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A Chimeric Enhancer-of-split Transcriptional Activator Drives Neural Development and *achaete-scute* Expression

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Drosophila melanogaster **neurogenesis requires the opposing activities of two sets of basic helix-loop-helix (bHLH) proteins: proneural proteins, which confer on cells the ability to become neural precursors, and the Enhancer-of-split [E(spl)] proteins, which restrict such potential as part of the lateral inhibition process. Here, we test if E(spl) proteins function as promoter-bound repressors by examining the effects on neurogenesis of an E(spl) derivative containing a heterologous transcriptional activation domain [E(spl) $m7^{Act}$ ($m7^{Act}$)]. In contrast to the wild-type E(spl) proteins, $m7^{Act}$ efficiently induces neural development, indicating that it binds to and activates target genes normally repressed by E(spl). Mutations in the basic domain disrupt $m7^{Act}$ activity, suggesting that its effects are mediated through direct DNA binding. $m7^{Act}$ causes ectopic transcription of the proneural *achaete* and *scute* genes. Our results support a model in which E(spl) proteins normally regulate neurogenesis by direct repression of genes at the top of the neural determination pathway.**

Lateral inhibition is a pathway of local cell-cell signalling that operates in several developmental contexts to select individual cells from groups of equivalent cells (reviewed in references 1, 8, 19, and 45). It has been most studied during patterning of sensory organs (SOs; bristles and other types of sensilla) on the body surface of the adult *Drosophila melanogaster*. SOs usually comprise four specialized cells that originate through two consecutive divisions from a single neural precursor, or sensory mother cell (SMC) (reviewed in reference 30). SMCs arise during late larval and early pupal stages within the imaginal discs, the epithelial sheets of cells that generate the cuticle of the adult fly. The emergence of SMCs for large bristles (macrochaetae) has been analyzed in detail and occurs in two steps (reviewed in references 8 and 19). First, groups of imaginal disc cells ("proneural clusters") express so-called proneural genes and thereby acquire the potential to become neural precursors. The best-characterized proneural genes belong to the *achaete-scute* complex (AS-C) and encode basic helix-loop-helix (bHLH) transcriptional regulators that are thought to activate the expression of genes required for the neural differentiation program (9, 63) (see below). Subsequently, one or two isolated cells accumulate higher levels of proneural gene products and become prospective SMCs. These cells then signal to their neighbors via lateral inhibition to suppress their neural potential and restrict the number of SMCs specified from a proneural cluster. Lateral inhibition also acts on the progeny of SMCs to ensure their differentiation into distinct cell types (23).

Lateral inhibition requires a heterogeneous group of genes that, when mutated, lead to a common phenotype characterized by overproduction of neural tissue (the "neurogenic" phenotype). The neurogenic genes *Delta* and *Notch* encode a transmembrane ligand and receptor, respectively, that mediate cell-cell signalling during lateral inhibition (17, 26, 38, 62, 65). Nascent neural precursor cells express Delta, which binds to and activates Notch on neighboring cells and so inhibits their neural determination. Other neurogenic genes encode components required to transduce the effects of Notch activation into the nucleus, thereby influencing target gene expression and activity.

Of particular importance among the neurogenic genes are members of the *Enhancer-of-split* gene complex [E(spl)-C] (16, 25, 39, 53), which have been shown by genetic analysis to function at the end of the neurogenic pathway (14, 40) and which may therefore directly mediate neural suppression. Consistent with their role as negative regulators of neurogenesis, overexpression of E(spl)-C genes leads to a reduction in the number of neural precursors and/or SOs (12, 46, 56). The E(spl)-C includes seven related genes (*m3*, *m5*, *m7*, *m8*, *mα*, *mγ*, and *mδ*) with similar patterns of expression and overlapping functions during neurogenesis (15, 16, 25, 34, 36, 37, 53). These encode bHLH repressors related to the products of the *hairy* (*h*) and *deadpan* genes, which act in segmentation and sex determination, respectively (4, 50, 66). bHLH proteins include two adjacent subdomains: a cluster of basic residues involved in sequence-specific binding to DNA and an HLH domain that mediates protein dimerization (43, 44). E(spl), Hairy, and Deadpan all have similar bHLH domains, a further putative helical domain, and the C-terminal tetrapeptide WRPW, which has been shown to mediate repressor activity in Hairy (11, 18, 32, 48, 64).

To understand how lateral inhibition ultimately suppresses neurogenesis, it is essential to identify the mechanism of action of E(spl) proteins. A favored model for their function is that they interfere with proneural gene activity (22, 54; reviewed in reference 8). This could result from an ability of E(spl) proteins to act as direct, promoter-bound repressors of either neural differentiation genes activated by AS-C proteins or the AS-C genes themselves. Such roles would be consistent with the presence of a conserved basic region in the E(spl) proteins and with their ability to bind DNA in vitro and directly repress reporter constructs in tissue culture cells (47, 52, 58, 61). Moreover, the related Hairy protein has been shown to repress proneural transcription through direct binding to the *achaete* (*ac*) promoter (47, 61), and we have provided evidence that it also acts as a promoter-bound regulator during embryonic segmentation (32). An alternative possibility, as both AS-C and E(spl)-C gene products are bHLH proteins, is that the latter antagonize proneural action by forming inactive HLH het-

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erodimers with the AS-C proteins. This idea is supported by the finding that AS-C and E(spl) proteins can be coexpressed in cells of the proneural cluster whose neural potential is inhibited (31) and by experiments suggesting that DNA binding may not be essential for E(spl) activity (46, 59).

To help distinguish between the above alternatives, we have explored the ability of m7, a typical E(spl) family member, to function as a promoter-bound regulator in vivo by expressing a derivative of this protein containing a heterologous transcriptional activation domain ($m7^{Act}$) (see Fig. 1). We find that $m7^{Act}$ efficiently induces neural development and activates transcription of the proneural genes *ac* and *scute* (*sc*). Our results support a model in which E(spl) proteins normally mediate lateral inhibition by directly repressing proneural gene expression.

