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Two modes of recruitment of E(spl) repressors onto target genes

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SUMMARY

The decision of ectodermal cells to adopt the sensory organ precursor fate in *Drosophila* is controlled by two classes of basic-helix-loop-helix transcription factors: the proneural Ac and Sc activators promote neural fate, whereas the E(spl) repressors suppress it. We show here that E(spl) proteins m7 and mγ are potent inhibitors of neural fate, even in the presence of excess Sc activity and even when their DNA-binding basic domain has been inactivated. Furthermore, these E(spl) proteins can efficiently repress target genes that lack cognate DNA binding sites, as long as these genes are bound by Ac/Sc activators. This activity of E(spl)m7 and mγ correlates with their ability to interact with proneural activators, through which they are probably tethered on target enhancers. Analysis of reporter genes and sensory organ (bristle) patterns reveals that, in addition to this indirect recruitment of E(spl) onto enhancers via protein-protein interaction with bound Ac/Sc factors, direct DNA binding of target genes by E(spl) also takes place. Irrespective of whether E(spl) are recruited via direct DNA binding or interaction with proneural proteins, the co-repressor Groucho is always needed for target gene repression.

Key words: Basic-helix-loop-helix, Proneural, HES, Transcriptional repression, Neurogenesis, Lateral inhibition, *Drosophila*, E(spl)

INTRODUCTION

Basic helix-loop-helix (bHLH) proteins constitute a large family of transcriptional regulators that are characterized by a basic DNA-binding domain contiguous with a dimerization domain consisting of two amphipathic α-helices separated by a loop. Members of this family are implicated in a multitude of biological functions, from proliferation to response to toxic stress (Ledent and Vervoort, 2001; Massari and Murre, 2000). Most notable is a class of bHLH proteins, termed Class II, which are capable of directing cells towards specific fates; well-studied examples are the myogenic and the proneural factors (Hassan and Bellen, 2000). These bHLH proteins dimerize (via their HLH domains) with ubiquitous bHLH Class I co-factors, also known as E-proteins, as a prerequisite to DNA binding (Murre et al., 1989). The heterodimer acts as a transcriptional activator of multiple target genes, some of which encode transcription factors, thus setting off a cascade of gene regulation that implements the particular developmental programme. Ac, Sc and L'sc are among the proneural bHLH proteins in *Drosophila* and together with the E-protein Daughterless (Da) are responsible for specifying most CNS and external sensory neural precursors (Campuzano and Modolell, 1992).

Within the anlagen of the CNS and PNS, proneural genes are initially expressed in groups of cells termed proneural clusters (Campuzano and Modolell, 1992). From these broad domains, only a subset of cells will commit to the neural fate. These neural precursors transiently upregulate proneural gene expression and activate a number of neural differentiation genes, such as *ase*, *sens*, *dpn* and others (Bier et al., 1992; Dominguez and Campuzano, 1993; Jarman et al., 1993; Nolo et al., 2000), which are direct transcriptional targets of proneural bHLH activators. The remaining cells of the proneural cluster are inhibited from embarking into a neural pathway and will either continue proliferation or differentiate to alternative cell types, such as epidermis (Artavanis-Tsakonas and Simpson, 1991). This is the outcome of intercellular signaling within the proneural cluster, which is mediated by the Notch pathway (Artavanis-Tsakonas et al., 1999) and is termed lateral inhibition. Cells that receive a high level of Notch signal cannot turn on the proneural target genes (such as *ase*, *dpn*, etc.); this block requires the activity of members of yet another class of bHLH proteins, named Class VI or HES proteins (Fisher and Caudy, 1998). The seven clustered E(spl) genes in *Drosophila*, *m8*, *m7*, *m5*, *m3*, *mβ*, *mγ* and *mδ* (Delidakis et al., 1991; Schrons et al., 1992), encode Class VI bHLH proteins and are directly turned on (transcriptionally) by Notch signaling (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Their products accumulate in all cells of the proneural cluster, but are minimal within the neural precursors (Jennings et al., 1994); they can be therefore considered 'anti-neural' proteins. Indeed, deletion of the entire E(spl) locus results in severe overcommitment of neural precursors (Lehman et al., 1983). However, mutations in individual *E(spl)* genes display no phenotypic defects, as a

result of partial functional redundancy, a fact that prohibits forward genetic dissection of E(spl) protein function (Delidakis et al., 1991; Ligoxygakis et al., 1999; Schrons et al., 1992).

The link between proneural bHLH proteins, Notch signaling and HES proteins is evolutionarily conserved, as it is encountered also in vertebrates, where the cellular events of neurogenesis are very distinct from those in insects (Kageyama and Nakanishi, 1997). In both phylogenetic groups, allocation of neural versus non-neural fates is the outcome of two antagonistic bHLH activities: proneural proteins that promote neurogenesis and HES proteins that inhibit it. As in *Drosophila*, in vertebrates some HES genes are direct transcriptional targets of Notch. Despite the central importance of these bHLH transcription factors in early neural commitment, there are many gaps in our knowledge of the regulatory circuits underlying neurogenesis, both in terms of the target genes of proneural and HES genes, and in terms of the mechanisms of gene activation and repression by these bHLH proteins. It was originally proposed that E(spl) proteins might block neurogenesis in *Drosophila* by repressing proneural genes (Martin-Bermudo et al., 1995; Skeath and Carroll, 1992). More recent data suggest that this is true only for specific enhancers of the proneural genes that are autostimulatory and sensory organ precursor (SOP) specific (Culi and Modolell, 1998), while the major function of E(spl) proteins is to repress downstream target genes of the proneural proteins (Culi and Modolell, 1998; Nakao and Campos-Ortega, 1996). HES proteins are indeed transcriptional repressors. Key amino acid differences between the basic domains of HES and Ac/Sc proteins endow these different bHLH factors with distinct target site specificities: Da-Ac/Sc heterodimers bind the E_A box GCAGSTG (Singson et al., 1994), whereas E(spl) homodimers preferentially bind to E_B-boxes (CACGTG) and variants thereof, the C and N boxes (CACGCG and CACNAG, respectively) (Jennings et al., 1999; Oellers et al., 1994; Ohsako et al., 1994; Tietze et al., 1992; Van Doren et al., 1994). E_A, E_B, C and N boxes are encountered clustered in enhancers of proneural target genes, such as *ase* and *dpn*, which are expressed strongly in the neural precursor and repressed in the remaining proneural cluster cells. The importance of E_A sites in such enhancers has been confirmed by mutagenesis; ablation of E_A boxes leads to loss of transcriptional activity (Culi and Modolell, 1998; Jarman et al., 1993). The same does not hold true, however, for E_B/C/N boxes; mutation of these does not lead to derepression of reporter genes – mutant versions of the *sc SMC* enhancer lacking all E(spl) binding sites are still expressed only in the SOPs (Culi and Modolell, 1998). Furthermore, E(spl) proteins retain residual activity after disruption of their DNA-binding basic domain (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996; Oellers et al., 1994), although this is still somewhat controversial (Jiménez and Ish-Horowicz, 1997). As a result, alternative models regarding the mechanism of target gene repression by E(spl) have been suggested. One proposes that E(spl) can sequester activator complexes away from DNA (Alifragis et al., 1997; Kageyama and Nakanishi, 1997). A second model proposes that E(spl) proteins may be recruited to target enhancers indirectly, via interactions with other uncharacterized DNA bound factors (Culi and Modolell, 1998).

The simplest explanation for the fact that proneural target enhancers can be repressed by E(spl) in the absence of cognate DNA-binding sites is that E(spl) proteins use a DNA-binding-independent mechanism for proneural target gene repression, instead of, or in addition to, a DNA-binding-dependent one. In the present work, we ask if this is indeed the case. We present in vivo data that strongly support protein-tether-mediated recruitment of some E(spl) repressors onto DNA – interestingly, this is achieved via protein-protein interactions with proneural activators. We demonstrate that direct DNA binding also contributes significantly to E(spl) activity, while activator sequestering is unlikely to be used by E(spl) proteins to counteract proneural function.

MATERIALS AND METHODS

Constructs

pUAST-E(spl)m7, pUAST-E(spl)mγ and pUAST-mδ have been described previously (Ligoxygakis et al., 1999). pUAST-E(spl)m7VP16 and pUAST-E(spl)m7KNEQVP16 have been described previously (Jiménez and Ish-Horowicz, 1997).

