

# Cell type-specific regulation of the *Drosophila FMRF-NH<sub>2</sub>* neuropeptide gene by Apterous, a LIM homeodomain transcription factor

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## SUMMARY

We describe the direct and cell-specific regulation of the *Drosophila FMRFa* neuropeptide gene by Apterous, a LIM homeodomain transcription factor. *dFMRFa* and Apterous are expressed in partially overlapping subsets of neurons, including two of the seventeen *dFMRFa* cell types, the Tv neuroendocrine cells and the SP2 interneurons. Apterous contributes to the initiation of *dFMRFa* expression in Tv neurons, but not in those *dFMRFa* neurons that do not express Apterous. Apterous is not required for Tv neuron survival or morphological differentiation. Apterous contributes to the maintenance of *dFMRFa* expression by postembryonic Tv neurons,

although the strength of its regulation is diminished. Apterous regulation of *dFMRFa* expression includes direct mechanisms, although ectopic Apterous does not induce ectopic *dFMRFa*. These findings show that, for a subset of neurons that share a common neurotransmitter phenotype, the Apterous LIM homeoprotein helps define neurotransmitter expression with very limited effects on other aspects of differentiation.

Key words: *Drosophila*, Neuropeptide gene, FMRFamide, *apterous*, LIM homeodomain

## INTRODUCTION

LIM homeoproteins are a family of transcription factors with diverse roles in developmental regulation (Curtiss and Heilig, 1998; Pfaff and Kintner, 1998). They are notable for their involvement in mechanisms of cellular specification and their analysis offers the prospect of defining the organization of regulatory mechanisms that underlie the differentiation of single cell types. To date, genetic analysis has revealed diversity in the scope of LIM homeodomain regulation of neuronal phenotypes. In *Caenorhabditis elegans* and *Drosophila* LIM homeoproteins regulate aspects of late neuronal differentiation, including the guidance of neuronal growth cones (Lundgren et al., 1995; Thor and Thomas, 1997), the establishment of proper synaptic connections (Way and Chalfie, 1988; Hobert et al., 1997, 1998) and the selection of appropriate neurotransmitters (Thor and Thomas, 1997). Five different LIM homeoproteins are dynamically expressed by postmitotic motoneurons of the vertebrate CNS (Tsuchida et al., 1994), consistent with a hypothesis that their combinatorial expression helps co-ordinate the diversification of motoneuron subtypes.

Understanding what aspects of cell differentiation LIM homeodomain proteins control, and under what limits and compensatory mechanisms they operate, is a major challenge in developmental neurobiology. Developmental analyses have also suggested that neural cell differentiation represents several separate regulatory programs within individual cells, each

responsible for producing distinct cell properties (Desai et al., 1988; Lo et al., 1998). Therefore we also wish to define the influences of LIM homeoproteins in this context. To address these questions regarding regulatory mechanisms, we have studied the function of the Apterous (Ap) LIM homeoprotein, a developmental regulator in both neural and non-neural tissues of *Drosophila*. Outside the central nervous system (CNS), Ap is involved in wing disk patterning (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994) and in muscle cell specification (Bourgouin et al., 1992). Within the CNS, Ap regulates axonal projections by a subset of interneurons (Lundgren et al., 1995). Although viable, *ap* mutant adults eclose at low frequency, and are infertile and unco-ordinated: these features suggest both endocrine and neural deficits (Altartatz et al., 1991). The present work considers these critical Ap functions at a cellular level. In particular, we have investigated the hypothesis that *ap* participates in the differentiation of one neuronal cell type, the neuroendocrine Tv neurons that express the neuropeptide gene *dFMRFa* (Schneider et al., 1991).

The *FMRFamide* gene of *Drosophila* encodes multiple functional neuropeptides (Hewes et al., 1998) and is expressed in only ~44 neurons out of 10,000 in the larval CNS. This restricted pattern of expression represents at least 17 different cell types and derives largely from transcriptional regulation (Schneider et al., 1991, 1993b). In vivo analysis has indicated that the neuropeptide gene promoter is highly mosaic and includes at least three non-overlapping enhancers that independently activate transcription in different *dFMRFa* cells

(Schneider et al., 1993b; Benveniste and Taghert, 1999). More detailed analysis of the Tv neuron-specific enhancer suggested the participation of multiple *cis*-elements, each making partial contributions to the activity of the larger fragment. These experiments suggest the generalization that a common neurotransmitter phenotype may be regulated by multiple transcription factors within a single cell type, and within different cell types, by different sets of transcription factors. This pattern of transcriptional regulation represents a useful model for analyzing mechanisms that generate spatially diverse neuronal gene expression. However, no *trans*-acting factors have yet been identified that regulate *dFMRFa* in any cell type *in vivo*.

Our study of *ap* has generated three principal conclusions. We show that Ap is necessary for the initiation of neuropeptide expression in Tv neurons – which are only a subset of the neurons that normally share the *dFMRFa* neuropeptide transmitter phenotype – and does so without affecting their survival or morphogenesis. Further, we find that neuropeptide gene expression in the postembryonic Tv neurons remains sensitive to the levels of Ap, although the influence of the regulator appears weaker than during embryonic stages. Finally, we deduce that control of the neuropeptide transmitter phenotype by the Ap protein includes mechanisms of direct regulation. These findings define principles of LIM homeoprotein action and support the view that neuronal cell differentiation represents an assembly of separable genetic programs each regulating distinct cellular properties.

## MATERIALS AND METHODS

### Immunohistochemistry

18–20 hour embryos were dissected with sharpened tungsten needles on double-sided tape in phosphate-buffered saline (175 mM NaCl, 10 mM PO<sub>4</sub> pH 7.4). CNS were transferred to a polylysine-covered slide and fixed 20–25 minutes at room temperature in 4% paraformaldehyde in PBS. They were incubated overnight at 4°C in anti-β-galactosidase (Cappel) at 1:10,000 or rabbit anti-dFMRFa propeptide at 1:1000. The anti-dFMRFamide propeptide antiserum was generated to a synthetic 19-mer comprising the final nineteen amino acids of the pro-dFMRF precursor (Chin et al., 1990). The CNS were washed in PBS + 0.1% Triton X-100, incubated for 2 hours at room temperature in 1:1500 biotinylated anti-rabbit (Vector Laboratories). After further washes, color was developed using the Vectastain kit (Vector Laboratories). In some experiments, FITC-conjugated avidin was used at 1:400 (Vector Laboratories). Embryonic tissues were then dehydrated in ethanol and mounted in Canada Balsam.