MATERIALS AND METHODS

DNA constructs. Plasmid manipulations were carried out by following standard protocols (2, 51). As starting material for $m7^{Act}$ expression constructs, we used a pBluescriptII plasmid (Stratagene) containing a *Bam*HI-*Xba*I insert coding for the VP16 activation domain (amino acids 412 to 490) (60). This plasmid was digested with *Hin*dIII and *Bam*HI and ligated to a *Hin*dIII-*Bam*HI fragment coding for amino acids 1 to 143 of m7, thereby creating $pBSm7^{Act}$. $pBSm7^{1-143}$ was made by replacing the VP16 coding sequences in $pBSm7^{Act}$ with a *Bam*HI-*Xba*I synthetic linker containing an in-frame stop codon. $pBSm7^{\Delta basicAct}$ and $pBSm7^{KNEQAct}$ were constructed by replacing the *Hin*dIII-*Bam*HI fragment in $pBSm7^{Act}$ with equivalent mutagenized fragments synthesized by recombinant PCR (27). Sequences coding for the m7 derivatives were recovered from the corresponding pBS plasmids as *Eco*RI-*Xba*I fragments and cloned into pUAST (7). Segments generated by PCR were sequenced to ensure the fidelity of the amplification reactions. (Additional details on the construction of the plasmids are available on request.)

P-element transformation and crosses. Germ line transformation was carried out as described previously (55) by DNA injection into *y w* embryos and selecting for rescue of *w* eyes. Several transformant lines were obtained for each construct. *UAS-m7* flies were a gift of S. Bray. Misexpression was achieved by crossing *GAL4* and *UAS* lines at 22 to 25°C. Results from different lines of the same construct were qualitatively similar.

Patterns of A101 expression were examined in third-instar imaginal discs dissected from the progeny of the crosses *UAS-m7^{Act}* × *ptc-GAL4*/+; *A101*/+ or *UAS-m7^{Act}* × *GAL4-455.2*/+; *A101*/+. Expression of *ac*- and *sc-lacZ* reporters (kindly provided by J. Modolell) was monitored by using crosses similar to those described for *A101* analysis. In all cases, mutant discs were identified by their abnormal *lacZ* staining.

Crosses examining the effects of $m7^{Act}$ in *ac* and/or *sc* mutant backgrounds were as follows (female genotypes first):

Df(1)sc$^{10-1}$/*y w*; *UAS-m7^{Act}*/+ × *w*/*Y*; *GAL4-455.2*

In(1)y^{3PL}sc^{8R}/*w*; *GAL4-455.2*/+ × *w*/*Y*; *UAS-m7^{Act}*

sc^{M6}/*y w*; *UAS-m7^{Act}*/+ × *w*/*Y*; *GAL4-455.2*

In(1)sc^{8L}sc^{4R}/*y w*; *UAS-m7^{Act}*/+ × *w*/*Y*; *GAL4-455.2*

Df(1)sc$^{10-1}$/*y w*; *UAS-m7^{Act}*/+ × *w*/*Y*; *GAL4-30A*

Appropriate genotypes were identified by the *y* marker and according to the pattern of endogenous bristles. Where necessary, the presence of the *UAS-m7^{Act}* chromosome was confirmed by PCR on single flies with primers for the VP16 domain.

Histochemistry. β-Galactosidase staining was carried out as described previously (20). Whole-mount in situ hybridization was performed essentially as described previously (35, 57). Briefly, larval heads were inverted to expose imaginal discs, fixed in 4% formaldehyde–phosphate-buffered saline (PBS) for 15 min, and then dehydrated and rehydrated with graded ethanol-PBS solutions. After being rinsed three times in 0.1% Tween 20–PBS (PTW), the heads were digested with proteinase K (50 μg/ml in PTW) for 2 min at room temperature. Digestions were stopped by rinsing twice in glycine (2 mg/ml in PTW). After being washed in PTW, the heads were refixed in 4% formaldehyde–PBS for 5 min, washed in PTW, and hybridized at 60°C (final salt concentration, 1× SSC [0.15 M NaCl plus 0.015 M sodium citrate]) with digoxigenin-labelled *ac* and *sc* RNA probes (Boehringer). Signals were detected with an anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer), and the discs were dissected in PTW and mounted in methacrylate (JB-4; Polyscience).

For notum, wing, and leg preparations, the dissected tissue was incubated in 10% KOH for 5 to 10 min at 100°C (only in the case of nota), dehydrated in isopropanol, and mounted in Euparal (BDH/Merck).

Samples were photographed on Ektachrome 160T or Fujichrome 64T film, scanned, and digitized, and photographs were compiled by using Adobe Photoshop.

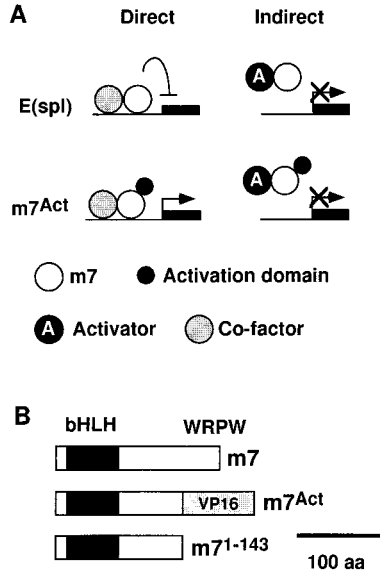

FIG. 1. (A) Diagram of possible mechanisms of repression by E(spl) proteins and predicted effects of $m7^{Act}$. E(spl) proteins could function as direct, promoter-bound repressors, in which case $m7^{Act}$ should cause activation of target genes via the heterologous activation domain (left). In contrast, if E(spl) proteins inhibit transcription indirectly by sequestering activator proteins, $m7^{Act}$ should be unable to recruit the activation domain to target promoters and could still behave as a repressor (right). (B) Diagram of the m7 protein and two derivatives, $m7^{Act}$ and $m7^{1-143}$. $m7^{Act}$ is a chimeric protein containing the first 143 amino acids of m7 fused to the VP16 transcriptional activation domain. $m7^{Act}$ and $m7^{1-143}$ lack the 43 C-terminal amino acids of m7, including the terminal WRPW tetrapeptide required for repressor activity of the related Hairy bHLH protein and for binding to Gro (see Discussion).

