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Ingrowth by photoreceptor axons induces transcription of a retrotransposon in the developing *Drosophila* brain

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SUMMARY

The development of the lamina, the first optic ganglion of the fly visual system, depends on inductive cues from the innervating photoreceptor axons. *lacZ* expression from a P-element insertion, A72, occurs in the anlage of the lamina coincident with axon ingrowth from the eye imaginal disc. In eyeless mutants lacking photoreceptor axons, *lacZ* expression did not occur.

The P-element was found to have inserted within the 3′ long terminal repeat (LTR) of a '*17.6*' type retrotransposon. The expression pattern of *17.6* transcripts in the brain in wild-type and eyeless mutants paralleled the expression of the *lacZ* reporter. Analysis of *17.6* *cis*-regulatory sequences indicates that the lamina-specific expression is due to the combined action of an enhancer element in the LTR and a repressor element within the internal body of the retrotransposon. The regulation of the *17.6* retrotransposon provides a model for the study of innervation-dependent gene expression in postsynaptic cells during neurogenesis.

Key words: visual system development, lamina, innervation, long terminal repeat (LTR), *Drosophila*

INTRODUCTION

During development of the visual systems of all organisms, precise synaptic connections between the eye and the brain are established. Proper establishment of these connections depends on coordinate regulation of the development of the retinal photoreceptor cells and their postsynaptic targets in the central nervous system. As a rule, differentiation of the target cells depends upon innervation. For example, in the frog (Kollros, 1953) and chick (Bondy et al., 1978), surgical elimination of the retina prior to innervation results in abnormal development of the optic tectum. Laser ablation of developing ommatidia in *Daphnia* (Macagno, 1979) results in the defective development of the underlying optic ganglia. These studies suggest that the cellular interactions between innervating photoreceptor axons and their postsynaptic targets are fundamental to brain development in both vertebrates and invertebrates.

In *Drosophila*, during the third larval instar, axons originating from the clusters of photoreceptor cells forming in the eye imaginal disc innervate the brain and project into the anlage of the first optic ganglion, the lamina. In each brain hemisphere, the lamina develops as a crescent array of cells close to the connection of the optic stalk (Meinertzhagen, 1973; Trujillo-Cenoz and Melamed, 1973). The region is demarcated by two grooves on the surface of the brain; an arc of proliferating cells, termed lamina precursor cells (LPCs), occupies the anterior margin (Selleck and Steller, 1991).

Development of the lamina in *Drosophila* depends on innervation of the brain by the retinal axons. In mutants lacking eyes, adults have no lamina and exhibit a reduction in size of the deeper ganglia (Power, 1943; Meinertzhagen, 1973; Fischbach, 1983). Analysis of adult flies mosaic for such mutations demonstrates that the requirement for normal gene function is in the retina and not in the optic lobes (Meyerowitz and Kankel, 1978; Fischbach and Technau, 1984). Progressive innervation of the third instar larval brain by the photoreceptor axons is associated with a wave of mitosis in a crescent-shaped cluster of cells thought to be lamina precursors (Hofbauer and Campos-Ortega, 1990; Selleck and Steller, 1991) In larval brains of eyeless mutants, this mitotic wave is absent, suggesting that innervation plays an inductive role, triggering the birth of these cells. In *Daphnia*, physical contacts resembling gap junctions, between the incoming axon fibers and the lamina precursor cells, have been demonstrated by electron microscopy (Lopresti et al., 1973, 1974). While direct contact has not yet been demonstrated in *Drosophila*, light microscopic studies suggest that a similar interaction may occur between incoming photoreceptor cell axons and lamina precursors in the G_1 stage of the cell cycle (Selleck et al., 1992)

We describe the characterization of the A72 'enhancer trap' P-element insertion strain in which *lacZ* expression in the larval brain coincides with photoreceptor axon ingrowth. Such expression depends on innervation; it is absent in eyeless mutants. Analysis of the DNA flanking the insertion site identified a 7.5 kb region of repetitive DNA that contains a copy of a '*17.6*' type retrotransposon (Kugimiya et al., 1983). In situ hybridization using probes containing *17.6* sequences detected transcripts showing the same pattern and innervation-

dependent expression as *lacZ*. Expression of the *17.6* element at the anterior margin of the lamina anlage suggests that transcription of this retrotransposon may be an early step in lamina cell differentiation. Using *lacZ* fusions and P-element transformation, we identified the *cis*-regulatory sequences within the *17.6* element that are required for the lamina-specific expression. These DNA sequences may be binding sites for transcription factors that initiate a differentiation program for the lamina cells in response to innervation.

MATERIALS AND METHODS

Drosophila cultures

All fly strains were grown on standard fly food at room temperature. Enhancer trap lines were generated using the A and B P[*lacZ, w*$^+$] insertions on the X chromosome (Bier et al., 1989). The P-elements were mobilized using the delta 2-3 chromosome (Robertson et al., 1988) to generate autosomal insertions. These were identified as w^+ males amongst the progeny from the cross *yw*, P[*lacZ, w*$^+$] ; *Sb ry*^{506} P[*delta 2-3, ry*$^+$]/+ males to *yw*; *TM3, Sb* / *D* females. To determine which chromosome carried the insertion and to establish lines, single w^+ male flies were outcrossed to females carrying dominantly marked third chromosomes. Each insertion chromosome was made homozygous by sib mating and homozygous flies were scored for visible phenotypes. Lethal insertions were identified using the dosage of the *white* gene (second chromosome) or the dominant markers on the third chromosome balancer. Lethal chromosomes were maintained over the balancer chromosomes *CyO* or *TM3, Sb* (for details see Lindsley and Zimm, 1992). The A72 insertion-bearing chromosome was homozygous female sterile. However, excision of the P[*lacZ, w*$^+$] element was never associated with reversion of the female sterility, indicating that the sterility was due to a separate mutation.

