The Drosophila basic helix-loop-helix protein DIMMED directly activates PHM, a gene encoding a neuropeptide-amidating enzyme

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The Drosophila Basic Helix-Loop-Helix Protein DIMMED Directly Activates PHM, a Gene Encoding a Neuropeptide-Amidating Enzyme

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The basic helix-loop-helix (bHLH) protein DIMMED (DIMM) supports the differentiation of secretory properties in numerous peptidergic cells of Drosophila melanogaster. DIMM is coexpressed with diverse amidated neuropeptides and with the amidating enzyme peptidylglycine α-hydroxylating monooxygenase (PHM) in approximately 300 cells of the late embryo. Here we confirm that DIMM has transcription factor activity in transfected HEK 293 cells and that the PHM gene is a direct target. The mammalian DIMM orthologue MIST1 also transactivated the PHM gene. DIMM activity was dependent on the basic region of the protein and on the sequences of three E-box sites within PHM’s first intron: the sites make different contributions to the total activity. These data suggest a model whereby the three E boxes interact cooperatively and independently to produce high PHM transcriptional activation. This DIMM-controlled PHM regulatory region displayed similar properties in vivo. Spatially, its expression mirrored that of the DIMM protein, and its activity was largely dependent on dimm. Further, in vivo expression was highly dependent on the sequences of the same three E boxes. This study supports the hypothesis that DIMM is a master regulator of a peptidergic cell fate in Drosophila and provides a detailed transcriptional mechanism of DIMM action on a defined target gene.

† Supplemental material for this article may be found at http://mcb.asm.org.

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Neurons release numerous biologically active transmitters, including neuropeptides, which are derived from larger precursor proteins. Certain neurons termed neurosecretory cells (NSCs) are specialized in neuropeptide production and have greatly amplified secretory capabilities that are akin to those of peripheral endocrine cells. NSCs play essential roles in animal development prior to terminal differentiation (e.g., cell migration and neurogenesis) and are characterized by their ability to release biologically active peptides that can act at long distances. They represent a distinctive neuronal class specialized to produce, package, and release large amounts of such signaling molecules. Additionally, NSC properties can be strongly modified in response to changes in environment or internal homeostasis (9). Examining the intracellular regulatory pathways that organize and modulate these specialized properties is therefore critical to understanding NSC physiology.

In vertebrates, several transcription factors have been identified that are required for the proper differentiation of neuroendocrine cells in the brain and periphery, including Mash1, Sim1, Otp, and others (1, 8, 24, 26, 31, 32, 36, 47, 52). In the case of Sim1, this requirement involves early aspects of development prior to terminal differentiation (e.g., cell migration [56]). In Drosophila melanogaster, only the basic helix-loop-helix (bHLH) protein DIMMED (DIMM) has so far been implicated in the development of peptidergic cells (2, 17, 20, 21). The Drosophila genome contains at least 30 neuropeptide precursor-encoding genes; remarkably, more than 90% of the known or predicted Drosophila neuropeptides are amidated (22). In the Drosophila central nervous system (CNS), neuropeptides are expressed by cells at all axial levels and individual neuropeptide genes are typically expressed by small subsets among the approximately 10,000 neurons of the larval CNS. DIMM displays a highly regulated pattern of expression in approximately 300 diverse larval CNS neurons. DIMM neurons (and the few DIMM-positive peripheral cells) are chemically heterogeneous but share certain properties: (i) individual DIMM cells contain large amounts of neuropeptides (as indicated by immunocytochemistry), and (ii) DIMM cells all express high amounts of the critical neuropeptide biosynthetic enzyme peptidylglycine α-hydroxylating monooxygenase (PHM), which is required for peptide α-amidation.

The results of previous studies of DIMM have paralleled those for regulatory factors for other transmitters. For example, PET-1 (an ETS domain transcription factor) coordinates the terminal differentiation of the serotonergic phenotype and is precisely expressed by the precursors to the vast majority of serotonergic neurons, but not elsewhere (18). Likewise, the paired-like homeodomain proteins Phox2a and Phox2b have been implicated as essential determinants of the noradrenergic phenotype (35, 41). However, in contrast to the situations for serotonergic and noradrenergic cell fates, in which all cells sharing the same fate express the same transmitter, the NSC cell fate represents the expression of many different secretory peptides by diverse cells. We estimate that there are at least 31
different neuropeptide genes in the Drosophila genome (51). Thus, NSC cell differentiation must include both cell type-specific features (e.g., neuropeptide selection) and generic features that support the expression, packaging, and release of amitidated neuropeptides. Reflecting this fact, we previously concluded that, within single neurons, DIMM operates in two distinct mechanisms: one is termed combinatorial and the other a form of master regulation (3).