RESULTS

$m7^{Act}$ promotes neural development. We have previously shown that fusing Hairy to the transcriptional activation domain from the herpesvirus VP16 protein converts it from a repressor to an activator ($Hairy^{Act}$) (32). This protein activates expression of genes that are directly repressed by wild-type Hairy. We decided to use a similar approach to investigate the function of E(spl) proteins during lateral inhibition. We generated an equivalent derivative of the E(spl) m7 protein ($m7^{Act}$) by fusing the 143 N-terminal amino acids of m7 to the VP16 transcriptional activation domain (Fig. 1B) (60). $m7^{Act}$ contains the bHLH and adjacent putative helical domains from m7 but lacks the last 43 amino acids of the protein, including the C-terminal WRPW tetrapeptide required for repressor activity of the related Hairy protein (11, 18, 32, 48, 64). If E(spl) proteins suppress neurogenesis by acting as direct, promoter-bound repressors of transcription, $m7^{Act}$ should recruit the VP16 activation domain to promoters of target genes and activate their expression. This should have effects on neurogenesis opposite those of the normal E(spl) proteins; i.e., it should lead to an increase in the number of SMCs and SOs. By examination of which genes become activated by $m7^{Act}$, the approach would also clarify whether E(spl) proteins act on AS-C gene transcription or on downstream neural differentiation genes. In contrast, if E(spl) proteins act by sequestering AS-C proteins, $m7^{Act}$ should still be a repressor, or it should be nonfunctional if it has lost the ability to antagonize those proteins (Fig. 1A).

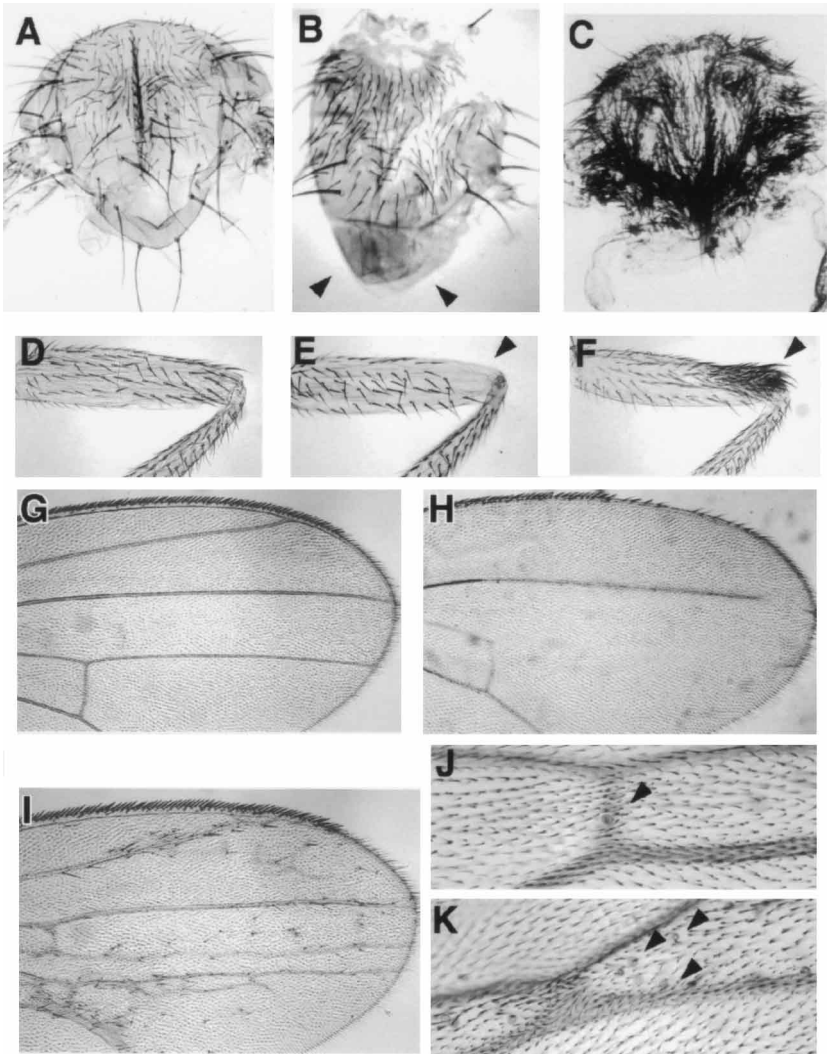

FIG. 2. Phenotypes induced by expression of m7 and $m7^{Act}$ under the control of the GAL4-UAS system. Shown are a wild-type notum (A), leg (D), and wing (G) and the effects of m7 (B, E, and H) and $m7^{Act}$ (C, F, and I) under the control of the *GAL4* drivers *h-GAL4* (B and C) (7), *GAL4-30A* (E and F) (7), and *GAL4-69B* (H and I) (7). Ectopic m7 leads to loss of bristles in the scutellum (B; arrowheads) and the leg (E; arrowhead) and suppresses wing veins L2 and L4 (H). By contrast, expressing $m7^{Act}$ causes the development of extra bristles in the notum (C), leg (F; arrowhead), and wing (I), as well as ectopic wing vein tissue (I). (J) Wild-type campaniform sensillum (arrowhead) at the anterior crossvein. (K) Ectopic campaniform sensilla (arrowheads) induced by *ptc-GAL4*; *UAS-*$m7^{Act}$ (28). These phenotypes are not generated by expression of $m7^{1-143}$ (data not shown). The domains of expression for lines *h-GAL4*, *GAL4-69B*, and *ptc-GAL4* are shown in Fig. 5I and J and 3E, respectively.

