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Role of the Sc C Terminus in Transcriptional Activation and E(spl) Repressor Recruitment*[S]

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Neurogenesis in all animals is triggered by the activity of a group of basic helix-loop-helix transcription factors, the proneural proteins, whose expression endows ectodermal regions with neural potential. The eventual commitment to a neural precursor fate involves the interplay of these proneural transcriptional activators with a number of other transcription factors that fine tune transcriptional responses at target genes. Most prominent among the factors antagonizing proneural protein activity are the HES basic helix-loop-helix proteins. We have previously shown (1) that two HES proteins of *Drosophila*, E(spl)mγ and E(spl)m7, interact with the proneural protein Sc and thereby get recruited onto Sc target genes to repress transcription. Using *in vivo* and *in vitro* assays we have now discovered an important dual role for the Sc C-terminal domain. On one hand it acts as a transcription activation domain, and on the other it is used to recruit E(spl) proteins. *In vivo*, the Sc C-terminal domain is required for E(spl) recruitment in an enhancer context-dependent fashion, suggesting that in some enhancers alternative interaction surfaces can be used to recruit E(spl) proteins.

Sc (Scute) and E(spl)m7 (Enhancer of split m7) are two Drosophila bHLH[1] transcription factors, characterized by the basic-helix-loop-helix structural motif, which is responsible for dimerization and DNA binding (2). This large family of transcription factors contains evolutionarily conserved subfamilies, which participate in a variety of biological processes. Sc belongs to the Class II bHLH proteins, more specifically to the achaete-scute branch of the proneural family, which also includes vertebrate Ash proteins (achaete-scute homologues). These proteins play a central role in initiating neural development in all metazoans studied so far (3). E(spl)m7, on the other hand, belongs to the HES (Hairy/Enhancer-of-split) family of bHLH proteins, also known as Class VI, which are structurally distinct from the proneural family (4). Although their biological effects encompass a great number of processes, one of their roles is to inhibit neurogenesis; in this context they are expressed downstream of a Notch-mediated signaling pathway termed lateral inhibition (5). HES proteins inhibit neurogenesis by repressing target genes of the proneural proteins or even the expression of proneural genes themselves. Consistent with this biological activity, it has been shown that although proneural proteins are transcriptional activators, HES proteins are repressors. Because of structural differences in their bHLH domains, the two families have distinct target site specificities. Proneural proteins make obligate heterodimers with the ubiquitous bHLH protein Da (Daughterless) and preferentially bind to the so-called E_A boxes, CASCTG (6). HES proteins, on the other hand, homodimerize or heterodimerize among themselves and bind preferably to E_B or C boxes, CACGTG and CACGCG, respectively (7).

A model for neural commitment proposes that genes initiating this process are targets of both proneural and HES proteins, and the relative activity of these two mutually antagonistic bHLH factors ultimately determines whether a cell will commit to the neural pathway or not. One way through which a gene could be a target of both proneural and HES proteins is by having target sites for both (*i.e.* both E_A and E_B/C boxes) in its regulatory regions. This, albeit generally true, is an oversimplification, because it has been appreciated for some time that E_B/C boxes are dispensable in some target enhancers (8). We have recently described a second mechanism through which target genes of proneural proteins can be repressed by HES proteins. This relies on the ability of E(spl) proteins to interact with Sc, detected both in a yeast two-hybrid system (9) and in *Drosophila* tissues (1). E(spl) proteins are thereby recruited onto the Da-Sc heterodimer and repress transcription of target genes, even in the absence of direct DNA-E(spl) binding. We have now characterized the mechanism of interaction between Sc and E(spl)m7 in detail. Interestingly, this interaction does not involve typical HLH-HLH dimerization; rather E(spl)m7 binds to the C-terminal domain of Sc, which we show to be its transactivation domain.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assays—Bait cDNAs were PCR-cloned downstream of the LexA gene fragment between the EcoRI (5′) and XhoI (3′) sites of the pEG202-NLS vector (OriGene) (10). Primer sequences are available upon request. Preys were also constructed as 5′ EcoRI-3′ XhoI fragments into the pJG4–5 vector.