In Drosophila, the selection of an appropriate neuropeptide gene is controlled by combinations of transcription factors. The compositions of codes are different for different cells, but many include DIMM as a constant element to help drive the selection of different neuropeptide precursors. This DIMM activity represents the first mechanism by which it regulates NSC development. For example, in identified Ty neuroendocrine neurons of the CNS, dFMRFa neuropeptide expression depends on DIMM but also on the LIM homeodomain protein Apterous (AP) (6) and the zinc-finger protein SQUEEZE (SOZ) (3, 40). No single member of this three-factor code produces ubiquitous dFMRFa expression when broadly misexpressed, and the triple coexpression of dimm, ap, and sqz is more effective at producing ectopic dFMRFa. Thus, different sets of positively acting factors combine with DIMM to drive specific neuropeptide gene expression in different neurons (19, 33, 40).

Independent of combinatorial codes, DIMM exerts a second critical influence, its so-called master regulatory role, on developing NSCs (2). DIMM displays singular control of properties held in common by diverse NSCs and has the ability to impose them onto non-NSCs. For example, DIMM misexpression throughout the entire CNS confers PHM expression (a common NSC property) on all neurons. Other common NSC features controlled by a master regulator like DIMM could involve mechanisms of precursor processing and routing and of dense-core secretory granule generation, trafficking, and accumulation. Therefore, the current conception of NSC cell organization supposes two interlocked regulatory networks (combinatorial codes and single master regulator) operating within single cells.

Previous work has demonstrated that DIMM controls the expression of the neuropeptide biosynthetic enzyme PHM (15, 28). This monoxygenase is most closely related to dopamine-β-hydroxylase (DBH) and is selective and required for secretory peptide C-terminal amidation (25). Loss-of-function genetic studies indicate that DIMM normally controls PHM (21), while gain-of-function studies show that it can confer ectopic PHM expression efficiently on all neurons (2). We used in vitro and in vivo experiments to show that DIMM directly activates PHM via three specific E boxes located in the first intron of PHM. Furthermore, we extend these results by showing that the same cis mechanism operates robustly in vivo and can explain in large part the specific high-level expression of PHM in peptidergic neurons and endocrine cells. Finally, we demonstrate that the mammalian orthologue Mist1 is capable of transactivating Drosophila PHM both in cell lines and in transgenic flies. Thus, DIMM and Mist1 share functional as well as sequence attributes, and the regulatory features we describe for specialized secretory cells in Drosophila are likely to be broadly applicable across animal phyla.

MATERIALS AND METHODS

Fly stocks. The following fly lines were used in this study: dimm mutant allele (Rev4 and Rev8); UAS (upstream activation sequence)-dimm-myc (21); UAS-2X EGFp, UAS-mist1, and ap-gal4 lines, PHM-gal4, PHM-GFP, PHM-E1M-gal4, PHM-E2M-gal4, PHM-E4M-gal4, PHM-E2A4M-gal4, and PHM-E124M-gal4 lines were established for this study and are described below. Two plasmids, pGT-gal4 and pStinger (4), were used for gal4 and green fluorescent protein (GFP) constructs, respectively. Some PHM-gal4 transgenic flies were generated by a microinjection into w1118 embryos, and the other transgenic flies were established commercially by Model Systems Genomics (http://www.biology.duke.edu/model-systems/).

Luciferase assay. PHM fragments were subcloned into the pGL3 and sv40-pGL3 luciferase (luc) vectors (Promega, Madison, WI). To avoid nonsensed-mediated decay, the translation start site of PHM was mutated by site-directed mutagenesis. Drosophila bHLH proteins (DIMM, DIMM-MB, Daughterless [DA], Atonal [Ato], HLH4C, and NAUTILUS [NAU]), the LIM homeodomain protein APTEROUS (AP), and the mammalian bHLH Mist1 were subcloned into the hemagglutinin (HA)-tagged pCDNA3 vector. dimm-mb encodes a DIMM protein with a mutated basic region that is projected to display minimal DNA binding. Three amino acids of the basic region of DIMM were changed to glycines (R164G, E165G, and R165G); the primers used for the site-directed mutagenesis are listed in Table S1 in the supplemental material. For luciferase assays, HEK 293 cells were transiently transfected with 2.1 μg of DNA using Lipofectamine 2000 (Invitrogen, San Diego, CA) and the activity of cell lysates was measured with a Victor-Wallac2 plate reader (Perkin-Elmer, CA). For each experiment, a vector containing the thymidine kinase-Renilla luciferase gene (TK-RLuc) was cotransfected for normalization, and each transfection was performed at least three times independently.

Site-directed mutagenesis. We performed site-directed mutagenesis following the manufacturer’s protocols (Stratagene) using the set of primers listed in Table S1 in the supplemental material. Mutagenesis was performed in the pGEM-TA vector, and sequence variants were then subcloned into the pGL3 vector (both from Promega, Madison, WI). All constructs were confirmed by sequencing.