We used the GAL4-UAS system (7) to express $m7^{Act}$ during imaginal disc development and examine its effects on adult neurogenesis. In this two-gene system, GAL4-expressing flies are crossed to flies containing transgenes in which *m7*^{Act} transcription is under the control of UAS enhancer elements. Persistent expression of wild-type E(spl) proteins causes loss of neural precursors and sensory bristles and also suppresses wing vein formation (12, 46, 56) (Fig. 2B, E, and H). By contrast, $m7^{Act}$ efficiently induces supernumerary external SOs, as predicted if E(spl) proteins function as direct repressors. Ectopic SOs arise in regions of GAL4-driven $m7^{Act}$ expression. Examples are shown of ectopic macro- and/or microchaetae (bristles) on the notum (Fig. 2C), the leg (Fig. 2F), and the wing blade (Fig. 2I) and of extra campaniform sensilla on the wing blade (Fig. 2K). Ectopic bristles are also observed on the abdomen, the pleura, and the halteres (data not shown). $m7^{Act}$ also produces ectopic wing vein tissue (Fig. 2I), consistent with the role of the neurogenic pathway, and *E(spl)* in particular, in regulating wing vein cell fates (13, 25). Some *GAL4* lines induce a high level of mortality during larval and pupal stages (e.g., >95% with *h-Gal4*; see Table 1); in these cases, flies dissected out of the pupal cases show dramatic transformations of cuticle into bristles (Fig. 2C). Although phenotypic strength is partly dependent on the particular *UAS-*$m7^{Act}$ line tested, presumably due to variable expression of insertions at different chromosomal positions, variability among the progeny of a given cross is low. We do not observe phenotypes apparently unrelated to the role of E(spl) in lateral inhibition, implying that $m7^{Act}$ retains the functional specificity of native m7. Overall, the results are consistent with $m7^{Act}$ functioning as a promoter-bound activator of genes normally repressed by the parental m7 protein. These target genes must be able to establish a complete program of neural development and should include proneural and/or neural differentiation genes.

An alternative explanation of the above results is that $m7^{Act}$ does not function as an activator but acts as an antimorphic

(dominant-negative) protein that causes a neurogenic-like phenotype by passively interfering with endogenous E(spl) activity. This could result from alterations in the normal balance of E(spl) HLH dimerization, or if the mutant protein occludes binding sites on DNA or on other proteins normally recognized by E(spl). One argument against this idea is that $m7^{Act}$ can generate ectopic SOs in regions that lack endogenous bristles and where E(spl) should therefore not be required for neural patterning (Fig. 2I). To test further whether $m7^{Act}$ indeed acts as an activator, we expressed an equivalent m7 truncation lacking the VP16 activation domain ($m7^{1-143}$) (Fig. 1B). This derivative has no phenotypic effects (data not shown), indicating that the activity of $m7^{Act}$ depends on the VP16 domain and involves a transcriptional activation function. This experiment also shows that $m7^{Act}$ does not act as a passive antagonist of other HLH negative regulators of neurogenesis, such as Hairy or Extramacrochaetae (5, 10), and that the 43 C-terminal amino acids of m7, which include the WRPW motif, are required for the ability of ectopic E(spl) to suppress SO development (12, 46, 56) (Fig. 2).

The results indicate that $m7^{Act}$ behaves as a proneural factor, forcing cells which would normally develop as epidermis to follow a neural pathway. To test this idea directly, we analyzed the pattern of SMCs in mutant discs by using the enhancer trap line *A101* (3, 29). This line marks all SMCs by expression of a *lacZ* gene inserted in the *neuralized* locus (6, 49). Expression of $m7^{Act}$ in a stripe along the anteroposterior boundary of the imaginal wing disc by using the *ptc-GAL4* driver (Fig. 3E) leads to ectopic SMCs, as shown by the induction of extra *lacZ*-expressing cells at the same location (Fig. 3B). Ectopic bristles (Fig. 3G) and campaniform sensilla (Fig. 2K) form in the corresponding region of the wing blade that develops from these discs. Similarly, $m7^{Act}$ efficiently generates ectopic *A101*-positive cells in the presumptive scutellum region of the wing disc (*GAL4-455.2*; *UAS-*$m7^{Act}$) (Fig. 3C and F; see Fig. 6A for the associated bristle phenotype). These results demonstrate that $m7^{Act}$ induces the specification of supernumerary SMCs.

Expression of $m7^{Act}$ under the control of certain *GAL4* drivers (e.g., *h-GAL4* and *ptc-GAL4*) leads to very strong phenotypes. Ectopic bristles occupy relatively large territories and form dense tufts with little or no intervening cuticle (Fig. 2C). Accordingly, $m7^{Act}$ can give rise to patches of cells showing ectopic *A101* expression (Fig. 3D), suggesting that the mutant protein induces the emergence of groups of SMCs adjacent to one another. These effects are similar to those produced by loss-of-function mutations in the neurogenic genes, suggesting that $m7^{Act}$ can block the singling out of neural precursors by lateral inhibition. We do not believe that this is due to dominant-negative interference with endogenous E(spl) action because $m7^{Act}$ is also able to induce ectopic neural precursors and SOs in regions where lateral inhibition is not normally active (e.g., Fig. 3B and G). Since $m7^{Act}$ behaves as an activator, we believe the neurogenic phenotypes reflect an ability of the chimeric protein to activate transcription of target genes so efficiently that it overcomes repression by the endogenous E(spl) proteins.