Immunochemistry

Brains were dissected from third instar larvae of the A72 strain and fixed in PLP (McLean and Nakane, 1974) for 15 minutes, followed by two 10 minute washes in BSS (Ashburner, 1989). The fixed brains were blocked with BSN (BSS +3% normal goat serum) plus 0.4% NP-40 for 30 minutes and incubated with monoclonal antibodies for 3 hours at room temperature or overnight at 4°C. Unbound antibody was removed by two 10 minute washes in BSS. Brains were incubated in horseradish peroxidase-linked (BioRad) or alkaline phosphatase-linked anti-mouse secondary antibody for 2 hours at room temperature. After two 10 minute washes in BSS, followed by one 10 minute wash in 1× PBS, brains were fixed for 15 minutes in 1% glutaraldehyde in 1× PBS (horseradish peroxidase only). After two 10 minute washes in PBS, brains were incubated an additional 10 minutes in 1× PBS, 0.5 mg/ml diaminobenzidene (DAB) and 0.04 % NiCl. Peroxidase activity was detected as a brown/black precipitation product by the addition of H_2O_2 to 0.0015%. Double staining with two mouse primary antibodies was done sequentially. Tissue was incubated with the first antibody and detected by alkaline phosphatase-linked secondary antibody and color reaction. After extensive washing in PBS, the sample was incubated with the second primary and an HRP-linked secondary. Anti-β-gal antibody (Promega) was diluted 1:250 in BSN. Monoclonals 24B10 and 22C10 hybridoma supernatants were diluted 1:1 in BSN. *lacZ* expression in dissected larval brains was assayed by X-gal staining following Bellen et al. (1989).

Molecular cloning

The A72 line contained a single P-element insertion as shown by in situ hybridization to polytene chromosomes and by genomic Southern analysis. The insertion site was mapped to the 77B region of the left arm of the third chromosome. Plasmid rescue of DNA flanking the insertion site was done with restriction enzymes *Xba*I and *Sac*II,

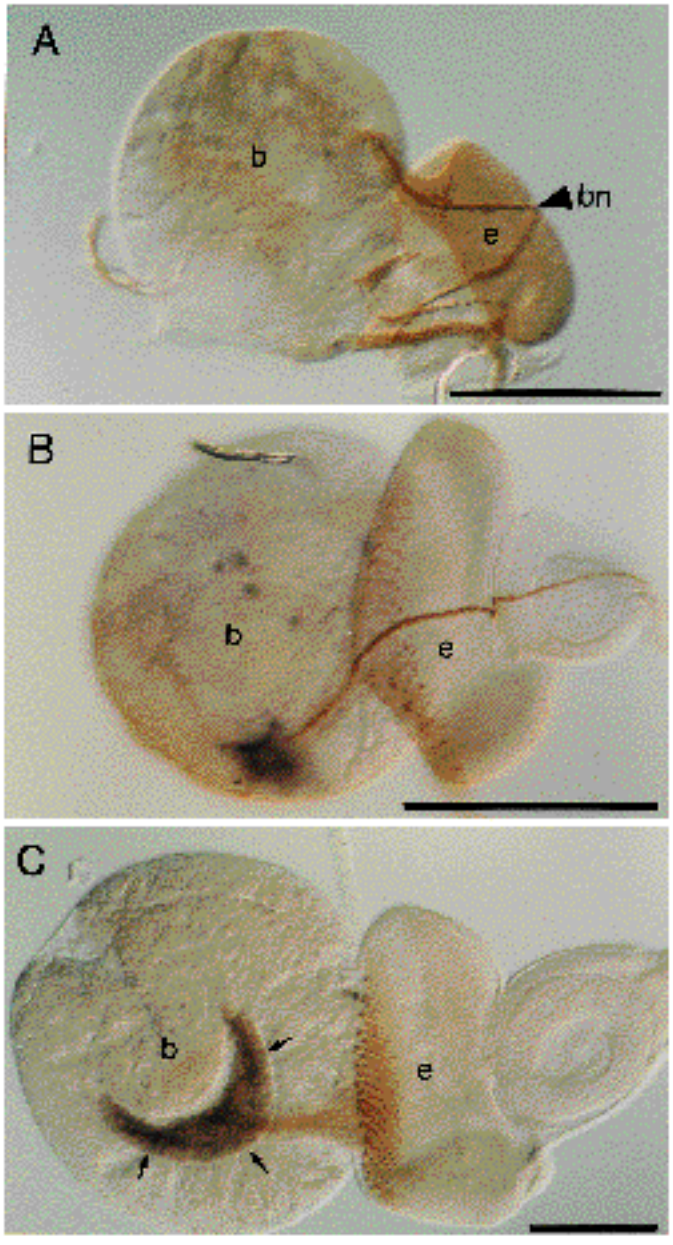

Fig. 1. Temporal expression of *lacZ* from the A72 insertion during the 3rd larval instar stage is coincident with innervation. Brain-eye disc complexes (orientation is anterior to posterior right to left) were stained with anti-β-gal antibodies (all) plus mAb22C10 (A, B) or mAb24B10 (C). mAb22C10 recognizes a neural-specific antigen while mAb24B10 is specific for photoreceptor cells and their axons (Zipursky et al., 1984). Photoreceptor cell axons (stained brown) begin forming in the eye disc during the early third instar. *lacZ*-expressing cells stain blue-black. (A) Early third instar, prior to photoreceptor cell axon formation. Prominent staining of Bolwig's nerve from the larval photoreceptor is evident. (B) Mid-third instar larval brain and eye disc. Early axon formation in the disc coincides with a patch of *lacZ*-positive cells in the developing lamina. (C) Late third instar larval brain and eye disc. *lacZ*-expressing cells are detected throughout the crescent-shaped lamina anlagen. The groove that marks the anterior margin of the anlage is labeled with arrows. e, eye disc; b, brain; bn, Bolwig's nerve. Bar, 100 μm.

essentially as described (Pirrotta, 1986). Two cosmid clones (2-1, 4-1) were isolated from the iso-1 library provided by J. Tamkun, using nonrepetitive restriction fragment probes derived from the rescued plasmids. Each cosmid contained a complete copy of the *17.6* element. To identify repetitive DNA, genomic clone blots were probed with random primed ^{32}P-labeled total *Drosophila* genomic DNA. The restriction map presented in Fig. 3 was derived from the cosmids and the rescued plasmids. DNA sequencing of the A72 P-element breakpoints was done with primers specific for the 5′ and 3′ ends of the P element, using the rescued plasmids as template. DNA cloning and plasmid manipulations were done by standard techniques (Sambrook et al., 1989).

In situ hybridization to whole mounts of larval brains

Digoxigenin probes were generated from genomic restriction fragments by random priming or single-stranded PCR. Third instar larval eye discs and brains were dissected and fixed in 4% paraformaldehyde in PBS for 15 minutes on ice then fixed again for 15 minutes in 4% paraformaldehyde, 0.6% Triton X-100 in PBS at room temperature. After three 5 minute washes in PBT (PBS, 0.1% Tween-20), brains were digested with Proteinase K (10 μg/ml) for 3-5 minutes at room temperature. Protease treatment was stopped by two 5 minute washes in PBT+2 mg/ml glycine and three 5 minute washes in PBT. Brains were postfixed for 20 minutes in 4% paraformaldehyde, 1% glutaraldehyde in PBS. Subsequent hybridization, washes and visualization of the probe were performed essentially as described (Tautz and Pfeifle, 1989) Brains were mounted in glycerol.