Ract-E(spl)mγ, Ract-E(spl)mδ and Ract-E(spl)m7 were constructed by subcloning the relevant *Bgl*II-*Xho*I fragments from pUAST constructs (Ligoxygakis et al., 1999) into the *Bam*HI/*Sal*I sites of RactHAdh, an *actin5C* promoter-containing plasmid (Swevers et al., 1996). pT5-0.9wt/luc (*ac* proximal promoter luciferase reporter), as well as its C-box mutated version pT5-0.9mut/luc have been described elsewhere (Ohsako et al., 1994). pAc-Da and pAc-Sc have been described previously (Van Doren et al., 1992).

pUC-E(spl)m7KNEQ was constructed by simultaneous ligation of an *Eco*RI/*Bam*HI 5′-terminal fragment of E(spl)m7KNEQVP16 [from a pBluescript KSII/*Eco*RI/*Xba*I clone – the *Bam*HI site, a naturally occurring site within *E(spl)m7* at codon G142 is the junction between E(spl)m7 and VP16 coding regions – (Jiménez and Ish-Horowicz, 1997)] with a *Bam*HI/*Sal*I C-terminal fragment of E(spl)m7 (from a pBluescript KSII/*Eco*RI/*Xho*I clone) into pUC18/*Eco*RI/*Sal*I. Then, it was subcloned in pUASTmod vector [pUASTmod is a modified pUAST vector that contains a synthetic oligonucleotide bearing an optimized translation start site just before the cloning sites (Ligoxygakis et al., 1999)] and digested with EcoRI/XhoI.

E(spl)mδVP16 was released from pHK3N-E(spl)mδVP16, kindly provided by B. Jennings and S. Bray, as a *Bam*HI/*Bgl*II fragment and subcloned into pUAST/*Bgl*II. The VP16 domain is fused to amino acid 169.

Ract-E(spl)mγ was subjected to mutagenesis in order to create a *Bam*HI site, followed by a stop codon, just after the R154 codon. A *Bam*HI/*Sal*I VP16 fragment was then inserted at this site to create Ract-E(spl)mγVP16. E(spl)mγVP16 was released from Ract E(spl)mγVP16 as an *Eco*RI/*Pst*I fragment and subcloned into pBluescipt SKII. pUASTmod E(spl)mγVP16 was then constructed by inserting E(spl)mγVP16 into pUASTmod/*Eco*RI/*Xba*I.

pBluescript KSII-E(spl)mγKNEQ was produced by mutagenizing pBluescript KSII-E(spl)mγ [E(spl)mγ cloned into the *Eco*RI site]. The E(spl)mγKNEQ mutagenesis primer (sequence available upon request) was based on the E(spl)m7KNEQ construct described elsewhere (Jiménez and Ish-Horowicz, 1997); it changes two conserved basic domain amino acids: K17 to N and E24 to Q. pBS-E(spl)mγKNEQ-VP16 was constructed by replacing an *Eco*RV fragment of pBluescript SKII E(spl)mγVP16 with a *Sma*I/*Eco*RV fragment from pBluescriptKSII E(spl)mγKNEQ, which corresponds to the 5′ terminal part of E(spl)mγKNEQ that bears the desirable point mutations. E(spl)mγKNEQ-VP16 was isolated with *Eco*RI and *Xba*I and cloned into pUASTmod.

All DNA manipulations were carried out using standard techniques.

Mutagenesis reactions were performed using the Gene Editor kit by Promega according to the manufacturer's instructions. Every construct was sequenced prior to injection into *Drosophila* embryos or transfection into S2 cells to verify success of mutagenesis procedures and integrity of the constructs.

Cell culture, transient transfections, luciferase and β-galactosidase assays

Drosophila Schneider S2 cells were cultured at 25°C in M3 medium supplemented with 10% heat inactivated fetal bovine serum and gentamycin. Transient transfections of approx. 2.5×10^6 cells/2.5 ml were performed with the $Ca_3(PO_4)_2$ co-precipitation method. All plasmids were purified with Qiagen columns, according to the manufacturer's instructions. Plasmids and amounts used per well are listed in the panels of Fig. 1; all effectors were expressed under the *actin5C* promoter using either the pAc or the RactHAdh vectors. Additionally 100 ng hs-*lacZ* plasmid was added for normalization. Empty vectors were used to bring the total DNA amount per transfection to 5 μg. β-Galactosidase assays were conducted in order to measure the efficiency of the transfections and to normalize luciferase measurements. Luciferase assays were performed using the luciferase kit (Promega) according to the manufacturer's instructions. Luminescence was measured using a Turner TD-20/20 luminometer.

Drosophila strains and crosses

All transformants were obtained in a yw^{67c23} background. All crosses were kept at 25°C, unless otherwise stated.

EE4-lacZ and *UAS-sc* transgenic flies have been described previously (Culi and Modolell, 1998). *UAS-E(spl)m7VP16* and *UAS-E(spl)m7KNEQVP16* flies have been described previously (Jiménez and Ish-Horowicz, 1997). *Gbe-B1-lacZ* flies have been described previously (Jennings et al., 1999). $Df(1)sc^{10\text{-}1}$ (abbreviated as $sc^{10\text{-}1}$), gro^{E48}, $Df(3R)gro^{b32.2}$ (deletion of the entire *E(spl)* locus), *Df(3R)P709* and *Df(3R)Espl22* are described in FlyBase (flybase.bio.indiana.edu).

We use the following abbreviations for Gal4 lines: *omb-Gal4* for $P[Gal4]bi^{omb\text{-}Gal4}$, *pnr-Gal4* for $P[GawB]pnr^{MD237}$ and *ap-Gal4* for $P[GawB]ap^{md544}$, all described in FlyBase (flybase.bio.indiana.edu).

In mosaic analysis experiments, clones were induced by heat shocking larvae (1 hour at 38°C) 48-96 hours after egg laying (AEL) of the following genotypes:

omb-GAL4/hs-FLP; EE4-lacZ/UAS-sc UAS-E(spl)m7; FRT82B hs-πMyc87E97E/FRT82B kar^2 ry^{506} gro^{E48} (experiment shown in Fig. 8A) or

hs-FLP/+; EE4-lacZ/+; FRT82B hs-πMyc87E97E/FRT82B kar^2 ry^{506} $P[gro^+]$ *Df(3R)* $gro^{b32.2}$ (experiment shown in Fig. 2M).

Larvae were picked at wandering third instar, heat shocked again for 90 minutes (38°C) to induce πMyc expression and then allowed to recover at 25°C for 90 minutes before dissection.

X-gal staining and immunocytochemistry

For X-gal staining, larvae were dissected in phosphate buffer and fixed in 1% glutaraldehyde/1×PBS for 9 minutes at room temperature. After extensive washing with 1×PBS, they were placed in colorization buffer [10 mM Na-PO_4 buffer (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_4[Fe_{II}(CN)_6]$, 3 mM $K_4[Fe_{III}(CN)_6]$, 0.3% Triton X-100], pre-warmed at 65°C, containing 0.2% X-gal and they were incubated at 37°C in a humid chamber for 15 minutes to overnight. For the experiment in Fig. 2K,L, larvae from the following cross were used:

EE4-lacZ/EE4-lacZ; TM6B, Tb/ Df(3R)P709 x $P[gro^+]/P[gro^+]$*; TM6B, Tb/ Df(3R)Espl22.*

Tb^+ larvae have the viable deficiency combination $P[gro^+]$*; Df(3R)E(spl)22 /Df(3R)P709*, which is null for *E(spl)m7* and *m8* (Delidakis et al., 1991), whereas TM6B (Tb^-) carry a wild-type copy of the *E(spl)-C*. To measure β-galactosidase activity semi-quantitatively, both Tb and Tb^+ larvae were fixed and incubated in the same test tubes. They were distinguished by leaving a piece of gut on one of the genotypic classes at dissection. X-gal development lasted only 20 minutes to avoid saturation.

Immunocytochemistry was performed as described previously (Pavlopoulos et al., 2001). Antibodies were from the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City), Cappel, Jackson Immunochemicals and Molecular Probes. Special conditions were used for the anti-Ac antibody: Dissected larvae were fixed in 1×PEM [100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$ (pH 6.9) corrected with KOH] 1% Triton X-100, 1% PFA, for 1 hour at 8°C. Subsequent washes and incubations were carried out in a 50 mM Tris-Cl (pH 6.8), 150 mM NaCl, 0.5% NP40 buffer, supplemented, where needed, with 1-5% normal goat serum. Specimens were observed either on a Leica Diaplan microscope or on a Leica SP confocal microscope (University of Crete).

RESULTS

Some E(spl) proteins can repress target genes without direct DNA binding

Our first indication that E_B/C sites are dispensable for E(spl)-mediated repression came from reporter gene analysis in transfected Schneider S2 cells. We used T5-0.9wt/luc, a luciferase reporter driven by the proximal 5′ regulatory region of the *ac* gene (Ohsako et al., 1994). This fragment probably constitutes an autoregulatory element, as it contains three E_A boxes and can be activated by Da/Sc or Da/Ac; it also contains one C-box needed for repression by the E(spl)-related protein Hairy (Ohsako et al., 1994; Van Doren et al., 1994). When we included an *E(spl)* expression plasmid in addition to those expressing *da* and *sc* in a transient transfection experiment, we observed repression of T5-0.9wt/luc; Fig. 1 shows the results for *E(spl)m7*, *mγ* and *mδ*. We also used a mutant version of the same reporter, T5-0.9mut/luc, in which the C box had been mutated, disabling repression by Hairy (Ohsako et al., 1994). As shown in Fig. 1, E(spl)m7 and mγ were still capable of repressing the mutant reporter, whereas E(spl)mδ had lost the ability to repress, in fact it somewhat activated transcription [an unexplained result, also observed with Hairy (Ohsako et al., 1994)]. It thus appears that different members of the HES family of repressors may use different mechanisms of repression, with Hairy and E(spl)mδ being strictly dependent on a DNA target site, versus E(spl)m7 and mγ retaining activity in the absence of direct DNA binding.