Mutations in the basic region of $m7^{Act}$ impair its activity. The above results suggest that m7 is able to target the VP16 domain to specific promoters. This could be mediated by interactions of the m7 moiety either with DNA or with other promoter-bound factors. We therefore examined the effects of expressing two $m7^{Act}$ derivatives with mutated basic domains ($m7^{\Delta basicAct}$ and $m7^{KNEQAct}$) that should be unable to bind DNA (Fig. 4A). $m7^{\Delta basicAct}$ includes an 8-amino-acid deletion that eliminates most of the basic region, whereas $m7^{KNEQAct}$ contains two mutations that replace Lys-15 and Glu-22 with Asn and Gln, respectively. The character of these two amino acids is conserved in almost all bHLH proteins, and they have been shown in the case of MyoD to play a direct role in contacting specific bases within the major groove of DNA (41). Therefore, they are unlikely to mediate interactions with other proteins. The ability of both proteins to induce ectopic SOs is markedly reduced, indicating that an intact basic domain is required for the activity of $m7^{Act}$. For example, expression of $m7^{Act}$ under the control of the *h-GAL4* driver leads to very strong bristle phenotypes that are not induced by the mutant derivatives (cf. Fig. 2C and 4B and C). In addition, neither of the two constructs causes the high levels of mortality associated with expression of $m7^{Act}$ in this cross (Table 1). $m7^{\Delta basicAct}$ and $m7^{KNEQAct}$ are not completely inactive; they retain some ability to induce ectopic bristles (Fig. 4). These residual phenotypes, which appear to be induced by an E(spl) m8 derivative lacking the basic region (46), could result from an antimorphic interference with endogenous E(spl) proteins (see Discussion).

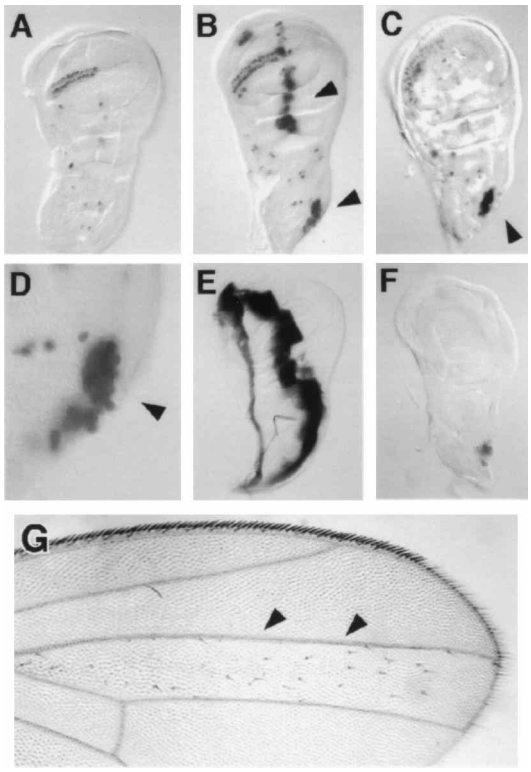

FIG. 3. $m7^{Act}$ induces ectopic SMCs. Shown are the wild-type pattern of SMCs in third-instar imaginal wing discs revealed by *lacZ* expression in the enhancer trap *A101* (A) and *A101* patterns after expression of $m7^{Act}$ driven by the *ptc-GAL4* (B) and *GAL4-455.2* (C) lines. Ectopic labelling is observed in regions of the disc coincident with the domains of GAL4 activity (arrowheads): along the anteroposterior boundary of the disc (*ptc-GAL4*) and in the presumptive scutellum (*GAL4-455.2*). (D) Magnification of the presumptive scutellum of the disc in panel B to illustrate that *A101* expression is induced in adjacent cells. Domains of GAL4 activation are revealed by β-galactosidase staining of *ptc-GAL4*; *UAS-lacZ* (E) and *GAL4-455.2*; *UAS-lacZ* (F) discs. *ptc-GAL4*; *UAS-*$m7^{Act}$ flies display a high level of mortality; survivor individuals show many ectopic macro- and microchaetae in the scutellum (data not shown), as well as ectopic microchaetae (G) and campaniform sensilla (Fig. 2K) along the anteroposterior axis of the wing blade. *GAL4-455.2*; *UAS-*$m7^{Act}$ individuals show ectopic macro- and microchaetae in the scutellum (see Fig. 6A) but are usually viable.

$m7^{Act}$ induces ectopic *ac-sc* expression. The phenotypes induced by $m7^{Act}$ are reminiscent of those generated by equivalent expression of proneural AS-C proteins (28), suggesting

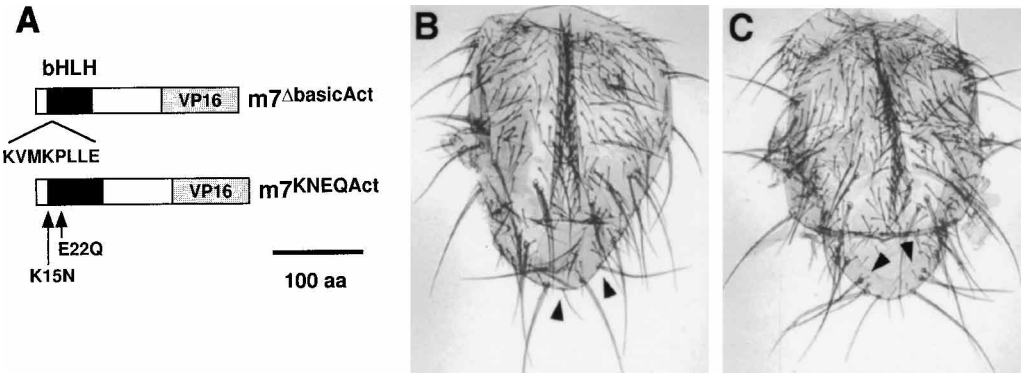

FIG. 4. Mutations in the basic domain of $m7^{Act}$ disrupt its activity. (A) Diagram of two $m7^{Act}$ derivatives with altered basic regions: $m7^{\Delta basicAct}$, which contains an 8-amino-acid deletion in the basic region, and $m7^{KNEQAct}$, in which residues Lys-15 and Glu-22 are replaced by Asn and Gln, respectively. (B and C) Representative phenotypes obtained after expression of $m7^{\Delta basicAct}$ (B) and $m7^{KNEQAct}$ (C) driven by the *h-GAL4* line. Although some ectopic bristles are observed (arrowheads), we do not see the very strong phenotypes induced by $m7^{Act}$ (Fig. 2C). Also, most flies are viable, whereas *h-GAL4*; *UAS-*$m7^{Act}$ individuals usually die at the larval or pupal stage (Table 1).

