case, the active protease(s) would cause cellular and tissue damage and lead to reduced viability. In the *nec; spz* double mutant, levels of the NEC target protease would remain at the basal level found in unchallenged wild-type flies. This basal

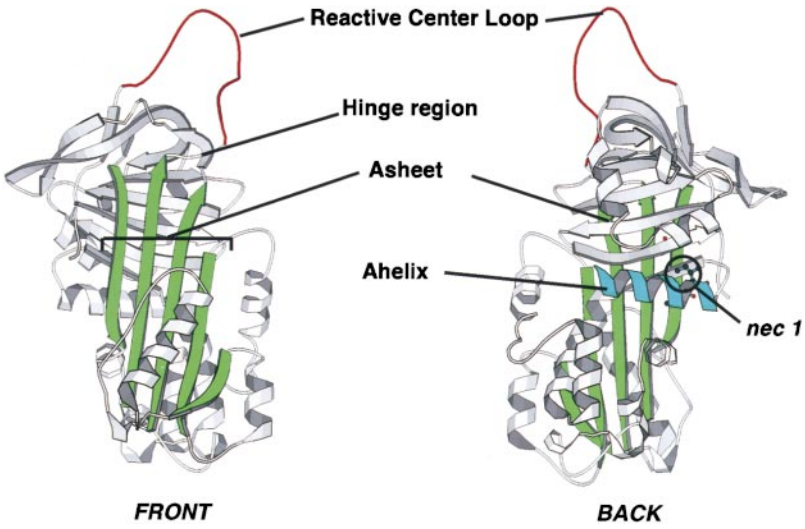


FIGURE 9.—A homology model of SPN43Ac based on the X-ray crystal structures of *M. sexta* serpin 1K and human α1-antitrypsin. Views from front and back are shown. The A-β-sheet is green, the reactive center loop is red, and the A-helix is cyan. The unique N-terminal extension of SPN43Ac is omitted. The position of the $nec^1$ deletion is indicated on the back view by a ball-and-stick marker. The picture was produced using Molscript (KRAULIS 1991).

level of protease activity could still cause limited damage, given that the *nec; spz* double mutant flies lack functional NEC inhibitor.

The insect serpin most closely related to the cluster of *Spn43A* transcripts is the *M. sexta* serpin1-B (JIANG *et al.* 1996) with 25–30% conservation at the amino acid level (KANOST *et al.* 1989; JIANG *et al.* 1994, 1996). This level of conservation is characteristic within the serpin family, with the C-terminal half of the protein tending to show the greatest conservation (SOMMER *et al.* 1987). The protease-binding specificity of a particular serpin depends on the amino acid sequence of its reactive center (BOSWELL and CARRELL 1988; HUBER and CARRELL 1989; CARRELL and EVANS 1992). The critical importance of the reactive center loop is underlined by the genomic organization of *M. sexta serpin-1* (KANOST *et al.* 1989). This transcription unit contains 12 variants of the exon that encodes the reactive center loop, exon 9. Mutually exclusive splicing of exon 9 generates multiple serpin isoforms (JIANG *et al.* 1994). These variants show differing proteinase affinities, which has been interpreted as suggesting that the protein isoforms of *M. sexta serpin-1* gene regulate different aspects of the wound healing and antimicrobial defense (JIANG *et al.* 1996). While this is an attractive possibility, most serpins will inhibit a broad range of proteinases *in vitro*, so that biochemical assays of serpin/proteinase specificity give a poor indication of physiologically significant interactions. The Drosophila *Spn43A* cluster of serpins shows no trace of the genomic organization of the Manduca serpin-1, but could regulate some of the activities suggested for the hornworm protein. In particular, the nec phenotype is suggestive of activation of a phenol oxidase cascade, causing epithelial necrosis with subsequent regeneration.