Larval CNS were dissected in saline (180 mM KCl, 46 mM NaCl, 2.2 mM CaCl<sub>2</sub>, 10 mM Tris pH 7.2), fixed 1 hour at room temperature in 4% paraformaldehyde in PBS, and stained overnight at 4°C in rabbit anti-dFMRFa propeptide and mouse anti-β-gal (Promega) in PBTN (PBS + 0.1% BSA + 0.3% Triton X-100 + 5% NGS-Gibco). Following secondary antibody incubations, they were cleared in 70% glycerol and mounted in Vectashield (Vector Labs).

### Histochemical detection of β-gal

Animals were dissected in saline, fixed 15 minutes at room temperature in 0.04% glutaraldehyde, 0.1 M cacodylate and 0.02% Triton X-100, and stained with X-Gal overnight at 37°C.

### Microscopy and imaging

For confocal imaging, we used an Olympus confocal microscope and

Fluoview software; images were adjusted and assembled in Adobe Photoshop.

### Heat-shock experiments

We recombined the *hs-ap-1b* transgene (described in Bourgouin et al., 1992) with the *ap<sup>P44</sup>* allele and mated the resulting animals to animals homozygous for a *Tv-lacZ* insertion (see Fig. 5). Flies were maintained at 24°C and eggs collected on agar plates. Following larval hatching, animals were heat shocked in a bacterial incubator (15 minutes at 37°C) then returned to 24°C for subsequent recovery until dissection.

### Electrophoretic mobility shift assays

We used a truncated form of the Ap protein (lacking the LIM domains) for these experiments, because LIM domains may inhibit binding of the homeodomain to DNA *in vitro* (e.g., Xue et al., 1993). A PCR-derived product from base 1322 of the *ap* cDNA (Bourgouin et al., 1992) to base 2171 (a *Hind*III site in the 3' UTR) was cloned into the bacterial expression plasmid pQE30, incorporating a 6xHIS tag (Qiagen). Truncated Ap protein was expressed in JM107 cells, purified under non-denaturing conditions and stored in aliquots at –20°C until use. Double-stranded 25 bp (wild-type) oligonucleotides (called A, B and C) were labeled by filling in 5' overhanging ends with Klenow enzyme (Promega) and α-<sup>32</sup>P-dCTP (Amersham). Double-stranded 17 bp (mutant) oligonucleotides were labeled by annealing, then trimming 3' overhanging ends and treating with Klenow enzyme and α-<sup>32</sup>P-dCTP. The sequences of wild-type and mutant probes were as follows, with their positions given in bp relative to the *dFMRFa* gene transcription start site (mutated sequences, a *Sac*I restriction site, are shown in bold):

A (–752 to –728): 5'-GCCGGCCGTAATTACAGACTTCCG-3'  
 mA: 5'-GGCCGGAGCTCCAGACT-3'  
 B (–574 to –550): 5'-GGCAATGGCAAATTATAACGCATAC-3'  
 mB: 5'-GGCAAGAGCTCACGCAT-3'  
 C (–529 to –505): 5'-GGCTAGAAGGCTAATTGGACGTGCC-3'  
 mC: 5'-AAGGCGAGCTCGACGTG-3'

2 pmoles probe were incubated with 140 ng purified Ap protein in a solution containing 150 mM KCl, 10 mM Hepes pH 7.8, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA pH 8, 1 mg/mL BSA, 1 mM dithiothreitol, 12% glycerol, and 250 ng poly(dI-dC)·poly(dI-dC) (Sigma). Following a 20 minute incubation at 30°C, samples were electrophoresed approximately 45 minutes, at 150 V at 4°C in a 4% (79.5:0.5 acrylamide:bisacrylamide) gel in Tris-glycine buffer (Ausubel et al., 1993).

### Synthesis of enhancer testing constructs

PCR was used to mutate the homeodomain binding motifs to *Sac*I restriction sites (TAATXX to GAGCTC). The primers used were the following (mutated sequences shown in bold):

mA, sense: 5'-GGCCGGAGCTCCAGACTTCCGTCTTT-3'  
 mA, antisense: 5'-GTCTGGAGCTCCGGCCCGCAAAAGG-3'  
 mB, sense: 5'-GGCAAGAGCTCACGCATACGGACACG-3'  
 mB, antisense: 5'-TGCGTGAGCTCTTGCCATTGCCGTCG-3'  
 mC, sense: 5'-AAGGCGAGCTCGACGTGCCCGGCCAG-3'  
 mC, antisense: 5'-ACGTCGAGCTCGCCTTCTAGCCAGTG-3'

Fragments containing mutated elements was cloned into the enhancer testing construct *hs43-CaSpeR-lacZ* (Schneider et al., 1993b) and sequenced.

### Transgenic *Drosophila*

We purified P-element DNA using Qiagen tips and transformed embryos using procedures described in Spradling (1986). The number of P-element insertions in each transgenic line was determined by Southern blot and only animals with one (haploid) copy of the P element were used. Two independent transgenic lines representing each mutant construct were tested.

## RESULTS

Co-expression of *ap* and *dFMRFa*

The fixation conditions required for the antiserum to Ap precluded double labeling with antisera to the *dFMRFa* propeptide. Therefore, to look for co-expression of Ap with *dFMRFa* in the CNS, we used two different *ap* enhancer trap lines. The first was *ap<sup>GAL4</sup>*, a *pGawB* insertion in the *ap* locus (Calleja et al., 1996). 18-20 hour embryos carrying the *ap<sup>GAL4</sup>* insertion and a *UAS-*lacZ** reporter gene were double labeled with anti-Ap and anti- $\beta$ -gal to confirm that *ap<sup>GAL4</sup>* reports expression of the *ap* gene. Confocal imaging demonstrated that the patterns of Ap and  $\beta$ -gal expression were identical (Fig. 1A). The second enhancer trap line was *ap<sup>rk568</sup>*, containing an element inserted 5' of the *ap* gene (Cohen et al., 1992). Previous work showed that *ap<sup>rk568</sup>* accurately reports Ap cells in embryos and larvae (Cohen et al., 1992). In addition, double labeling of *ap<sup>rk568</sup>/ap<sup>GAL4</sup>* embryos demonstrated that *ap<sup>rk568</sup>* and *ap<sup>GAL4</sup>* are active in the same neurons (S. T., data not shown).