The pEG202 constructs were transformed into yeast strain EGY48 (*Mata trp1 his3 ura3 leu2*::*6LexAop-LEU2*); the pJG4–5 ones along with the LexAop-lacZ reporter plasmid pSH1834 (URA3) into strain FT4 (*Matα trp1 his3 ura3 leu2*) (9). After mating, diploids were tested

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; TAD, transcriptional activation domain; GST, glutathione *S*-transferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

on galactose/raffinose/leucine/X-gal plates. The plates were photographed after 24 h of growth at 30 °C, and the strength of the interaction was qualitatively judged by the extent of the blue color. LexA constructs were excluded from analysis if they were constitutively active or inactive with all preys tested. The preys were selected using the same criteria.

GST Pull-downs—For *in vitro* transcription/translation of *sc* and *E(spl)m7*, pBluescript KSII subclones were made of the EcoRI-XhoI inserts from pJG4–5 or pEG202 clones. To generate GST fusion genes, full-length and subfragments of *E(spl)m7* were cloned in pGex4T-1 (Amersham Biosciences) as EcoRI-XhoI fragments.

GST fusion proteins were expressed in *Escherichia coli* BL21plys (Stratagene). After induction and lysis, soluble fractions were stored at −80 °C, and fusion protein content was estimated by Coomassie Blue staining of a polyacrylamide gel. To detect interactions, 1–5 μg of various GST fusions (equal amounts) were incubated on 30 μl of glutathione-agarose beads and mixed with *in vitro* translated proteins. The latter were produced using the coupled TnT/T7 reticulocyte lysate transcription/translation system (Promega) in the presence of [^{35}S]methionine according to the manufacturer's instructions. After incubation at 4 °C for 3–4 h and extensive washing, affinity resin-bound proteins were run on a denaturing polyacrylamide gel. The gel was fixed and treated with Amplify fluorographic reagent (Amersham Biosciences) before imaging on a Molecular Dynamics phosphorimaging device.

Immobilized Oligonucleotide Pull-down—Approximately 100 pmol of biotinylated double-stranded oligonucleotide containing an E_A box was immobilized on 50 μl of streptavidin-coated magnetic beads (Dynal), according to the manufacturer's instructions. After extensive washes to remove unbound DNA, the beads were blocked in a buffer containing 20 mM Hepes, pH 7.9, 150 mM KCl, 5% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 2.5 μg/μl bovine serum albumin, 1 μg/μl sonicated salmon sperm DNA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. [^{35}S]Methionine (Amersham Biosciences) radiolabeled proteins were synthesized by *in vitro* transcription/translation using the TnT system of Promega and subsequently incubated with the DNA-coated beads at 4 °C in blocking buffer. The bound proteins were analyzed by PAGE and autoradiography.

Transient Transfection Assays—The reporter gene used in all assays, UAS-tk-luc, contained five tandem UAS_{GAL4} sites fused upstream of a minimal herpes simplex virus *thymidine kinase* (HSV-tk) promoter driving the *luciferase* gene. The UASx5 enhancer was excised from pUAST (11) as BamHI-HpaI and inserted in SmaI of pGL3tk. For effector protein expression we used the RactHAdh vector, bearing a constitutive *Drosophila act5C* promoter (12). Ract-m7 is described in Giagtzoglou *et al.* (1). Ract-m7KNEQ was constructed by PCR *in vitro* mutagenesis, cloned in pGEM-Teasy (Promega), and sequence-verified (mutagenic primer sequences available upon request). Gal4-DBD fusions were constructed as follows: a fragment encoding the Gal4 DNA-binding domain (amino acids 1–147) was released from pBXG1 (13) as a HindIII-XbaI fragment and inserted by blunt ligation into SalI of RactHAdh to generate RactGDBD. The Sc coding region, or segments thereof, were released as SmaI-XhoI inserts from their respective pBluescript-KSII clones (see above) and inserted into the SmaI-SalI sites of RactGDBD. The VP16 activation domain was cloned as a filled-in BamHI-XbaI fragment from pBluescript-E(spl)m7-VP16 (14) into RactGDBD/PstI/blunt.

S2 cell transfections and luciferase assays were performed as described in Giagtzoglou *et al.* (1). 1 μg of total DNA was used per 0.5-ml well, of which 0.4 μg were taken by the RactHAdh effector plasmids (topped up by empty vector), the remainder being 0.4 μg of UAS-tk-luc reporter and 0.2 μg of hs-lacZ used for normalization. The averages and standard deviations of experiments repeated at least in triplicate are shown.