To gain more insight into this novel repression mechanism of E(spl)m7 and mγ, we turned into an in vivo system. We decided to study an artificial reporter gene in the fly driven solely by E_A boxes to avoid the possibility of E(spl) proteins binding to atypical sites, a behavior for which there is ample precedent (Chen et al., 1997; Culi and Modolell, 1998; Yang et al., 2001), and may have been the cause of repression of T5m-luc in our transfection experiments. The *EE4-lacZ* reporter, consisting of eight tandem E_A boxes in front of a minimal promoter (Table 1), was shown by Culi and Modolell (Culi and Modolell, 1998) to respond to proneural proteins by turning on in all proneural cluster cells in the wing disk. We assayed the response of *EE4-lacZ* in larval imaginal disks in response to E(spl) proteins expressed using the *Gal4/UAS* system. Overexpression of *E(spl)m7* abolished *EE4-lacZ*

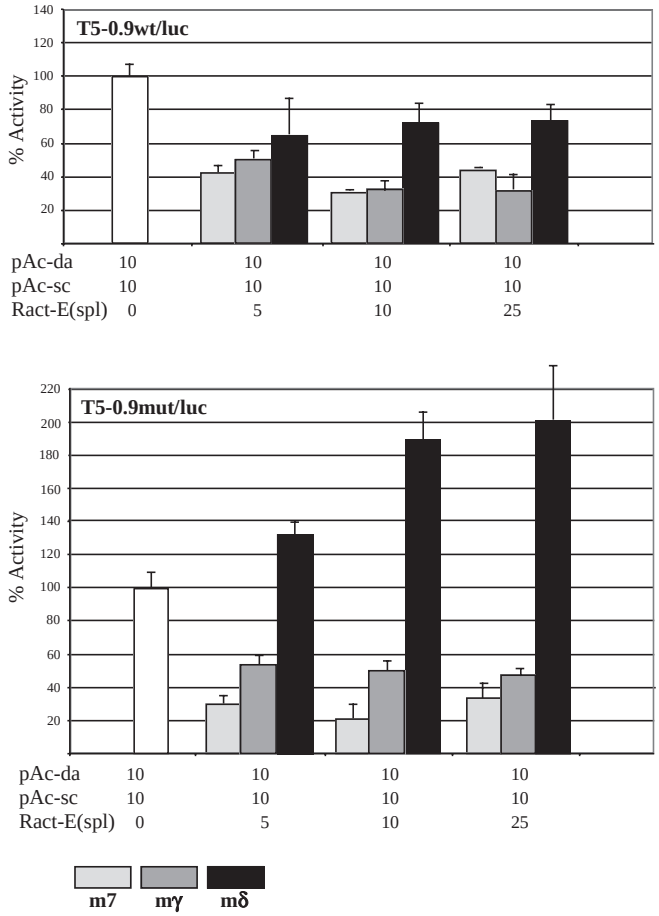

Fig. 1. Response of T5-0.9wt/luc and T5-0.9mut/luc reporters to bHLH effectors E(spl) m7, mγ and mδ transiently transfected into S2 cultured cells. Amounts of transfected plasmids per well are shown in ng. Graphs show averages of four replicates with standard deviations as error bars. Luciferase activity obtained in the da+sc transfection (no repressors) is arbitrarily defined as 100%.

activity, whereas *E(spl)mδ* only moderately reduced expression (Fig. 2A-C). This was somewhat surprising, given that E(spl) proteins do not recognize the E_A target site (Culi and Modolell, 1998; Jennings et al., 1999; Oellers et al., 1994). Thus, we entertained the possibility that the repression by E(spl) was not a direct effect on the *EE4* enhancer, rather it could have arisen from the fact that overexpression of E(spl) repressed endogenous proneural genes, which in turn are needed to activate *EE4*. We therefore visualized Ac protein in wing disks overexpressing E(spl)m7 (Fig. 2I,J). The overall proneural pattern of Ac was not altered, but expression levels were variably reduced within the overexpression domain. Strongly expressing SOP cells within proneural clusters were never seen (Fig. 2J arrow), in agreement with the well-established sensory-organ suppressive activity of E(spl) proteins (Culi and Modolell, 1998; Giebel and Campos-Ortega, 1997; Ligoxygakis et al., 1999; Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995).

In order to test more rigorously the mechanism of E(spl)-mediated repression of *EE4-lacZ* and to avoid the fluctuation of endogenous proneural protein levels caused by E(spl) overexpression, we decided to bypass the need for endogenous proneural proteins altogether by providing excess Sc exogenously. A *UAS-sc* transgene was expressed alone (Fig. 2E) or together with *UAS-E(spl)* transgenes (Fig. 2F,G). Ectopic Sc gave the expected broad, yet patchy, ectopic activation of *EE4-lacZ*. Patchy activation of proneural target genes has been observed before (Hinz et al., 1994) and apparently reflects stochastic damping of Sc activity, at least partly because of induction of endogenous *E(spl)* genes (Cooper et al., 2000; Nellesen et al., 1999), which inhibit Sc activity (Giebel and Campos-Ortega, 1997; Hinz et al., 1994) (this work). Co-expression of *E(spl)m7* resulted in strong repression of the *EE4* enhancer (Fig. 2E,F), whereas *E(spl)mδ* did not affect activation by *UAS-sc* (Fig. 2E,G). We observed the same effects using two different *GAL4* lines, *pnr-GAL4* (Fig. 2) and *omb-GAL4* (data not shown), which drive expression in a central wing pouch region (visualized in Fig. 5). It thus appears that E(spl)m7, but not mδ, can repress transcription of *EE4-lacZ* without directly binding to DNA, consistent with the different behavior of these proteins in transfection assays. mδ still weakly represses *EE4-lacZ* transcription (Fig. 2C), most probably through repression of activators, such as *sc*. Another *UAS-E(spl)* transgene, *E(spl)mγ*, was able to repress *UAS-sc*-driven activation of *EE4-lacZ*, similar to *E(spl)m7* (data not shown).

If direct DNA binding is dispensable for the repression by E(spl)m7 and mγ of *EE4-lacZ*, mutant versions that lack the DNA-binding basic domain should be functional. We therefore generated E(spl)m7KNEQ, a double point mutation in the basic domain, which abolishes DNA binding (Jiménez and Ish-Horowicz, 1997), and tested it in transgenic flies. *UAS-E(spl)m7KNEQ* had strong repressive activity on *EE4-lacZ* when expressed either alone or together with *UAS-sc* (Fig. 2D,H), confirming the dispensability of the basic domain in this assay. *UAS-mγKNEQ*, which bears the same basic domain inactivating mutations as *m7KNEQ*, was also capable of repressing EE4-lacZ, even in the presence of exogenous *UAS-sc* (data not shown).

In a converse experiment, we examined the activity of the *EE4-lacZ* reporter in loss-of-function backgrounds for *E(spl)*. *EE4-lacZ* was consistently more active in a mutant background lacking *E(spl)m7* and *m8* (see Materials and Methods) compared with wild type (12 disks of each genotype scored in three repeats of the experiment; Fig. 2K,L). This happens even though the

Table 1. Sequences of artificial reporter genes

Reporter	Insert	Reference
EE4-lacZ	(GATCCAAATCCAGCCCAAAGAACTAAATA**CACCTGC**GAGCTAAATA**CACCTGC**A)$_4$	Culi and Modolell, 1998
Gbe-B1-lacZ	(CTAGAGCGAT***TGAACCGGTCCT***GCGGT)$_3$, a 21 bp polylinker, (TCGAGGGTGG**CACGTG**CCATTG)$_3$	Jennings et al., 1999

Inserts shown were cloned upstream of a basal *hsp70* promoter and the *lacZ*-coding region. E_B boxes are in bold, E_A boxes are in bold-underline and Grh-binding sites are in bold italic.