DNA-protein interaction assays. Electrophoretic mobility shift assays (EMSA) were performed as previously described (29). A double-stranded DNA probe corresponding to the E1 E box (see below) was radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP and then column purified. In vitro-translated proteins were prepared following the manufacturer’s protocols using a TNT kit (Promega, Madison, WI). The binding reaction mixtures contained 5 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM MgCl2, 1 mM dithiothreitol, 2% glycerol, 0.5 μg of poly(dI-dC), 3 μl of in vitro-translated protein, and radiolabeled DNA probe (25-μl final volume) were incubated at room temperature for 30 min. For competition assays, the reaction mixtures included 20 pmol of cold competitor. For supershift assays, the reaction mixtures included 1 μl of antibody and were preincubated for 30 min on ice. After incubation, all reaction mixtures were analyzed by 6% nondenaturing gel electrophoresis, which was run at 200 V at 4°C for 4 h. Signals were detected by autoradiography. The primers are listed in Table S1 in the supplemental material.

Antibodies, immunocytochemistry, and fluorescence imaging. Affinity-purified guinea pig anti-Dimm (1:200) (2), mouse monoclonal anti-GFP 3E6 (1:100; Molecular Probes, Carlsbad CA), rabbit anti-Mist1 (1:250) (29), rabbit anti-FMRAmide (1:1,000) (50), and rabbit anti-GFP (1:500, rabbit polyclonal, catalogue no. AB3080; Chemicon, Temecula, CA) as primary antibodies and Cy3-conjugated or Alex-488-conjugated secondary antibodies (Jackson Immuno- research, West Grove, PA) were used for immunocytochemistry. Immunostaining methods were as previously described (21). Images were acquired on an Olympus FV500 confocal laser scanning microscope and manipulated by Adobe Photoshop software to adjust contrast and/or levels.

RESULTS

DIMM activates PHM in vitro. Our previous data indicated that dimm activity is required for the expression of PHM. To test the possibility that such regulation is direct, we tested four genomic regions around the PHM locus for enhancer activity by creating luciferase fusion constructs and transfecting them into mammalian HEK 293 cells (Fig. 1A). These regions included the complete intergenic domain (249 bp) between PHM and its nearest annotated neighbor and the first intron, which includes an unexpectedly large number of E boxes (see below).
The two larger constructs (containing ~0.8 and 1.0 kb of \( \text{PHM} \), respectively) displayed fivefold or greater increases in luciferase activity when \( \text{dimm} \) was cotransfected. In contrast, the two smaller constructs (~0.2 and 0.4 kb of \( \text{PHM} \)) did not respond to cotransfection of \( \text{dimm} \). We then asked whether cotransfection of \( \text{dimm} \) and the proneural E protein bHLH daughterless gene \( \text{da} \) would increase \( \text{PHM} \) transactivation since \( \text{da} \) encodes a putative heteromeric binding partner (10, 11, 13, 14). DA did not interact with DIMM in our previous in vitro biochemical measures (2). While the cotransfection of \( \text{dimm} \) with \( \text{da} \) increased \( \text{PHM} \) transactivation compared to the levels in controls, it was always to a level lower than that of the response to transfection of \( \text{dimm} \) alone (Fig. 1B). Next, we evaluated the specificity of DIMM’s transcriptional activity by comparing the response to this \( \text{PHM} \) fragment to that for cotransfection with other transcription factors. All four additional bHLH proteins tested (DA, NAU, ATO, and HLH4C) failed to activate the 0.8-kb \( \text{PHM} \) fragment, as did the LIM-HD protein AP (Fig. 1C). The mammalian protein Mist1 includes a bHLH domain with ~90% identity to that of DIMM (29). Mist1 increased \( \text{PHM} \)-luc activity significantly, although not to the same extent as DIMM (Fig. 1C). Finally, to ask whether DIMM’s DNA binding properties were required for its specific transactivating properties, we mutated the basic region within the binding domain of DIMM; we observed little residual activity (Fig. 1D).

In summary, we found that a small \( \text{PHM} \) fragment is transactivated by DIMM following transient transfection in a heterologous cell line. Furthermore, these activities are specific, they are not augmented by cotransfection of a class I (E class) bHLH, and they appear to require DNA binding by DIMM.

**Three E boxes within the first intron of \( \text{PHM} \) are necessary for activation by DIMM.** Next, we searched for critical cis-regulatory elements within the \( \text{PHM} \) genomic fragment by functional assay and by DIMM binding. We found that the ~400-bp first intron of the \( \text{PHM} \) gene, when placed upstream of a heterologous promoter, displayed significant, orientation-independent enhancer activity (Fig. 2B). While both orientations were active, the antisense construct was more so: in response to DIMM transactivation, the \( \text{PHM}-\text{E71} \) construct (antisense) produced an increase similar to that of the wild-type \( \text{PHM} \) promoter; however, the \( \text{PHM}-\text{E17} \) construct (sense) produced approximately half that level of activity. Generally, most bHLH proteins are known to bind a canonical 6-bp sequence, CANNTG, called the E box (7), although noncanonical sites have also been observed (54). Therefore, we searched for and found seven E boxes (putative DIMM binding sites) within this \( \text{PHM} \) intronic fragment; by random estimate, less than two E boxes are expected within this ~400-bp domain. We named them E1 to E7, from 5’ to 3’ within the intron (Fig. 2A). E boxes E1 to E7 display at least some sequence conservation among related \( \text{Drosophila} \) species (see Fig. S1 in the supplemental material).