P-element transformations using *lacZ* reporter constructs

To assay enhancer/promoter activity a transformation vector, pCanB10 was constructed. pCanB10 contains the *white*$^{+}$ gene and the *lacZ* gene under the control of a minimal heat-shock promoter. *lacZ* activity encoded by this vector is localized to the nucleus, due to a nuclear targeting sequence. To construct pCanB10, the 3 kb *Pst* (blunt) *Bam*H1 fragment containing the heat-shock promoter/*lacZ* gene cassette from pBn27.1 (Riddihough and Ish-Horowicz, 1991) was cloned into the *Bam*HI and *Eco*RI (filled in) sites of pCaSpeR1. The 173 bp *Bss*HII (filled in) polylinker fragment from pBSIISK(+) (Stratagene) was cloned into the *Bam*HI (filled in) site of this plasmid to produce pCanB10. Restriction fragments containing *17.6* element DNA were cloned into the polylinker, 5′ to the *lacZ* cassette. PCR-derived inserts (L5, L1, CS3, Δ33, Δ34, Δ64, Δ65, Δ40, Δ41, Δ42, Δ43, Δ36, Δ22, Δ11) were sequenced in their entirety before microinjection. Transformants were generated as described (Rubin and Spradling, 1982) by injecting a mixture of 500 μg/ml of construct and 100 μg/ml of helper plasmid (pΠ 27.1) into *w*^{1118} hosts.

Some of the insertion lines showed position effects on the pattern of *lacZ* expression. These patterns in dissected larval tissues were assayed in multiple, independent transformed lines for each construct. The number of lines assayed per construct was (parentheses): Xb7 (3), B5 (6), S1 (2), S14 (8), L1 (11), L5 (7), Δ33 (15), Δ34 (12), Δ41 (4), Δ42 (10), Δ11 (9), Δ22 (11), Δ40 (8), Δ36 (10), Δ43 (12), Δ64 (10), Δ65 (10) and CS3 (7). The expression pattern common to the majority of transformant lines for a particular construct is shown.

Oligonucleotide primers

Oligonucleotide primers used to generate the LTR and deletion constructs were designed based on the nucleotide sequence of the 3′ LTR defined by the A72 insertion. They contained *Bam*HI sites at their 5′ ends to allow cloning into the *Bam*HI site of pCanB10. LTR fragments and deletion derivatives were cloned after PCR amplification with the appropriate primers, using a plasmid subclone containing the 3′ LTR of the *17.6* element. The CS3 construct was made as follows: Canton-S genomic DNA was PCR-amplified using oligos 25 and 26. Two major PCR products of 513 and 568 bp were resolved by agarose gel electrophoresis. The 513 bp band was purified from the gel, digested with BamHI and cloned in pCanB10. Following are the sequences of the oligos used.

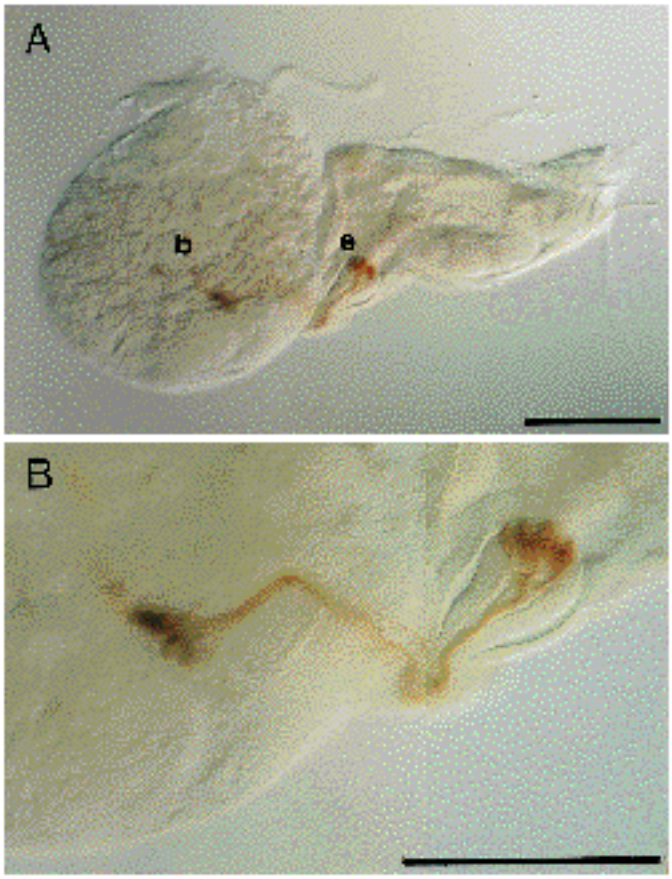

Fig. 2. *lacZ* expression from the A72 insertion depends on photoreceptor axon innervation. *lacZ*-expressing cells and photoreceptor axons were visualized using anti-β-gal antibodies and mAb24B10 in third instar larval brains from eyeless mutants. Double-stained *sine oculis* brains vary in the degree of innervation from the eye disc and the expression of the *lacZ* marker. The majority of *so* brains were not innervated and did not express *lacZ*. However, in partially innervated *so* brains, patchy expression of the *lacZ* marker was always associated with incoming axon fibers. (A) *so* mutant brain and eye disc. A small cluster of *lacZ*-expressing cells (blue) is associated with axon fibers from a few ommatidial clusters in the eye disc. (B) Higher magnification of the brain shown in (A). The close proximity of axon fibers to cells expressing the *lacZ* marker suggests that physical contact may be involved in the activation of gene expression in the developing lamina. e, eye disc; b, brain. Bar, 100 μm.