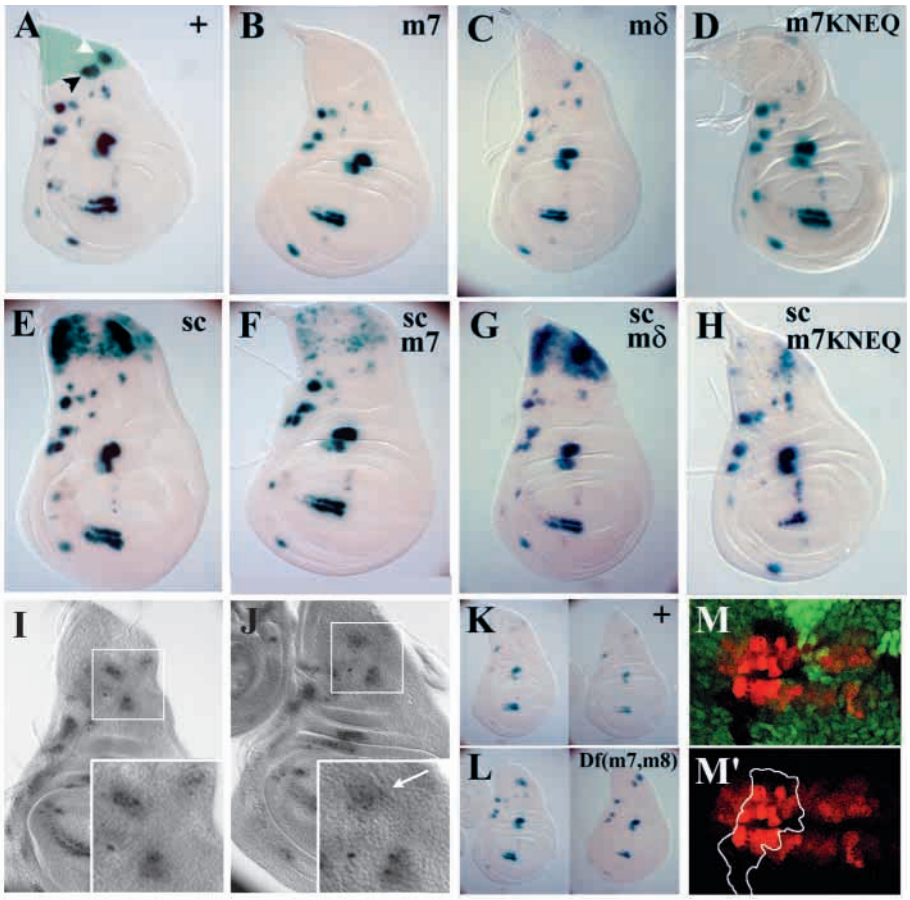

Fig. 2. Response of *EE4-lacZ* to bHLH transgene expression and *E(spl)* loss of function in third instar wing disks. (A-H) *UAS* transgenes (as noted on each panel) were driven by *pnr-Gal4*, which expresses in the proximal notum (region shaded green in A; note SC and DC proneural clusters, white and black arrowheads, respectively). (A) Wild-type pattern, which corresponds to the proneural clusters present at this stage (compare with Ac accumulation in I). (B) *EE4-lacZ* was abolished by *UAS-E(spl)m7* expression. (C) *EE4-lacZ* was significantly reduced by *UAS-E(spl)mδ* expression. (D) *UAS-E(spl)m7KNEQ* also repressed strongly. (E) *EE4-lacZ* was activated when *UAS-sc* is present, but severely diminished when *E(spl)m7* (F) or *E(spl)m7KNEQ* (H) were co-expressed. Note that weak patchy expression remains. (G) Co-expression of *UAS-E(spl)mδ* did not suppress *EE4-lacZ* activation by *UAS-sc*. (I) Wild-type and (J) *UAS-E(spl)m7; pnr-GAL4* disk immunostained for Ac. Accumulation in proneural clusters was seen in both cases – despite E(spl)m7 expression. Over-accumulation in SOPs of the dorsocentral cluster was abolished by E(spl)m7 (arrow). Insets show the boxed region of the notum at twice the magnification. (K,L) *EE4-lacZ* disks developed lightly with X-gal to compare levels of expression between wild-type disks (K – compare with wild-type disk developed longer in A) and disks null for *E(spl)m8* and *m7* (L). Although the *E(spl)* mutation does not affect expression pattern, it results in a more intense signal in all proneural clusters. (M,M′) A mitotic clone null for the entire *E(spl)-C* is visualized by absence of green nuclear πMyc staining. β-galactosidase (*EE4-lacZ*) is visualized in red. The clone (outlined in M′), which overlaps the distal wing margin, shows more intense *EE4-lacZ* staining, consistent with loss of repression due to the absence of *E(spl)* function. (M′) Red channel only.

number and pattern of SOPs in this mutant background is identical to wild type, presumably owing to the activity of the remaining *E(spl)* genes (Delidakis et al., 1991). We conclude that activity of *E(spl)m7* and *m8*, the two most highly expressed *E(spl)* genes in wing disk proneural clusters (de Celis et al., 1996), attenuates *EE4-lacZ* expression. As *E(spl)* genes other than *m7* and *m8* were still present in the above genetic background, we tested the response of *EE4-lacZ* in homozygous clones of a deficiency removing the entire *E(spl)* locus (Fig. 2M). Increased levels of β-galactosidase expression were again observed within mutant patches, confirming the response of *EE4-lacZ* to E(spl) activity, despite the absence of E(spl) binding sites on this reporter. A caveat in interpreting these experiments is that *E(spl)* loss-of-function may increase *sc* expression, which would then act on the *EE4-lacZ* reporter.

Ectopic expression of *sc* in flies is known to induce formation of supernumerary chetae (Hinz et al., 1994; Rodriguez et al., 1990), reflecting induction of endogenous Sc target genes. We tested individual *UAS-E(spl)* transgenes for their ability to block ectopic cheta production by *sc*. When expressed alone by *pnr-Gal4*, all *UAS-E(spl)* genes inhibited formation of both macro- and micro-chetae, resulting in a bald stripe in the center of the thorax (Fig. 3A,C,E), in agreement with previous findings (Culi and Modolell, 1998; Giebel and Campos-Ortega, 1997; Ligoxygakis et al., 1999; Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995). This was even true for *UAS-E(spl)m7KNEQ* (Fig. 3G) and *E(spl)mγKNEQ* (data not shown), suggesting that, under the conditions of this assay, direct DNA binding (to presumably natural target genes controlling SOP fate) is dispensable. When co-expressed with *UAS-sc*, *UAS-E(spl)m7* and *mγ*, as well as *E(spl)m7KNEQ* and *mγKNEQ*, still produced completely bald thoracic stripes (Fig. 3B,D,H), indicating that these proteins can inhibit the activity of both endogenous and overexpressed Sc on (endogenous) target genes very effectively. By contrast, *UAS-E(spl)mδ* only partially suppressed the ectopic bristle phenotype of *UAS-sc* (Fig. 3F). This behavior was essentially the same as that documented above using *EE4-lacZ* and was further confirmed by assaying the expression of two target genes, *SMC-lacZ* and *ase* (data not shown). The sole difference was that E(spl)mδ could partially decrease the number of ectopic bristles (Fig. 3F), while having no effect on *EE4-lacZ* activation (Fig. 2G). We attribute the bristle/SOP suppressive activity of E(spl)mδ to DNA-binding-dependent repression of proneural target genes (see Discussion). Taken together, reporter and bristle repression assays demonstrated that E(spl)m7 and mγ, but not mδ, can repress an E_A-driven artificial reporter gene, as well as endogenous target genes, despite the overexpression of *sc*. Based on the fact that basic domain mutated versions of E(spl)m7 and mγ are much more potent repressors than mδ,

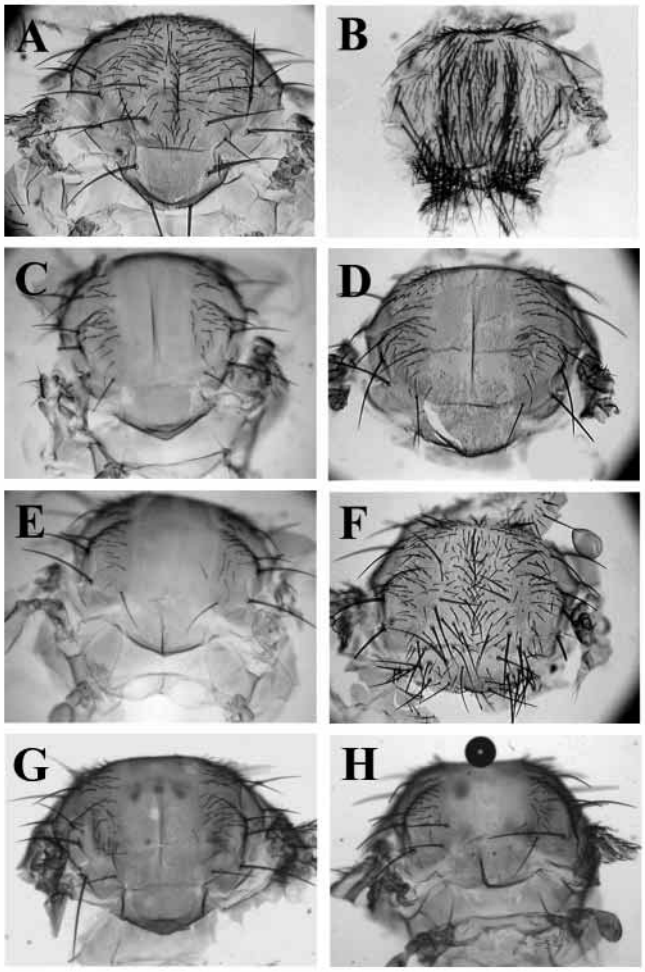

Fig. 3. Adult phenotypes caused by bHLH transgene expression via *pnr-Gal4*. The *UAS* transgenes were as follows: (A) none, (B) *UAS-sc*, (C) *UAS-E(spl)m7*, (D) *UAS-sc, UAS-E(spl)m7* co-expression, (E) *UAS-E(spl)mδ*, (F) *UAS-sc, UAS-E(spl)mδ* co-expression, (G) *UAS-E(spl)m7KNEQ* and (H) *UAS-sc, UAS-E(spl)m7 KNEQ* co-expression.

