**INTRODUCTION**

The attainment of a complete understanding of axon guidance presupposes that we know, for specific guidance decisions of identified neurons, the identities of the guiding molecule(s) and their receptors as well as the mechanism by which these molecules come to be expressed at appropriate times and places. One approach that has been used to identify many genes involved in a single regulatory hierarchy has been to screen for unlinked mutations that give rise to a common mutant phenotype (Anderson and Nusslein-Volhard, 1984; Lehman et al., 1983; Nusslein-Volhard et al., 1987). Such an approach has been extremely valuable in dissecting the logic underlying guidance decisions in the *C. elegans* nervous system (Hedgecock et al., 1990; McIntire et al., 1992). In that system, mutant phenotypes have been grouped into distinct classes, affecting specifically anteroposterior axon extension or dorsoventral extension. Mutations disrupting dorsoventral processes were further divided into those perturbing just dorsalward growth of axons (eg *unc-5*), just ventralward growth (eg *unc-40*), or growth in both directions (eg *unc-6*). Two of the genes required for dorsoventral axon extension have recently been cloned. *unc-6* encodes a protein related to the vertebrate extracellular matrix molecule, laminin (Ishii et al., 1992). *unc-5* encodes an apparent transmembrane receptor with repeats related to the vertebrate protein, thrombospondin, which is known to interact with laminin (Leung-Hagesteijn et al., 1992).

Interestingly, mutations in these genes also disrupted directed cell migrations in the embryo. As yet, the nuclear regulatory hierarchy that orchestrates dorsoventral axon guidance in the worm has remained obscure.

Recently, a genetic approach to identify decision points in axon guidance in the *Drosophila* embryonic CNS was taken by Seeger et al. (1993). Their analysis revealed four classes of mutant phenotypes, differentially affecting guidance towards and away from the ventral midline of the animal or along the longitudinal tracts of the CNS. One class of mutations described by Seeger et al. (1993) and exemplified by the mutations *longitudinal lacking (lola)* and *longitudinal gone (logo)*, prevents the growth of axons along the longitudinal tracts connecting the segmental ganglia in the CNS. This phenotype had been described previously as also arising from appropriate temperature shifts of temperature-sensitive mutations of the genes *Delta* and *Notch* (Giniger et al., 1993). The regulatory decision revealed by these mutations is a promising candidate for genetic dissection, due to the number of loci already known to affect it and the existence of a number of additional loci, currently being characterized, that give similar mutant phenotypes (E. G., unpublished data). We therefore undertook the cloning of a gene, in retrospect identical to the gene *lola*, which we identified by lethal transposon insertions that gave rise to a reduction of longitudinal CNS fibers.

We show here that *lola* encodes a nuclear protein, putatively...

**SUMMARY**

Mutations in the gene *longitudinals lacking (lola)* lead to defects in the development of axon tracts in the *Drosophila* embryonic central nervous system. We now show that *lola* mutations also cause defects of axon growth and guidance in the peripheral nervous system, and causes a particular cluster of embryonic sense organs (lch5) to be oriented improperly. Axonal aberrations caused by *lola* are similar to those caused by mutations of three other genes, *logo*, *Notch* and *Delta*, raising the possibility that *lola* works in the same genetic pathway as do these other molecules. The *lola* gene encodes at least two nuclear protein products, apparently by differential RNA splicing. The predicted proteins contain an amino-terminal motif similar to that recently described for a family of transcription factors, including the products of the *Drosophila* genes *tramtrack* and the Broad Complex. Like *Ttk* and *BR-C*, one of the two characterized products of the *lola* locus bears sequences similar to the zinc-finger motif, but the other (neuronal) form of the protein has no recognizable DNA-binding motif.

Key words: intersegmental nerve, PNS, Tramtrack, zinc finger, PlacW, axon pathfinding

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**lola encodes a putative transcription factor required for axon growth and guidance in *Drosophila***

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a transcription factor, which is required for axon growth and
guidance in the Drosophila embryo. In addition to the charac-
terized CNS aberrations found in lola embryos, mutations in
this gene also cause defects in pathfinding of the peripheral
intersegmental nerve (ISN) of the embryo. The combination of
CNS and PNS pathfinding defects in lola mutants is reminis-
cent of the axonal aberrations caused by temperature-sensitive
mutations of Delta and Notch. In addition, the lola mutation
causes abdominal chordotonal organs of the PNS to be oriented
improperly. Cloning of the lola locus and determination of the
nucleotide sequences of encoded RNAs predicts synthesis of
at least two nuclear proteins. These are related to a family of
transcription factors which includes Tramtrack (Ttk), the
products of the Broad Complex (BR-C), the GAGA transcrip-
tion factor and the vertebrate protein, KUP, though only one of
the characterized lola products includes an identifiable
DNA-binding motif. The tissue distribution of Lola protein is
determined, and its nuclear localization is established, by
immunocytochemistry of embryos. We suggest that isolation of
genes regulated by lola is apt to identify other molecules
that are key to the proper guidance of the axons whose
extension is dependent on Delta, Notch and lola.