To examine the functional contributions of these individual E-box elements to transactivation by DIMM, we performed selective site-directed mutagenesis and tested the resulting lev-
FIG. 2. DIMM transactivates the PHM via the subsets of E boxes in its first intron and binds the E1 box directly. (A) A schematic of the first PHM intron indicating the positions of seven E boxes, E1 to E7 (see Table 1 for sequences of wild-type and mutated E boxes). Arrows indicate the original orientation of the DNA fragments. (B) Activity of the two constructs and the control vector diagrammed in panel A. In response to DIMM cotransfection, the first intron of PHM displays strong enhancer activity when fused to a heterologous simian virus 40 (SV40) promoter. (C to F) Effects on PHM transactivation by DIMM following site-directed mutagenesis of different E-box sequences within PHM’s first intron. A gray box indicates a particular E-box mutation site. Ratios were calculated by dividing values resulting from dimm cotransfection by those resulting from no cotransfection. The histogram represents the means and standard errors of the means of the results. *, P < 0.05; **, P < 0.01. P values in comparison to the wild type were obtained by Student’s t test.
Table 1. Wild-type and mutant sequence probes representing the seven E box sites present in the first PHM intron

<table>
<thead>
<tr>
<th>E box(es) and probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>E1 box: 5′-cgc ta tggag 3′</td>
</tr>
<tr>
<td>E1M</td>
<td>E1 box: 5′-cgc ta tggag 3′</td>
</tr>
<tr>
<td>E1 to 2 box</td>
<td>E1 to 2 box: 5′-cgc ta tggag 3′</td>
</tr>
<tr>
<td>E1 to 7 box</td>
<td>E1 to 7 box: 5′-cgc ta tggag 3′</td>
</tr>
<tr>
<td>E2 and E3</td>
<td>E23 box: 5′-tcc ggtgccg 3′</td>
</tr>
<tr>
<td>E23M</td>
<td>E23M box: 5′-tcc ggtgccg 3′</td>
</tr>
<tr>
<td>E3M</td>
<td>E3M box: 5′-tcc ggtgccg 3′</td>
</tr>
<tr>
<td>E4 and E5</td>
<td>E45 box: 5′-ttt ctgccct 3′</td>
</tr>
<tr>
<td>E45M</td>
<td>E45M box: 5′-ttt ctgccct 3′</td>
</tr>
<tr>
<td>E5M</td>
<td>E5M box: 5′-ttt ctgccct 3′</td>
</tr>
<tr>
<td>E45 to 7 box</td>
<td>E45 to 7 box: 5′-ttt ctgccct 3′</td>
</tr>
<tr>
<td>E6</td>
<td>E6 box: 5′-cca cgaag 3′</td>
</tr>
<tr>
<td>E6M</td>
<td>E6 box: 5′-cca cgaag 3′</td>
</tr>
<tr>
<td>E7</td>
<td>E7 box: 5′-act ccatgaa 3′</td>
</tr>
<tr>
<td>E7M</td>
<td>E7 box: 5′-act ccatgaa 3′</td>
</tr>
</tbody>
</table>

The 6-bp E-box sequences are in uppercase and in order as named in column 1. Sequence alterations are shown in bold. Underlines indicate the final extent of sequence matching to the target E-box site (e.g., E1 to 2 box is the 10 bp surrounding E2).

