Development 118, 1291-1301 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

expanded: a gene involved in the control of cell proliferation in imaginal discs

Michael Boedigheimer and Allen Laughon*

Laboratory of Genetics, University of Wisconsin, Madison, 445 Henry Mall, Madison, WI 53706, USA

*Author for correspondence

SUMMARY

The *expanded* gene was first identified by a spontaneous mutation that causes broad wings. We have identified an enhancer-trap insertion within *expanded* and used it to generate additional mutations, including one null allele. *expanded* is an essential gene, necessary for proper growth control of imaginal discs and, when mutant, causes either hyperplasia or degeneration depending on the disc. Wing overgrowth in *expanded* hypermorphs is limited to specific regions along the anterior-posterior and dorsal-ventral axis. *expanded* encodes a novel 1429 amino acid protein that is localized to the apical surface of disc cells and contains three potential SH3-binding sites. Together, these observations suggest that the Expanded protein engages in protein-protein interactions regulating cell proliferation in discs.

Key words: *Drosophila*, imaginal disc, tumor suppressor, *expanded* gene, SH3-binding site, cell proliferation

INTRODUCTION

Imaginal discs are the progenitors of adult integument in holometabolous insects. They consist of a single-cell layer epithelium that arises as an infolding of the embryonic ectoderm. After exponential increase in cell number during the three larval instars, cell division slows and the discs evaginate (Bryant and Levinson, 1985; Madhavan and Schneiderman, 1977). Cessation of cell division occurs in a spatially and temporally defined pattern across the disc (Schubiger and Palka, 1987; O'Brochta and Bryant, 1987), and is controlled by a disc autonomous mechanism that is dependent on intercellular contact (Bryant and Schmidt, 1990; Bryant and Simpson, 1984).

Mutants in seven loci have been found that interfere with cessation of cell division in imaginal discs (Bryant and Schmidt, 1990; Mechler et al., 1991). These have been called tumor suppressor genes because of the resulting overgrowth of discs, which occurs during an extended larval period. Tumor suppressor genes have been divided into two groups based upon the type of disc overgrowth. Mutations in one group lead to neoplastic overgrowth. In these mutants, the discs lose their single-layer epithelial organization and their ability to differentiate. The genes in this group are *discs large-1 (dlg)* (Woods and Bryant, 1989; Stewart et al., 1972) and *lethal(2) giant larvae (lgl)* (Gateff and Schneiderman, 1974). Mutations in the second group lead to hyperplastic overgrowth. In these mutants, the discs retain their single-layer epithelial structure and their ability to differentiate adult cuticular structures. The genes in this group are *discs overgrown (dco)* (Jursnich et al., 1990), *lethal(2)fat (fat)* (Bryant et al., 1988), *lethal(2) giant discs (lgd)* (Bryant and Levinson, 1985), *lethal(3)c43 (c43)* (Martin et al., 1977; Shearn, 1977), and *tumorous discs (tud)* (Gateff and Mechler, 1989). In addition to causing disc overgrowth, mutants in the tumor suppressor genes also cause some cell death. The most extreme example of degeneration occurs in *dco* mutants, in which certain allelic combinations lead to complete degeneration, while others lead to overgrowth of discs (Jursnich et al., 1990).

The Fat protein is a member of the cadherin superfamily (Mahoney et al., 1991). Vertebrate cadherins are calcium-dependent cell adhesion molecules, indicating Fat may be involved in cell adhesion. The Fat protein also contains four epidermal growth factor repeats and is a transmembrane protein. The Lgl protein is also somewhat similar in sequence to cadherins (Lützelschwab et al., 1987; Klambt et al., 1989) and is membrane associated (Strand et al., 1991). *dlg* encodes a guanylate kinase that is localized in a lateral belt near the apical cell surface (Woods and Bryant, 1991). Mutations in *dco* and *c(43)* reduce the number of gap junctions on cell membranes (Jursnich et al., 1990; Ryerse and Nagel, 1984a,b). The net function of these genes is to inhibit cell proliferation, possibly via cell-cell communication mechanism.

Here we provide evidence that the *expanded (ex)* gene affects the growth of imaginal discs. The original *ex* mutation (Stern and Bridges, 1926) causes wide wings. Waddington (1940) characterized the *ex* phenotype in greater detail and concluded that the wing defect was probably due to effects on cell division. We have isolated an enhancer-trap transposon insertion in the *ex* gene, and

used it to generate an allelic series of *ex* mutations that cause varying degrees of hyperplastic overgrowth of discs. We have also cloned *ex*, determined the sequence of its predicted protein product and characterized its expression pattern with antibodies raised against an Ex fusion protein. *ex* encodes a protein with three potential SH3-binding sites and may therefore interact with SH3-containing proteins such as Dlg, *Drosophila* Abl (Dabl) or *Drosophila* Src (Dsrc).

MATERIALS AND METHODS

Fly stocks

Flies were reared on standard cornmeal molasses medium at room temperature. *lethal(2)eyeless* (*l(2)ey*) a mutant isolated in an EMS screen by Huettner and Bryant (1988), and *l(3)dco* were provided by P. Bryant. *Df(2L)al* was provided by J. Kennison. ds^{38k} and ds^{33k} were provided by A. Dingwall. (Complementation tests were done using both alleles.) In the text, *ush* refers to the allele ush^{IIA102}. Complementation tests with *al* were done with alleles al^{1} and al^{2}. *ush* and *al* stocks were obtained from the Bloomington Stock Center. Canton S (CS) and balancer chromosomes were laboratory stocks. Except the new *ex* mutants described in the text, all mutants used here are described in Lindsley and Zimm (1992).

Enhancer-trap insertion characterization

A new set of *P*[lacW] insertions on the 2nd and 3rd chromosomes was generated by the mobilization of an X-chromosome insert as described by Bier et al. (1989). The starting X-chromosome *P*[lacW] insert was generously provided by E. Bier. New inserts on the autosomes were selected on the basis of w^{+}. Lines containing inserts were screened for β-gal activity in imaginal discs.

Polytene chromosome squashes and in situ hybridizations were performed according to the protocol of Ashburner (1989). The probes were labelled with digoxigenin-dATP (Boehringer Mannheim) using random primers.

Isolation of *ex* clones

Screening of recombinant DNA libraries, subcloning, Southern and northern blot analysis was performed according to Sambrook et al. (1989). Genomic DNA clones were isolated from the *Drosophila* genomic library of Maniatis et al. (1978) using an amplified aliquot provided by B. Ganetzky. 50,000 plaques were plated in each round of screening. cDNAs were isolated from a third instar imaginal disc library kindly provided by G. Rubin. 300,000 plaques from this library were plated in each round of screening. All probes used to screen libraries were labelled with [α-^{32}P]dATP using the random primer method to a specific activity of at least 10^{8} cts/minute/μg of DNA. 500,000 cts/minute/ml of the labelled probe was used for each hybridization.

Sequencing

Nested deletion series for DNA sequencing were created using an Erase-a-base kit (Promega) following the manufacturer's recommended procedures. DNA templates were prepared by the small-scale alkaline lysis method (Sambrook et al., 1989). Sequencing was done using Sequenase version 2.0 (US Biochemicals) following the manufacturer's recommended procedures. Compressions and premature terminations were resolved using deaza-dGTP with Sequenase and high temperature reactions with Pfu DNA polymerase (Stratagene), respectively. Deletion series constructs and several relevant subclones were sequenced to span the 6.1 kb of cumulative cDNA. Except for 253 bases in the 3′ untranslated region, all sequence was determined for both strands.