Oligo 25 (5′ end of LTR)	CGGGATCCAGTGACATATTCACATACAC
Oligo 26 (3′ end of LTR)	CGGGATCCAATTGCAAATAAATGGTTG
Oligo 29 (Δ33, Δ34)	CGGGATCCTGCTTAAAGAATATGTTAG
Oligo 34 (Δ41, Δ42)	CTGGATCCTCAGAGAGAGAGCAGG
Oligo 35 (Δ11)	CTGGATCCCATACTGCGCAGTAGGC
Oligo 36 (Δ64, Δ65)	CTGGATCCATACTGTATGCCATCT
Oligo 37 (Δ22)	CTGGATCCTGTTATTTGATCC
Oligo 38 (Δ36)	CTGGATCCCTTTTCGGCGTAG
Oligo 39 (Δ40)	CTGGATCCGCATGGTGGTCAC
Oligo 40 (Δ43)	CTGGATCCCCAGATCATAACG

RESULTS

We sought to identify genes that are expressed in the lamina anlage in response to innervation, using the enhancer trap technique (O'Kane and Gehring, 1987; Bellen et al., 1989; Bier et al., 1989). Histochemical screening of larval brain and eye disc preparations of 1600 autosomal P[*lacZ*, w^+] insertion lines identified a collection of lines that express *lacZ* in the developing lamina (Mozer, Choi and Benzer, unpublished data). The A72 insertion, because of its temporal and spatial specificity of expression in the developing visual system, is the subject of this study.

lacZ expression in the A72 line is coincident with photoreceptor axon innervation

Brain/eye disc complexes from A72 third instar larvae were double-stained with an anti-β-gal monoclonal antibody and neuron-specific mAb22C10 or photoreceptor cell-specific mAb24B10 (Zipursky et al., 1984) to determine the extent of innervation. In early third instar larval brain, prior to axon outgrowth from the eye disc, only a few scattered *lacZ*-positive cells were detected; these were outside the region of the developing lamina (Fig. 1A). At a slightly later stage, a group of *lacZ*-positive cells was seen, associated with the arrival of axons from photoreceptor cell clusters that had differentiated in the posterior of the eye disc (Fig. 1B). In brains from late third instar larvae (Fig. 1C), *lacZ* expression was seen throughout the entire crescent-shaped anlage.

Expression of the *lacZ* marker depends on photoreceptor cell innervation

The influence of innervation on *lacZ* expression from the A72 insertion was examined in late third instar larval brains in animals homozygous for the eyeless mutants, *sine oculis* (*so*) (Ransom, 1979) and *eyes absent* (*eya*) (Bonini et al., 1993). The *so* mutant is important in this context because mosaic analysis has demonstrated that the lamina defect depends on the genotype of the eye (Fischbach and Technau, 1984). *lacZ*-positive cells and photoreceptor cells and their axons were visualized with anti-β-gal antibody and mAb24B10.

In the eya^2 mutant, which has no photoreceptor axons (Bonini et al., 1993), *lacZ* expression was never detected in 20 brains examined (data not shown). In the *so* mutant, the eye disc defect is variable; each brain hemisphere varies in the extent of innervation from the corresponding eye disc. Of 23 individuals examined, 9 (39%) showed no *lacZ* expression in the lamina anlage. Most of these brains were not innervated as judged by antibody staining of photoreceptor axons. The remaining 14 (61%) exhibited a range of *lacZ* expression patterns, from small clusters of *lacZ*-positive cells to a full crescent pattern resembling wild type. An example of a partially innervated *so* brain is shown in Fig. 2. In such partially innervated brains, *lacZ*-positive cells in the lamina were always associated with photoreceptor cell axons.

These results demonstrate a strong correlation between innervation and expression of the *lacZ* marker in the lamina. The close proximity of the *lacZ*-positive cells to the photoreceptor axon termini closely parallels the behavior of the LPCs in the developing lamina, as assayed by BrdU labeling (Selleck and Steller, 1991). These data and the expression of *lacZ* along the advancing anterior margin of the lamina anlage suggest that transcription of the *17.6* element may occur in the LPCs, with the absence of *lacZ* expression in noninnervated brains being due to failure of lamina precursor cells to be induced to enter S-phase. To confirm this, higher resolution imaging will be needed.

Molecular analysis of the A72 insertion

The *lacZ* expression pattern of the A72 line suggests that this insertion reflects the activity of a gene that plays an early role in lamina development. To isolate the gene, DNA surrounding the insertion site was cloned by plasmid rescue and overlapping cosmid clones from the region were isolated, using a non-repetitive restriction fragment as probe. The partial restriction map of the genomic region surrounding the insertion is shown in Fig. 3A. Reverse genomic Southern blot analysis, using as a probe total *Drosophila* DNA, showed that the 7.5 kb *Xba*I insertion fragment is repetitive. Restriction mapping of this fragment and DNA sequence analysis of the insertion breakpoints revealed that the P-element had inserted within the 3′ long terminal repeat (LTR) of a *17.6* transposable element, related to the *gypsy* family of retrotransposons, with an estimated copy number of 40 per haploid genome (Kugimiya et al., 1983).

Pattern of transcript expression in developing lamina

To determine the spatial pattern of expression of sequences adjacent to the A72 insertion, genomic restriction fragments were labeled with digoxigenin and used to probe whole third instar larval brains. Only DNA fragments containing *17.6* sequences (Probe 2, Fig. 3A) detected transcripts in wild-type brain in a crescent pattern in the region of the lamina anlage (Fig. 3B). Using the same probe on *eya* mutant brains, no transcripts were detected (data not shown). In *so* mutant brains, reduced or no expression was observed, consistent with the variable extent of innervation in this mutant (Fig. 3C). Antisense cRNA probes made from probe 2 DNA detected the same crescent expression pattern while sense probes did not (data not shown). These experiments demonstrate that expression of *17.6* RNAs is pari passu with *lacZ* expression from the A72 insertion.

The LTR of the 17.6 element itself contains *cis*-regulatory sequences for lamina expression

We sought to determine whether the lamina anlage expression is an intrinsic property of the *17.6* element or depends upon *cis*-regulatory sequences in the flanking DNA. Restriction fragments from the 7.5 kb *Xba*I fragment, which contains a complete copy of the *17.6* element, were fused to the 5′ end of a minimal heat-shock promoter/*lacZ* gene cassette in a P-element vector (pCanB10, see methods). The ability of restriction fragments from the *17.6* element to confer lamina anlage expression on the promoter was assayed in multiple, independent germline transformant strains for each construct.