In the ventral ganglion, *ap* is expressed in up to ten of the ~350 neurons in each hemi-segment (Lundgren et al., 1995; Fig. 1B). In each of six thoracic hemisegments and in the third subesophageal hemisegments, the *ap* neurons include a ventrolateral cluster of four or five cells (Fig. 1B). Double-labeling with antiserum to the *dFMRFa* propeptide showed that one of the neurons in each cluster was the Tv neuron (Fig. 1C,D). Double-labeled Tv neurons showed cytoplasmic *dFMRFa* immunoreactivity and nuclear  $\beta$ -gal immunoreactivity (Fig. 1E). The *ap* gene is also expressed in several brain cells, one of which is the *dFMRFa*-positive SP2 neuron (Fig. 1F). Other larval brain *dFMRFa* neurons, such as the neighboring SP1 neuron, do not express *ap* (Fig. 1F). The restriction of *ap* and *dFMRFa* co-expression to the Tv and SP2 neurons was constant throughout mature larval stages; in the

adult, additional neurons begin to express *dFMRFa* (O'Brien et al., 1991), and some of these, including the Tv and several subesophageal neurons, also express *ap* (R. J. B., unpublished observations).

*ap* regulates neurotransmitter phenotype in Tv neurons

We tested the hypothesis that loss of *ap* gene function would affect *dFMRFa* expression in the Tv neurons. SP2 peptide expression was not yet strong enough at the late embryonic/early larval stages to evaluate the role of Ap in the differentiation of that neuron. We tested two independently generated *ap* alleles as homozygotes and as *trans*-heterozygotes. The first is *ap<sup>P44</sup>*, which has a deletion of the 5' exon of the *ap* gene and is transcriptionally null (Bourgouin et al., 1992). The second allele is the *ap<sup>GAL4</sup>* P-element insertion described above, which acts as a protein null allele (O'Keefe et al., 1998). We assayed immunostaining of a *dFMRFa-lacZ* fusion transgene or of *dFMRFa* propeptide in wild-type and in *ap* mutant embryos. The ~8 kb *dFMRFa-lacZ* fusion gene, called pWF3 (Schneider et al., 1993b), included ~5 kb of flanking DNA and was expressed in a pattern very similar to that of *dFMRFa* immunoreactivity (Schneider et al., 1993a; R. J. B. and P. H. T., unpublished data).

We examined *dFMRFa-lacZ* expression in *ap* mutant embryos. Tv neurons first stain with antibodies to the tetrapeptide FMRFa during stage 17 (Schneider et al., 1991). In 18-20 hour embryos (stage 17) heterozygous for the *ap<sup>P44</sup>* allele, *dFMRFa-lacZ* is active in 98% of Tv neurons, assayed by labeling with anti- $\beta$ -gal (Fig. 2; Table 1). In *ap* mutant embryos, the percentage of  $\beta$ -gal<sup>+</sup> Tv neurons ranged from 45% to 57% depending on the specific mutant background. The loss of *dFMRFa-lacZ* activity was restricted to Tv neurons, as  $\beta$ -gal immunoreactivity was observed in other *dFMRFa* cell types not expressing *ap*, like the strongly labeled SE2 neurons

**Table 1. Occurrence of *dFMRFa* and *dFMRFa* reporter activities in Tv and SE2 neurons of wild-type and *ap* mutant embryos and larvae**

	$\frac{ap^{P44}}{+}$	$\frac{ap^{P44}}{ap^{P44}}$	$\frac{ap^{GAL4}}{ap^{GAL4}}$	$\frac{ap^{P44}}{ap^{GAL4}}$
<i>dFMRFa-lacZ</i> (embryos)-Tv	129/132 (98)	73/144 (50)	38/64 (45)	58/102 (57)
<i>dFMRFa-lacZ</i> (embryos)-SE2	46/46 (100)	42/42 (100)	26/26 (100)	36/36 (100)
<i>dFMRFa-lacZ</i> (larvae)				
T1v		16/28 (57)	17/28 (61)	11/19 (58)
T2v		21/28 (75)	26/28 (93)	18/19 (95)
T3v		16/28 (57)	26/28 (93)	18/19 (95)
Total	132/138 (96)	53/84 (68)	69/84 (82)	47/57 (82)
anti- <i>dFMRFa</i> (larvae)-Tv	142/148 (96)	37/60 (62)	54/84 (64)	46/57 (81)
anti- <i>dFMRFa</i> (larvae)-SE2	21/22 (95)	24/24 (100)	23/24 (96)	22/22 (100)
<i>Tv-lacZ</i> (embryos)	99/102 (97)	16/120 (13)	N.D.	N.D.
<i>Tv-lacZ</i> (larvae)				
T1v		3/54 (6)		
T2v		25/54 (46)		
T3v		45/54 (83)		
Total	332/356 (93)	82/180 (46)	N.D.	N.D.

The ratios represent the numbers of marker-positive Tv or SE2 neurons and the total number of hemisegments scored; percentages of marker-positive neurons are shown in parentheses. T1v, T2v and T3v refer to activities in Tv neurons in the first, second and third thoracic segments, respectively. SE2 values indicate those hemisegments with at least 1 positive neuron; in a small number of cases, more than one SE2 neuron was stained. Values not determined are marked as N.D.

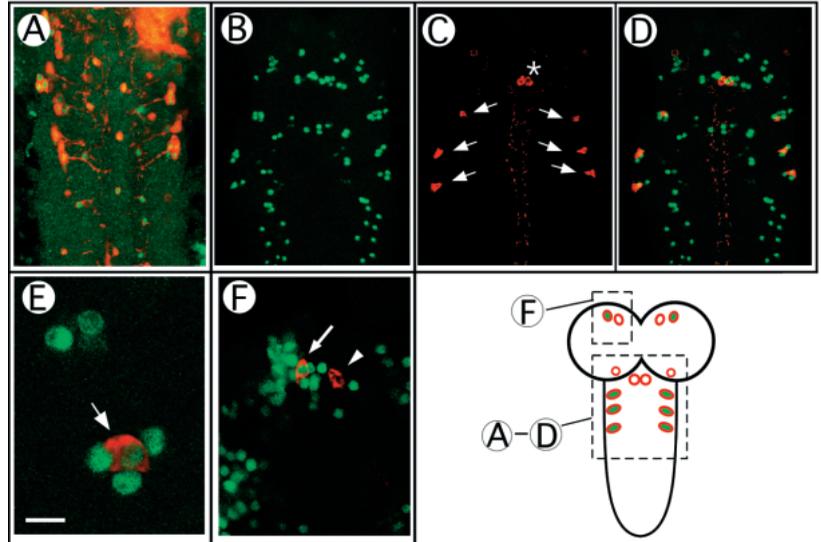
(Table 1) and other, weakly labeled neurons (Fig. 2). We obtained similar results with antiserum to the dFMRFa propeptide in larvae (Table 1). In these experiments, rostral Tv neurons were more affected than caudal Tv neurons.