Antibody production and staining

A 2405 bp *Pst*I-*Bam*HI fragment from cDNA pL3 was cloned into a T7 expression vector (Rosenberg et al., 1987). Cells carrying this construct were induced, harvested and lysed. Inclusion bodies were spun down and washed four times with RIPA buffer (150 mM NaCl, 1% Tween, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5), resuspended in PBS (10 mM KPO_4, 150 mM NaCl, pH 7.2) and 2 mg were injected intradermally into New Zealand White Rabbits. Monthly subcutaneous boosts were given. Antibodies were affinity purified on an Ex-Actigel (Sterogene) affinity column according to the supplier's instructions. Staining was done as follows. Fixed imaginal discs were blocked with PBS supplemented with 3% BSA for 1 to 4 hours, and incubated with a 1:8000 dilution of anti-Ex in PBTw (PBS + 0.1% Tween) with 0.3% BSA for 4 hours. The material was washed in PBTw and then incubated overnight in a 1:500 dilution of horseradish peroxidase-congjugated goat anti-rabbit IgG (Fischer-Biotech). The discs were washed and equilibrated in reaction buffer. Detection was done following the manufacturer's recommendations.

Comparative analysis of wings

Photographs of mounted wings were traced by hand then scanned and imported into a drawing program. Wings were rotated about the vertex of veins L2 and L3 to align veins L2 or L3. After wings were aligned, they were scaled so the aligned veins were the same length from the vertex to the margin. To obtain an average wing shape the three flattest wing mounts for each genotype were aligned and scaled along vein L2, printed and retraced. Tracings of wing hairs were taken from photographs of the dorsal and ventral wing surfaces between veins L4 and L5. The dorsal and ventral images were coded so their identities were unknown until after the analysis was complete.

Database searches

The predicted protein was used in searches of NBRF-PIR release 33 and SwissProt release 22 using Proscan software (DNAstar) with PAM matrices. PATMAT version 2.21 was used to search the Blocks database (release 4.1) for matches with conceptual proteins translated from all six frames of the cDNA sequence. The Prosite database was also searched to identify any matches to known protein motifs.

RESULTS

Isolation of *ex* mutant alleles

In a search for genes involved in imaginal disc development, a collection of *P*[lacW] enhancer-trap transposon insertions was generated and screened for β-galactosidase expression in discs. Insert 697 expresses *lacZ* throughout discs and also disrupts disc development, causing overgrown, missing, or improperly differentiated adult limbs. The most obvious defect in 697 mutants is broad, arced wings. A single *P*[lacW] insert at 21C1-4, *P*[lacW](21C1-4), was detected in polytene chromosomes squashes prepared from the 697 stock after probing with pUC sequences contained in *P*[lacW]. A deficiency spanning this region, *Df(2L)al21B8-C1;21C8-D1*, failed to complement the 697 defect, confirming the cytological mapping. *Df(2L)al* uncovers mutations in several known genes: *al*, *ex*, *l(2)ey*, *ds*, and *ush*. *697*, *ex* and the previously identified mutant *l(2)ey* fall into one complementation group. Since *ex* was the first allele named, it will be referred to as ex^{1}, and *697* and *l(2)ey* will be called ex^{697} and $ex^{l(2)ey}$, respectively.

Southern blot analysis confirmed the existence of a single insert in the ex^{697} stock. To test whether the ex^{697} phenotype was due to *P*[lacW](21C1-4), 100 independent lines were created from dysgenic crosses that lacked the *white*$^{+}$ marker contained in *P*[lacW]. As expected, most of the lines (89) retained the ex^{697} phenotype. These presumably contained an internal excision of the enhancer-trap that disrupts the white marker and were therefore discarded. Five were full phenotypic revertants, providing evidence that insert 697 causes the ex^{697} phenotype. Six gave a more severe phenotype, which may have arisen due to excision of DNA flanking the insert. These were designated ex^{e1}-ex^{e6}, and were tested inter se and against *al*, *ds*, *ush*. ex^{e1} through ex^{e6} fall into a single *ex* complementation group. We have further characterized ex^{e1}, ex^{e2} and ex^{e6}.

ex^{e1}-ex^{e6} were originally classified as recessive lethals because no homozygous progeny were recovered among at least 100 scored. However, during routine stock maintenance escapees in the ex^{e6} and ex^{e2} stocks were observed, implying that ex^{e2} and ex^{e6} retain some *ex* function. Escapees have not been observed in *Df(2L)al*, ex^{e1} or $ex^{l(2)ey}$ stocks. The allele strength was estimated by viability, eclosion rate and penetrance of the *ex* phenotype (Table 1). All the data are consistent with the following ranking (from most to least severe): *Df(2L)al*= $ex^{e1} \geq ex^{l(2)ey} > ex^{e2} > ex^{e6} > ex^{697} > ex^{1}$. ex^{e1} is a small deficiency that removes 5′ sequences of the expanded transcription unit (see below) and behaves like *Df(2L)al* in all heteroallelic tests, and is therefore classified as a genetic null allele.

ex mutant phenotypes

The *ex* mutant phenotype is incompletely penetrant and expressive (Table 1). Weak mutant alleles, such as ex^{1}, generally show only a broad wing phenotype, which occasionally have an upward or downward arc. Intermediate mutants such as ex^{697} or mutant combinations such as $ex^{l(2)ey}/ex^{697}$ display phenotypes affecting legs, thorax, head and wings. Occasionally, entire legs are missing, but more commonly, legs are kinked or have swollen distal tarsal segments, with completely separated internal vesicles of unknown origin and composition (Fig. 1G). In intermediate mutant combinations such as $ex^{l(2)ey}/ex^{697}$, about 50% of the individuals display leg defects. In these flies, only one or two legs are typically affected. The thoracic defects include duplication of scutellar bristles and sensilla on the wing. Defects in the head capsule are variable. Eyes are reduced in size and occasionally split. Duplicated antennae or vibrissae occur in about 50% of $ex^{l(2)ey}/ex^{697}$ heterozygotes, apparently at the expense of peripheral eye tissue. In addition to being broad, the wings are arced down and have incomplete crossveins (Fig. 1C). The wing phenotype of intermediate mutants will be discussed in detail below.

Compared to the intermediate alleles, ex^{e1} pharate adults display a highly penetrant and expressive phenotype. ex^{e1} mutants survive until the pharate adult stage and show massive head, wing and leg defects (Fig. 1D, G). All ex^{e1} pharate adults have leg defects and usually all legs of an individual are defective (Fig. 1F). Some legs are entirely missing, but the most common defect is missing distal tarsal segments including the claw organs. The legs usually terminate beyond the second tarsal segment. The remaining proximal tarsal segments have supernumerary bristles. The antennae are enlarged and are missing aristae, but otherwise appear normal (Fig. 1D). The reduction of eye tissue seen in intermediate mutant alleles is exaggerated in ex^{e1} pharate adults, such that eyes fail to differentiate ommatidia (Fig. 1D). Unlike intermediate mutants, antennal duplications do not generally occur.