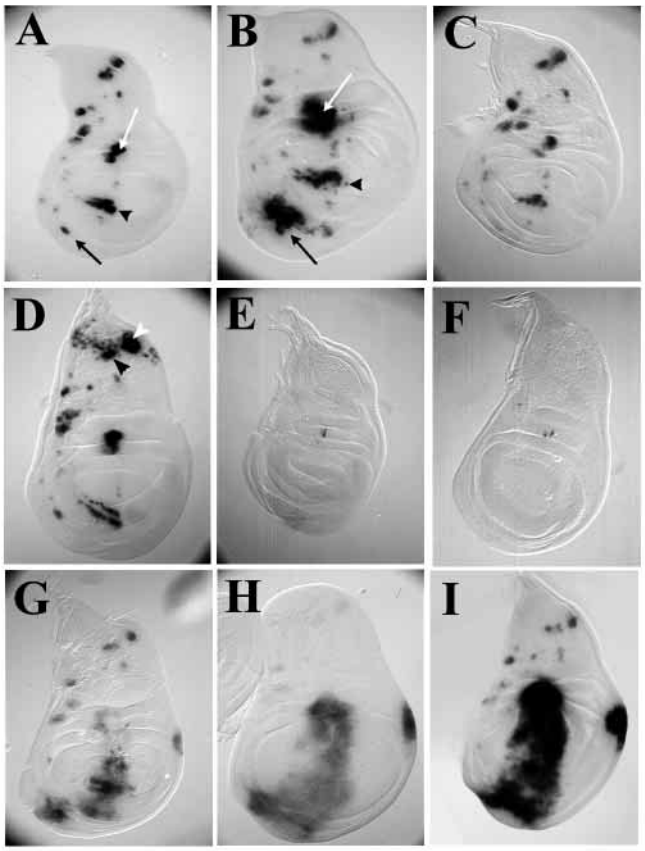

Fig. 4. Response of *EE4-lacZ* to E(spl)VP16 variants. All panels show wing disks carrying one copy of the *EE4-lacZ* reporter. (A) Wild type. (B) *omb-Gal4; UAS-E(spl)m7VP16*. Note the intense staining in three proneural clusters: dorsal radius (white arrow), wing margin (arrowhead) and ventral radius (black arrow). The extent of the *omb-Gal4* expression domain is visualized in Fig. 5C-E. (C) *omb-Gal4; UAS-E(spl)mδVP16*. No activation is observed. (D) *UAS-E(spl)m7VP16; pnr-Gal4*. In addition to intense staining in the SC (white arrowhead) and DC (black arrowhead) proneural clusters, dispersed individual cells are expressing *EE4-lacZ*. (E) $sc^{10\text{-}1}/Y$ background essentially abolishes *EE4-lacZ* activity. (F) $sc^{10\text{-}1}/Y$*; UAS-E(spl)m7VP16; pnr-Gal4*. The intense response of *EE4-lacZ* observed in D is absent due to absence of Ac and Sc. (G) *omb-Gal4; UAS-sc*. Note weak and somewhat patchy response of *EE4-lacZ* to uniform expression of Sc. (H,I) *omb-Gal4; UAS-sc UAS-E(spl)m7VP16*. Note a more uniform and much more intense response. H is under-stained, whereas I is stained to the same extent as G.The notum proneural clusters (top of each panel), where *omb-Gal4* is not expressed, can be used to judge the extent of color development in A-C and G-I.

we conclude that in this assay some activity of E(spl) proteins other than their direct DNA binding ability is most important in target gene repression.

m7 is tethered to E_A-boxes via proneural protein complexes

We have previously shown that E(spl) proteins interact selectively with proneural ones in a yeast two-hybrid assay (Alifragis et al., 1997); E(spl)m7 and mγ interact with Ac, Sc and Da, whereas mδ interacts with none. In the light of results presented in the previous section, the interesting possibility arose that the ability of E(spl) proteins to interact with activator bHLH proteins might underlie the ability of the former to repress target genes in the absence of direct DNA binding and enhance their potency in neural fate suppression. The question arises as to how interaction with proneural proteins might help realize this potent repressive activity: do E(spl) proteins sequester proneural activators off the target DNA or do they use the proneural complexes as tethers to bind to DNA? A way to approach the question of whether a repressor works on or off DNA has been devised by Jiménez and Ish-Horowicz (Jiménez and Ish-Horowicz, 1997), whereby a fusion of a strong transcriptional activation domain (VP16) to a repressor is tested for its ability to activate transcription, which can only happen if the VP16 domain is tethered to the DNA. If, however, the repressor works by sequestering activators off DNA, the VP16-tagged repressor should still be able to repress (rather than activate) target genes. We expressed a hybrid E(spl)m7VP16 protein (Jiménez and Ish-Horowicz, 1997) in wing disks and assayed its effect on *EE4-lacZ*. In both *pnr-Gal4* and *omb-Gal4* expression domains, we observed strong activation of *EE4-lacZ* (Fig. 4A,B,D), suggesting that

E(spl)m7VP16 is somehow tethered to this artificial enhancer. Rather than being ubiquitous, activation by E(spl)m7VP16 was patterned in a way that strongly resembled the proneural pattern, suggesting that E(spl)m7VP16 was tethered to *EE4-lacZ* via proneural complexes. To demonstrate this we assayed the same effector-reporter combination in both loss-of-function and gain-of-function backgrounds for proneural genes. $sc^{10\text{-}1}$ is a null allele for both *ac* and *sc*, the only proneural proteins expressed in the wing disk. In $sc^{10\text{-}1}$ wing disks, *EE4-lacZ* was not expressed and could not be activated by E(spl)m7VP16 (Fig. 4E,F). In the converse experiment, we supplied ectopic Sc by co-expressing *UAS-sc* with *UAS-m7VP16* (Fig. 4G-I); in this case, the pattern of *EE4-lacZ* activation was broadened to encompass the whole expression domain and was not restricted to proneural clusters (compare Fig. 4B with 4I). It therefore appears that it is the availability and spatial distribution of proneural proteins, which determines the pattern of activation of *EE4-lacZ* by E(spl)m7VP16. The simplest way to account for this finding is to propose that E(spl)m7VP16 is recruited onto DNA using the proneural complexes (and not some other DNA-bound factor) as tethers. This was confirmed by testing the ability of two other E(spl)VP16 variants: E(spl)mγVP16 and mδVP16. Whereas the former behaved identically to E(spl)m7VP16 (data not shown), E(spl)mδVP16 had no effect on *EE4-lacZ* expression (Fig. 4C). We attribute the inability of E(spl)mδVP16 to become recruited onto *EE4-lacZ* to its inability to interact with the proneural protein-tethering factors.

It is possible that proneural cluster restriction of *EE4-lacZ* activation by E(spl)m7VP16 and mγVP16 was due to some regional inactivation (by protein modification) of the VP16 effector itself, and not to its recruitment onto DNA via proneural complexes. We therefore asked whether the E(spl)-VP16 variants were inherently capable of transcriptional activation in all cells by assaying their ability to activate another artificial enhancer [*Gbe-B1-lacZ*; Table 1 (Jennings et al., 1999)] that bears three E_B boxes (recognized by HES-family proteins) in addition to binding sites for Grh, an activator ubiquitously present in wing disk cells. In a wild-type background, *Gbe-B1-lacZ* is expressed very weakly and cannot be activated by *UAS-sc* [as Sc only weakly binds the B1 E_B box (Jennings et al., 1999); Fig. 5A,B]. In the presence of *UAS-E(spl)m7VP16*, *mγVP16* or *mδVP16*, strong ubiquitous activation was observed (Fig. 5C-E), indicating that all three E(spl)VP16 variants are strong activators when directly tethered to DNA and their activity does not seem to be spatially modulated. We therefore favor that the variable activation of *EE4-lacZ* (Fig. 4) reflects selective recruitment of the VP16 proteins onto the EE4 enhancer and is not a result of post-translational modulation of their transactivation ability. This result also strengthens our conclusion from Fig. 4C that E(spl)mδVP16 cannot become recruited onto *EE4-lacZ*.