MATERIALS AND METHODS

Genetics
All five P-element-induced alleles of lola (1A4, 2B13, 4D4, 5D2 and
4E4) were identified among the collection of 550 P-element-induced
lethal mutations described by Bier et al. (1989). 5D2 and 2B13
chromosomes were cleaned by recombination with ar dp pr Bl c px sp;
followed by backcrossing to y w iso 2,3Oregon R to remove these
markers, and balancing over CyO. Linkage of the mutant phenotype
to the transposon insertion was verified by excising the P-element
with P-transposase, supplied by crossing-in the chromosome
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markers, and balancing over CyO. Linkage of the mutant phenotype
followed by backcrossing to
mosomes were cleaned by recombination with
lethal mutations described by Bier et al. (1989).

Histocchemistry and microscopy
Chromosomal localization of P-element insertions and of cloned DNA
was determined by in situ hybridization to squashes of larval salivary
glands (Ashburner, 1989), using probes labelled with biotin or digox-
igenin and visualized by peroxidase staining following incubation
with an appropriate secondary reagent. In situ hybridization to whole-
mount embryos was performed by standard methods (Ashburner,
1989), using digoxigenin-labelled DNA or RNA probes made as
suggested by the manufacturer (Boehringer Mannheim) and alkaline
phosphatase visualization. The in situ hybridization protocol for RNA
probes was essentially the same as for DNA probes (Rao et al., 1990),
except hybridization was performed at 55°.

Immunohistochemistry was performed on embryos fixed with 4%
formaldehyde by standard methods (Bier et al., 1989; Bodmer and
Jan, 1987). Antibodies used were as follows: anti-Cut (Blochinger et
al., 1988), mAb 44C11 (anti-Elav)/Bier et al., 1988), anti-HRP (Jan
and Jan, 1982), mAb 22C10 (Zipursky et al., 1984), anti-Prospero
(Vaessen et al., 1991), mAb 6G5D3 (recognizes an anonymous
antigen of the tracheal lumen; (Giniger et al., 1993)) and anti-Lola
(see below). In all cases, detection was with an HRP-conjugated
antibody, using peroxidase cytochemistry. Secondary anti-
bodies were from BioRad (EIA grade) or from Jackson Immunolog-
icals, and were preabsorbed against WT fly embryos prior to use
(1:40-1:100 dilution; 100 µl packed embryos per 1 ml diluted
antibody). For some experiments, lola mutant embryos were identi-
fied by their failure to stain with anti-Lola antibody. Stained samples
were observed under Nomarski optics, using a Nikon Optiphot micro-
scope. Staging of embryos was as described by Campos-Ortega and
Hartenstein (1985).

DNA cloning and analysis
Standard protocols were used for all molecular cloning experiments,
including plasmid rescue (Roberts, 1986), subcloning of rescued
DNA, isolation of genomic and cDNA clones by plaque hybridization,
Southern and northern blotting, DNA sequencing and construction of
bacterial expression vectors (Maniatis et al., 1982) and PCR (Innis et
al., 1990). All cloned DNAs were analyzed by in situ hybridization
to salivary gland chromosomes, to verify that they derived from 47A
9-14 (PlocW-associated sequences obtained by plasmid rescue, as
well as cdNA and genomic clones). Genomic bacteriophage clones
were isolated from a library constructed in the vector λ DASH (Strat-
agenae); cDNA clones were in λgt10 and were isolated from the
Kauer E library (derived from 3-10 hour embryonic mRNA; (Poole et
al., 1985)).

DNA sequence homology analysis was performed using public
email servers: BLAST (NCBI, Bethesda), BLOCKS (FHCRC,
Seattle), BLITZ (EMBL, Heidelberg) and FASTA (DNA Data Bank
of Japan, National Institute of Genetics, Mishima). All other sequence
analysis, including multiple sequence alignment, was performed using
the commercial GCG package, version 7 (Devereux et al., 1984).

The sequences of both characterized lola splice variants have been
deposited in Genbank. The accession number for cDNA 8 (repre-
senting lola 3.8) is U076606; the accession number for cDNA 4 (re-
presenting lola 4.7) is U07607.

anti-Lola antibodies
cDNA 8.13 (derived from lola 3.8) was digested with BcII and
HinDIII to produce a 1.45 kb fragment encoding amino acids 19-467
of the short form of Lola. This was cloned into the bacterial expression
vector pUR289 (Ruthier and Muller-Hill, 1983) to generate a fusion
to the C-terminal end of E. coli β-galactosidase. To produce protein
for use as immunogen or for affinity-purification of antibodies, log
phase cultures of bacterial strain BSJ 72, transformed with the
expression vector (pEG110), were induced with 1 mM IPTG for 4
hours, cells were harvested by centrifugation, and resuspended, 20-
fold concentrated, in 50 mM sodium phosphate pH 7.2, 10 mM β-
mercaptoethanol, 10 mM EDTA. Resuspended cells were lysed by
freezing in liquid nitrogen, thawing and sonicating on ice in the
presence of a cocktail of protease inhibitors (final concentrations:
PMSF, 1 mM; leupeptin, 10 µg/ml; aprotinin, 20 µg/ml; benzamidine, 100 µg/ml; TPCK, 10 µg/ml; prepared as a 100x
cocktail in DMSO). NaCl was then added to a final concentration of
2 M, and after a 10 minute incubation on ice, insoluble protein was
collected by centrifugation at 16.5×103 revs/minute for 20 minutes.
The bulk of the fusion protein was found in this insoluble fraction.
Pellet was resuspended in 25% the lysate volume of lysis buffer plus
8 M urea. One half volume of Laemml sample buffer was added to the
extract, the sample was boiled 4 minutes and aliquots containing
approximately 1 mg total protein were separated by SDS-polyacyr-
lamide gel electrophoresis (6.5% acrylamide). The band of fusion
protein was identified by staining with Coomassie Blue and cut out
of the gel. Acrylamide strips were sent either in this form, or after 1
hour fixation with 4% formaldehyde in 0.1 M sodium phosphate, pH
7.2 and extensive washing with water, to Babco, Berkeley, CA, for
injection into two rabbits.