Fig. 4 summarizes the initial series of constructs and their expression in third instar larval brain and eye disc. The 7.5 kb *Xba*I fragment contains a complete copy of the *17.6* element and a small amount of flanking genomic DNA. *lacZ* expression in transformant lines carrying this fragment (construct Xb7) was identical to the A72 expression pattern (data not shown), suggesting that the 7.5 *Xba*I fragment contains the *cis*-acting

sequences required for lamina-specific expression in the third instar larval brain. A smaller, 2.4 kb *Sal*I-*Xba*I genomic fragment, containing the 3′ third of the *17.6* element and approximately 200 bp of flanking genomic DNA (construct S14) was likewise sufficient for lamina-specific expression. The ability of this fragment to activate *lacZ* expression in the lamina anlage depended on its orientation; there was no *lacZ* expression in transformant lines harboring the reverse orientation (construct S1). Similarly, construct B5 in reverse orientation was also ineffective. To define further the regulatory sequences and to rule out a contribution from the flanking DNA, the enhancer activity of the *17.6* LTR was assayed by deletion analysis. Constructs containing a complete copy (L1, L5) in either orientation, direct *lacZ* expression in the lamina anlage in transformant lines. Although *lacZ* expression within the brain from these constructs was still restricted to the developing lamina, additional expression was observed in the eye imaginal disc. In L5 transformants, *lacZ* expression in the lamina anlage was noticeably less intense than from the L1 construct. The *lacZ* expression pattern typical of an L5 transformant is shown in Fig. 5B. Expression in the eye disc is restricted to the posterior margin and to two patches of cells that may correspond to precursors of the ocelli. In the L1 construct (Fig. 5C), *lacZ* expression was intense in the lamina anlage and, in addition, robust expression occurred throughout the posterior region of the eye disc, behind the morphogenetic furrow.

A 54 bp sequence in the 5′ end of the LTR is sufficient for lamina anlage and eye disc expression of *lacZ*

To define further the regulatory sequences, 5′ and 3′ terminal deletions of the LTR were constructed and their regulatory activity assayed in transformant lines (Fig. 6). Constructs containing the 5′ terminal 363 bp (Δ33, Δ34), 266 bp (Δ41, Δ42), 184 bp (Δ11), and 114 bp (Δ22) of the LTR showed lamina and eye disc expression. In contrast, *lacZ* expression from the Δ40 and Δ36 constructs, which contain the 5′ terminal 95 and 60 bp of the LTR, was only detected in the eye disc, suggesting that sequences between positions 60 and 114 are essential for lamina expression. To test whether these sequences are sufficient for expression, their activity was assayed in transformant lines. *lacZ* expression from the Δ43 construct, which contains the 54 bp from position 60 to 114 of the LTR, was detected in the lamina and the eye disc in 10/10 independent insertion lines.

Constructs containing LTR sequences from positions 146 to 363 (Δ64, Δ65) had no *cis*-regulatory activity and transformant lines failed to express *lacZ* in the brain or eye disc. These results point to the importance of sequences within the 5′ end of the LTR, and also exclude a possible artifactual role of the vector sequences in contributing to the expression pattern.

A variant 17.6 LTR also contains the enhancer

Many of the estimated 40 or so *17.6*-related elements in the *Drosophila* genome are structurally heterogeneous containing terminal and/or internal deletions. In addition, there is considerable variability of the DNA sequences of their LTRs: most are single base pair changes, but others include a common set of additional internal sequences that resemble duplications (Inouye et al., 1986). The LTR pinpointed by the A72 insertion is a member of the latter class (see Fig. 6 for details). We therefore made a construct, CS3, containing a 511 bp LTR with a sequence identical to that of the prototype form (λ*hist17.6*) which lacked these additional sequences (Kugimiya et al., 1983). In all transformant lines harboring this construct, *lacZ* expression was detected in the lamina anlage. Therefore, the additional sequences present in our LTR are not necessary for the *cis*-regulatory activity that we have studied. The fact that two versions of the LTR are both effective, suggests that many, if not all of the *17.6*-like sequences in the genome containing the LTR, may also be transcriptionally active in the lamina anlage.

DISCUSSION

We have shown that, in third instar larval brain, transcripts derived from one or more of the *17.6* class of retrotransposons are expressed in the developing lamina. To our knowledge, ours is the first demonstration of tissue-specific expression of a retrotransposon in the *Drosophila* nervous system. Meinertzhagen (1973) proposed that the anterior-posterior positions of cells within the lamina anlage reflect a gradient of differentiative events analogous to those occurring behind the morphogenetic furrow in the eye imaginal disc. Lamina precursor cells divide in response to an inductive cue from the incoming photoreceptor axons. The mitotic products of these divisions subsequently differentiate into neurons, as suggested by the onset of expression of the neural-specific antigen Elav (Selleck and Steller, 1991). The onset of expression of the *17.6* element at the anterior margin of the lamina anlage, and the close physical association of *17.6*-expressing cells with photoreceptor axon terminals, is consistent with the hypothesis that *17.6* transcription, like mitosis, is an early response to the inductive cues.

The lamina-specific expression of *17.6* is an intrinsic property of the element, mediated by the combined action of multiple *cis*-regulatory sequences. *lacZ* fusion constructs containing the 3′ LTR along with 1.9 kb of internal *17.6* element DNA are lamina-specific while constructs containing the LTR or deletion derivatives of the LTR alone show additional expression in the eye imaginal disc. This suggests that lamina-specific expression may depend on a positively acting enhancer sequence in the LTR, plus a second 'silencer' sequence upstream of the LTR required to repress transcription in the developing eye. Tissue-specific regulatory sequences containing positive and negative elements have been described for the zebra stripe element of the pair-rule gene *ftz* (Dearolf et al., 1989); they are a common feature of gene regulation in eukaryotes (Renkawitz, 1990)

The orientation of restriction fragments containing *17.6 cis*-regulatory sequences relative to the minimal heatshock promoter in pCanB10, affected the *lacZ* expression pattern. The 2.4 kb *Sal*I-*Xba*I restriction fragment conferred lamina-specific *lacZ* expression in one orientation (S14) but failed to express in the opposite orientation (S1, B5). This effect may be due to the presence of upstream sequences that interfere with the activation of the heat-shock promoter by the LTR enhancer, or simply due to a distance effect. Constructs containing the LTR enhancer element alone (Figs 5 and 6) displayed quantitative and qualitative effects on *lacZ* expression that depended on orientation.