The localized patterns of expression of the *Spn43Aa* and *Spn43Ab* serpins in imaginal discs are hard to interpret given that the transcripts appear to encode secreted proteins. To some extent, these transcription patterns may reflect ectopic regulation by enhancer elements in the adjacent *pk* promotor and be irrelevant to the function of the serpins. The expression of *Spn43Aa* transcripts in the presumptive vein 3 sensillae, however, is not seen with *pk* (GUBB *et al.* 1999), although the expression pattern of these two transcripts along the dorsoventral boundary of the wing is similar. *Spn43Ab* expression in the wing disc is absent during stages when the *Spn43Aa* and *pk* transcripts, on the opposite DNA strand, are being expressed. The expression of *Spn43Ab* at the segmental boundaries in the leg and at the site of the presumptive tarsal claw in the center of the disc is distinctive and may mark a critical region for the control of leg disc growth. Like MENT, SPN43Ab is extremely basic and might have a similar function in stabilizing heterochromatic domains. This possibility was not confirmed by genetic tests, as none of the *Spn43A* serpins show the expected dominant suppression of position effect variegation. Even when homozygous, the *Df(2R)pk-30* deletion, including the *pk* 5′ exon and *Spn43Ab*, does not modify the $w^{m4}$ phenotype. In addition, the putative signal peptide in SPN43Ab would not be expected in a nuclear protein.

The lack of phenotypes other than nec and pk with deletions that remove all three serpins implies that the *Spn43Aa* and *Spn43Ab* serpins are redundant, with other genetic functions mapping elsewhere in the genome. Within the *Spn43A* cluster itself, however, the three serpin genes do not complement each other. The visible nec phenotype implies an essential role for the NEC serpin and will allow direct mutagenesis screens to identify critical regions within serpin molecules.

We thank Reine Klock for the northern blot experiments; Daniel Zachary for the electron microscopy; Darin Coulson, Annie Meunier, and Glynnis Johnson for skilled assistance in fly pushing; Robin Carrell and David Lomas for discussion and critical reading of the manuscript; John Tamkun, Nick Brown, and Steve Russell for cDNA and genomic libraries; and the FlyBase consortium, particularly Rachel Drysdale,

for rulings on the *Spn* nomenclature. This work was funded by Medical Research Council programme grants to Michael Ashburner, David Gubb, and Steven Russell, with E.L. and J.-M.R. being supported by the CNRS and grants from the Marie Curie Research training Program (EU) and the NATO Scientific Research Program.

The EMBL accession numbers for the SPN43A serpins are SPN43Aa (AJ245442), SPN43Ab (AJ245443), and NEC (SPN43Ac) (AJ245444).

## LITERATURE CITED

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

Aulak, K. S., E. Eldering, C. E. Hack, Y. P. Lubbers, R. A. Harrison *et al.*, 1993 A hinge region mutation in C1-inhibitor (Ala436→Thr) results in nonsubstrate-like behavior and in polymerization of the molecule. J. Biol. Chem. **268:** 18088–18094.

Bairoch, A., P. Bucher and K. Hofmann, 1997 The PROSITE database, its status in 1997. Nucleic Acids Res. **25:** 217–221.

Baumann, U., R. Huber W. Bode, D. Grosse, M. Lesjak *et al.*, 1991 Crystal structure of cleaved human $\alpha_1$-antichymotrypsin at 2.7 Å resolution and its comparison with other serpins. J. Mol. Biol. **218:** 595–606.

Baumann, U., W. Bode, R. Huber, J. Travis and J. Potempa, 1992 Crystal structure of cleaved equine leucocyte elastase inhibitor determined at 1.95 Å resolution. J. Mol. Biol. **226:** 1207–1218.

Bayer, C., L. Von Kalm and J. W. Fristrom, 1996 Gene regulation in imaginal discs and salivary gland development during Drosophila metamorphosis, pp. 321–361 in *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*, edited by L. I. Gilbert, J. R. Tata and B. G. Atkinson. Academic Press, San Diego.

Boswell, D. R., and R. W. Carrell, 1988 Genetic engineering and the serpins. BioEssays **8:** 83–87.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

Brown N. H., and F. Kafatos, 1988 Functional cDNA libraries from *Drosophila* embryos. J. Mol. Biol. **203:** 425–437.