The loss of *dFMRFa* gene activity in *ap* mutant embryonic Tv neurons was more pronounced when assayed by the activity of a smaller, Tv-specific enhancer. This *Tv-lacZ* transgene consists of a 446 bp Tv neuron-specific enhancer sequence located within the first kB of *dFMRFa* 5' flanking region (Benveniste and Taghert, 1999), linked to a heterologous *hs43* promoter and a *lacZ* reporter. Like *dFMRFa-lacZ*, *Tv-lacZ* was active in 98% of Tv neurons in control embryos, but only 13% of Tv neurons in *ap<sup>P44</sup>* mutant embryos (Fig. 5; Table 1). Together these results indicate that Ap is required for normal initiation of neuropeptide expression by the Tv neurons.

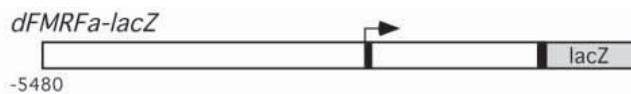
***ap* is not required for the survival or morphological differentiation of the Tv neuron cluster**

Tv neurons may lose *dFMRFa* gene activity in the *ap* mutant background because they require Ap for their generation or early survival. To test this hypothesis, we examined embryos (18-20 hours after egg laying, AEL) that carried both the *ap<sup>GAL4</sup>* insertion and a *UAS- $\tau$ myc* axon-targeted, reporter transgene (Thor and Thomas, 1997). In *ap<sup>GAL4/+</sup>;UAS- $\tau$ myc* embryos, antiserum to the myc epitope labeled the cell bodies and axonal processes (Fig. 3A) of the four to five cells in the clusters that contain the Tv neurons (Fig. 3B). Each cluster included an average of 4.1 *myc<sup>+</sup>* cells. In *ap<sup>GAL4/ap<sup>P44</sup></sup>;UAS- $\tau$ myc* embryos (Fig. 3C), these clusters also included an average of 4.1 *myc<sup>+</sup>* cells (Fig. 3D), suggesting that Ap is not required for generation or survival of the cluster of cells that normally includes the Tv neuron. Furthermore, the somata of the neurons in *ap* mutant clusters

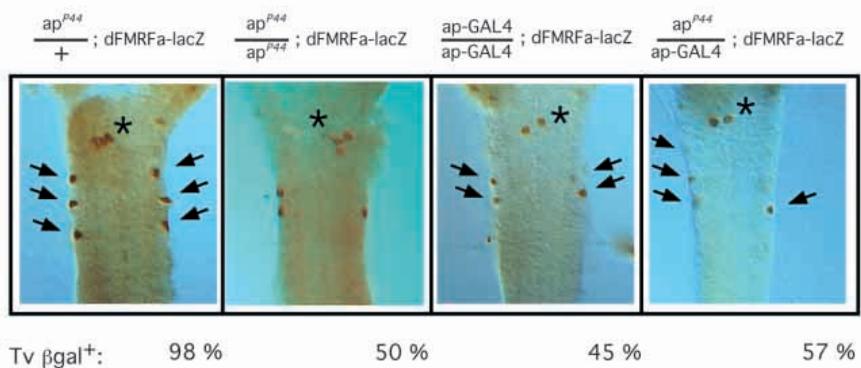
appear to be in the proper locations, and of normal size and shape (Fig. 3D). In addition, we observed labeled axons projecting from the lateral cluster that projected to the midline (Fig. 3C) and that reached the dorsal aspect of the ganglion (data not shown) where they normally enter the neurohemal



**Fig. 1.** Co-expression of *apterous* and dFMRFa in 2 of 17 *dFMRFa* neuronal cell types. (A) The CNS of a *ap<sup>GAL4</sup>/CyO;UAS-*tlacZ** embryo double-labeled with anti-Ap (green) and anti- $\beta$ -gal (red): the *ap<sup>GAL4</sup>* expression pattern accurately represents Ap expression. (B-E) The CNS of an *ap<sup>rk568</sup>/CyO* first instar larva, double-labeled with anti- $\beta$ -gal (green) and anti-dFMRFa propeptide (red). (B) A small number of neurons (~3%) in the nerve cord express Ap. (C) dFMRFa is expressed in Tv neurons (arrows) and in other neurons like the SE2 neurons (asterisk). (D) The merged image shows that Tv neurons co-express Ap and dFMRFa, while the SE2 and other dFMRFa neurons do not. (E) A higher magnification view of the ventrolateral Ap-positive cluster that includes the Tv neuron (arrow); the cytoplasm is stained with anti-dFMRFa and the nucleus is stained with anti- $\beta$ -gal. (F) Protocerebral region of the brain of *ap<sup>rk568</sup>/CyO* larva, double-labeled with anti- $\beta$ -gal (green) and anti-dFMRFa propeptide (red). The SP2 interneuron is double-labeled (arrow), while the SP1 interneuron is not (arrowhead). The drawing illustrates the positions of A-D and F on a schematic of the larval CNS. The positions of dFMRFa cells visible in this figure are shown in red outline; those dFMRFa neurons filled with green are *ap*-positive. Scale bar equals 40  $\mu$ m (A-D), 4  $\mu$ m (E) and 10  $\mu$ m (F).



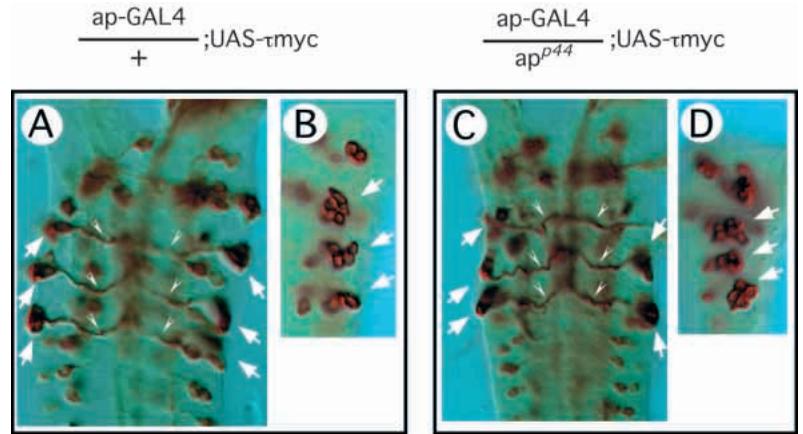
**Fig. 2.** Effects of *ap* mutations on *dFMRFa-lacZ* expression. Whole-mount immunostains of CNS from 18-20 hour embryos carrying the *dFMRFa-lacZ* transgene, labeled with anti- $\beta$ -gal. The schematic indicates the fragment of *dFMRFa* included in the construct; -5480 refers to bp before the transcription start site. In *ap<sup>P44</sup>* heterozygotes (left), the six Tv neurons (arrows) as well as the other neurons that express *dFMRFa*, including the SE2 neurons (asterisks), are nearly always  $\beta$ -gal<sup>+</sup>. In *ap<sup>P44</sup>* homozygotes, *ap<sup>GAL4</sup>* homozygotes, or *ap<sup>P44/ap<sup>GAL4</sup></sup>* trans-heterozygotes, fewer Tv neurons are  $\beta$ -gal<sup>+</sup>, but other neurons, such as the SE2 neurons, are not affected.