To understand better the function of *ex*, the phenotype of null mutants was examined at earlier stages. No visible defects were seen in ex^{e1} mutant embryos and they hatched at a rate comparable to wild type (data not shown). The first obvious defects occur in the imaginal discs at about mid-third instar, by which time the wing disc is noticeably enlarged (data not shown). The wing disc continues to grow, mainly in the presumptive wing pouch region and, by day

Table 1. Summary of inter se crosses with *ex* mutants

	e1	*l(2)ey*	*e2*	*e6*	*697*†	*ex*†
Df(2L)al	lethal 0.0 (73)*	lethal 0.0 (58)	lethal 0.0 (69)	lethal 0.0 (90)	b,cv,ea,l 0.16 (106)	b,cv,ea,l 0.25 (71)
e1	nt	lethal 0.0 (123)	lethal 0.0 (217)	b,cv,ea,l 0.01 (450)	b,cv,ea,l 0.13 (195)	b,cv,ea,l 0.16 (68)
l(2)ey		nt	lethal 0.0 (61)	b,cv,ea,l 0.01 (83)	b,cv,ea,l 0.13 (168)	b,cv,ea,l 0.43 (79)
e2			nt	b,cv,ea,l 0.06 (148)	b,cv,ea,l 0.18 (72)	b,cv 0.45 (199)
e6				b,cv,ea,l nt	b,cv,ea,l 0.28 (109)	b,cv 0.43 (79)
697					b,cv,ea nt	b nt
ex						b nt

†stocks maintained as homozygotes; other stocks maintained over SM6
*fraction homozygous viable progeny (total scored)
nt - viability not scored
b - broad wings
cv - greater than 10% of the mutant progeny have incomplete posterior cross vein
ea - greater than 10% of the mutant progeny have defects in eye-antennal disc derivatives
l - greater than 10% of the mutant progeny have swollen or missing leg segments

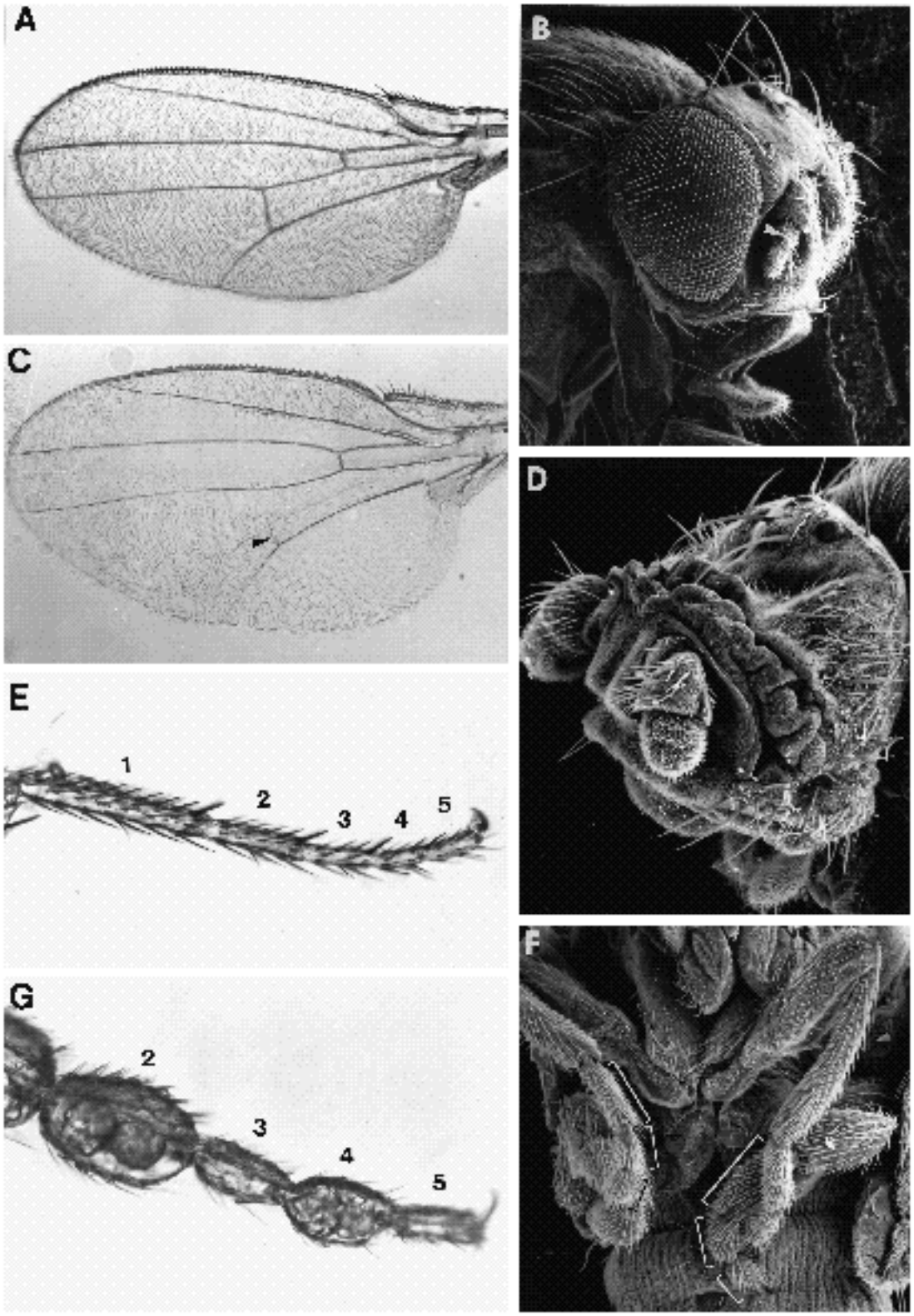

Fig. 1. Adult *ex* phenotype. (A) Wing from Canton S fly. (B) Canton S head capsule showing normal eyes and antennae with aristae (arrowhead). (C) Wing from ex^{697} fly. Arrowhead points to incomplete crossvein. (D) An ex^{e1} pharate adult head capsule with missing eyes and aristae. (E) Canton S with tarsal segments numbered. (F) SEM of ex^{e1} pharate adult showing 2 missing legs and 4 legs with truncated tarsal segments. The remaining tarsal segments are bracketed. (G) ex^{697} tarsal segments with characteristic swelling and internal vesicles. Tarsal segments are numbered in panels E and G.