For affinity purification of antibodies, a preparative gel of fusion
protein was transferred to nitrocellulose, the band of fusion protein
was identified by staining with Ponceau S and cut-out. The
nitrocellulose strip was blocked as for a western blot (1 hour at room
temperature with PBS containing 0.5% Tween 20, 2% BSA and 2% goat
serum). Typically, 2 ml of serum was incubated with a strip bearing
100-500 µg fusion protein (1 hour, room temperature), washed 3-4
times with PBS+Tween, and eluted 30 minutes with 2 ml 0.2 M

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glycine, pH 1.5-2, 0.5% Tween 20. Eluate was immediately neutralized with Tris base. To remove antibodies against contaminating bacterial proteins, including β-galactosidase, affinity-purified antibodies were then counter-selected against a column (BioRad AffiGel 10 and 15, mixed 1:1) bearing total protein extract of BSt 72 E. coli transformed with the parent expression vector, pUR289, and induced with IPTG. Counter-selection column was prepared as per manufacturer's protocol, and contained ~50 mg total protein coupled to 2 ml AffiGel. Final antibody preparation was diluted with an equal volume PBS containing 4% BSA and 4 mM NaN₃, and frozen in aliquots at −80°C. For whole-mount embryo immunocytochemistry, anti-Lola antibodies were used at dilutions between 1:10 and 1:1000; identical staining patterns were observed regardless of concentration. Typically, antibodies were used at 1:100 dilution for analyses of the expression pattern, and at 1:400 dilution to identify mutant embryos in double stainings with other antibodies. Three lines of evidence suggest that affinity-purified anti-Lola antibodies specifically recognized Lola protein: antibodies from both rabbits gave identical patterns; purified antibodies preabsorbed against a nitrocellulose strip bearing the fusion protein did not stain WT embryos, and mutant embryos did not stain with purified antibodies (lola<sup>2B13</sup>, lola<sup>4D4</sup> and lola<sup>3B2</sup>).

### RESULTS

A collection of 550 homozygous lethal insertions of the synthetic transposon PlacW (Bier et al., 1989) was screened by visualizing the nervous system with antibody against HRP (Jan and Jan, 1982) to identify mutations that disrupted, in concert, pathfinding of the intersegmental nerve (ISN) in the lateral part of the fly embryo, and formation of the connective fibers between the segmental ganglia in the embryonic CNS. This combination of pathfinding defects had previously been observed in studies of Delta and Notch mutants (Giniger et al., 1993). Among the mutants that we identified that fit these criteria were five independent P-element insertions that were mapped to chromosomal position 47A 9-14 by hybridization of transposon sequences to polytene chromosomes in situ. These were l(2)1A4, l(2)2B13, l(2)5D2, l(2)4D4 and l(2)4E4. The mutations were crossed inter se and found not to complement, as assayed by lethality (no non-balancer progeny obtained greater than 150 offspring examined for each cross). Two of the mutant alleles, 5D2 and 2B13, were selected for further characterization. Transposase-induced excision of the P-element reverted both the lethality and the mutant neuronal phenotypes from each of these two lines (data not shown). The chromosome arms were exchanged by crossing on genetic markers along the length of the chromosome and crossing them back off. These ‘cleaned’ chromosomes were then used for the detailed phenotypic analysis of the mutations.

We noted that the chromosomal location of the mutations that we had isolated overlapped that described for the gene, lola. Moreover, the CNS phenotype observed in mutant embryos (see below) was strikingly similar to the published lola phenotype. Finally, l(2)5D2 failed to complement bona fide lola alleles (G. Tear, M. Seeger and C. S. Goodman, personal communication). We therefore conclude that the locus defined by the five P-element alleles described above is lola.

### Mutant phenotype

A common set of neuronal aberrations was observed in all alleles of lola, and these are displayed in Figs 1 and 2. Most readily apparent was disruption of the axon tracts in the CNS (Fig. 1). There appear to be two parts to this phenotype. The connective fibers are commonly missing between the segmental ganglia, and the two commissures in each ganglion are often partly or fully fused (Fig. 1A; compare WT, Fig. 1B). The former of these phenotypes has been seen previously in embryos bearing temperature-sensitive mutations of the neurogenic genes Delta or Notch (Giniger et al., 1993). The latter, fused-commissure phenotype can arise from absence or improper development of midline glial cells (Klambt et al., 1991). In lola, the fusion is exacerbated by CNS condensation at late embryonic stages (data not shown). The CNS effects of lola mutations can also be observed with reagents specific for subsets of CNS axons. Staining embryos with mAb 22C10 (Zipursky et al., 1984); provided by Dr S. Benzer) reveals that the MP fascicle, an early element of the longitudinal fibers that connect the segmental ganglia (Thomas et al., 1984), fails to form properly in some segments of mutant embryos (Fig. 1C; WT Fig. 1D) and, at later stages, there is a general reduction of 22C10-positive axons throughout the affected sections of the connective (Fig. 1E; WT Fig. 1F). Furthermore, in some segments, the VUM neurons are born and differentiate, but fail to form a well-defined posterior root to the anterior peripheral fascicle (Fig. 1E). It appears that they either fail to extend their axons fully or else exit the CNS via the posterior fascicle. For this experiment, and for most of the phenotypic characterization, mutant embryos were identified by their failure to stain with anti-Lola antibodies (see below). The mutant CNS phenotypes that we observe thus essentially recapitulate the effects of published lola alleles (Seeger et al., 1993).