organs (NHO; Schneider et al., 1993a,b; Gorczyca et al., 1994). In *ap* mutant embryos and larvae, we observed myc-stained processes in the NHO, consistent with the hypothesis that one or more neurons, presumably including the Tv cells, are capable of reaching their normal point of axonal termination. Together, these observations are consistent with the hypothesis that Tv neurons show proper axonal pathfinding and morphological differentiation in *ap* mutant embryos.

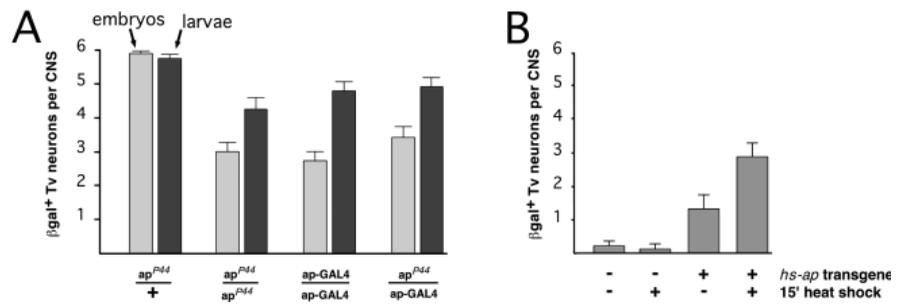
***ap* is required for maintenance of neuropeptide expression in Tv neurons**

We asked whether Ap continues to regulate *dFMRFa* expression in Tv neurons during postembryonic stages by increasing and decreasing Ap levels in separate experiments. We first analyzed *ap* loss-of-function phenotypes. *dFMRFa-lacZ* was active in 97% of Tv neurons in wild-type first instar larvae that were within 10 hours of hatching. In comparable *ap* mutant larvae, the percentage of  $\beta$ -gal<sup>+</sup> Tv neurons ranged from 68% to 82% in different mutant backgrounds (Fig. 4A; Table 1). Some Tv neurons in *ap* mutants scored as  $\beta$ -gal positive expressed reporter more



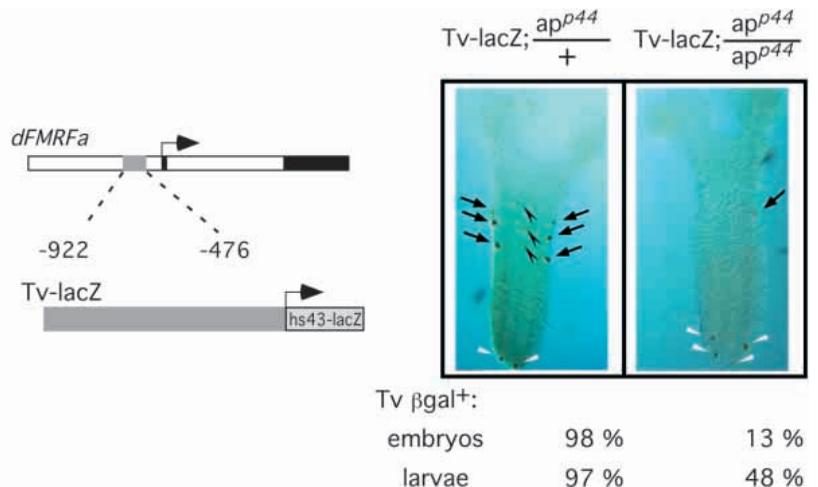
**Fig. 3.** Survival and morphology of Tv neurons in *ap* mutant embryos. (A,B) Whole mount of a CNS from an 18-20 hour AEL *ap*<sup>GAL/+</sup>; *UAS-τmyc* embryo, labeled with anti-myc. (A) The thoracic ventrolateral clusters (arrows) that include the Tv neurons are stained. All neurons in this cluster project axons in a single bundle (arrowheads); two axons, including the Tv axon, diverge to the dorsal midline. (B) Higher magnification view of clusters (arrows) in an *ap*<sup>GAL/+</sup>; *UAS-τmyc* embryo. An average of 4.1 cells was counted in each of 21 clusters. (C-D) Whole mount of a CNS from an 18-20 hour AEL *ap*<sup>GAL/ap<sup>p44</sup></sup>; *UAS-τmyc* embryo, labeled with anti-myc. (C) The ventrolateral clusters show proper positioning (arrows) and axonal bundles (arrowheads) appear normal. (D) Higher magnification view of clusters (arrows) in an *ap*<sup>GAL/ap<sup>p44</sup></sup>; *UAS-τmyc* embryo. An average of 4.1 cells was counted in each of 15 clusters, the same number as in *ap* heterozygotes.

**Fig. 4.** Ap regulates *dFMRFa* expression in postembryonic Tv neurons. (A) Histograms showing the proportions of Tv neurons that express the *dFMRFa-lacZ* fusion gene in 18-20 hour embryos (light bars) and in ~10 hour first instar larvae (dark bars). Values shown are means ±SE. Both *ap* mutant embryos and larvae show decreased *dFMRFa-lacZ* activity in Tv neurons relative to heterozygous controls, but the phenotype is less pronounced in larvae than in embryos. Values were compared by a two-tailed, heteroscedastic *t*-test. For each