Results. The single E box E1 strongly reduced responsiveness to DIMM. In contrast, sequence alteration to either E6 or E7 had no such effect (Fig. 2C). Likewise, the alteration of either E3 or E5 did not lessen DIMM responsiveness (Fig. 2D), and in fact, the result with the E5 variant was significantly elevated over that with the wild-type sequence control (Fig. 2D). Simultaneously altering four E boxes (E2, E3, E4, and E5) strongly reduced responsiveness to DIMM, to a level equal to that from altering E1 alone (Fig. 2C). Sequence alteration of E2 or of E4 individually resulted in a decrease of approximately 50% (Fig. 2D). Thus, the three E boxes E1, E2, and E4 appeared to make the biggest contributions; we therefore compared their activities directly to the activity of the vector only (Fig. 2E). The single E2 or E4 mutation displayed half the activity of the wild-type sequence, while the single E1 mutation and the double E2/E4 mutation had even greater effects and displayed only about 20% of that level. All the activities were significantly different from that of the empty vector control. Finally, to determine whether (i) the E-box position or (ii) its specific sequence was the critical determinant of functionality, we tested additional E-box sequence variants (Fig. 2F). Exchanging the sequence of E1 (plus its four adjacent bp [TCCATATGGA]) for that of the nonfunctional E7 (CCCATTTGAA) (Table 1) resulted in the loss of all DIMM responsiveness. Likewise, the exchange of both 3′ and 3′′ with E1 produced a PHM-enhancer fragment with half of its normal levels of enhancer activity in HEK 293 cells. Different E boxes made different contributions to DIMM activity. Sequence alteration of the single E box E1 strongly reduced responsiveness to DIMM. In contrast, sequence alteration to either E6 or E7 had no such effect (Fig. 2C). Likewise, the alteration of either E3 or E5 did not lessen DIMM responsiveness (Fig. 2D), and in fact, the result with the E5 variant was significantly elevated over that with the wild-type sequence control (Fig. 2D). Simultaneously altering four E boxes (E2, E3, E4, and E5) strongly reduced responsiveness to DIMM, to a level equal to that from altering E1 alone (Fig. 2C). Sequence alteration of E2 or of E4 individually resulted in a decrease of approximately 50% (Fig. 2D). Thus, the three E boxes E1, E2, and E4 appeared to make the biggest contributions; we therefore compared their activities directly to the activity of the vector only (Fig. 2E). The single E2 or E4 mutation displayed half the activity of the wild-type sequence, while the single E1 mutation and the double E2/E4 mutation had even greater effects and displayed only about 20% of that level. All the activities were significantly different from that of the empty vector control. Finally, to determine whether (i) the E-box position or (ii) its specific sequence was the critical determinant of functionality, we tested additional E-box sequence variants (Fig. 2F). Exchanging the sequence of E1 (plus its four adjacent bp [TCCATATGGA]) for that of the nonfunctional E7 (CCCATTTGAA) (Table 1) resulted in the loss of all DIMM responsiveness. Likewise, the exchange of both E2 and E3 with E7 or both E4 and E5 with E7 produced a PHM-enhancer fragment with half of its normal
DIMM responsiveness (Fig. 2F), mimicking the effect of the loss of the paired sites.

In summary, the first intron of the PHM gene has seven E boxes. Three of these, E1, E2, and E4, proved especially important for the transactivation by DIMM protein in HEK 293 cells, but our experiments indicated that they make different contributions (E1 > E2 > E4). Additional data implicated the specific sequences within each of these E boxes, and not simply their positions, as critical determinants of their specific contributions. Together, these data indicate that the three cis regions within the first intron represent critical elements of a putative DIMM response element.

DIMM binds the E1 box of the PHM gene in vitro. Next, we examined DIMM’s ability to bind the relevant E boxes within the PHM intron (Fig. 3A to C). We performed EMSAs to address this question, using an E1-box probe. In vitro-translated DIMM bound the E1 probe specifically: the wild type, but not the sequence-mutated E1 probe, was able to compete such binding efficiently (Fig. 3B, lanes 2 to 4). Likewise, we observed poor competition by a cold E1 box probe in which the E-box sequence had been switched to that of E7 (Fig. 3B, lane 5). Supershift assays using either anti-HA or anti-DIMM antibodies confirmed the presence of DIMM protein (Fig. 3C).

We also examined the degree to which the DIMM-E1 interaction could be competed by the six other PHM intronic E-box sequences, E2 to E7. DIMM-E1 interaction was dose dependent (see Fig. S2 in the supplemental material). DIMM binding with E1 was competed efficiently by probes containing both E2 and E3 and also ones containing both E4 and E5 (Fig. 3D). Within the E2/E3 pair, the competition derived primarily but not exclusively from E2; within the E4/E5 pair, all the competing activity derived from E4 (Fig. 3D). We note that E2 and E3 share the same core 6-bp sequence, while the E4 and E5 sequences are different from each other (Table 1). The E6 and E7 oligonucleotides competed poorly with E1 binding and at levels that did not differ when they were tested following mutation of their specific E-box sequences (Fig. 3D). Next, we investigated the sequence requirements within the 10-bp E1 sequence (NNCANNTGNN) in greater detail by using site-directed mutagenesis. We found that specific sequences outside the canonical 6-bp E box (CANNTG) were required for activation by DIMM. Specifically, the nucleotides at the 1 position, but not at the 2 region, could not be altered without loss of DIMM activity. The results also indicated that, within the 6-bp sequence, DIMM prefers TA (and to a lesser extent, GC) for the internal NN sequences (see Fig. S3 in the supplemental material).