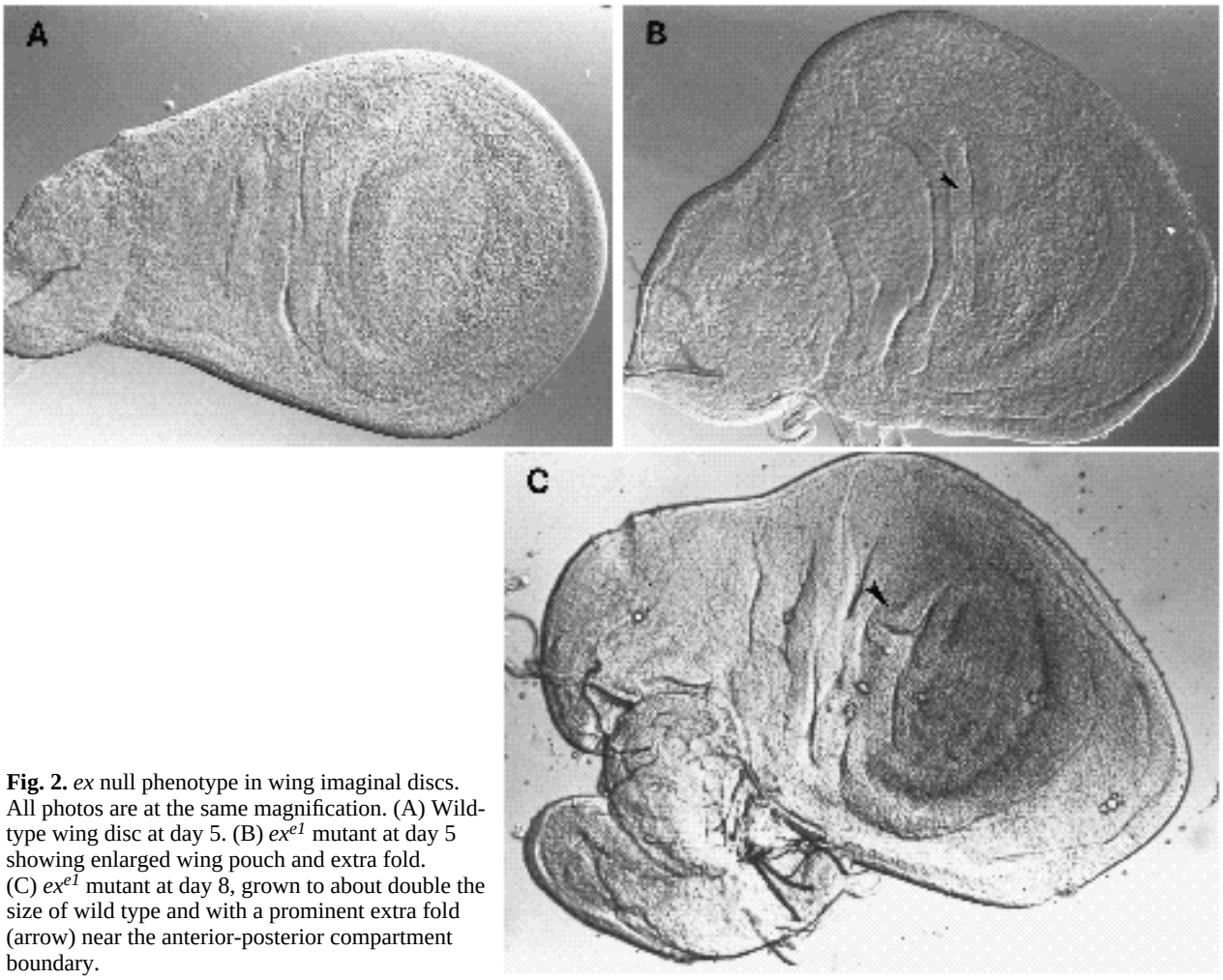

Fig. 2. *ex* null phenotype in wing imaginal discs. All photos are at the same magnification. (A) Wild-type wing disc at day 5. (B) ex^{e1} mutant at day 5 showing enlarged wing pouch and extra fold. (C) ex^{e1} mutant at day 8, grown to about double the size of wild type and with a prominent extra fold (arrow) near the anterior-posterior compartment boundary.

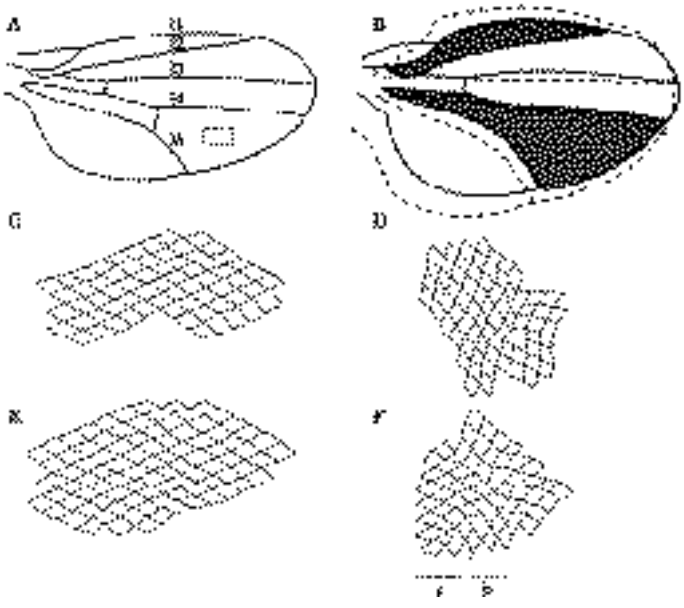

Fig. 3. Wing shape phenotype in *ex* mutants. (A) Average CS wing with veins labelled (L1-L5). (B) Average ex^{697} mutant wing, solid black line, showing the hyperplastia between L1 and L2 and between L4 and L5 (shaded regions). ex^{e1} pharate adult wings, dashed lines, showing addional hyperplasia between L5 and the margin. Panels C-F show position of hairs at intersections of diagonal lines. Dorsal (C) and ventral (E) wing surfaces of wild-type (taken from region near boxed region in A) are well organized compared to the dorsal (D) and ventral (F) surfaces of mutant wing. C and E show that cells in the wild-type wing are elongated in the long axis of the wing, while B shows cells elongated predominantly in the broad axis of the wing. D shows both types of elongation, above region a is more similar to wild-type than above region b. The difference in dorsal and ventral cell shapes may account for the downward arc of the wing seen in intermediate *ex* alleles.

five, begins to form an extra fold approximately at the anterior-posterior compartment boundary (Fig. 2B). Growth continues during an extended larval period (2-3 extra days in uncrowded conditions), usually until the wing disc reaches double the wild-type size (Fig. 2C), although occasionally continuing until they are many times the size of wild type. Overgrowth also occurs in the haltere disc (data not shown). In the eye-proper and the leg discs, degeneration is visible (data not shown). The eye region of the eye-antennal disc is largely atrophied, presumably due to a lack of ommatidial development, as evidenced by the lack of a morphogenic furrow or organized photoreceptor cells and by the missing eye in pharate adults (Fig. 1D). The presumptive head capsule is still intact (data not shown). The leg discs appear to be lacking distal segments (data not shown). All discs examined retained a single-cell layer epithelium.

ex affects the number, size and shape of cells in the wing

Since each cell in the wing blade is associated with a single hair, the hairs can serve as markers for cell number and position (Dobzhansky, 1929). An examination of *ex* hypomorphic mutant wings revealed that the increased size of the wing is due to hyperplasia in two regions of the wing. In one of these regions, there is an increase in cell density, an elongation of cells in the broad axis of the wing and a greater elongation in dorsal surface than ventral surface.

The wing phenotype of a viable *ex* mutant is shown in Fig. 3. To analyze their shape, the wings were aligned along a wing vein and scaled to the same size. After scaling, all mutant wings were essentially superimposable (as are wild type), showing that the mutant wing phenotype is highly reproducible. This was true regardless of the heteroallelic combinations tested, allowing a comparison of wing shape to be made between scaled mutant and wild-type wings. Moreover, the overgrowth phenotype is completely recessive, indicating that overgrowth is not due to dominant allele-specific affects. The shape of *ex* mutant wings is broader than wild type, and rounder at the tip. The distal tip of wild-type wings occurs where L3 meets the margin, whereas the distal tip of *ex* wings is at the margin between L3 and L4 (Fig. 1A, C). The sizes of intervein regions were compared separately between wild-type and *ex* mutant wings. This analysis revealed that only specific regions of the wing are larger in expanded mutants. The area between L1 and L2, and between L4 and L5 are increased the most (shaded regions in Fig. 3B). The area bounded by L3, L4 and the anterior crossvein is not significantly different in the mutant. Although the figure shows ex^{697} wings, essentially identical results were obtained for ex^{1}, $ex^{l(2)ey}/ex^{697}$, ex^{e1}/ex^{697}, ex^{e2}/ex^{697} and ex^{e6}/ex^{697} mutant wings. The posterior crossvein is about the same length in mutants and wild type and does not span the intervein region in *ex* mutants. Thus, it is possible that the overgrowth is limited to a region anterior to the incomplete crossvein. From this analysis, it cannot be determined whether overgrowth is limited in the proximal distal axis. To determine whether overgrowth is spatially limited in null mutants, adult wings were dissected from pharate adults and carefully flattened. There was considerably more variation in shape among mutant pharate wings. The mutant pharate wings were generally larger than wild type with excessive growth between L1 and L2, and between L4 and the margin (dotted lines in Fig. 3B). These results indicate that regional hypertrophy occurs in complete as well as partial loss-of-function *ex* mutations.