We went on to investigate the effects of lola mutations on the development of the peripheral nervous system. In the PNS of mutant embryos (Fig. 2), we found various aberrations of the intersegmental nerve (compare WT, Fig. 2C), either failure of the nerve to grow (Fig. 2A), ‘wandering’ of axons into adjacent segments (Fig. 2B) or defasciculation of ISN sensory axons (not shown). This phenotype was substantially confined to the lateral region of the embryo, that is, that region in which axons of the ISN normally grow along the transverse branch of the trachea (Hartenstein, 1988). Again, similar mutant phenotypes are produced by appropriate temperature-shifts of N<sup>ts</sup> and D<sup>fl</sup> mutants (Giniger et al., 1993). In a typical experiment, ISN defects were observed in nearly one-quarter of the progeny from lola<sup>3B2</sup>/+ parents stained with mAb 22C10 (25/115 appropriately oriented embryos between late stage 15 and early stage 17), suggesting nearly complete penetrance of the phenotype as regards sensory axons of the ISN. The expressivity of the ISN phenotype was incomplete in this sample, however: 2.3±1 mutant abdominal segments were observed per half-embryo (mean ± standard deviation; mode = 2 mutant segments). We also observed that, in mutant animals, the lateral chordotonal (ch) organs of the abdomen were sometimes displaced dorsally from their wild-type position (penetration varied from 25 to 50 % in different experiments). The expressivity of this phenotype was such that it was seldom seen in more than one or two segments of a single mutant animal. Dorsally displaced chordotonal neurons still formed a tight linear array, as in wild-type embryos, but in a subset of mutant embryos (10-25%) the orientation of displaced arrays was aberrant, with the dendrites of these neurons directed posteriorly (Fig. 2E), in contrast to the extremely consistent
dorsally-directed orientation of wild type abdominal chordotonal organs (Fig. 2F). All of the mutant PNS phenotypes were observed in all abdominal segments at similar frequencies; we did not observe any obvious segment-specific differences in sensitivity to the effects of the mutation.

Failure of the ISN to develop appropriately may reflect indirect effects such as an absence of the cellular substratum for the nerve or alteration in neuronal identity, or it may be directly due to defects in the guidance system. The experiments described below are consistent with the latter idea. We and
**Fig. 2.** PNS phenotypes of *lola* mutants. Lateral views of embryos derived from *lola*<sup>D2</sup>/*+* parents, stained with the indicated antibodies and visualized by HRP cytochemistry. (A-C) Stage 15/16 embryos stained with α-HRP to label all neurons. Arrows in A and B highlight axonal aberrations in mutant embryos, either failure of the ISN to extend across the lateral part of the embryo or crossing of sensory axons between two segments, respectively. Compare with the ISN of a non-mutant embryo, highlighted by arrow, in C. Dorsal, lateral and ventral clusters of peripheral neurons are marked d, l and v, respectively; c denotes the CNS. (D) Stage 16 *lola* mutant embryo, stained with mAb 68G5D3 to label the tracheal lumen. The pattern of tracheal development is indistinguishable from that of wild type. The bracket denotes the portion of the transverse tracheal branch that guides the growth of ISN axons through the lateral part of the embryo. (E,F) Lateral chordotonal neurons visualized by staining with α-HRP. In wild-type embryo (F), note the consistent dorsalward orientation of chordotonal dendrites (bracket). Similarly, most segments of mutant embryos have dorsally oriented chordotonal organs, but a subset of segments have displaced chordotonal organs with their dendrites pointing posteriorly (middle segment of E, highlighted with bracket). (G,H) Stage 16 embryos, mutant and wild type, respectively, stained with α-Prospero to label the inner support (sheath) cells of external and internal sensory organs. The number of inner support cells per hemisegment is the same in mutant as in wild type, suggesting that *lola* does not alter the lineages of cells within the sensory organ. Note that the positions of the chordotonal sheath cells reflect the aberrant location and orientation of a displaced chordotonal cluster (bracket). As above, dorsal, lateral and ventral sensory organ clusters are denoted d, l and v, respectively, while CNS is denoted c. Anterior is to the left and dorsal at the top of all panels. For most experiments, mutant embryos were identified by their failure to stain with α-Lola antibodies; in some cases, the distinctive CNS phenotype was used to identify the mutants.
others have shown previously that disruption of tracheal development can give rise to ISN aberrations similar to those observed in lola (Giniger et al., 1993; Hartenstein and Hartenstein, 1993), so we examined tracheal integrity in the mutant embryos. We find no obvious morphological abnormalities in the tracheal tree in these embryos (Fig. 2D). We also examined PNS differentiation to begin to address the issue of possible effects of the mutation on neuronal identity. The total number of PNS neurons is normal in the mutants, as is their distribution into various neuronal subtypes (es, ch and md), as assayed both by overall morphology (Fig. 2A,B and E), and by the presence of distinctive α-HRP immunoreactive dot and scolopale structures in es and ch neurons, respectively (Fig. 2A,B and E and data not shown; for description of wild-type PNS, see Bodmer and Jan, 1987; Ghysen et al., 1986; Hartenstein, 1988). Other than the lateral chordotonal neurons (see above) the positions and orientations of peripheral neurons in mutant embryos are not consistently different from that seen in WT. Moreover, the differentiation of sense organs appears normal as assayed by two criteria, expression of Prospero protein (Matsuzaki et al., 1992; Vaessin et al., 1991) in the nuclei of inner support (sheath) cells of es and ch organs (Fig. 2G; WT, Fig. 2H), and expression of Cut protein (Blochlinger et al., 1988, 1990) in all cells of es organs, and also in a subset of md neurons, but not in any ch organ cells (data not shown). Similarly, there is no evidence that the lola mutation leads to failure of substratium differentiation or alterations of neuronal identity in the CNS of mutant embryos (Seeger et al., 1993; EG, data not shown).