that $m7^{Act}$ drives expression of either ectopic AS-C proteins or their neural differentiation targets. We therefore tested if $m7^{Act}$ induces expression of *ac* and *sc*, the two proneural genes that act as primary regulators of neural cell fate decisions in imaginal discs (9, 19). Expression of $m7^{Act}$ driven by the *h-GAL4* and *GAL4-69B* lines, which generates extra bristles in both the notum and the wing blade (Fig. 2C and I and data not shown), leads to ectopic *ac* and *sc* transcript accumulation in many scattered cells of discs (Fig. 5E and F). To confirm that this ectopic expression results from de novo transcription and not, for example, from increased *ac* or *sc* mRNA stability, we examined the pattern of *ac*- and *sc-lacZ* reporter constructs in the mutant discs. These reporter constructs contain 3.8- and 3.7-kb promoter fragments from the *ac* and *sc* genes, respectively (42). As shown in Fig. 5G, $m7^{Act}$ causes a dramatic induction of *ac-lacZ* expression throughout most of the wing disc, and ectopic *sc-lacZ* activation is observed in many cells (Fig. 5H). These results indicate that $m7^{Act}$ functions as a promoter-bound activator upstream of the *ac* and *sc* genes.

Endogenous *ac* and *sc* genes mediate the effects of $m7^{Act}$. The ability of $m7^{Act}$ to induce *ac* and *sc* transcription, and the similarity of phenotypes produced by ectopic proneural expression (28) and by $m7^{Act}$, suggest that the latter are due to ectopic *ac* and *sc* expression. We therefore examined the effects of $m7^{Act}$ in the absence of the endogenous *ac* and/or *sc* genes. For these experiments, we used the *GAL4-455.2* insertion (28), which leads to extra SMCs and bristles in the scutellum (Fig. 3C and 6A) but otherwise allows for the recovery of a high proportion of adult mutant flies. Such extra bristles are not induced in flies lacking both *ac* and *sc* gene activities [*Df(1)*$sc^{10\text{-}1}$; *GAL4-455.2*; *UAS-*$m7^{Act}$] (Fig. 6B), implying that *ac* and *sc* mediate the proneural effects of $m7^{Act}$. A deletion of *ac* [*In(1)*y^{3PL} sc^{8R}] or a nonsense mutation in *sc* (sc^{M6}) reduces the number of ectopic bristles (Fig. 6C and D, respectively), indicating that both genes contribute to the $m7^{Act}$ phenotype. Surprisingly, a deficiency of both *sc* promoter and coding sequences [*In(1)*$sc^{8L}sc^{4R}$] does not suppress the formation of ectopic bristles (Fig. 6E), suggesting that the absence of *sc* regulatory sequences somehow increases the efficiency with which $m7^{Act}$ activates transcription of other target genes (see Discussion).

TABLE 1. Mutations in the basic domain reduce $m7^{Act}$-associated mortality

Male parental genotype	No. of progeny[a] with genotype:	
	TM3; *UAS*	*h-Gal4*; *UAS*
UAS-$m7^{Act}$	66	0
UAS-$m7^{\Delta basicAct}$	87	84
UAS-$m7^{KNEQAct}$	71	66

[a] From the cross *h-Gal4*/*TM3* (female) × *UAS*/*UAS* (male). Data correspond to a single line of *UAS* transformants; similar results were obtained with another independent line for each construct.

Although our results indicate that E(spl) proteins function upstream of the *ac-sc* genes, it is possible that E(spl) proteins act additionally on downstream genes. In support of this idea, we find that $m7^{Act}$ driven by the *GAL4-30A* insertion induces ectopic leg bristles independently of *ac* and *sc* (Fig. 2F and 6F). This suggests that $m7^{Act}$ can activate alternative targets in that tissue, raising the possibility that E(spl) proteins also function downstream of proneural genes to regulate neural differentiation genes.

DISCUSSION

Previous analyses of loss- and gain-of-function phenotypes have demonstrated a negative role of E(spl) in regulating the neural decision, but the molecular basis of this function remains unclear. Here, we have converted E(spl) m7 from a repressor to an activator, $m7^{Act}$, and used this novel protein to demonstrate the ability of E(spl) proteins to act as promoter-bound regulators during neurogenesis. By showing that $m7^{Act}$ functions as a transcriptional activator which efficiently induces neural development and leads to ectopic *ac* and *sc* transcription, we provide evidence that E(spl) proteins normally function as promoter-bound repressors upstream of the *ac-sc* genes.

Our results argue that $m7^{Act}$ elicits neural fates as a transcriptional activator, not as an antimorph. First, we find that a C-terminal m7 truncation, equivalent to that used to construct $m7^{Act}$ but lacking the VP16 activation domain ($m7^{1\text{-}143}$), has no phenotypic effects. This indicates that the activity of $m7^{Act}$ depends on its ability to activate transcription and is not due merely to passive antagonism of endogenous E(spl) proteins. Indeed, $m7^{Act}$ induces bristles in regions that normally lack SOs (e.g., the wing blade) (Fig. 2I and 3G), whereas loss of E(spl) activity in those locations does not produce ectopic bristles (25). Thus, extra bristles must result from de novo