Hair distribution was used to examine the shape and distribution of cells in wild type and $ex^{l(2)ey}/ex^{697}$ in the most severely affected region, between L4 and L5 (Fig. 3D,F). The density of hairs on mutant wings is increased and the hairs are more disorganized than on wild-type wings. The ratio of the average distance between hairs in the broad and long axis of the wing is larger in the mutant than in wild type. The ratio in wild-type wings is the same for the dorsal and ventral wing blade surfaces, 0.53±0.05 (n=20) and 0.53±0.07 (n=24), respectively. However, in mutants the

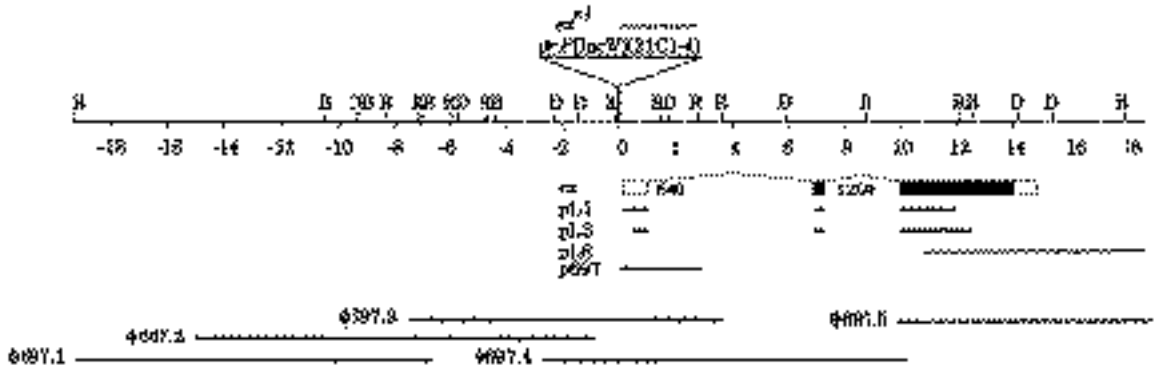

Fig. 4. *ex* Genomic and cDNA maps. The limits of the ex^{e1} deletion are shown by an open box with dotted lines representing a region of uncertainty. Below this is the genomic restriction map, where B is *Bam*HI, R is *Eco*RI and D is *Hin*dIII. The triangle shows the position of the 697 insert (*P*[lacW](21C1-4)). The direction of transcription of β-galactosidase (β-gal), mini-white and ampicillin resistance gene within the construct are indicated. The boxes labeled *ex* shows the positions of exon relative to the genomic DNA. The numbers next to the boxes refer to exon-intron junctions relative to the cDNA. Open boxes refer to the non-coding regions and filled boxes refer to the coding regions. The positions of the cDNA subclones, pL1, pL3, and pL6 are indicated. *ex* is transcribed to the right. p697 indicates the 2.8 kb of rescued flanking DNA. At the bottom of the figure are the approximate positions of 5 genomic phages that span the region.

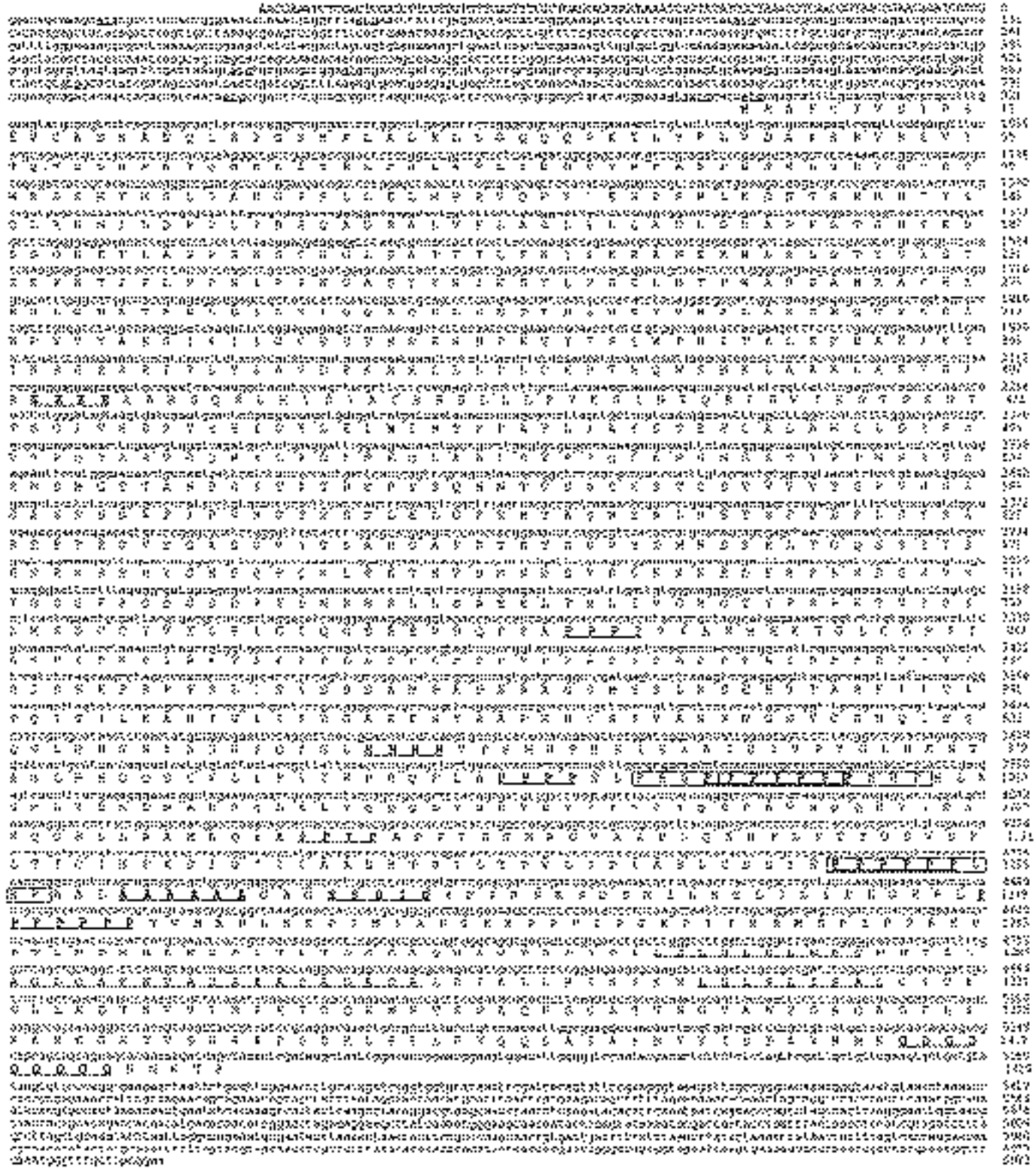

Fig. 5. *ex* and protein sequence. Genomic sequence is in capital letters followed by the cDNA sequence in lowercase. The *P*[lacW](21C1-4) is at position −1. Below the cDNA sequence is a conceptual translation in single letter amino acid code starting at the ATG at position 892. The 9 upstream ATGs are underlined. The 697 insert is at position −1 in this figure. Three basic regions 306-408, 840-879, 1194-1270 are separated by neutral or slightly acidic regions. Homopolymeric runs, and QA and LX repeat regions are in bold underlined text. Three putative SH3-binding domains are boxed.

ratio is larger on the dorsal surface than on the ventral surface (1.59±0.22 (n=15) and 0.90±0.41 (n=42), respectively). The abnormal hair distribution indicates that, in this region, the cells in *ex* mutants are elongated along the broad axis of the wing. This type of cell elongation does not occur in the region between L5 and the margin in $ex^{l(2)ey}/ex^{697}$ mutants (data not shown).