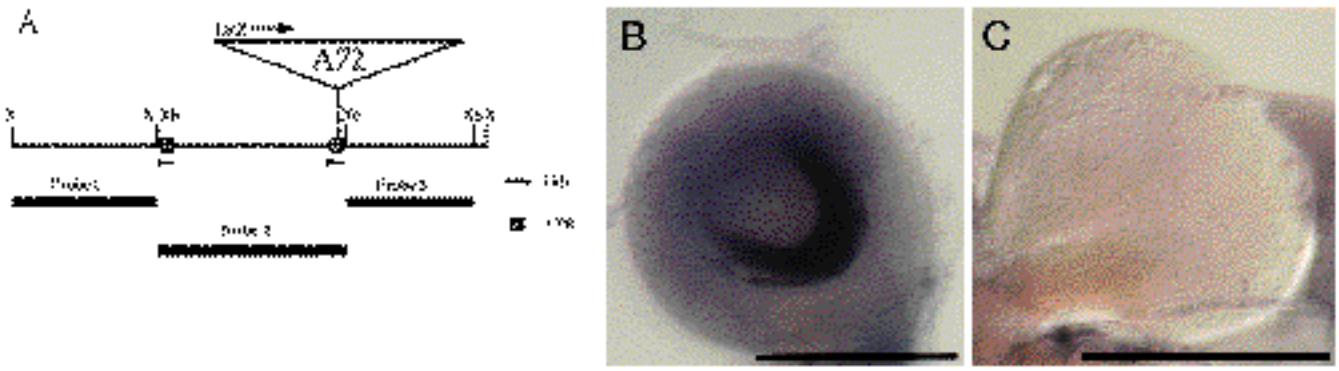

Fig. 3. RNAs of the *17.6* retrotransposon identified by the P-element insertion are expressed in brain of wild type but not in *so*. (A) Partial restriction map of the genomic region surrounding the A72 insertion. Molecular analysis of the insertion site revealed that the P element had inserted within the 3′ LTR of a *17.6* retrotransposon, a member of the *gypsy*-like family of repetitive elements. The 7.5 kb *Xba*I genomic fragment (probe 2) contains a complete copy of the *17.6* element. LTRs are depicted as stippled boxes and their orientation is indicated with arrows. X, *Xho*I; Xb, *Xba*I. (B). Spatial pattern of transcripts detected by probe 2 in brain from wild-type third instar visualized by in situ hybridization using the digoxigenin technique. (C) Expression of RNAs detected by the same probe as in B in *so* mutant brain. Only probes containing retrotransposon sequences detected transcripts in the developing lamina (probes 1 and 3 were negative) suggesting that the expression of the *lacZ* reporter in the A72 insertion reflects the transcriptional activity of the *17.6* element. e, eye disc; b, brain. Bar, 100 μm.

Deletion analysis of the LTR suggests that sequences from positions 60 to 114 are necessary and sufficient for expression in the developing lamina. A computer search of this interval revealed no significant homology to known *Drosophila* transcription factor binding sites present in the TFSITES (release 4.0) data base. The 54 bp sequence that we have defined contains two different short repeats (GATCA and AATAA, dotted in Fig. 6C). In addition, the sequence, GCTAATGT is present at positions 93 to 100 (underlined, Fig. 6C) that contains the TAAT motif, the conserved core sequence found in the binding sites of most homeodomain-containing proteins (Scott et al., 1989). The TAAT motif has been found in *cis*-regulatory elements from several *Drosophila* genes including the R7 cell-specific opsins, Rh3 and Rh4 (Fortini and Rubin, 1990) and DOPA decarboxylase (Lundell and Hirsch, 1992).

A 19 bp deletion that removes the TAAT sequence and one copy of each short repeat converts an active enhancer (Δ22) to an inactive one (Δ40), suggesting that the TAAT sequence is required for enhancer function and that a single copy of the two repeats is not active. Molecular genetic analysis of *Bicoid* and *Antennapedia* protein-binding sites in yeast indicates that the nucleotide directly following the last T of the motif is a critical determinant of specificity (Hanes and Brent, 1991). The presence of a G nucleotide at this position in the lamina enhancer suggests that this site may bind a novel homeodomain-containing protein distinct from *Ubx*, *Bicoid* and the *AntP* class of homeodomain proteins. Thus, transcriptional activation of the *17.6* element in the developing lamina may be controlled in part by a novel homeodomain-containing protein.

The *lacZ* expression pattern of the A72 insertion

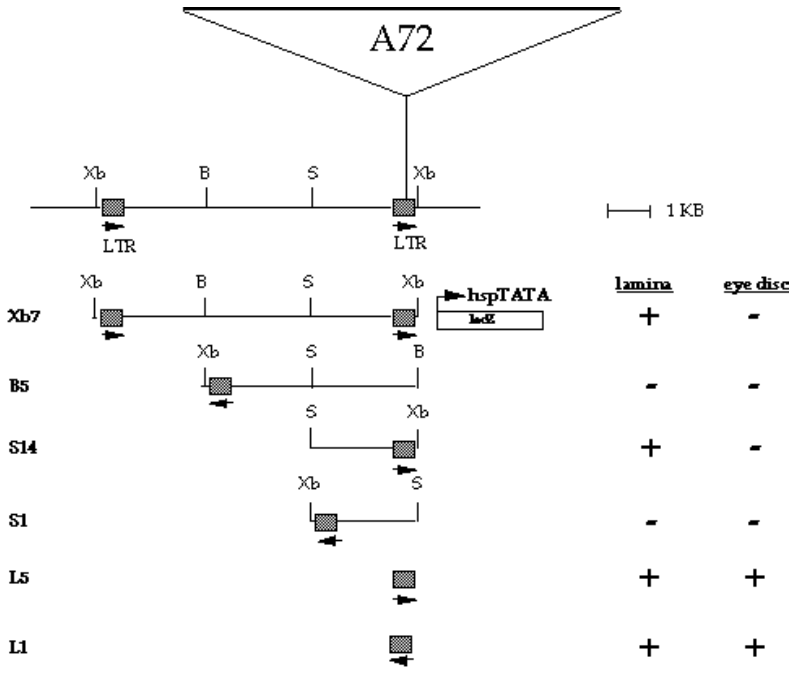

Fig. 4. DNA sequences within the *17.6* element can confer lamina-specific expression on a *lacZ* reporter gene. Restriction fragments containing retrotransposon sequences were cloned into the enhancer detection vector pCanB10 and their enhancer activity assayed in the developing visual system by germline transformation. Constructs containing a complete copy (Xb7) or the 3′ third of the element (S14) and some additional flanking DNA gave specific expression in the lamina anlage. *lacZ* expression from constructs containing the LTR alone was not restricted to the lamina. Constructs containing only the 3′ LTR (L5, L1) expressed *lacZ* in the lamina anlage independently of orientation but showed orientation-dependent expression patterns in the eye imaginal disc. These results suggest that the LTR of the *17.6* element contains an enhancer-like sequence and that the lamina-specific expression of the *17.6* element in the developing visual system requires multiple *cis*-regulatory sequences. X, *Xho*I; Xb, *Xba*I; B, *Bam*HI; S, *Sal*I.