An E(spl)m7VP16 variant with mutated basic region should behave in a manner complementary to E(spl)mδVP16, as it should lack direct DNA-binding activity but should retain the ability to be indirectly tethered to targets via proneural proteins. The behavior of a *UAS-E(spl)m7KNEQ-VP16* transgene showed that this was indeed the case. First, this effector was unable to activate the *Gbe-B1-lacZ* reporter, confirming disruption of its basic region (Fig. 5F). By contrast, it was able to activate the *EE4-lacZ* reporter to the same extent as wild type E(spl)m7VP16 (Fig. 6A-C). One interesting difference was that the activity of E(spl)m7KNEQ-VP16 was restricted to proneural clusters (where *ac* and *sc* are expressed), whereas E(spl)m7VP16 gave additional patchy activation of *EE4-lacZ* in non-proneural cells of the *pnr-Gal4* domain. This was accompanied by marked ectopic accumulation of the Ac proneural protein, something not seen with E(spl)m7KNEQ-VP16 (Fig. 6G-I). Ectopic activation of endogenous proneural genes by E(spl)m7VP16 is probably achieved by directly binding to enhancers that contain E_B/C/N boxes (such as the autoregulatory ones), because it is abolished by mutation of the basic region. The resulting ectopic proneural protein is subsequently used as a tether to bring E(spl)m7VP16 onto the *EE4-lacZ* reporter. To bypass this feedback loop involving endogenous proneural genes, we supplied Sc via co-expression of a *UAS-sc* transgene. As shown before, *UAS-sc* alone resulted in patchy activation of *EE4-lacZ* (Fig. 6D). However, in the presence of E(spl)m7VP16 or m7KNEQ-VP16 activation became ubiquitous and much stronger (compare Fig. 6D with 6E,F), reflecting ubiquitous tethering of the E(spl)m7VP16 effector regardless of the integrity of its basic domain.

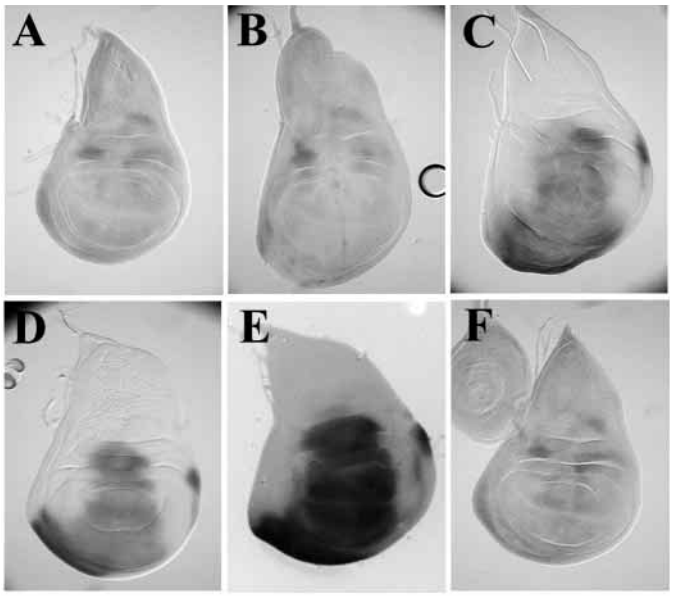

Fig. 5. Response of *Gbe-B1-lacZ* to bHLH activators. All panels show wing disks carrying one copy of the *Gbe-B1-lacZ* reporter. (A) Wild-type expression pattern, developed for 5 hours. (B) *omb-Gal4; UAS-sc*, developed for 5 hours. (C) *omb-Gal4; UAS-E(spl)m7VP16*, developed for 10 minutes. (D) *omb-Gal4; UAS-E(spl)mδVP16*, developed for 10 minutes. (E) *omb-Gal4; UAS-E(spl)mγVP16*, developed for 30 minutes. F: *omb-Gal4; UAS-E(spl)m7KNEQ-VP16*, developed for 5 hours.

Direct versus protein-mediated binding to target genes by E(spl) proteins

The data presented so far have highlighted a novel mechanism of target gene repression by E(spl), one that requires recruitment on DNA via protein-protein interactions with proneural proteins. What role, if any, does direct DNA binding play in the activity of E(spl) proteins? We addressed this question by assaying the ability of E(spl)VP16 variants to activate endogenous target genes in the absence of *ac* and *sc*

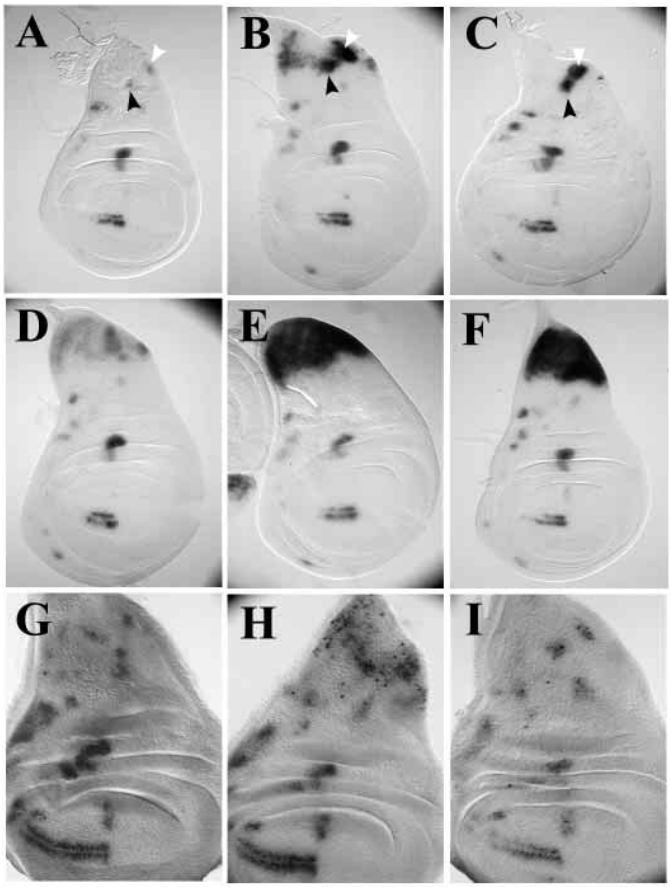

Fig. 6. Comparison of *E(spl)m7VP16* with *E(spl)m7KNEQ-VP16* in the activation of *EE4-lacZ* and Ac expression. (A-F) X-gal staining of *EE4-lacZ* wing disks. (G-I) immunostaining for Ac protein. (A,G) Wild type. (B,H) *UAS-E(spl)m7VP16; pnr-GAL4*. (C,I) *UAS-E(spl)m7KNEQ-VP16; pnr-GAL4*. Reporter activation at the SC (white arrowhead) and DC (black arrowhead) proneural clusters is seen at equally high levels in B and C. (D) *UAS-sc; pnr-GAL4*. (E) *UAS-sc UAS-E(spl)m7VP16; pnr-GAL4*. F: *UAS-sc UAS-E(spl)m7KNEQ-VP16; pnr-GAL4*. X-gal development in D-F is at equivalent levels, as judged by the wing-margin proneural clusters, where *pnr-Gal4* is not expressed. Note the increased transcriptional response of the *EE4-lacZ* reporter in E and F compared with D.

(Fig. 7A,F), which eliminates the possibility of proneural-protein-mediated recruitment. All E(spl)m7VP16, mγVP16 and mδVP16 induced bristles when driven by *pnr-Gal4* in a $sc^{10\text{-}1}$ background (Fig. 7C,E,H,J and data not shown). This suggests that these E(spl)VP16 variants can bypass the requirement for endogenous proneural genes and trigger the sensory organ pathway, presumably by directly activating one or more proneural target genes. Indeed direct binding of target genes must be involved, since cheta production in a $sc^{10\text{-}1}$ background was abolished by mutating the basic domain of E(spl)m7VP16 (Fig. 7D,I). In a wild-type background, E(spl)m7KNEQ-VP16 induces fewer ectopic bristles than its wild-type counterpart (Fig. 7C,D), which suggests a lower activity, consistent with its ability to activate target genes only via protein-mediated recruitment, whereas E(spl)m7VP16 can also directly bind to its target genes. mγKNEQ-VP16 behaved identically to m7KNEQ-VP16 (data not shown). Therefore,