An estimate of the number of extra cell divisions occurring between L4 and L5, N_{ex}, was made using the formula $N_{ex}=\ln R / \ln 2$, where R is the ratio of cell densities in mutant versus wild-type wings. This calculation assumes that wild-type and mutant wing discs start with the same number of cells and that the amount of cell death is unchanged in the mutant. R was estimated as 1.7 for the region between L4 and L5 in $ex^{l(2)ey}/ex^{697}$ mutants, yielding an N_{ex} of 0.7. According to this estimate, an average increase of 0.7 cell divisions per cell occurs in the region between L4 and L5. This is a minimum estimate, since it is quite possible that increased cell death occurs in *ex* mutants, as has been described for other tumor suppressor mutants (Jursnich et al., 1990). Also, $ex^{l(2)ey}/ex^{697}$ is a viable allelic combination, and a qualitative difference in size exists between mutant wing discs from this allelic combination and null alleles. Based on visual examination of mutant wing discs from null mutants and not accounting for possible cell death, we estimate that the N_{ex} is slightly greater than 1.

Molecular characterization of *ex*

The *ex* gene was unambiguously identified by the position of the *P*[lacW](21C1-4) and by a small deletion, ex^{e1}, that removes part of the *ex* transcript. Genomic DNA flanking one side of the ex^{697} *P*[lacW] insert was recovered by plasmid rescue. The resulting plasmid, p697, contained 2.8 kb of flanking DNA and was used as a probe to polytene chromosomes from salivary glands of wild-type larvae. A single band was detected at 21C1-4, confirming the identity of the rescued plasmid. p697 was then used as a probe to identify *ex* clones in genomic and cDNA libraries. Probes generated from the ends of the phage insert were used to extend the walk to about 40 kb (Fig. 4).

Using p697 as a probe to northern blots revealed a prominent band at 6.9 kb (data not shown). This same probe was used to retrieve cDNAs from a larval disc cDNA library. Eight cDNAs, which together span 6.1 kb, were analyzed by restriction mapping and sequencing. No differences were found in the overlapping regions and thus appear to be derived from a single species of transcript.

The composite sequence of the three *ex* cDNAs was determined (Fig. 5). There is a single large open reading frame of 4287 bases, encoding a predicted protein of 1429 amino acids. A good in-frame match to the *Drosophila* consensus translational start (Cavener, 1987) is present at base 892. There are nine other ATGs present upstream. The longest potential protein encoded by an upstream ORF is relatively short, 137 amino acids in length. We conclude that the 1429 amino acid protein is the major *ex* product.

The approximate positions of the *ex* exons were determined by hybridization of cDNAs to genomic DNA fragments on Southern blots. The precise locations of intron-exon junctions were determined from comparison of cDNA and genomic DNA sequences at those junctions (Fig. 5). Southern blots of ex^{e1} mutant DNA shows that a small deletion is associated with a partial excision of the *P* element. This deletion removes at least the rightmost 1.9 kb of the *P*[lacW] insert plus 3-12 kb of flanking genomic DNA, including the first exon. The deletion does not extend as far as the second exon (Fig. 4). Because no other transcripts are affected by the deletion, the 6.9 kb transcript belongs to *ex*. Absence of the first exon is consistent with genetic evidence that ex^{e1} is a null allele.

Ex protein contains potential SH3-binding sites

Comparisons of the predicted Ex protein sequence to protein databases and translated DNA databases reveals no striking similarities to known proteins. A lack of hydrophobic segments suggests that the Ex protein is probably not secreted or membrane spanning. Overall the predicted protein is basic, with three positively charged amino acid segments (isoelectric point greater than 9.0) separated by neutral or slightly acidic regions. The C-terminal half of the protein contains many short homopolymeric runs of proline, serine, alanine, histidine and glutamine. Three consensus binding sites for Src homology 3 (SH3) domains, defined as PXXPPPXXP (Ren et al., 1993), are boxed in Fig. 5. SH3-binding sites have also been found in 3BP1, a mammalian protein that also has homology to GAP-rho outside the SH3-binding site (Cicchetti et al., 1992), in formins, which are products of the limb deformity gene in mice (Woychik et al., 1990), and in the muscarinic acetylcholine receptor genes (Bonner et al., 1987). Homopolymeric runs of glutamine, proline and histidine have been associated with

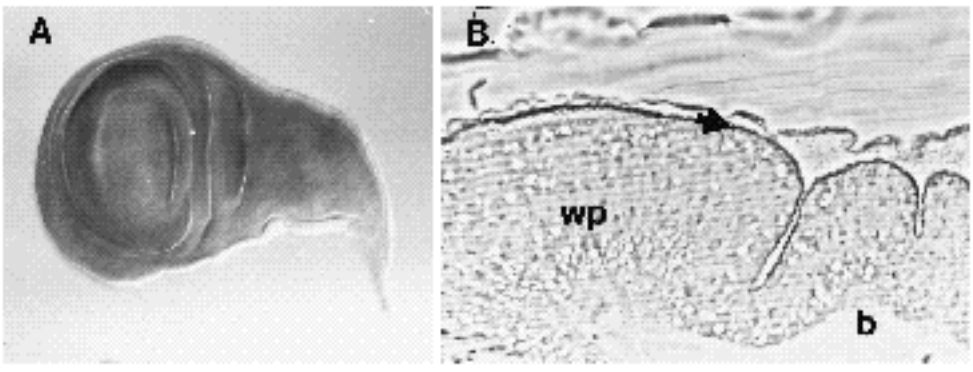

Fig. 6. *ex* protein expression. (A) Staining of a third instar wing disc with anti-*ex* shows the appearance of more intense staining in folds. (B) Longitudinal section of an anti-*ex*-stained third instar wing disc showing that the *ex* protein is tightly localized to the apical cell surface. wp marks the wing pouch and b marks the basal cell surface.

protein-protein interactions in transcription factors (Mitchel and Tjian, 1989). Ex also contains QA and LX repeats of unknown significance. Searches of Prosite revealed 51 potential serine-threonine phosphorylation sites and 1 potential tyrosine phosphorylation site.

Expression pattern of Ex

Antibodies generated against the Ex protein were used to detect its expression in imaginal discs (Fig. 6). The Ex protein is detected by early third instar (the youngest discs examined). Expression is relatively uniform throughout leg discs, but is intensified in the presumptive wing pouch relative to elsewhere in wing disc. Sections of stained mature third instar discs showed that the protein was localized to the extreme apical cell surface. *ex* mRNA also appears to be apically localized in whole-mount and sectioned discs that were hybridized with digoxigenin-labelled probe from the first *ex* exon (data not shown).

DISCUSSION

The phenotypes seen in the *ex* mutants resemble those in some tumor suppressor mutants. To date, seven tumor suppressor gene affecting imaginal discs have been identified (Bryant and Schmidt, 1990). Five of these, when mutant, cause hyperplastic growth of discs during an extended larval period. Based on the disc overgrowth phenotype, we propose that *ex* be assigned to the hyperplastic group of tumor suppressor genes. The discs of these tumor suppressor mutants, including *ex,* continue to grow during an extended larval period. Overgrowth in the previously characterized tumor suppressor mutants is not due to the extended larval period, but rather has been interpreted to be due to a disruption of a signal normally required to limit growth (Bryant and Schmidt, 1990). The hyperplastic phenotype suggests that *ex* may also be required to generate or respond to growth-limiting signals. Overgrowth of mid-third instar discs suggests that *ex* must be needed before this time (as are other tumor suppressor genes). This overgrowth could be due to extra cell divisions or decreased cell death. Since there is normally little cell death during development of the wing disc (James and Bryant, 1981), we infer that the increase in cell number is due primarily to extra cell divisions starting before mid-third instar and continuing throughout an extended larval period.