In summary, we found that DIMM directly binds E box E1 located within the first intron of PHM; this binding was efficiently competed by oligonucleotides corresponding to E2 and E4, but not by those corresponding to E3, E5, E6, or E7. These results further demonstrate differential E-box contributions to transactivation by DIMM, ranking them in an order consistent with the results from the luciferase reporter assays.
A 1-kb enhancer fragment from the first intron of PHM recapitulates the expression of the endogenous gene in vivo and is dependent on DIMM. We next asked whether the PHM first intronic fragment contains sufficient cis-regulatory information to drive a normal PHM-like expression in vivo, particularly within NSCs that are strongly DIMM expressing. We established transgenic flies that contain the ~1.0-kb PHM fragment as a GAL4 fusion (PHM-WT-GAL4) and crossed them into a UAS-GFP reporter background. Gene expression was studied by immunocytochemistry in the third instar larval brain. We found that PHM-WT-GAL4 was expressed normally throughout the brain (Fig. 4). GFP-expressing cells were predominantly DIMM positive (Fig. 4B). Forty-one percent of GFP+ cells in the brain and 91% of GFP+ cells in the ventral nerve cord (VNC), respectively, were colocalized with DIMM (Fig. 4D). Notably, most DIMM-positive cells were strongly GFP positive (in one representative specimen, we counted 189 GFP-positive cells among the 192 DIMM-positive cells [98.4%] throughout the entire CNS). The DIMM-negative, GFP-positive brain cells were primarily Kenyon cells of the larval mushroom bodies (MBs). PHM is found in moderate levels in both larval and adult MBs (49).

Next, we wished to determine whether DIMM protein regulates the expression of the PHM transgene as it does the endogenous PHM locus, and so performed dimm loss-of-function analyses. The strongest dimm loss-of-function alleles display lethality in the early larval stages (21): in the second instar larval CNS, we observed a strong correspondence between DIMM protein expression and PHM-GAL4 transgene expression (Fig. 5A to C). This activity was greatly reduced in a strong dimm mutant background (Fig. 5D to F). Some residual GFP activity was detectable in a few neurons in mutant tissues; several of these cells were DIMM positive, likely reflecting that, even in the severe dimm hypomorphic background, a small number of DIMM-positive neurons remain. PHM transgene activity in Kenyon cells was not apparent at that age and so could not be scored in the dimm loss-of-function state. Together these data indicate that the DIMM-responsive ~1.0-kb PHM fragment also drives patterned gene expression in vivo. The reporter activities are largely coincident with the expression of DIMM and are largely dependent upon normal DIMM expression.

The PHM transgene also responds strongly to DIMM overexpression in vivo. To analyze the effects of DIMM gain-of-function in vivo, we constructed PHM-GFP transgenic reporter lines using the same ~1-kb PHM fragment. In the larval CNS, we again found high-level GFP expression largely coincident with and dependent on DIMM expression (see Fig. S4 in the supplemental material). Second, we found that within the CNS, misexpressed DIMM protein produced strong, ectopic PHM transgene expression (Fig. 6). In the VNC, AP is expressed in three cell groups: (i) the four-cell Tv cluster (Tv, Tva, Tvb, and Tvc), (ii) the ventral AP pair (vAP), and (iii) the dorsal single Aplet cell (dAP) (2, 40). Within theTv cluster, DIMM is normally found in two cells, Tv and Tvb, but not in Tva or Tvc (the latter are here called Tv3 and Tv4 due to lack of identifying markers [Fig. 6]). Likewise, DIMM is normally found in the dAP, but not in vAP cells. Using an ap-GAL4 driver, we found that PHM-GFP expression was responsive to DIMM overexpression in vivo throughout all four neurons of the Tv cluster (Fig. 6D) and in both vAP neurons (data not shown). This is consistent with the results of previous studies demonstrating that DIMM misexpression throughout all these AP neurons directs ectopic PHM protein in them all (2, 16). Notably, the mammalian homologue, MIST1, also showed an ability to activate the PHM-GFP reporter in vivo throughout all four cells of the Tv cluster (Fig. 6E and F) and in the vAP cells (data not shown).

In summary, we found that in vivo, dimm regulated the activity of an ~1-kb PHM regulatory fragment in a manner consistent with its regulation of the endogenous PHM protein. This further validates this ~1-kb fragment as a primary response element mediating the transcriptional control of PHM by DIMM. Additionally, the mammalian orthologue Mist1 also activated this same PHM fragment in vivo, supporting the hypothesis that these related factors share functional properties.

Specific E-box sequences within the first PHM intron are essential for the activity of the PHM transgene. We further asked which cis-regulatory sequences within this ~1-kb PHM fragment contribute to its activity as a transgene in vivo. Based
on the functional analysis in HEK 293 cells, three E boxes (E1, E2, and E4) are required for activation by DIMM. We therefore asked whether these specific cis-regulatory sequences also contribute to the activity of this PHM fragment in vivo. In the third instar larval brain, all activity was lost in flies bearing a GAL4 fusion of the 1-kb PHM fragment in which E1, E2, and E4 E boxes were mutated (n = 10; Fig. 7A to C). We note that activity was lost equally from the DIMM-positive and DIMM-negative (primarily MB) cells.