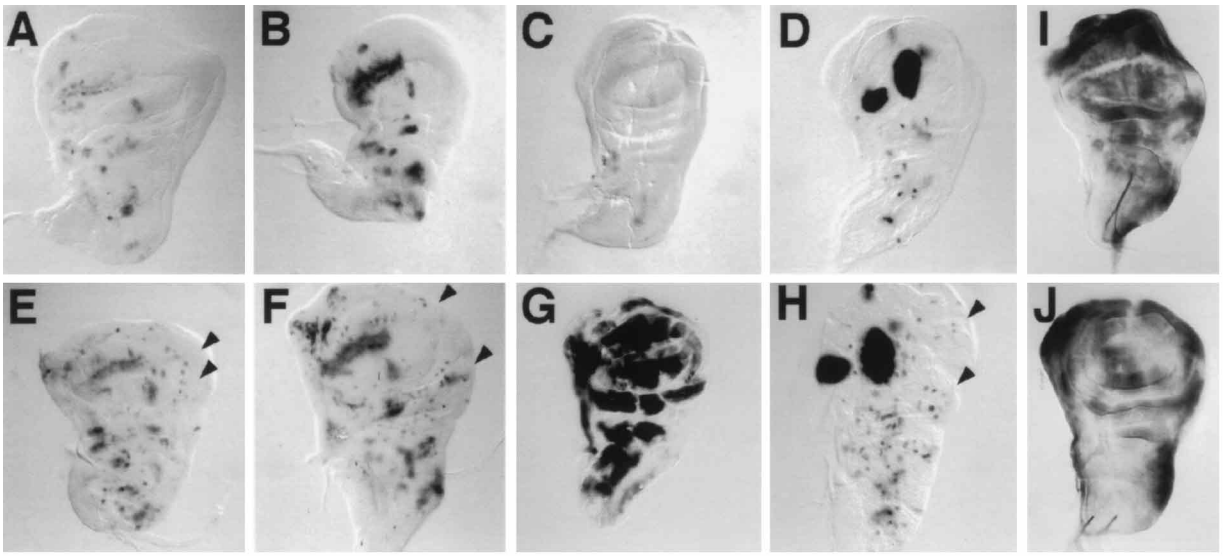

FIG. 5. $m7^{Act}$ activates *ac* and *sc* transcription. Shown are expression patterns of *ac* (A and E), *sc* (B and F), *ac-lacZ* (C and G), and *sc-lacZ* (D and H) transcripts in wild-type (A through D), *h-GAL4*; *UAS*-$m7^{Act}$ (E and H), and *GAL4-69B*; *UAS*-$m7^{Act}$ (F and G) imaginal wing discs. Note the presence of many ectopic sites of *ac* and *sc* expression in the *GAL4/UAS* discs (arrowheads), consistent with the relatively broad domain of *lacZ* expression driven by *h-GAL4* (I) and *GAL4-69B* (J).

activation of proneural and/or neural differentiation genes at ectopic sites of the imaginal disc. Together, these results show that $m7^{Act}$ induces neural fates by activating transcription of genes controlling neural commitment and/or differentiation, not by passively interfering with the normal roles of E(spl) proteins in lateral inhibition.

We have shown that expression of $m7^{Act}$ under the control of strong *GAL4* lines (e.g., *h-GAL4*) leads to closely spaced SOs, as well as to adjacent *A101* positive neural precursors. Thus, it appears that under certain circumstances (presumably high levels of expression), the ability of $m7^{Act}$ to activate genes normally repressed by wild-type E(spl) proteins is so effective that it overcomes the function of the latter during lateral inhibition. We therefore envision $m7^{Act}$ acting in two ways: (i) it induces neural potential on ectopic groups of cells, and (ii) it can impede the singling out of neural precursors within such groups by overriding repression by the endogenous E(spl) proteins.

Our data are consistent with m7 interacting with target promoters through DNA binding rather than by association with other promoter-bound factors. Thus, either deletion of the basic region or the simultaneous mutation of amino acids Lys-15 and Glu-22 strikingly reduces the SO-inducing properties of $m7^{Act}$. Crystal structure analyses of a MyoD bHLH domain-DNA complex (41) predict that residues Lys-15 and Glu-22 directly contact DNA, making it unlikely that they mediate protein-protein recognition. The $m7^{Act}$ derivatives with mutations in the basic domain retain, however, some ability to generate ectopic bristles (Fig. 4). This may reflect weak binding of the mutant proteins to target promoters via protein-protein interactions with E(spl) cofactors. Alternatively, the mutant derivatives could be completely unable to interact with target promoters but could give rise to residual phenotypes by exerting a dominant-negative effect on the endogenous E(spl) proteins. This interpretation is supported by the finding that an E(spl) protein lacking the basic domain can also induce ectopic SOs (46) and by our recent observation that a mutated Hairy derivative without a basic region behaves as an antimorphic factor during segmentation (32). According to this view, $m7^{\Delta basicAct}$ and $m7^{KNEQAct}$ could form inactive HLH dimers with E(spl) or E(spl)-interacting factors and poison them by interfering with their ability to bind DNA.

In contrast, the C-terminal truncation, $m7^{1-143}$, which is not antimorphic, either is unable to form complexes with endogenous factors or does not disrupt activity when incorporated into such complexes. We favor the latter possibility because $m7^{Act}$ appears to activate target genes normally repressed by m7, implying that the C-terminal region is not required for