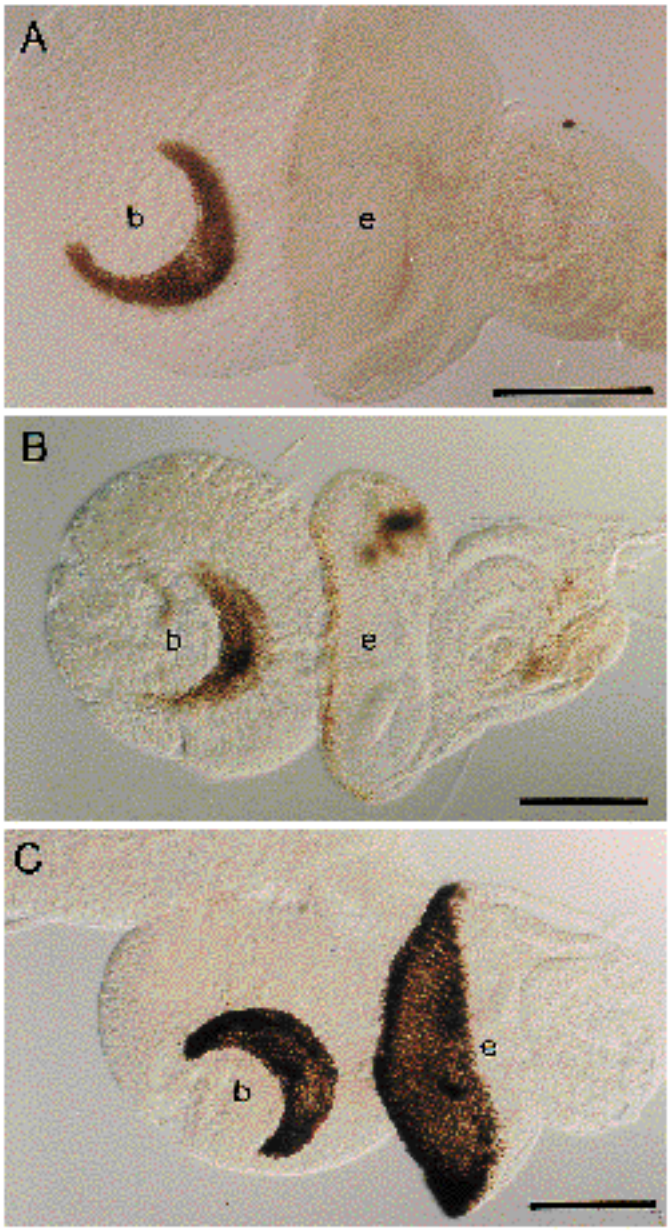

Fig. 5. *lacZ* expression patterns in third instar larval brain and eye disc from transformants. *lacZ* expression for each construct was assayed in multiple transformant lines, using a monoclonal antibody visualized by HRP. *lacZ* fusion constructs containing a 2.4 kb *Sal*I-*Xba*I fragment in one orientation reproduce the lamina-specific expression. Constructs containing a complete LTR or an LTR deletion show expression in the lamina and in the eye disc. Both the pattern and intensity of *lacZ* expression in these lines was affected by orientation. (A) Lamina-specific expression. Constructs Xb7 and S14. (B). Expression in lamina anlage, the posterior edge of the eye disc, and in the region of the ocelli precursors. Constructs L5, Δ33, Δ41 and Δ43. (C) Intense *lacZ* expression in the lamina, throughout the eye disc posterior to the morphogenetic furrow and in the ocelli precursors. Constructs L1, Δ34, Δ42, Δ11, Δ22. e, eye disc; b, brain. Bar, 100 μm.

is remarkably specific to the developing visual system. This specificity may be a property of the particular *17.6* element defined by the enhancer trap insertion in the A72 line and not necessarily a common characteristic of all *17.6* elements in the genome. *lacZ* expression in the A72 line was not detected at any stage of embryogenesis. This is in contrast to the finding that certain *17.6*-type transcripts can be detected in mRNA from embryonic and other stages (Parkhurst and Corces, 1987). On northern blots, *17.6* transcripts are heterogeneous, although there are major size classes including a prominent 7.5 kb transcript corresponding to the full-length element (Parkhurst and Corces, 1987; Mozer and Benzer, unpublished data). *17.6* transcripts have also been detected in the *Drosophila* cell line GM2 where many of the *17.6*- RNA start sites were heterogeneous, mapping upstream of the 5′ LTR (Inouye et al., 1986). This heterogeneity of expression of various *17.6*-type retrotransposons could be due to misexpression of rearranged elements in the genome from promoters in adjacent genes. Our studies of *17.6* expression in the developing visual system suggest that internal *cis*-regulatory sequence elements are responsible for transcriptional repression. Rearranged elements that have lost such control sequences would be expected to display altered temporal and spatial expression patterns.

The genomic organization of the *17.6* element resembles that of a retrovirus; it contains an internal region predicted to encode several proteins including a reverse transcriptase (Saigo et al., 1984). Direct long terminal repeats (LTRs) at each end of the element contain sequences necessary for transcriptional regulation (Kugimiya et al., 1983). Although a functional role for *17.6* encoded gene products in lamina development remains to be demonstrated, regulatory sequences in the *17.6* element may be involved in the innervation-dependent expression of adjacent cellular genes. Evolutionary refinement of the steps in neurogenesis of a species may in part consist of transposition of *17.6* to locations that enhance or suppress transcription of appropriate groups of genes at the proper time. The molecular analysis of an insecticide-sensitive allele of the *Drosophila* P450-B1 gene, suggests that sequences in the *17.6* LTR also exert negative regulatory effects (Waters et al., 1992). Vertebrate retrovirus and retrotransposon sequences have been implicated in the regulation of a number of endogenous genes including the calcium-binding protein gene *oncomodulin* (Banville and Boie, 1989), the mouse *Sex Limited Protein* gene (Stavenhagen and Robins 1988) and the activation of the *myc* oncogene in certain cancers (Hayward et al., 1981). We are currently investigating the role of other 17.6 elements of the genome in regulating cellular genes during lamina development.