To investigate contributions by individual E boxes or subsets of them, we also established four different PHM-gal4 lines harboring either single (E1M, E2M, and E4M) or double (E24M) E-box mutations. Analysis of the single E-box mutations revealed that reporter activity was very low in each, with, typically, a few weakly expressing cells at most (Fig. 7). The reporter activity in the double-mutant line was likewise very low. These results suggest that all three E boxes (E1, E2, and E4) are required for the normal activities of this PHM fragment in vivo and that the contribution of these E boxes is crucial.

**DISCUSSION**

The experiments reported here address the mechanisms underlying DIMM’s regulatory functions within peptidergic NSCs in Drosophila. We have shown that the tissue-restricted bHLH factor DIMM works as a transcriptional activator and that PHM is a direct DIMM target both in heterologous mammalian cells and in vivo. We have also resolved the enhancer activity of PHM to a region that contains its first intron and further shown that three tandem E boxes within that intron are essential for full DIMM activation.

**DIMM is a transcription factor that regulates PHM directly.** Class I bHLH factors (e.g., E12, E47, and HEB) are widely expressed, while class II bHLH proteins (e.g., MyoD, myogenin, Mash1, and NeuroD) exhibit tissue-restricted expression profiles. Class I and II bHLH proteins function as heterodimer complexes to regulate the expression of target genes by binding to E-box (CANNTG) DNA elements (16, 30). Some bHLH proteins also form homodimers; for example, HAIRY is thought to form homodimers exclusively (39, 46). Likewise, the Drosophila mesoderm regulator TWIST forms heterodimers with DA but also forms homodimers: these different TWIST molecular pairs produce different transcriptional readouts in vivo (12).

At present, we favor the hypothesis that the master regulatory functions of DIMM (such as its control of PHM) represent actions as a homomeric dimer or oligomer. Four lines of evidence support this conclusion. (i) Cotransfection of PHM-luc with *dimm* was sufficient to transactivate PHM in heterologous (mammalian) cells, (ii) whereas cotransfection with *dimm* and *da* produced a less robust transactivation (Fig. 2). (iii) In vitro, DIMM did not bind DA, but did bind itself efficiently when tested as a GST fusion protein or via coimmunoprecipitation (2). (iv) In the absence of a class I bHLH, DIMM was nevertheless able to bind to the PHM E1 probe in vitro, presumably as a homodimer (Fig. 2). It may be significant that the three E-box sequences indicated by functional analysis display palindromic core sequences, which is consistent with binding by a homomorphic bHLH dimer. We also note that the DIMM mammalian sequence orthologue (called Mist1) forms heterodimers with class I bHLH factors under certain conditions but also forms homodimers to directly regulate the fate of developing acinar cells of the pancreas (29, 45, 56).
The significance of PHM as a direct regulatory target of the prosecretory factor DIMM. In the context of establishing transmitter phenotypes, there is special significance to having established direct transcriptional regulation of PHM by DIMM. DIMM and PHM, among those neurons that display high levels of neuropeptide expression, display highly congruent patterns of expression within the nervous system (2, 21). PHM is a monooxygenase that is required for neuropeptide amidation and whose closest homologue is the hydroxylase DBH. Phox2a and Phox2b are critical regulators of the noradrenergic phenotype and have been shown to directly regulate DBH transcription (27, 48, 53, 55). Likewise, PET-1, which is a critical regulator of the serotonergic phenotype, directly regulates the transcription of tryptophan hydroxylase, which encodes the rate-limiting enzyme for serotonin biosynthesis (18). In each case cited, genes encoding biosynthetic hydroxylases appear to be critical points of regulation. Thus, direct transcriptional control of such enzymes by highly dedicated developmental regulators represents a mechanistic parallel between aminergic and peptidergic transmitter cell fates.

DIMM binding sites on PHM. We propose that the high level of PHM expression found in Drosophila NSCs results from direct control by DIMM acting via specific PHM cis-regulatory elements (Fig. 8A). The full activity of Class 2 bHLH proteins often requires interactions with multiple tandem E boxes (37), although single E boxes are sometimes sufficient (54). In the current study, we identified a cluster of seven E boxes in the enhancer region of PHM, of which at least three (E1, E2, and E4) are critical. bHLH proteins exhibit specificity as to which E-box sequences they utilize (44). The loss of these three motifs in PHM resulted in a substantial reduction in luciferase reporter assays and transgenic flies, indicating that there is an essential requirement of multiple E boxes for PHM activation by DIMM. We observed small differences in the activities of mutated PHM regulatory regions when tested in vitro versus in vivo. Focusing on the in vivo results, we propose a simple model of synergistic or cooperative interactions between DIMM homodimers bound to E1, E2, and E4 (Fig. 8A).