We suspect the set of phenotypes described above to be the zygotic null phenotype of lola for two reasons. First, all five lola alleles give essentially the same phenotype and, second, Lola protein was not detectable in embryos homozygous for lola5D2, lola2B13 or lola4D4 (see below). We note that Seeger et al. (1993) also report incomplete expressivity for the mutant phenotypes of both EMS (ethyl methane sulfonate)- and P-induced lola alleles. This incomplete expressivity may account, in part, for the apparent discrepancy between our finding of reproducible ISN defects in lola embryos versus the finding by Seeger et al. (1993) that aCC usually succeeds in reaching its dorsal muscle targets in mutant embryos. In this connection, it may also be relevant that ISN sensory axons appeared to be more sensitive to the effects of lola mutations than were motor axons (data not shown).

Cloning of the lola locus

Genomic DNA from the lola locus was isolated by plasmid rescue of PlacW and adjacent sequences from all five lola alleles (Bier et al., 1989; Roberts, 1986). Rescued DNA from the line lola5D2 was used to screen a genomic library (λDASH) and a restriction map was constructed of the genomic region surrounding the five P-element insertions (Fig. 3). These insertions were found to lie in two clusters, separated by approximately 1 kb. The entire cloned region was screened for transcripts by hybridization of cloned fragments of genomic DNA to blots of poly(A)+ RNA of various embryonic stages. Two transcription units were identified, using probes indicated in Fig. 3 as α, β, γ and δ. One transcription unit comprised a family of three related mRNAs (3.8, 4.7 and 4.9 kb) and was closely associated with the lethal P-element insertion sites. In Fig. 3, β and γ indicate sequences encoding two different 5′ ends that were found to be associated with a common sequence encoded by region δ. The temporal pattern of expression of these RNAs is shown in Fig. 4. All three RNA species are present at egg deposition, though the 4.9 kb RNA rapidly disappears. The 3.8 kb RNA continues to be present throughout embryogenesis, whereas the 4.7 kb transcript decays at about 9 hours, the time at which the 4.9 kb RNA reappears. Expression of the 4.9 kb RNA then persists at low levels throughout the remainder of embryonic life. In addition to these major transcripts, several minor RNA species of uncertain significance were observed (see Fig. 4 and legend). The second transcription unit (encoded by genomic region α in Fig. 3) was approximately 4 kb away from the lethal insertions and was associated with a cluster of independent, homozygous-viable P-element insertions that complemented lola mutations (A4 2nd 51, B1 2nd 45 and B4 2nd 29). This distal transcription unit generated a 4 kb RNA which had no apparent protein-coding potential when the DNA sequence was determined (E. Bier, E. G., L. Y. J. and Y. N.J., unpublished data), and this transcript was not analyzed further. (Hereafter, we will refer to the 3.8, 4.7 and 4.9 kb transcripts as lola 3.8, lola 4.7 and lola 4.9, respectively.)

We used the cloned genomic sequences to obtain by plaque hybridization cDNAs representing the 3.8 and 4.7 kb lola transcripts (from the Kauvar E library representing RNA from 3-10 hour embryos (Poole et al., 1985)). The sequences of these

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**Fig. 3.** Genomic region surrounding lola P-element insertions. The approximate positions of mapped P-insertions in the 47A 9-14 region are indicated with inverted triangles. Insertions marked l(2) are lola’s, viable insertions map to the EcoRI-HindIII restriction fragment denoted by the solid bar. mRNAs species map to the region as follows: α indicates the EcoRI restriction fragment that hybridizes to a non-protein coding message upstream of lola; β indicates the genomic DNA that encodes the 5′ end of the cDNA representing the 3.8 kb lola message (cDNA lola 8); γ indicates the genomic DNA that encodes the 5′ end of the cDNA representing the 4.7 kb lola RNA (cDNA lola 4); δ indicates the genomic DNA coding for the exon shared by the various lola RNAs. Sequences encoding the 3′ exon(s) of the longer lola RNAs are more than 19 kb downstream of the promoter (not shown). The position of the insertion in l(2)5D2 was arbitrarily selected as the zero position of the map and distances (in kbp) are indicated by the scale at the bottom of the figure. R, EcoRI; H, HindIII.
cDNAs were determined (see below). cDNAs were placed relative to the genomic map by a combination of hybridization of cDNA fragments to cloned genomic sequences, preparation of isoform-specific oligonucleotides and their use to probe Southern blots of the cloned DNA, and determination of partial genomic sequences, as required (data not shown). We have no data to suggest a special role for any of these minor RNAs, as none of the characterized cDNAs preferentially labelled any of the minor transcripts. They could conceivably represent products of aberrant or incomplete RNA processing.