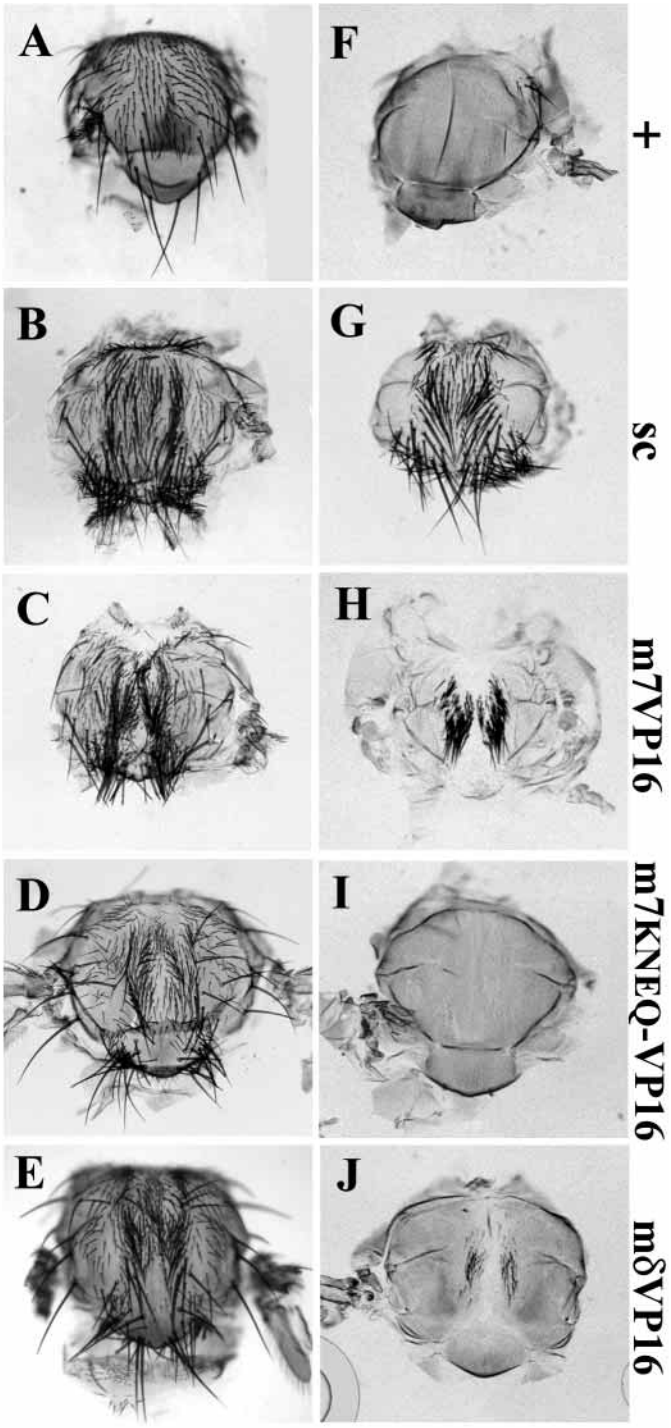

Fig. 7. Effect of bHLH activator transgene expression on bristle production in the absence of endogenous *ac* and *sc*. Adult nota of flies carrying *pnr-Gal4*. (A-E) Wild type for the X chromosome. (F-J) $sc^{10\text{-}1}/Y$ results in a bald notum except for the central *pnr-Gal4* domain, whenever sensory organ-inducing bHLH proteins were expressed. Effector transgenes expressed are as follows. (A,F) none. (B,G) *UAS-sc*. Sc expression induces bristles within the *pnr-Gal4* domain. (C,H) *UAS-E(spl)m7VP16*. Bristles are induced even in the absence of *ac* and *sc*. (D,I) *UAS-E(spl)m7KNEQ-VP16*. Excess bristles are induced only in the ac^{+} sc^{+} background. (E,J) *UAS-E(spl)mδVP16*. Bristles are induced even in the absence of *ac* and *sc*.

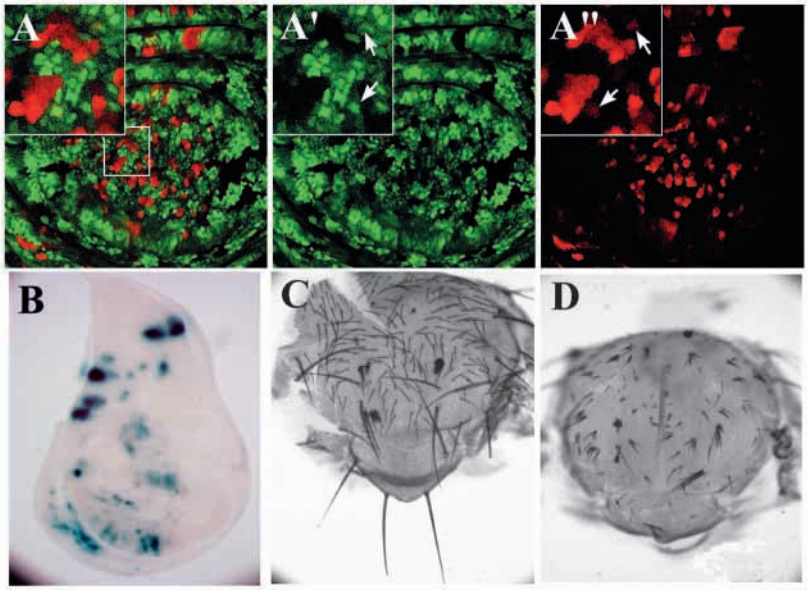

Fig. 8. E(spl)-mediated repression requires Gro. (A-A″) Wing pouch of *omb-Gal4/hsFLP; UAS-sc UAS-E(spl)m7/EE4-lacZ; FRT82B gro^{E48}/FRT82B πMyc*. Homozygous clones for *gro^{E48}* are marked by the absence of πMyc (green) and β-galactosidase is visualized in red. Intense *EE4-lacZ* expression is autonomously induced within *gro* mutant cells, indicating lack of repression. Arrows indicate wild-type cells that express low levels of *EE4-lacZ*. This low-level patchy expression in wild type is expected, as the overview of an *omb-Gal4; UAS-sc UAS-E(spl)m7/EE4-lacZ* (non-mosaic) disk stained by X-gal shows in B. It indicates that E(spl)m7-mediated repression is strong, yet not complete; compare with Fig. 2E-H. (C) *hsFLP; FRT82B gro^{E48}/FRT82B πMyc* mosaic notum. Even though the clones are unmarked, we presume that they correspond to patches exhibiting bristle tufting. (D) *hsFLP; ap-Gal4/UAS-E(spl)m7; FRT82B gro^{E48}/FRT82B πMyc* mosaic notum. Similar bristle tufts are observed, presumably corresponding to *gro^{E48}* homozygous territories, within a bald background because of the overall expression of E(spl)m7, which suppresses bristles within the *gro^{+}* territories.

both mechanisms, direct DNA contact and interaction with the pre-bound proneural activators, seem to play a role in the recruitment of E(spl) proteins to their target genes. It should be noted that in a wild-type background both E(spl)m7- and mδ-VP16 variants produced a larger number of excess bristles than that produced in a *sc$^{10-1}$* background (Fig. 7C,E,H,J), indicating synergy between the hybrid E(spl) activators and the proneural ones, which is in part due to protein-mediated recruitment of the former onto the latter (see Discussion).

Proneural-mediated repression by E(spl)m7 involves an active repression mechanism

E(spl) proteins are known to recruit the co-repressor Groucho in order to silence target genes (Fisher and Caudy, 1998). It is conceivable that when E(spl) exert their repressive effect by interacting with proneural proteins, a different mechanism might be at play, such as occlusion of the transcriptional activation domain of proneural activators. We therefore wanted to address whether Gro is needed to mediate repression when E(spl) proteins are indirectly bound to DNA. To this end, we drove expression of *UAS-sc* together with *UAS-E(spl)m7* in a mosaic background containing patches homozygous for the severe *gro^{E48}* allele and assayed the response of the *EE4-lacZ* reporter. As described in a previous section, this reporter is repressed by E(spl)m7 exclusively via protein-mediated recruitment. Indeed in *gro^{+}* territory little or no expression was observed, as expected (Fig. 8A, cells stained green; Fig. 8B); however, within mutant clones *EE4-lacZ* was strongly expressed (Fig. 8A, cells lacking green). Therefore, E(spl) proteins employ a Gro-dependent repression mechanism regardless of mode of recruitment on target genes.

The requirement for Gro was corroborated by cuticle phenotype: *gro^{E48}* clones produce tufts of bristles on the notum (Fig. 8C), a result of the breakdown of lateral inhibition during SOP commitment. Although ubiquitous expression of E(spl)m7 abolishes bristles (Fig. 3C,D), when we induced *gro^{E48}* clones in an *ap-Gal4; UAS-E(spl)m7* background (which abolishes bristles throughout the notum), we recovered patches of high bristle density in a bald notum (Fig. 8D). This suggests that ectopic (as well as normally expressed) E(spl)m7 cannot repress endogenous target genes in the absence of Gro, just as it cannot repress the artificial *EE4-lacZ* target (Fig. 8A). Finally, a *UAS-E(spl)m7ΔW* transgene, which lacks the C-terminal tryptophane of the Gro-binding WRPW motif, was completely inactive in both bristle suppression and reporter gene repression (results not shown). A corollary from these experiments is that E(spl)m7 does not function by sequestering proneural activators off DNA. The latter activity should have no requirement for a co-repressor like Gro, as physical removal of activators should suffice to turn target genes off.

DISCUSSION

It has long been appreciated that two families of bHLH proteins, the proneural activators and the HES repressors act antagonistically to each other: the former promoting neural development and the latter suppressing it. This interplay happens in insects as well as vertebrates, probably reflecting an evolutionarily ancient regulatory circuit. We have presented a detailed analysis of the mechanism underlying this antagonism in *Drosophila* through an approach that employs in vivo study of reporter-effector transgene combinations in different genetic backgrounds. Our most important conclusions are the following: (1) targets of E(spl) repression are the target genes of the proneurals, and to a lesser extent the proneural genes themselves; (2) E(spl) recruitment onto target genes can occur via direct DNA binding (to E_B/C/N boxes), but also via interactions with E_A-box-bound proneural activators; (3) sequestration of the proneural activators off DNA, a mechanism employed by the Emc/Id family of HLH proteins, does not seem to operate in the case of E(spl); and (4) in both DNA-mediated and activator-mediated modes of E(spl)

tethering to target genes Groucho recruitment is required for repression.