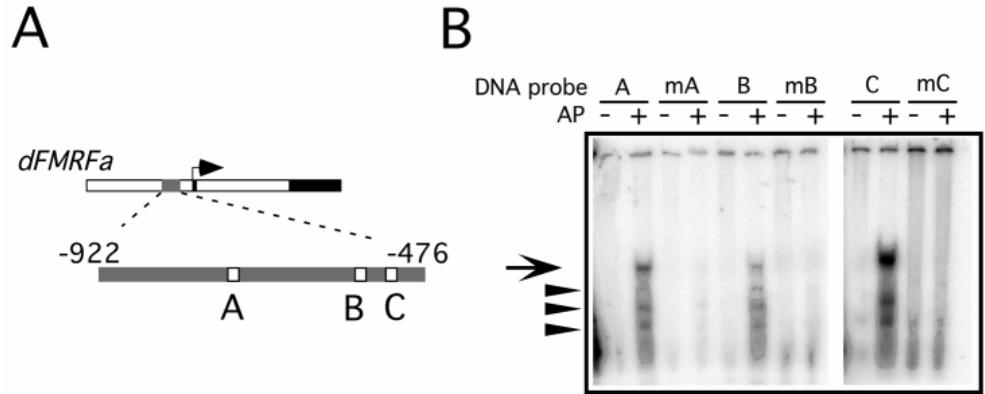
comparison of *ap* mutants with *ap*<sup>p44</sup> heterozygotes, *P*<0.01; for all comparisons of *ap* mutant embryos with *ap* mutant larvae of the same genotype, *P*<0.01. (B) Histogram showing the proportions of larval Tv neurons that show *Tv-lacZ* reporter activity, as assayed with X-Gal histochemical staining. 2 hours after a 15 minute heat shock, activity in the Tv neurons increased in larvae carrying the *hs-ap* transgene, but not in larvae that do not have the *hs-ap* transgene (*P*<0.01 by two-tailed, heteroscedastic *t*-test). The number of animals studied ranged from 15 to 24.

**Fig. 5.** Effects of *ap* mutations on expression of the *Tv-lacZ* transgene. Left: Schematic of the *dFMRFa* gene, showing the 446 bp *Tv*-specific enhancer (gray) located between bp -922 and -476 relative to the transcription start site (bent arrow). Right: Whole mounts of 18-20 hour AEL embryonic CNS carrying the *Tv-lacZ* transgene, labeled with anti- $\beta$ -gal. Percentages of Tv neurons in embryos and larvae that are  $\beta$ -gal<sup>+</sup> are shown below. In *ap*<sup>p44</sup> heterozygotes (left image), the *Tv-lacZ* transgene is active in nearly all Tv neurons (arrows) and in two to four ectopic abdominal neurons that do not express *dFMRFa* (white arrowheads). Black arrowheads indicate immunoreactivity in the Tv axon terminals of the NHOs. In *ap*<sup>p44</sup> homozygotes (right), *Tv-lacZ* is active in only 13% of embryonic Tv neurons and 48% of larval Tv neurons, but expression in ectopic abdominal neurons (white arrowheads) is not affected.



**Fig. 6.** Direct interaction between Ap and the Tv-specific enhancer of the *dFMRFa* gene promoter.

(A) Schematic of the *dFMRFa* gene, showing the 446 bp Tv neuron-specific enhancer (gray) located between bp -922 and -476 relative to the transcription start site (bent arrow). The Tv-specific enhancer contains three TAAT motifs (labeled A, B and C). (B) EMSA showing that a truncated Ap protein (AP) binds in vitro to each of three oligonucleotide probes corresponding to wild-type A, B and C sequences, respectively (arrow), but not to mutated A, B or C sequences (mA, mB and mC).



Smaller binding complexes (arrowheads) may represent binding of incomplete Ap molecules.

weakly than normal (data not shown). As in embryos, the loss of *dFMRFa-lacZ* activity in larvae was restricted to Tv neurons and not to other *dFMRFa* cell types normally present at that stage. *Tv-lacZ* activity was also affected in larvae: in *ap<sup>P44</sup>* mutant larvae, *Tv-lacZ* was active in 46% of Tv neurons (as compared with 13% in *ap<sup>P44</sup>* mutant embryos; Table 1). The severity of the *dFMRFa-lacZ* and *Tv-lacZ* phenotypes were correlated with segmental identity; rostral Tv neurons were affected more frequently than caudal Tv neurons.

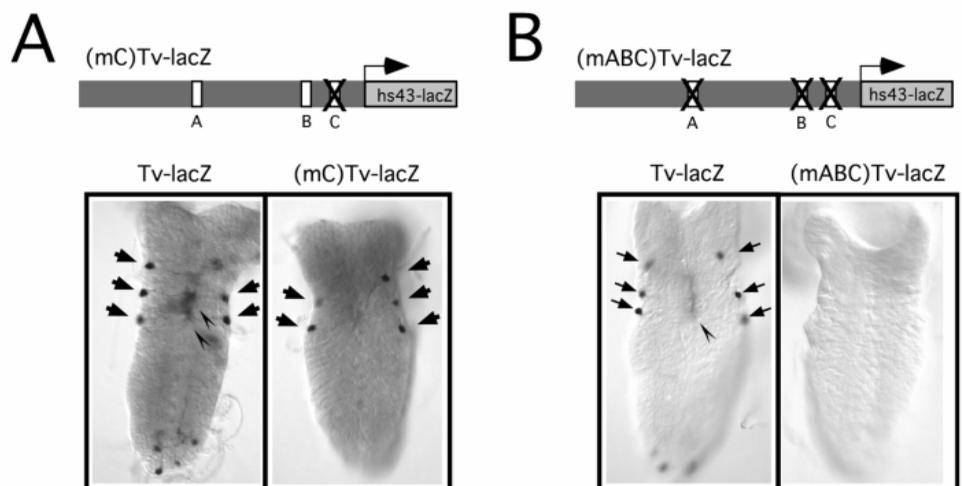
In a second set of experiments, we next asked if increased levels of Ap would increase moderate levels of *dFMRFa* gene activity in a wild-type genetic background. Under the staining conditions that we used, wild-type first instar larvae carrying one copy of a *Tv-lacZ* transgene showed  $\beta$ -gal activity in an average of 0.24 Tv neurons per CNS (Fig. 4B). At 24°C,  $\beta$ -gal activity was detected in an average of 1.3 Tv neurons in larvae carrying one copy of the *Tv-lacZ* transgene and one copy of a *hs-ap* transgene (Bourgouin et al., 1992); we ascribe this increase to low-level, constitutive expression of the *hs-ap* at 24°C. Following a 15 minute induction and 2 hour recovery period,  $\beta$ -gal activity increased to an average of 2.8 Tv neurons in larvae carrying the *hs-ap* transgene, but did not increase in *Tv-lacZ* larvae that lacked the *hs-ap* transgene (Fig. 4B). We did not observe ectopic *dFMRFa* gene activity when Ap was expressed ubiquitously using the *hs-ap* transgene, or when Ap

was expressed throughout the CNS using a combination of *elav-GAL4* and *UAS-ap* transgenes (data not shown). Together these results indicate that in the wildtype Tv neuron, *dFMRFa* expression is sensitive to Ap levels.