Mutation of the E3 and E5 E boxes produced elevated PHM-luc levels upon dimm cotransfections. The simplest explanation is that these E boxes bind one or more bHLH proteins that can repress PHM expression. The spacing of these potentially “inhibitory” E boxes, approximately one-half turn away from the “activating” E2 and E4 boxes, respectively, may be significant in effecting such negative regulation. Interest-
ingly, Mist1 functions as a transcriptional repressor when bound as an E47 heterodimer to troponin E boxes (29). DA cotransfection reduced the DIMM transactivation of PHM:\n\none possible explanation for this effect is that DA may bind to one or more of the “inhibitory” PHM E boxes. Whether and how other factors modulate the level of DIMM’s activation by binding PHM E3 and/or E5 in vivo will require additional studies.

PHM regulatory elements tested in vivo. We extended the scope of our analysis by considering the behavior of the ~1-kb PHM fragment in vivo. Importantly, DIMM regulation of PHM in Drosophila cells paralleled its activity in HEK 293 cells. Although PHM regulation in vivo is complex and subject to multiple influences, these data suggest that much of PHM expression in Drosophila NSCs can be ascribed to DIMM direct regulation via the ~1-kb PHM fragment that includes its first intron. Significantly, most DIMM-positive cells were PHM-GAL4-positive cells, suggesting a strong correlation between DIMM and the same 1-kb PHM fragment that displayed transactivation in vitro. In addition, the promoter activity that could be assayed in second instar larvae (before dimm mutant animals die) was essentially all DIMM dependent: the few resilient GFP-positive cells that remained in dimm mutants were those that also retained DIMM protein—the strongest dimm alleles are not protein nulls. As found in HEK 293 cells, specific E boxes appeared to be especially important in vivo as well. The same three PHM E boxes that were implicated from in vitro experiments (E1, E2, and E4) were again implicated as critical for supporting PHM regulatory activity in vivo. This work therefore defines the PHM enhancer region and the constituent cis-regulatory sequences therein that confer sensitivity to DIMM. The data also indicate additional complexity of PHM regulation. The reliable incidence of GFP-positive/DIMM-negative neurons (especially some or all MB cells in the brain lobes) indicated that other factors besides DIMM may regulate PHM expression in other domains of the CNS. Interestingly, the loss of PHM transgene expression by MB neurons in flies bearing mutated PHM E boxes suggests that the regulatory factor(s) driving PHM in MBs may also be a bHLH protein(s).

What is the precise role of DIMM in the differentiation of NSC? In mammals, several transcription factors have been implicated in directing the differentiation of neuroendocrine lineages (1, 8, 24, 26, 31, 32, 36, 47, 52). Based on information so far available, DIMM does not resemble any known mammalian regulator of neuroendocrine development. For example, while DIMM is activated postmitotically in NSCs, Mash1 appears required for the generation of pulmonary neuroendocrine cells (8). Like dimm, Sim1 and Otp are thought to act in parallel as determinants of cell fate in hypothyalamic neuroendocrine cells and are needed for cell generation or survival (1, 32, 52). Recently, Sim1 mutant cells were shown to reach their final cell division with normal display of their molecular phenotype but subsequently fail to differentiate as a function of altered cell migration (56). These factors, therefore, appear to
act early and prior to the terminal differentiation of the peptidergic cells.

Based on several observations of normal expression and from genetic analyses, we consider DIMM to be the major regulator of terminal cell differentiation in Drosophila NSCs. Our previous work proposed two, nonexclusive mechanisms to explain \textit{dimm} actions. One is a model of transcription factor combinatorial regulation to drive specific neuropeptide expression (e.g., \textit{dFMRFa} and \textit{NPLP1}) (2, 5) in different neurons. In this scenario, DIMM acts combinatorially with several locally expressed transcription factors to drive neuropeptide gene expression. In no case, however, has it been possible to detail the precise identities of any particular combination regulating one specific neuropeptide gene. DIMM also acts independently to regulate the transcription of genes that control common NSC traits. \textit{dimm} is a “master regulator,” as its overexpression confers high-level PHM expression in all neurons of the CNS (2).

In this dual manner, we speculate that \textit{dimm} contributes to specific cellular properties of diverse NSCs (e.g., neuropeptide gene selection—a “shell program” of neuroendocrine differentiation) and to more general ones involving generic NSC properties (e.g., PHM expression—the “core program” of neuroendocrine differentiation) (Fig. 8B). The present results in vitro and in vivo strongly support the second model of \textit{dimm} actions: we have shown that the control of PHM protein expression derives from the very strong, direct regulation of PHM transcription. Given its potent transcriptional activity, we suspect that DIMM promotes a general NSC phenotype by also activating target genes besides PHM, but this supposition will require further analysis.

The relationship of DIMM and the mammalian bHLH Mist1. Among mammalian bHLH proteins, Mist1 is the most similar to DIMM (34). At the sequence level, the bHLH regions of DIMM and Mist1 exhibit greater than 90% sequence identity. Both proteins are enriched in cells that are specialized in specific regions of DIMM and Mist1 exhibit greater than 90% sequence identity (34). At the sequence level, the bHLH re-

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