**lola encodes proteins related to transcription factors**

The DNA sequence of *lola* is presented in Fig. 5A,B and its relationship to known protein sequences is summarized in Fig. 5C. Both protein forms have a large protein-coding region in common. The sequence of the 3.8 kb RNA predicts a protein of 467 residues (49.5×10^3 M_r). The sequence of the 4.7 kb RNA diverges near the predicted C terminus of the ORF present in the 3.8 kb RNA, and is predicted to encode a substantially larger protein (894 residues; 96.5×10^3 M_r). The two different 5′ ends converge to a common sequence 5′ to the AUG. The presence of nonsense codons in all three reading frames 5′ to the first AUG and 3′ to the termination codons of both messages suggests that we have isolated cDNAs encoding the entire proteins.

The predicted isoforms of *lola* protein have, at their N terminus, a 115 amino acid motif found in a recently described family of transcription factors (Koonin et al., 1992), including three *Drosophila* loci; *tramtrack* (Harrison and Travers, 1990), the protein products of the Broad Complex (DiBello et al., 1991) and the GAGA transcription factor (Soeller et al., 1993), as well as the human transcription factor KUP (Chardin et al., 1991). The known members of this family typically show ~40-50% sequence identity in pairwise comparisons. In this domain, *lola* is 62% identical and 80% similar to *tramtrack*, 51% identical with the BR-C proteins, 41% with GAGA factor and 20% with KUP. The four known members of the *tramtrack* family are all DNA-binding proteins bearing two zinc fingers of the C2H2 type (Miller et al., 1985). In contrast, while the long isoform of *lola* is also predicted to include two sequences related to C2H2-class zinc fingers (amino acids 796-817 and 826-847, respectively), the final histidine of the first predicted finger is replaced by a third cysteine. While such a hist→cys substitution is somewhat uncommon, it is not unprecedented. Similar sequences have been reported, for example, in *Caenorhabditis elegans* SDC1 (Nonet and Meyer, 1991), in *Drosophila* Snail (Boulay et al., 1987), Escargot (Whiteley et al., 1992) and Serendipity β (Payre et al., 1990), and in *Xenopus* Snail (Sargent and Bennett, 1990), though the functional significance of the substitution has not been examined. We verified the structure and sequence of the zinc finger region by sequencing a PCR product corresponding to this region, amplified from genomic DNA. Surprisingly, the short form of *lola* (that which is expressed throughout embryogenesis) has no sequence similar to the zinc finger motif. We have been unable to identify any DNA-binding motif in the sequence of the smallest *lola* isoform. Proteins bearing a divergent *tramtrack* domain but no apparent DNA-binding motif have been identified in a number of mammalian viruses (Koonin et al., 1992), but their function is unknown. To ensure that the absence of a predicted DNA-binding unit in *lola* 3.8 was not due to a cloning artifact, we verified the structure of this part of the message by PCR amplification of a region of the transcript extending from within the sequence common to both *lola* cDNAs to beyond the stop codon of the short protein, using as template first-strand cDNA made from embryonic RNA (data not shown). Consistent with a role as a transcription factor, the region of *lola* common to both transcripts includes a short, basic sequence similar to known nuclear-targeting sequences (Chelsky et al., 1989).