Repression targets of E(spl) proteins

It is sometimes assumed that E(spl) proteins suppress neurogenesis solely by repressing proneural gene transcription. We have shown this not to be the case, as E(spl)m7 and mγ can completely block sensory organ commitment in a background of exogenously (transgenically) provided high levels of Sc. Target genes (genuine and artificial) that are activated by Da/Sc are still repressed by E(spl)m7 and mγ in the above genetic background. This is consistent with the earlier observation that E(spl) overexpression has only a moderate effect on *ac* expression, whereas it completely represses downstream targets, such as *SMC-lacZ* (Culi and Modolell, 1998) (see below), *ase* or *EE4-lacZ* (this study). Even though *ac* and *sc* are not the main targets of E(spl), some of their enhancers are repressible by E(spl). *ac* and *sc* genes elaborate expression pattern is dependent on a number of prepattern enhancers, which are controlled by patterning systems and are weakly, if at all, repressible by E(spl) (Gomez-Skarmeta et al., 1995). One enhancer each of *ac* [the proximal 900bp, used in the experiments whose results are shown in Fig. 1 (Martinez et al., 1993)] and *sc* (the *SMC* enhancer) (Culi and Modolell, 1998) has been described that is repressible by E(spl). Both of these are autoregulatory inasmuch as they contain E_A boxes and are activated by Da/Sc or Ac, hence they act to boost *ac/sc* levels after transcription has been initiated via the prepattern enhancers; in this context the *SMC* and *ac*-proximal enhancers can be viewed as 'target genes' of the proneural proteins.

Another piece of evidence in favor of regulation of proneural target genes (rather than proneural genes themselves) by E(spl) is that E(spl)m7VP16 can activate the neural pathway in genetic backgrounds mutant for *ac* and *sc*. Other than displaying aberrant spacing, bristles produced in such a background are normal, at least in external appearance. This is consistent with E(spl)m7VP16 binding and activating many, perhaps all, target genes of Ac/Sc (not just the autoregulatory *ac/sc* enhancers), bypassing the need for proneural proteins. One should be aware, however, that there are other bHLH proneural genes, besides *ac* and *sc*, in the fly genome; e.g. *l'sc* is not affected by the $sc^{10\text{-}1}$ allele used in the experiments whose results are shown in Fig. 7. Although *l'sc* is not normally expressed in the larval wing disk, it is conceivable that it is turned on by E(spl)VP16 activators and then takes over the task of activating the panel of downstream genes. Another potential candidate that might single-handedly mediate the sensory-organ promoting activity of E(spl)VP16 is *ase*, a SOP-specific gene that bears homology to the proneural genes of the *ac/sc* family and can act as a proneural gene itself (Dominguez and Campuzano, 1993). Thus, it is a matter of further research whether the bristle-induction ability of E(spl)VP16 in a $sc^{10\text{-}1}$ background is channeled through activation of a single E(spl) target gene or of a number of target genes.

Dual mechanism of E(spl) recruitment onto enhancers

All proneural target genes contain E_A boxes, via which the Da/proneural activators exert their effect. Our analysis of the *EE4-lacZ* enhancer has revealed that the same E_A boxes are sufficient for E(spl)-mediated repression, even though the latter bind a different class of target sites, the E_B/C/N-boxes. Based on the data presented in this work, we propose that this is achieved by enhancer recruitment of E(spl) proteins via protein-protein interactions with proneural activators. We focused on three E(spl) proteins. Two, m7 and mγ, have been shown to interact with both Da and Ac/Sc (Alifragis et al., 1997) and in the present study displayed equivalent ability to be indirectly recruited onto DNA by Da/Sc. The third, E(spl)mδ, showed no proneural-mediated recruitment activity, apparently because of its inability to interact with either Da or Sc. Perhaps this Da/Sc-binding activity of some of the E(spl) proteins has evolved to enable them to repress all proneural target genes effectively without the need for direct DNA binding. Ac and Sc seem to play a central role in this repression mechanism, as the ubiquitous Da was not sufficient to recruit E(spl)-VP16 proteins to *EE4-lacZ* and other proneural target enhancers (e.g. Fig. 4B,D).

Even though E(spl) proteins can be recruited onto their target genes via proneural complexes, all characterized proneural target enhancers (e.g. *SMC*, *ac*-proximal, *ase*, *dpn*, *neur*) do bear E_B/C/N-boxes in addition to E_A-sites (Culi and Modolell, 1998; Emery and Bier, 1995; Jarman et al., 1993; Ohsako et al., 1994; Van Doren et al., 1994) (M. Monastirioti and C. D., unpublished). Likewise, all E(spl) proteins possess well-conserved DNA-contacting basic domains. Two observations from our work strongly suggest that direct DNA binding is also used in the repression of target genes by E(spl). First, we observed a significant suppression of bristle formation by E(spl)mδ upon co-expression with Sc (Fig. 3F). This can only be interpreted as repression of Sc targets by E(spl)mδ by direct binding to their E_B/C/N-boxes, as we have established that E(spl)mδ is incapable of proneural-mediated enhancer binding. Second, E(spl)m7VP16, but not a basic region mutant version, turned on bristle commitment in the absence of proneural genes (Fig. 7H,I), pointing towards DNA-binding-dependent recruitment onto proneural target genes.

The realization that some E(spl) proteins can act as both repressors and co-repressors of the proneurals prompts reconsideration of the proneural proteins as dedicated transcriptional activators; they seem to be equally important in effecting repression of their target genes. Other transcriptional activators, such as Dorsal and HNF4 can act as repressors in certain contexts (Dubnicoff et al., 1997; Ktistaki and Talianidis, 1997), suggesting that this may be quite a widespread mechanism.

Implications for lateral inhibition

We have used a transgenic approach to establish the ability of E(spl) proteins to be recruited onto target genes by the two mechanisms discussed above. We cannot predict from our results whether in a wild-type background the two mechanisms are used exclusively of one another or simultaneously. The presence of E_B/C/N-boxes in close proximity to E_A-boxes in enhancers of proneural target genes favors the latter possibility, namely that proneural and E(spl) proteins each bind their cognate target sites and subsequently also interact at the protein level. Protein-protein interaction concomitant with DNA binding may enable cooperative enhancer binding, which would ensure a rapid response of target genes to changes in concentration of proneural and E(spl) proteins.

Having realized the plausibility for two (alternative or

simultaneous) modes of E(spl) recruitment onto target enhancers, we still do not have a complete picture of what it takes (in terms of transcriptional regulation) to achieve a robustly laterally inhibited response to proneural activity; in other words, to turn on a proneural target gene solely in the neural precursor. The artificial *EE4-lacZ* enhancer, though responsive to wild-type levels of E(spl) (Fig. 2K-M) is still not fully repressed, and is expressed in most cells of a wild-type proneural cluster. By contrast, another enhancer that also lacks E_B/C/N boxes has been reported to be fully repressible by wild-type levels of E(spl); *SMCN-Δ147-181* is a mutant version of the *SMC* enhancer lacking all E(spl)-binding sites, but containing two E_A-boxes; this enhancer expresses solely in the neural precursor (SOP) and not in surrounding proneural cluster cells (Culi and Modolell, 1998). One can hypothesize that additional factors binding *SMCN-Δ147-181* favor the formation of a repressive DNA-protein complex in the E(spl)-containing non-SOPs. Indeed this enhancer contains two copies each of conserved α and β boxes (bound by unknown factors) interspersed with the E_A boxes (Culi and Modolell, 1998). One or both of these factors may cooperate with low (wild-type) levels of E(spl) (bound to E_A via interaction with the proneural complex) to stabilize Gro binding to this enhancer; indeed Gro often has to simultaneously interact with more than one DNA bound factors to gain access to an enhancer (Valentine et al., 1998).

Natural proneural target enhancers contain E_A, E_B, C, N, α and β boxes, in addition to binding sites for other factors, such as the Zn-finger protein Senseless (Nolo et al., 2000). Some of these enhancers (e.g. *SMC*, *ase*, *dpn*, *neur*) are expressed solely in the neural precursor, whereas others [*ac* proximal, *sca*, various *E(spl)* enhancers] are expressed more widely within the proneural cluster (Cooper et al., 2000; Nellesen et al., 1999; Singson et al., 1994), apparently not responding (or less responsive) to lateral inhibition. Yet, the two types of enhancer are not obviously different with respect to types of target sites contained. Perhaps it is the exact number and arrangement of the various target sites and DNA-bound factors that defines the threshold level of lateral inhibition that each enhancer is responsive to. Seen in this light, it is conceivable that interaction of E(spl) with proneural factors (and perhaps other factors within a large protein-DNA complex) may bring about conformational changes, which are needed to fine-tune crosstalk of these transcription factors with co-activators, co-repressors and other components of the transcriptional machinery. Characterizing these regulatory interactions will improve our insight on the transcriptional mechanisms that mediate neural fate acquisition and will be a major challenge for the future.

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