Bruce, D., D. J. Perry, J. Y. Borg, R. W. Carrell and M. R. Wardell, 1994 Thromboembolic disease due to thermolabile conformational changes of antithrombin Rouen-VI (187 Asn→Asp). J. Clin. Invest. **94:** 2265–2274.

Carrell, R. W., and D. L. I. Evans, 1992 Serpins: mobile conformations in a family of proteinase inhibitors. Curr. Opin. Struct. Biol. **2:** 438–446.

Carrell, C., and J. Travis, 1985 $\alpha_1$-Antitrypsin and the serpins: variation and countervariation. Trends Biochem. Sci. **10:** 20–24.

Carrell, R. W., P. E. Stein, G. Fermi and M. R. Wardell, 1994 Biological implications of a 3 Å structure of dimeric antithrombin. Structure **2:** 257–270.

Clark, A. G., M. Agude, T. Prout, L. G. Harshman and C. H. Langley, 1995 Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. Genetics **139:** 189–201.

Davis, R. L., A. E. Shrimpton, P. D. Holohan, C. Bradshaw, D. Feiglin *et al.*, 1999 Familial dementia caused by polymerization of mutant neuroserpin. Nature **401:** 376–379.

Dunin-Borkowski, O. M., and N. H. Brown, 1995 Mammalian CD2 is an effective heterologous marker of the cell surface in Drosophila. Dev. Biol. **168:** 689–693.

Erdjument, H., D. A. Lane, M. Panico, V. Di Marzo and H. R. Morris, 1988 Single amino acid substitutions in the reactive site of antithrombin leading to thrombosis. J. Biol. Chem. **263:** 5589–5593.

Grigoryev, S. A., J. Bednar and C. L. Woodcock, 1999 MENT, a heterochromatin protein that mediates higher order chromatin folding, is a new serpin family member. J. Biol. Chem. **274:** 5626–5636.

Gubb, D., and A. Garcia-Bellido, 1982 A genetic analysis of the determination of cuticular polarity during development in *Drosophila melanogaster*. J. Embryol. Exp. Morphol. **68:** 37–57.

Gubb, D., C. Green, D. Huen, D. Coulson, G. Johnson *et al.*, 1999 The balance between isoforms of the Prickle LIM domain protein is critical for planar polarity in *Drosophila* imaginal discs. Genes Dev. **13:** 2315–2327.

Han, J., H. Zang, G. Min, D. Kemler and C. Hashimoto, 2000 A novel *Drosophila* serpin that inhibits serine proteases. FEBS Lett. **468:** 194–198.

Heitzler, P., D. Coulson, M.-T. Saenz-Robles, M. Ashburner, J. Roote *et al.*, 1993 Genetic and cytogenetic analysis of the 43A-E region containing the segment polarity gene *costa* and the cellular polarity genes *prickle* and *spiny-legs* in *Drosophila melanogaster*. Genetics **135:** 105–115.

Henikoff, S., 1990 Position-effect variegation after 60 years. Trends Genet. **6:** 422–426.

Huber, R., and R. W. Carrell, 1989 Implications of the three-dimensional structure of $\alpha_1$-antitrypsin for structure and function of serpins. Biochemistry **28:** 8951–8966.

Jiang, H., Y. Wang and M. R. Kanost, 1994 Mutually exclusive exon use and reactive center diversity in insect serpins. J. Biol. Chem. **269:** 55–58.

Jiang, H., Y. Wang, Y. Huang, A. B. Mulnix, J. Kadel *et al.*, 1996 Organization of serpin gene-1 from *Manduca sexta*. J. Biol. Chem. **271:** 28017–28023.

Kanost, M. R., S. V. Prasad and M. A. Wells, 1989 Primary structure of a member of the serpin superfamily of proteinase inhibitors from an insect, *Manduca sexta*. J. Biol. Chem. **264:** 965–972.

Kraulis, P. J., 1991 Molscript—a program to produce both detailed and schematic plots of protein structures. J. Appl. Crystal. **24:** 946–950.

Lemaitre, B., E. Nicolas, L. Michaut, J.-M. Reichhart and J. Hoffman, 1996 The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in Drosophila adults. Cell **86:** 973–983.