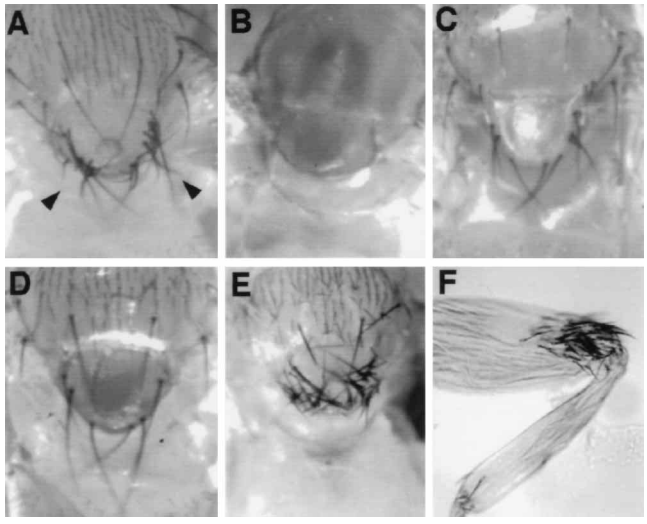

FIG. 6. Mutations in the *ac* and *sc* genes suppress the proneural effects of $m7^{Act}$. (A) Ectopic bristles in the scutellum of a *GAL4-G455.2*; *UAS-*$m7^{Act}$ male (arrowheads) (cf. wild-type pattern in Fig. 2A). (B through E) Phenotypes of *GAL4-G455.2*;*UAS-*$m7^{Act}$ males that are also mutant for both *ac* and *sc* [$Df(1)sc^{10\text{-}1}$] (B), *ac* [$In(1)y^{3PL}\ sc^{8R}$] (C), sc^{M6} (a nonsense mutation at position 114 of Sc) (D), or *sc* [$In(1)sc^{8L}sc^{4R}$] (E). Supernumerary bristles are totally absent in panel B and significantly suppressed in panels C and D, where the number of ectopic bristles is less than half of those in panel A. In contrast, no suppression is observed in panel E, in which the *sc* but not the *ac* promoter is deleted. (F) Ectopic leg bristles are not suppressed by the absence of endogenous *ac* and *sc* genes [$Df(1)sc^{10\text{-}1}$; *GAL4-30A*; *UAS-*$m7^{Act}$]. Note the lack of endogenous bristles due to $sc^{10\text{-}1}$.

accurate promoter specificity. Presumably, $m7^{Act}$ (and $m7^{1-143}$) retains the ability to recognize many of the factors that normally interact with and define the functional specificity of E(spl) (see reference 32 for further discussion).

We have shown that $m7^{Act}$ leads to ectopic transcription of *ac* and *sc*, the two proneural genes that normally control determination of most external SOs. Although we cannot completely exclude the possibility that $m7^{Act}$ induces an upstream activator(s) of *ac* and *sc*, the simplest interpretation is that m7 and other E(spl) proteins directly repress *ac* and *sc* by binding to their *cis*-controlling sequences. This could involve specific DNA sites in the proximal *ac* and/or *sc* promoters, such as a recently identified binding site for the related Hairy bHLH protein (CACGCG) present in the *ac* promoter (47, 61). However, mutation of this site in vivo leads only to *hairy* loss-of-function phenotypes, not to defects in lateral inhibition. Alternatively, E(spl) binding sites might be included within the more distant enhancers that drive simultaneous expression of *ac* and *sc* in specific regions of the imaginal disc (21) (see below). Clearly, the identification of potential E(spl) binding sites in the AS-C will require additional sequence information on the locus, as well as precise knowledge of the sites recognized by homo- and heterotypic combinations of E(spl) proteins.

E(spl) proteins are likely to have other activities that do not involve direct regulation of *ac-sc* expression. Thus, it has been shown that lateral inhibition can regulate the neural potential conferred by expression of proneural genes under the control of heterologous promoters (28). Also, we find that $m7^{Act}$ generates leg bristles in the absence of the *ac* and *sc* genes (Fig. 6F). In this case, $m7^{Act}$ could activate transcription of genes that function downstream of proneural genes to implement neural differentiation. Alternatively, it could act on other proneural genes that are targets of inhibition by E(spl). Finally, it is also possible that E(spl) proteins have a parallel activity in which they sequester bHLH proneural activators. Indeed, it has been shown that ectopic expression of E(spl) derivatives lacking a functional basic domain can suppress SO formation (46). However, our results argue that such an activity would necessarily be weak because it is not shown by the truncated $m7^{1-143}$ protein. Moreover, we have never observed phenotypes consistent with $m7^{Act}$ acting as an inhibitor of neural fate.

The finding that mutations in the *ac-sc* genes reduce or abolish the effects of $m7^{Act}$ argues that its proneural activity is largely mediated by ectopic *ac-sc* expression. Surprisingly, although a point mutation in the coding region of *sc* (sc^{M6}) clearly suppresses the $m7^{Act}$ phenotype (Fig. 6D), a complete deletion of the gene [$In(1)sc^{8L}sc^{4R}$] does not (Fig. 6E). Both mutations completely inactivate Sc: the sc^{M6} allele encodes a prematurely terminated Sc protein which lacks the HLH domain and downstream sequences and whose accumulation cannot be detected with a polyclonal anti-Sc antibody (21), and *sc* coding sequences are deleted in $In(1)sc^{8L}sc^{4R}$. Thus, the failure to suppress the proneural activity of $m7^{Act}$ in $In(1)sc^{8L}sc^{4R}$ flies could be due to enhanced activation of other proneural genes in this genotype. It has recently been shown that *ac* and *sc* are under the control of shared regulatory sequences within the AS-C (21). It is possible that $m7^{Act}$ bound to such sequences is shared between the *ac* and *sc* promoters and that deleting one transcription start site allows for more effective activation of the other.

Recently, the conserved C-terminal WRPW motif present in the E(spl) and Hairy proteins has been shown to mediate binding in yeast and in vitro to the Groucho (Gro) protein (18, 48). Gro is a nuclear protein required for neural suppression by lateral inhibition (16, 24, 25, 53). It does not contain a recognizable DNA-binding domain but includes repeated WD motifs also present in the yeast corepressor TUP1 (33). $m7^{Act}$ lacks the WRPW domain, arguing that neither this domain nor Gro is required for promoter binding by E(spl) proteins. Related Hairy-VP16 constructs also show promoter-specific transcriptional activity during segmentation (32) and bristle patterning (31a). Our results support a model in which m7 and other E(spl) proteins regulate neurogenesis by targeting the Gro corepressor to proneural promoters (48).

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