The LTRs of many vertebrate and invertebrate retrotransposons contain promoter and other *cis*-regulatory sequences required for tissue-specific expression (see review for *Drosophila* by Arkhipova and Ilyin, 1992) Positive and negative transcriptional regulation of many retrotransposons in vivo or in tissue culture cells by growth factors and hormones are mediated by regulatory elements in their LTRs. Two vertebrate examples are glucocorticoid induction of mouse mammary tumor virus (MMTV) (Beato, 1989) and gonadotropin induction of the VL30 element (Schiff et al., 1991). Nerve growth factor-dependent transcription of the NICER family of retrotransposons in PC-12 cells (Cho et al., 1990) suggests that growth factor-dependent expression of retrotransposons occurs in the vertebrate nervous system. In *Drosophila* tissue culture cells, the steroid hormone 20-hydroxyecdysone down-regulates the transcription of the

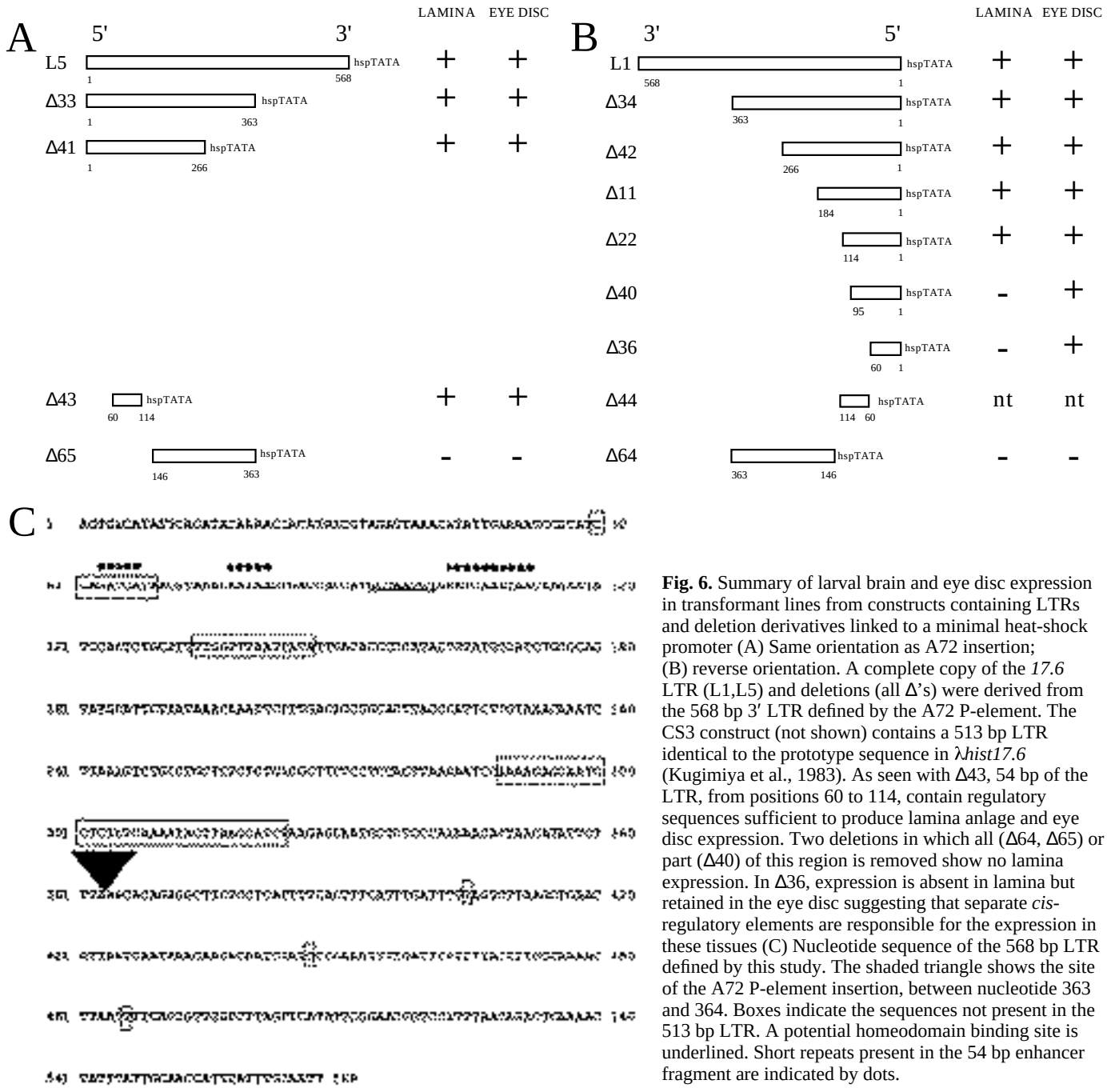

Fig. 6. Summary of larval brain and eye disc expression in transformant lines from constructs containing LTRs and deletion derivatives linked to a minimal heat-shock promoter (A) Same orientation as A72 insertion; (B) reverse orientation. A complete copy of the *17.6* LTR (L1,L5) and deletions (all Δ's) were derived from the 568 bp 3′ LTR defined by the A72 P-element. The CS3 construct (not shown) contains a 513 bp LTR identical to the prototype sequence in λ*hist17.6* (Kugimiya et al., 1983). As seen with Δ43, 54 bp of the LTR, from positions 60 to 114, contain regulatory sequences sufficient to produce lamina anlage and eye disc expression. Two deletions in which all (Δ64, Δ65) or part (Δ40) of this region is removed show no lamina expression. In Δ36, expression is absent in lamina but retained in the eye disc suggesting that separate *cis*-regulatory elements are responsible for the expression in these tissues (C) Nucleotide sequence of the 568 bp LTR defined by this study. The shaded triangle shows the site of the A72 P-element insertion, between nucleotide 363 and 364. Boxes indicate the sequences not present in the 513 bp LTR. A potential homeodomain binding site is underlined. Short repeats present in the 54 bp enhancer fragment are indicated by dots.

'1731' and '412' retrotransposons (Micard et al., 1988; Ziarczyk et al., 1989) Negative regulation by this steroid hormone of the 1731 element is mediated by hormone-responsive sequences in its LTR (Ziarczyk and Best-Belpomme, 1991). Expression of the *17.6* retrotransposon in the developing lamina may be a response to a novel growth factor the activity of which depends on photoreceptor cell axon innervation.

The study of the *trans*-acting factors that bind to the glucocorticoid response element in the MMTV LTR has contributed to the identification of the steroid receptors, molecules responsible for the regulation of networks of hormone-responsive genes (Beato, 1989). *17.6* expression in the developing visual system is likely a response to the transcriptional machinery in the postsynaptic cells that is activated by innervation. Transcription factors that interact with the LTR enhancer to activate *17.6* element expression may similarly be involved in the regulation of other genes whose expression in the brain depends on innervation. Identification of the *trans*-acting genes required for the expression of the *17.6* element in the lamina anlage might therefore provide clues to identification of the molecules that mediate the inductive events that occur upon innervation of the brain.

We tested two structural variants of the *17.6*-type LTR and found both to possess enhancer activity. This suggests the intriguing possibility that many of the 40 or so *17.6*-type

elements in the genome might contain the enhancer, in which case the *17.6* RNAs detected by in situ hybridization may be the result of transcription of multiple elements in the genome. Innervation might thus coordinately unleash a constellation of gene products that participate in the subsequent differentiation events.

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