#### Mechanisms of *dFMRFa* gene regulation by *ap*

We tested the hypothesis that Ap regulates *dFMRFa* in the Tv neurons directly by seeking potential Ap-binding sites within the *dFMRFa* gene regulatory sequences. We limited the search to the 446 bp Tv neuron-specific enhancer, which is highly responsive to Ap levels (Fig. 5) and located in the 5' flanking region. The 446 bp enhancer contains three sequences corresponding to the six-nucleotide consensus binding site for homeodomain proteins (TAATNN – Gehring et al., 1994; Fig. 6A). All three of these sequences are shared between the homologous regions of the *dFMRFa* genes of two *Drosophila* species, *D. melanogaster* and *D. virilis* (Taghert and Schneider, 1990). We used electrophoretic mobility shift assays (EMSA) to test the ability of Ap protein to bind in vitro to these three sequences, as represented by three different 25 bp oligonucleotide probes. Recombinant Ap homeodomain bound all three oligonucleotide probes with different affinities (Fig. 6B, lanes A, B and C), and at stoichiometries comparable to those observed for other LIM homeoproteins binding in vitro (Karlsson et al., 1990; Wang and Drucker, 1995). Ap binding

**Fig. 7.** In vivo activity of the *Tv-lacZ* transgene following mutagenesis of Ap-binding sequences. Whole mounts of X-Gal-stained CNS from first instar larvae homozygous for wild-type or mutant *Tv-lacZ* transgenes. The two schematics indicate the sites in the Tv-specific enhancer where mutations were introduced (marked by Xs). (A) Activity from wild-type *Tv-lacZ* sequence and (*mC*)*Tv-lacZ* with a mutated C element: both constructs display strong to moderate reporter gene expression in the Tv neuronal cell bodies (arrows) and terminals (black arrowheads). (B) Activity from wild-type *Tv-lacZ* and (*mABC*)*Tv-lacZ*. The latter construct contains mutations in all three Ap-binding elements (altering a total of 18 of 446 bp) and displays no enhancer activity in the CNS.



to these probes in vitro was sequence-specific: mutant oligonucleotide probes, with clusters of 6-point mutations replacing the TAATNN sequences did not bind Ap in these assays (lanes mA, mB and mC in Fig. 6B).

We then asked whether these Ap-binding sequences were functionally important in vivo. We synthesized two mutant *Tv-lacZ* constructs incorporating the same clustered point mutations in the Ap-binding sequences used in the EMSA. In first instar larvae, a construct containing mutations in Ap-binding site C [(mC)*Tv-lacZ*] showed slightly decreased activity in Tv neurons and in ectopic cells relative to the wild-type enhancer (Fig. 7A). Construct (mABC)*Tv-lacZ*, which included mutations in all three Ap-binding sequences, showed no detectable activity in Tv neurons or ectopic cells (Fig. 7B). These results show that at least two of the three elements within the Tv neuron-specific enhancer that bind Ap in vitro are critical for proper enhancer activity in vivo.

## DISCUSSION

In this study, we showed that Ap, a LIM homeodomain transcription factor, regulates expression of the neurotransmitter *dFMRFa* in a specific cell type, the Tv neuroendocrine cells. These results present an explanation for cell-type-specific *dFMRFa* regulation: it derives from partial overlap with restricted *ap* expression. The analysis also provides a basis for deducing more general principles of LIM homeodomain regulation of neuronal differentiation. *ap* displays partial expressivity and direct binding to the target neurotransmitter gene; it has little effect on properties of the Tv neurons other than transmitter phenotype, but has sustained effects in the mature neurons. These features are informative regarding the organization of regulatory cascades that coordinate the production of distinct cellular phenotypes.

### ***ap* regulates initial and maintained neurotransmitter expression in the Tv neurons**

Loss of *ap* function did not produce consistent loss of *dFMRFa* expression by all Tv neurons, but reliably caused a change in the transmitter properties of some. This observation suggests that several factors, of which Ap is one, normally contribute to the proper initiation of *dFMRFa* gene activity in Tv neurons. Alternatively, mechanisms that partially compensate for *ap* may operate in the mutant backgrounds. The first hypothesis is consistent with studies of other LIM homeodomain genes like *ttx-3* or *lin-11* of *C. elegans*, whose specific influences on neural phenotypes may be complete or partially penetrant (Hobert et al., 1997, 1998). Previous studies of *ap* mutant phenotypes have also indicated a range in severity (Bourgouin et al., 1992; Lundgren et al., 1995). Deficiencies in neuropeptide synthesis, stability, transport or release could explain decreased *dFMRFa* expression in *ap* mutant Tv neurons. We favor the hypothesis that the deficits were largely in neuropeptide synthesis because expression of a *dFMRFa-lacZ* fusion transgene was decreased in *ap* mutants. Both loss-of-function and gain-of-function experiments argue that *ap* exerts an influence in mature neurons, well past the stage of initial transmitter selection. Other LIM homeoproteins (e.g., Islet-1) regulate early stages of cellular differentiation (Ahlgren et al., 1997), and are suggested to participate in the

maintenance or modulation of gene expression in mature cells (Thor et al., 1991; Wang and Drucker, 1995). The *ap* mutant phenotype in postembryonic stages was less severe: such diminished *ap* influence indicates upregulation of alternative activating mechanisms postembryonically within the Tv neurons. The availability of conditional *ap* mutations (Stevens and Bryant, 1986) should allow further definition of these temporal changes in neurotransmitter regulation.

### ***ap* and *dFMRFa* expression patterns overlap partially**

We found that *ap* is expressed in more than 100 neurons in the larval CNS, but that *dFMRFa* is expressed in only eight of these. Therefore, co-factors must be required to activate *dFMRFa* transcription in the Tv neurons or to repress *dFMRFa* transcription in other neurons that express Ap. Two lines of evidence suggest that positively acting co-factors are required for *dFMRFa* gene activation by Ap. First, widespread ectopic expression of Ap (ubiquitously or throughout the CNS) did not induce ectopic *dFMRFa* expression. Second, Ap expression in embryonic Tv neurons begins soon after the birth of the cell (Lundgren et al., 1995) and precedes *dFMRFa* expression by at least 3–6 hours (Schneider et al., 1991). LIM homeoproteins heterodimerize with other transcription factors (e.g., Xue et al., 1993; Lichtsteiner and Tjian, 1995) and with adaptor proteins (e.g., Jurata et al., 1996; Morcillo et al., 1997). It will be useful to employ genetic methods to search for Ap-interacting proteins in Tv neurons that may participate in this cell-specific mechanism.