### Temporal and spatial distribution of *lola* gene products

The products of the *lola* gene were visualized both by in situ hybridization of common or isoform-specific probes to whole-mount embryos, and by raising an antibody against the common region of *lola* protein and using it to stain whole-mount embryos. The distribution of *lola* RNA is presented in Fig. 6. Both short (3.8 kb; Fig. 6A) and long (4.7 kb and 4.9 kb; Fig. 6F) forms of the *lola* cDNA are present uniformly at high levels in preblastoderm embryos and through germ band extension (stage 10). They appear to be supplied maternally, as they are present in zygotically mutant embryos. After germ band extension, a probe recognizing the 4.7 kb isoform preferentially labels mesodermal (Fig. 6G) and mesectodermal cells (Fig. 6H), while a common probe additionally labels ectoderm (Fig. 6B). The invading posterior midgut ceases to contain *lola* RNA (Fig. 6B). While the 4.7 kb message remains largely mesodermal after this stage, label associated with a common probe progressively concentrates in neural tissue between stages 11 and 13 (Fig. 6C,D), presumably
Fig. 5. lola sequence and its relation to known proteins. (A) Complete sequence of cDNA lola 8, representing the 3.8 kb mRNA, together with the predicted protein sequence. (B) Complete sequence of cDNA lola 4, representing the 4.7 kb mRNA, together with the predicted protein sequence. The exon shared by lola 4 and lola 8 extends from nt 180 to nt 1762 of the sequences presented. Asterisks in A and B represent translation termination codons. (C) Sequence motifs predicted for Lola protein. The top of the figure places sequence motifs on the map of the two lola cDNAs; the bottom of the figure displays the sequences of these protein motifs and aligns them with similar published motifs. BLAST score for highest match to Ttk domain is 390 (p(N)=2.5 e-48) for a comparison to Tramtrack. BLOCKS scores for zinc finger region are 1274 and 1332 for the first and second fingers, respectively; this represents the 99.01 percentile of shuffled queries. Nuclear localization signals do not fit a strict sequence consensus; the indicated peptide is the region of Lola most similar to known NLS elements. In alignments, Φ represents hydrophobic residues. Alignment of the Ttk domain was generated by the program PILEUP of the OCG sequence analysis package.
Fig. 6. Spatial distribution of *lola* RNAs during embryonic development. Embryos were hybridized with digoxigenin-labelled probes and visualized with alkaline phosphatase cytochemistry. A–E show the expression patterns observed with a probe that recognizes all *lola* RNA species; F–J show the pattern observed with a probe specific for *lola* 4.7 and *lola* 4.9. For detailed descriptions of expression pattern, see text. Staging and labelling of embryos is as follows: (A and lower right embryo in F) stage 5; (upper left embryo in F) stage 8, dorsal view; (B,G,H) stage 10/11; PMG, invaginating posterior midgut; E, ectoderm; M, mesoderm; ME, mesectodermal cells, H is a dorsal view; (C) stage 13; (D) stage 14; d and l, the dorsal and lateral clusters of sensory neurons, respectively; (I) stage 15; hb, histoblasts; id, imaginal disc precursors; (J) stage 16/17; b, brain lobe; (E) stage 17; c, CNS; ventral view. If not otherwise specified, embryos are oriented with anterior to the left and dorsal at the top.
reflecting primarily the distribution of the short RNA. Thus, at stage 12 and 13 expression is most evident in cells of the PNS and CNS. RNA ceases to be detectable in the CNS by stage 14, though it remains homogeneous in the CNS. At stage 17, the most evident RNA expression revealed by a common probe resolves into a discrete subset of CNS cells (Fig. 6E). However, concomitant with the reappearance of the 4.9 kb form in northern blots, RNA detectable with the long probe appears transiently in clusters of peripheral cells that are probably precursors of imaginal tissues (imaginal discs and abdominal and tracheal histoblasts, Fig. 6I), and in a small sector of the brain, possibly the optic lobe anlagen (Fig. 6J).

**Localization of Lola protein**

The distribution of Lola protein was determined using an antibody raised against the common portion of the protein. The localization of the protein is consistent with the RNA distribution, but reveals aspects of the expression pattern not evident from the pattern of RNA accumulation. During gastrulation, Lola protein appears to turn over at mitosis (Fig. 7B; Foe, 1989). Moreover, while the protein, like the RNA, is present at highest concentration in neurons at stage 13 (Fig. 7D), the presence of substantial protein product in epithelial cells and in sensory organ support cells is more readily apparent than might be predicted from the RNA analysis. Finally, while mesodermal expression of the RNA is no longer detectable by stage 15, the protein is rather abundant in mesodermal nuclei as late as stage 17. This can be seen in somatic muscles (Fig. 7G) and in the mesodermally-derived peritracheal cells (Hartenstein and Jan, 1992) that lie alongside the transverse branch of the trachea (Fig. 7H). These peritracheal cells are the tracheal cell type on which the ISN actually extends ((Giniger et al., 1993); EG unpublished observations).

Some general properties of the protein may be inferred from these expression patterns. In many cell types the protein persists well after the RNA decays. Also, the protein is nuclear, as predicted based on the sequence analysis. Finally, staining of homozygous mutant embryos with the affinity-purified antibody indicates, first, that the maternally-provided Lola product disappears rapidly at about stage 10 (germ-band extended), second that the *lola* locus is required for expression of Lola-immunoreactivity, thereby suggesting that the antiserum is specific for Lola protein, and third, that the 5D2, 2B13, and 4DM alleles are strong or null, as they are devoid of detectable Lola immunoreactive material (Fig. 7F).

**DISCUSSION**

We have shown that the *lola* gene, which is required in the *Drosophila* embryo for axon growth and guidance, encodes a protein that is related to the Tramtrack family of transcription factors. Mutations of *lola* lead to axonal defects in those axon tracts that have previously been shown to be sensitive to mutations in *Notch, Delta* and *logo*, raising the possibility that *lola* acts somewhere in the same pathway as do these other genes. One Lola isoform has two zinc fingers, as do other members of the Tramtrack gene family, while the other (neuronal) Lola isoform is unusual in that it lacks any recognizable DNA-binding motif.

Four lines of evidence argue that the gene we have cloned is responsible for *lola* function. First, lethal P-element insertions that map near *lola* and give rise to a *lola*-like mutant phenotype fail to complement an EMS-induced *lola* allele, and excision of such transposons co-reverts both the lethality and the mutant neuronal phenotypes. Second, the lethal insertions cluster around the two 5′ ends of the differentially spliced *lola* messages. Third, we have detected only one other transcript in the region, and this RNA is associated with a different cluster of viable P-element insertions that do not interact genetically with *lola* alleles; moreover, that RNA does not appear to encode a protein. Fourth, embryos homozygous for any of three *lola* alleles that we tested lack Lola protein. Attempts to rescue the *lola* mutant phenotype by heat-shock expression of the short cDNA have been unsuccessful, perhaps reflecting differing requirements for the various protein isoforms (data not shown). The large size of the *lola* locus (>35 kb) has so far prevented us from rescuing the mutation by transformation with a complete genomic clone.