Levashina, E. A., E. Langley, C. Green, D. Gubb, M. Ashburner *et al.*, 1999 Constitutive activation of the TOLL-mediated antifungal defense in serpin-deficient *Drosophila*. Science **285:** 1917–1919.

Li, J., Z. Wang, B. Canagarajah, H. Jiang, M. Kanost *et al.*, 1999 The structure of active serpin 1k from *Manduca sexta*. Structure **7:** 103–109.

Loebermann, H., R. Tokuoka, J. Diesenhofer and R. Huber, 1984 Human $\alpha_1$-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. J. Mol. Biol. **177:** 531–557.

Lomas, D. A., D. L. Evans, J. T. Finch and R. W. Carrell, 1992 The mechanism of Z $\alpha_1$-antitrypsin accumulation in the liver. Nature **357:** 605–607.

Lomas, D. A., P. R. Elliott, W.-S. W. Wardell and R. W. Carrell, 1995 Preparation and characterization of latent $\alpha_1$-antitrypsin. J. Biol. Chem. **270:** 5282–5288.

Marshall, C. J., 1993 Evolutionary relationships among the serpins. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. **342:** 101–119.

Mottonen, J., A. Strand, J. Smersky, R. M. Sweet, D. E. Danley *et al.*, 1992 Structural basis of latency in plasminogen activator inhibitor-1. Nature **355:** 270–273.

Muller, H. J., 1930 Types of visible variations induced by X-rays in Drosophila. J. Genet. **22:** 299–334.

Nielsen, H., J. Engelbrecht, S. Brunak and G. Von Heijne, 1997 Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:** 1–6.

O'Connell, P., and M. Rosbach, 1984 Sequence, structure and codon preference of the Drosophila ribosomal protein rp49 gene. Nucleic Acids Res. **12:** 5495–5513.

Potempa, J., E. Korzus and J. Travis, 1994 The serpin superfamily of proteinase inhibitors; structure, function and regulation. J. Biol. Chem. **269:** 15957–15960.

Reichhart, J.-M., and D. Ferrandon, 1998 Green balancers. DIS **81:** 201–202.

Ringo, J. M., R. Werczberger, M. Altaratz and D. Segal, 1991 Female sexual receptivity is defective in juvenile hormone-deficient mutants of the *apterous* gene of *Drosophila melanogaster*. Behav. Genet. **21:** 453–469.

Russell, S., and K. Kaiser, 1993 *Drosophila melanogaster* male germ line-specific transcripts with autosomal and *Y*-linked genes. Genetics **134:** 293–308.

Sali, A., and T. L. Blundell, 1993 Comparative protein modeling by satisfaction of spatial restraints. J. Mol. Biol. **234:** 779–815.

Sambrook, J., E. F. Frisch and T. Maniatis, 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schreuder, H. A., B. De Boer, R. Dijkema, J. Mulders, H. J. M. Theunissen *et al.*, 1994 The intact and cleaved human antithrombin III complex as a model for serpin-proteinase interactions. Nat. Struct. Biol. **1:** 48–54.

Sommer, J., S. M. Gloor, G. F. Rovelli, J. Hofsteenge, H. Nick *et al.*, 1987 cDNA sequence coding for a rat glia-derived nexin and its homology to members of the serpin superfamily. Biochemistry **26:** 6407–6410.

Spradling, A., 1986 *P*-element mediated transformation, pp. 176–197 in *Drosophila; A Practical Approach*, edited by D. B. Roberts. IRL Press, Oxford.

Takahashi, A., P. Y. Musy, L. M. Martins, G. G. Poirier, R. W. Moyer *et al.*, 1996 CrmA/SPI-2 inhibition of an endogenous ICE-related protease responsible for lamin A cleavage and apoptotic nuclear fragmentation. J. Biol. Chem. **271:** 32487–32490.

Wright, H. T., 1996 The structural puzzle of how serpin serine proteinase inhibitors work. BioEssays **18:** 453–464.

Zou, Z., A. Anisowicz, M. J. Hendrix, A. Thor, A. M. Neveu *et al.*, 1994 Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science **263:** 526–529.

Communicating editor: T. C. Kaufman