Previous studies of *dFMRFa* transcription have suggested a model in which different regulatory regions of the gene direct its expression by different neurons, termed cell-type-specific regulation (Schneider et al., 1993b, R. J. B. and P. H. T., unpublished data). Our study of *ap* is consistent with this general conclusion: neurons that share a common neurotransmitter phenotype but that occupy different positions within the nervous system, employ different mechanisms of transcriptional control. Control of neurotransmitter phenotypes in *C. elegans* also involves cell type-specific regulatory mechanisms: the UNC-30 homeodomain protein is required for normal expression of GABA by many GABAergic neurons, but several GABAergic cells do not normally express UNC-30 and are not affected by *unc-30* mutations (Jin et al., 1994). Likewise, in *Drosophila*, different transcription factors bind *DOPA decarboxylase* regulatory sequences in different neuronal cell types (Johnson and Hirsh, 1990; Lundell and Hirsh, 1992). Cell type-specific regulatory mechanisms do not preclude the participation of more widely expressed transcription factors like Ap. For example, *unc-30* is not exclusively associated with GABAergic cells, suggesting that co-factors in GABAergic neurons must provide necessary instructions for production of that transmitter phenotype.

### ***ap* is not required for survival or early morphogenesis of Tv neurons**

The observation of decreased *dFMRFa-lacZ* activity in Tv neurons of *ap* mutants was consistent with either a specific loss of neurotransmitter expression or a more general change in Tv neuronal cell fate, by *trans*-determination or death. Previous studies of LIM homeoproteins offer precedents for different possibilities (e.g., Pfaff et al., 1996; Porter et al., 1997; Way

and Chalfie, 1988; Thor and Thomas, 1997). We concluded that Tv neurons survived in *ap* mutants because the number of cells in the ventrolateral clusters of wild-type and *ap* mutant embryos was constant. Furthermore, in *ap* mutants, the four cells of the lateral clusters displayed normal sizes, shapes and positions, and continued to express an *ap*-transgene; these results suggest that the Tv neurons were not *trans*-determined in *ap* mutants. To what extent were other Tv cell properties affected by loss of *ap* function? The Islet LIM homeoprotein co-ordinately regulates both neurotransmitter phenotype and axon guidance in serotonergic neurons of *Drosophila* (Thor and Thomas, 1997); in other *Drosophila* neurons of the ventral ganglion, *ap* regulates axon guidance (Lundgren et al., 1995). In contrast, we deduced that axon guidance by the neurons of the Tv cell cluster was normal in *ap* mutant embryos. The four axon bundle displayed normal orientation to the dorsal midline and reached the target NHO: both observations indicate a normal morphology. However, because we have no suitable marker to identify Tv neurons individually and prior to the differentiation of their transmitter phenotype, we could not positively identify the *ap*-mutant Tv axon among the tightly fasciculated axons, nor could we exclude the possibility that the mutant Tv axon occasionally stalled within the bundle.

A relation between the proper formation of Tv axon terminals in the NHO and *dFMRFa* expression has previously been suggested: animals mutant for the *tinman* homeobox gene lack the NHO due to the absence of non-neuronal cells that normally form this structure. In that mutant background, very few FMRFa-positive Tv neurons are seen in the embryonic CNS prior to the death of the animals (Gorzyca et al., 1994), although whether Tv neurons survive in *tinman* mutant animals is not known. In addition, mutations in the *C. elegans ap* homologue, *ttx-3*, lead to formation of synaptic terminals of normal appearance though of abnormal physiologic function (Hobart et al., 1997). These findings suggest that a LIM homeoprotein may affect transmitter phenotype indirectly, via effects on the formation of axon terminals.

In the case of *ap* regulation of *dFMRFa* in the Tv neurons, we believe the hypothesis of indirect action is incorrect for four reasons. First, in *ap* mutant animals, neurons in the Tv cluster form axon terminals of normal appearance in the NHO. Second, failure of Tv *dFMRFa* expression in *tinman* mutant larvae may reflect cell death or a failure to maintain neuropeptide expression, and not necessarily a failure in its normal initiation. Third, Ap binds the *dFMRFa* promoter with sequence specificity in vitro. Fourth, our demonstration that *dFMRFa* expression in wild-type Tv neurons is sensitive to Ap levels suggests that Ap can influence neuropeptide gene expression in addition to any potential signals resulting from cell interactions. Future experiments, employing different selective markers of the Tv neurons, will help evaluate the relationship between connectivity and transmitter expression in Tv neurons. Although we emphasize caution in the interpretation, our current evidence strongly argues that Ap regulation of neurotransmitter expression in Tv neurons occurs independently of effects on cell morphology. We suggest that the role of *ap* in Tv neuron development is thus limited in scope and favor the hypothesis that Tv neuronal differentiation represents a mosaic of mechanisms each controlling separate differentiation events (Desai et al., 1988; Lo et al., 1998). In this regard, we propose that *apterous* contributes to a

regulatory cascade dedicated to transmitter production, and that it has little or no influence on other properties displayed by mature Tv neurons.

### ***ap* regulates *dFMRFa* directly**

We presented evidence that Ap regulates *dFMRFa* gene expression in Tv neurons at least in part by direct activation. Specifically, we demonstrated that Ap binds to each of three sequences within the 446 bp Tv neuron-specific enhancer in vitro and that these sequences are important in vivo. While these results are consistent with direct activation of the *dFMRFa* gene, they do not preclude the possibility that Ap also regulates transcription via other genes, as in the case of *even-skipped* regulation by *bicoid* (Struhl et al., 1989; Small et al., 1991). Mutation of one of the Ap-binding sequences decreased enhancer activity moderately in larval Tv neurons, while mutations of all three Ap-binding sites eliminated all enhancer activity. Mutagenesis of single binding sites in cell type-specific enhancers often preserves some activity in vivo (e.g., Small et al., 1992; Capovilla et al., 1994). Our observation that mutations in all three binding sites totally abolished *Tv-lacZ* activity was surprising, since wild-type *Tv-lacZ* was still active in 46% of Tv neurons in larvae homozygous for the *ap*<sup>P44</sup> null allele. To explain this result, we propose that other transcription factors expressed by the Tv neurons (probably of the homeodomain class – see Biggin and McGinnis, 1997) can bind to and activate at least one of the three Ap-binding sites in *Tv-lacZ*. Thus our results are consistent with the supposition that *ap* is one of several factors that bind to and regulate the Tv-specific enhancer in vivo.

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