Degeneration, instead of overgrowth, occurs in distal tarsi, aristae and eyes of *ex* null mutants. This may reflect a varying sensitivity to reduced *ex* activity rather than different functions of *ex* in these discs. It is possible that loss of *ex* function causes cell death in these structures, as seen in *dpp*, *vg* and *scalloped* mutants (James and Bryant, 1981), or simply a failure to grow, as has been postulated for *wg* mutants (Cousso et al., 1992). In either case, these defects may be mechanistically related to the hypertrophy seen in wing discs.

The *ex* phenotype closely resembles the phenotype of *dco* mutants. Both phenotypes include duplications of head structures, bulged leg segments and disc overgrowth and degeneration (Jursnich et al., 1990; this work). There is also a striking similarity in the abnormal folding pattern of wing discs from *ex* and *dco* mutants, whereas all the other tumor suppressor mutants have distinctly different abnormal disc morphologies. Therefore, we suggest that *ex* and *dco* may impinge on the same growth control pathway. Although the molecular function of *dco* is unknown, *dco* mutants have a reduced number of gap junctions. By extension, *ex* may be involved in gap junction formation or may be part of a signalling pathway dependent on gap junctions.

Recently, a consensus peptide was defined that binds to SH3 regions (Ren et al., 1993; Cicchetti et al., 1992). Analysis of the Ex protein sequence revealed three such SH3-binding sites. SH3 domains have been found in proteins involved in signal transduction pathways, including non-receptor tyrosine kinases and proteins in Ras signalling pathways (for review see Pawson and Gish, 1992; Clark et al., 1992). The SH3 domain has been shown to have a negative regulatory effect on the Abl and Src tyrosine kinases (Seidel-Dugan et al., 1992; Franz et al., 1989), and to be necessary for signal transduction involving guanosine triphosphatase activating protein (GAP) (Duchesne et al., 1993). Thus Ex may function by binding to proteins that are directly involved in a signal transduction pathway.

The SH3-containing proteins, *Drosophila* Abl (Dabl) (Bennet and Hoffman, 1992) and Dsrc29A (Katzen et al., 1990), are expressed in imaginal discs. Moreover, the Dabl protein, like Ex, is localized to the apical surface of cells in imaginal discs. p66, a product of Dsrc29A, is localized to the plasma membrane in embryonic cells, and may be membrane localized in imaginal disc cells as well. However, genetic analysis of *dabl* in flies has yet to reveal any connection to growth control in imaginal discs.

Another potential interaction is with the SH3-containing product of the tumor suppressor gene *dlg*, which encodes a guanylate kinase, and has therefore been postulated to affect growth by regulating the GTP:GDP ratio in the cell. A high GTP:GDP ratio is known to activate Ras and it was therefore postulated that Dlg could affect this pathway. The Dlg protein is expressed in a lateral belt at the apical end of the cell (Woods and Bryant, 1991) and may make some direct contact with Ex.

Overgrowth of wing tissue is limited to certain regions of the wing blade of *ex* mutants depending on the allelic combination, implying that multiple growth control mechanisms operate across the wing disc. An initial examination of other wing shape mutants indicates that different regions of the wing can also be affected. For example, *Broad* and *fat*^{Gull} seem to cause an increase in the area between L2 and L3 (unpublished data). Multiple growth control mechanisms may have provided a simple evolutionary mechanism accounting for differences in wing shape in *Drosophila* species. Since the Ex protein expression is not limited to the overgrown regions, it seems likely that an unidentified localized signal is directly responsible for regionalized growth control.

It remains to be determined whether region-specific overgrowth in *ex* mutants is related to compartment boundaries (Garcia-Bellido et al, 1973, 1976; Lawrence and Morrata, 1976; Blair, 1992), regions of neuronal competence (Simpson and Carteret, 1989), or patterns of gene expression, which run parallel to the proximal-distal axis (Kornberg et al., 1985; Brower, 1986; Orenic et al., 1990;

Cousso et al., 1993; Williams et al., 1993). Another possibility is that an redundant growth control system may be active in the presumptive medial wing blade, which could compensate for effects of *ex* mutations in this region. *dpp*, for example, is expressed in a proximal-distal stripe centered in the wing disc (Massucci et al, 1990; Blair, 1992), and, although *dpp* is needed throughout the wing disc, the region most sensitive to loss of *dpp* is where *dpp* expression is highest. Consistent with this idea is the observation that the regions sensitive to *ex* levels flank the regions of high *dpp* expression.

We thank Duane Price for his participation in carrying out the enhancer-trap screen. We thank Peter Bryant, Andy Dingwall, Jim Kennison and Matthew Scott for generously providing stocks and for valuable discussion of unpublished results. We appreciate helpful comments on the manuscript from Jim Williams, Brian Florence and Duane Price. We thank Bill Engels for use of a 4× objective. This work was supported by a grant to A. L. from the NSF and by an NIH predoctoral training grant (GM07133). This is publication number 3351 from the Laboratory of Genetics, University of Wisconsin, Madison.

REFERENCES

Ashburner, M. (1989). Drosophila*, A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Bennet, R. L. and Hoffman, F. M. (1992). Increased levels of the *Drosophila abelson* tyrosine kinase in nerves and muscles: subcellular localization and mutant phenotypes imply a role in cell-cell interactions. *Development* **116**, 953-966.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T. and Grell, E. (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. *Genes Dev.* **3**, 1273-1287.

Blair, S. (1992). *engrailed* expression in the anterior lineage compartment of the developing wing blade of *Drosophila*. *Development.* **115,** 21-33.

Bonner, T. I., Buckley, N. J., Young, A. C. and Brann, M. R. (1987). Identification of a family of *Muscarinic Acetylcholine Receptor* genes. *Science* **237,** 527-532**.**

Brower, D. L. (1986). *engrailed* expression in *Drosophila* imaginal discs. *EMBO J.* **5**, 2649-2656.

Bryant, P. J., Huettner, B., Held, L. I., Jr., Ryerse, J. and Szidonya, J. (1988). Mutations at the *fat* locus interfere with cell proliferation control and epithelial morphogenesis in *Drosophila*. *Dev. Biol.* **129**, 541-554.

Bryant, P. J. and Levinson, P. Y. (1985). Intrinsic growth control in the imaginal primordia of *Drosophila* and the autonomous action of a lethal mutation causing overgrowth. *Dev. Biol.* **107**, 355-363.

Bryant, P. J. and Schmidt, O. (1990). The genetic control of cell proliferation in *Drosophila* imaginal discs. *J. Cell Science.* **13**, 169-189.

Bryant, P. J. and Simpson, P. (1984). Intrinsic and extrinsic control of growth in developing organs. *Quart. Rev. Biol.* **59**, 387-415.

Cavener, D. R. (1987). Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Research*. **15**, 1353-1361.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992). *C. elegans* cell signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.

Cicchetti, P., Mayer, B. J., Thiel, G. and Baltimore, D. (1992). Identification of a protein that binds to the SH3 region of *abl* and is similar to *bcr* and *GAP-rho.Science* **257,** 803-806**.**

Cousso, J. P., Bate, M. and Martinez-Arias, A. (1993). A *wingless* dependent polar coordinate system in *Drosophila* imaginal discs. *Science* **259,** 484-490**.**

Dobzhansky, T. (1929). The influence of the quantity of chromosomal material on the size of the cells in *Drosophila melanogaster*. *Wilhelm Roux's Arch. EntwMech. Org.* **115**, 363-379.

Duchesne, M., Schweighoffer, F., Parker, F., Clerc, F., Frobert, Y., Thang, M. N. and Tocque, B. (1993). Identification of the SH3 domain of GAP as an essential sequence for Ras-GAP-mediated signaling. *Science* **259**, 525-528.