In order to interpret the *lola* phenotype, we must know in which cells the gene is required. Fusion of commissures like that seen in *lola* mutants can be caused by mutations that disrupt development of the midline glia (Klambt et al., 1991). The observation that *lola* 4.7 is expressed in mesectodermal cells that give rise to midline glia makes it attractive to speculate that the fusion of commissures in *lola* mutants is also due to defects in these cells, though this has not been tested directly. For the other axonal aberrations in *lola*, the requirement for the gene could in principle be either in the affected neurons or in their substrata, as expression of *lola* encompasses both of these tissues. It is striking, however, that the shortest form of Lola is found preferentially in neurons, while the substratum (at least for the ISN) primarily expresses a longer Lola isoform. Perhaps the various Lola proteins differentially regulate guiding molecules in the substratum and responding molecules in the axon, respectively. Resolution of this issue will require either mosaic analysis or tissue-specific expression of the various *lola* isoforms.

Mutations of *lola* presumably cause their effects by disrupting the expression of other genes. Ttk, the original member of this family, is believed to be a repressor of transcription, specifically a repressor of the transcription of the segmentation genes *fz* (Brown et al., 1991), *eve, h, odd, run* (Brown and Wu, 1993) and *en* (Xiong and Montell, 1993). It is thus conceivable that the Ttk domain is a transcription repressing region. Other models could equally well be proposed: GAGA transcription factor is an activator of transcription (Biggin and Tjian, 1988) and, genetically, the BR-C both activates and represses different aspects of the ec dysone response (Karim et al., 1993). The observation that other Ttk family proteins are direct regulators of transcription makes it particularly surprising that one form of Lola, the major neuronal form, has no recognizable DNA-binding motif. It is possible that the product of *lola* 3.8 acts as a negative regulatory element, titrating some other factor, though heat-shock expression of just this isoform is not obviously deleterious to flies (data not shown). Alternatively, the short protein may associate with a separate DNA-binding unit, thereby providing it with an effector domain (Gerster and Roeder, 1988; Keleher et al., 1992; Kristie and Sharp, 1990; Stern and Herr, 1991). In contrast with the nuclear Ttk-like proteins such as Lola, it has recently been shown that
Fig. 7. Distribution of Lola protein through embryogenesis. Embryos were stained with α-Lola antibodies and visualized with HRP cytochemistry. Affinity-purified antiserum from two different rabbits gave identical results. Embryos homozygous for lola532 did not stain with either antiserum (F). For detailed description of expression pattern, see text. Staging and labelling of panels is as follows: (A) stage 5; (B) stage 7; arrow indicates dividing cells; (C) stage 11; (D) stage 13; d, l and v denote, respectively, the dorsal, lateral and ventral clusters of developing peripheral sense organs; (E) stage 14, ventral view; c indicates CNS; (F) stage 14; (G) stage 17; lower brackets enclose ventral longitudinal muscle group, upper brackets enclose lateral transverse muscles; (H) stage 17; T indicates epithelial cells of the transverse tracheal branch, pt denotes mesodermally derived peritracheal cells. Unless otherwise specified, embryos are oriented with anterior to the left and dorsal at the top.
an actin-associated cytoplasmic protein, Kelch, includes a Ttk domain (Xue and Cooley, 1993). Perhaps the Ttk homology provides a protein-protein interaction surface and is not itself a transcriptional effector domain.

There are two basic ways in which a nuclear regulatory protein can affect axon guidance. The protein may be required to specify the identity of the neuron or its substratum, or it may be required more directly for expression of molecules involved in cell recognition and process extension. Examples of both of these have previously been described. In the first category, mutation of the homeobox genes ftz or eve transform the identity of motoneuron RP2 leading to incorrect pathway choices (Doe et al., 1988a,b), while mutation of the bHLH gene sim (Nambu et al., 1991) disrupts the differentiation of the midline glia (Crews et al., 1988) confusing those axons that normally cross the midline (Klambt et al., 1991). In the second category, it has been argued that prospero mutations block expression of the pathway of axonogenesis without affecting the identities of most neurons (Vaessin et al., 1991) (though see also Doe et al., 1991).

What is the place of lola in the genetic circuit that controls axon pathfinding? By the assays described above, it appears that the substrata for the disrupted nerves are present in lola mutants, and neither we nor Seeger et al. (1993) have found evidence for alterations in the identities or overall differentiation of neurons contributing to these nerves. (It should be noted, however, that these analyses are not exhaustive: there could be additional, as yet unidentified cells that serve a substratum function and require lola, or lola could cause subtle modifications of neuronal identity that are not revealed by the assays employed by us and by Seeger et al., 1993.) In principle, Lola could be a transcription factor required for expression of logo, Delta or Notch, since these molecules are necessary for the extension of those axons that also require lola. While not enough is known about logo to make any prediction regarding the regulation of logo expression, transcriptional control of Delta or Notch by lola seems unlikely, since lola mutants do not display any of the multitude of other phenotypes associated with mutations of Delta or Notch (Corbin et al., 1991; Hartenstein and Posakony, 1990); Figs 1 and 2 and data not shown). It is thus conceivable that lola controls the expression of effector molecules that transduce the signals by which Delta, Notch and logo regulate axon extension. Alternatively, if there are other cell-surface molecules that collaborate with Delta and Notch proteins in specifying the axon-substratum interaction, their expression could also be affected by lesions in lola. Isolation of genes that are regulated by lola may identify other elements of the control system that lays down the nerve scaffold in the fly embryo.

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