Franz, W. M., Berger, P. and Wang, J. Y. J. (1989). Deletion of an N-terminal regulatory domain of the *c-abl* tyrosine kinase activates its oncogenic potential. *EMBO J.* **8**, 137-147.

Garcia-Bellido, A., Morata, G. and Ripoll, P. (1973). Developmental compartmentalization of the wing disc of *Drosophila. Nature* **245**, 251-253.

Garcia-Bellido, A., Ripoll, P. and Morata, G. (1976). Developmental compartmentalization of the dorsal mesothoracic disk of *Drosophila. Dev. Biol.* **48**, 132-147.

Gateff, E. A. and Mechler, B. M. (1989). Tumor-suppressor genes of *Drosophila melanogaster. Crit. Rev. Oncogen.* **1**, 221-245.

Gateff, E. A. and Schneiderman, H. A. (1974). Developmental capacities of benign neoplasms of *Drosophila*. *Wilhelm Roux's Arch. EntwMech. Org.* **176**, 23-65.

Huettner, B. and Bryant, P. J. (1988). *l(2)ey: lethal(2)eyeless. Drosophila Information Service.* **67**, 110.

James, A. A. and Bryant, P. J. (1981). Mutations causing pattern deficiencies and duplications in the imaginal wing disk of *Drosophila melanogaster. Dev. Biol.* **85,** 39-54.

Jursnich, V. A., Fraser, S. E., Held, L. I., Jr., Ryerse, J. and Bryant, P. J. (1990). Defective gap-junctional communication associated with imaginal disc overgrowth and degeneration caused by mutations of the *dco* gene in *Drosophila. Dev. Biol.* **140**, 413-429.

Katzen, A. L., Kornberg, T. and Bishop, J. M. (1990). Diverse expression of *dsrc29a*, a gene related to *src*, during the life cycle of *Drosophila melanogaster.Development* **110,** 1169-1183.

Klambt, C., Muller, S., Lutzelschwab, R., Rossa, R., Totzke, F. and Schmidt, O. (1989). The *Drosophila melanogaster l(2)gl* gene encodes a protein homologous to the cadherin cell-adhesion molecule family. *Dev. Biol.* **133**, 425-436.

Kornberg, T., Siden, I., O'Farrell, P. and Simon, M. (1985). The *engrailed* locus of *Drosophila*: In situ localization of transcripts reveals compartment-specific expression. *Cell* **40**, 45-53.

Lawrence, P. A. and Morata, G. (1976). Compartments in the wing of *Drosophila:* A study of the *engrailed* gene. *Dev. Biol.* **50,** 321-337.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of* Drosophila Melanogaster. New York: Academic Press, Inc.

Lützelschwab, R., Klämbt, C., Rossa, R. and Schmidt, O. (1987). A protein product of the *Drosophila* recessive tumor gene, *lethal(2)giant larvae*, potentially has cell adhesion properties. *EMBO J.* **6**, 1791-1797.

Madhavan, M. and Schneiderman, H. A. (1977). Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **183**, 269-305.

Mahoney, P. A., Weber, U., Onofrechuk, P., Biessmann, H., Bryant, P. J. and Goodman, C. S. (1991). The *fat* tumor suppressor gene in *Drosophila* encodes a novel member of the cadherin gene superfamily. *Cell* **67**, 853-868.

Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. *Cell* **15**, 687-701.

Martin, P., Martin, A. and Shearn, A. (1977). Studies of *l(3)c43*^{hs1} a polyphasic, temperature-sensitive mutant of Drosophila melanogaster with a variety of imaginal disc defects. *Dev. Biol.* **55**, 213-232.

Massucci, J. D., Miltenberger, R. J. and Hoffman, F. M. (1990). Pattern-specific expression of the *Drosophila decapentaplegic* gene in imaginal disks is regulated by 3′ cis-regulatory elements. *Genes Dev.* **4**, 2011-2023.

Mechler, B. M., Strand, D., Kalmes, A., Merz, R., Schmidt, M. and Torok, I. (1991). *Drosophila* as a model system for molecular analysis of tumorigenesis. *Environ. Health Perspect.* **93**, 63-71.

Mitchell, P. J. and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-378.

O'Brochta, D. A. and Bryant, P. J. (1987). Distribution of S-phase cells during the regeneration of *Drosophila* imaginal wing discs. *Dev. Biol.* **119**, 137-142.

Orenic, T. V., Slusarski, D. C., Kroll, K. L. and Homgren, R. A. (1990). Cloning and characterization of the segment polarity gene *cubitus interruptus Dominant* of *Drosophila. Genes Dev.* **4**, 1053-1067.

Pawson, T. and Gish, G. D. (1992). SH2 and SH3 domains: from structure to function. *Cell* **71,** 359-362.

Ren, R., Mayer, B. J., Cicchetti, P. and Baltimore, D. (1993).

Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **259**, 1157-1161.

Rosenberg, A. H., Lade, B. N., Chui, D., Lin, S., Dunn, J. J., Studier, F. W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56,** 125-135.

Ryerse, J. S. and Nagel, B. A. (1984a). Gap junction distribution in the *Drosophila* wing disc mutants *vg, l(2)gd, l(3)c43*^{hs1}, *and l(2)gl*^{4}. *Dev. Biol.* **105**, 396-403.

Ryerse, J. S. and Nagel, B. A. (1984b). Changes in the distribution of gap junctions in *Drosophila melanogaster* wing discs during the third larval and early pupal stages of development. *Wilhelm Roux's Arch. Dev. Biol.* **193**, 187-196.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harvor Laboratory Press.

Schubiger, M. and Palka, J. (1987). Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. *Dev. Biol.* **123**, 145-153.

Seidel-Dugan, C., Meyer, B. E., Thomas, S. M. and Brugge, J. S. (1992). Effects of SH2 and SH3 deletions on the functional activities of wild-type and transforming variants of *c-Src.Mol. Cell. Biol.* **12**, 1835-1845.

Shearn, A. (1977). Mutational dissection of imaginal disc development in *Drosophila melanogaster*. *Amer. Zool.* **17**, 585-594.

Simpson, P. and Carteret, C. (1989). A study of *shaggy* reveals spatial domains of expression of *achaete-scute* alleles on the thorax of *Drosophila*. *Development* **106**, 57-66.

Stern, C. and Bridges, C. P. (1926). The mutants on the extreme left arm of chromosome two of *Drosophila melanogaster. Genetics* **11**, 503-530.

Stewart, M., Murphy, C. and Fristrom, J. (1972). The recovery and preliminary characterization of x-chromosome mutants affecting imaginal discs of *Drosophila melanogaster. Dev. Biol.* **27**, 71-83.

Strand, D., Torok, I., Kalmes, A., Schmidt, M., Merz, R. and Mechler, B. M. (1991). Transcriptional and translational regulation of the expression of the *l(2)gl* tumor suppressor gene of *Drosophila melanogaster*. *Adv. Enz. Reg.* **31**, 339-350.

Waddington, C. H. (1940). The genetic control of wing development in *Drosophila*. *J. Genetics* **41**, 75-139.

Williams, J. A., Paddock, S. W. and Carroll, S. B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. *Development* **117**, 571-584.

Woychik, R. P., Maas, R. L., Zeller, R., Vogt, T. F. and Leder, P. (1990). 'Formins': proteins deduced from the alternative transcripts of the *limb deformity* gene. *Nature* **346,** 850-853.

Woods, D. F. and Bryant, P. J. (1989). Molecular cloning of the *lethal(1)discs large-1* oncogene of *Drosophila*. *Dev. Biol.* **134**, 222-235.

Woods, D. F. and Bryant, P. J. (1991). The *discs-large* tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66**, 451-464.

(Accepted 3 May 1993)