Fly Transformation and Reporter Assays—The coding regions for $Sc_{1–260}$ and $Sc_{1–290}$ were excised from the corresponding pJG4–5 constructs (see above) by EcoRI-XhoI and subcloned into pUAST (11) cut with EcoRI-XhoI. Fly strains, transgenesis, crosses, and X-gal histochemistry were done as described in Giagtzoglou *et al.* (1). *EE4-lacZ* reporter gene is described by Culi and Modolell (8). *ac-lacZ* is described by Martinez *et al.* (15).

RESULTS

Identification of the Interaction Domains of Sc and E(spl)m7—We used the yeast two-hybrid system to identify the domains of Sc and E(spl)m7 responsible for their interaction (1, 9). E(spl)m7 fragments were fused with the LexA DNA-binding domain ("baits") and were tested against fusions of Sc fragments to the B42 activation domain ("preys") (10) (Fig. 1*A*). The full set of combinations tested is shown in Table I. The full-length Sc fusion, B42-$Sc_{1–345}$, interacted with three of the six LexA-E(spl)m7 fusions, those with the N terminus intact. Conversely the N-terminal two-thirds of E(spl)m7, LexA-$E(spl)m7_{1–137}$, interacted with those Sc preys that had their C terminus intact; the minimal interacting fragment contained only 25 C-terminal Sc amino acids. Based on these data we propose that the major domains mediating the Sc-E(spl)m7 interaction are the N-terminal 80 amino acids of E(spl)m7 and the C-terminal 25–55 amino acids of Sc. An additional weaker interaction between the C terminus of E(spl)m7 (constructs 61–186 and 81–186) with the N-terminal 260 amino acids of Sc was sometimes observed, but this seems to be suppressed when

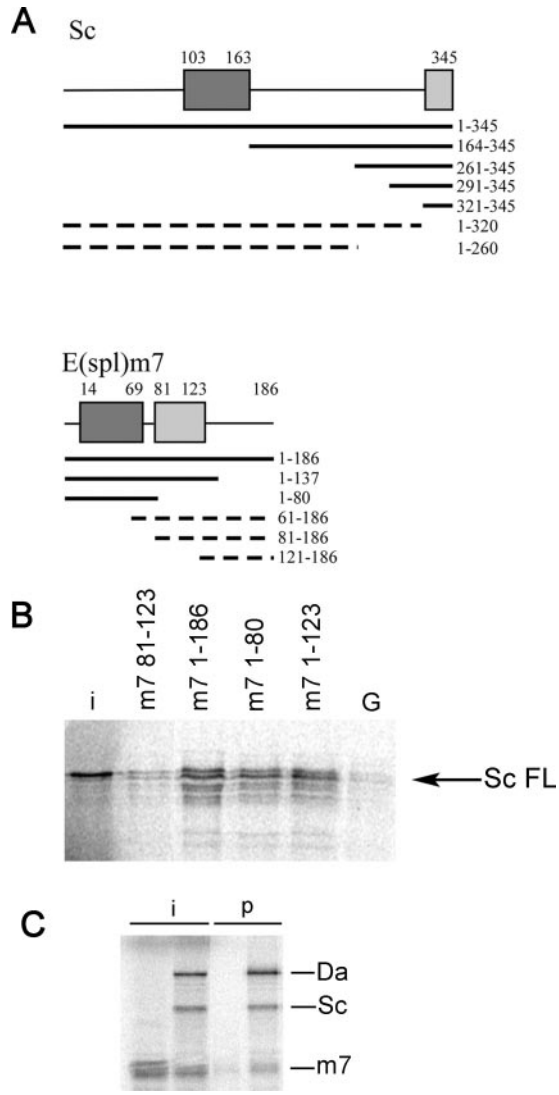

FIG. 1. **Molecular interactions between Sc and E(spl)m7.** *A*, the constructs tested by yeast two-hybrid are shown. The *numbers* refer to amino acids. *Continuous lines*, constructs that displayed interactions with the reciprocal full-length partner (E(spl)m7 or Sc; see also Table I); *dashed lines*, constructs that did not display interactions. *Dark gray*, bHLH domain; *light gray*, acidic C terminus of Sc or Orange domain of E(spl)m7. *B*, GST pull-down experiment using [^{35}S]met-labeled *in vitro* translated full-length Sc. *i*, 1/10 Sc input. The remaining lanes show Sc protein retained by GST fusions of fragments of E(spl)m7 as marked or GST alone (*G*). Appreciable amounts of Sc are retained only by GST fusion proteins carrying the N terminus of E(spl)m7 (*lanes 3–5*). *C*, DNA affinity pull-down experiment. E(spl)m7 was *in vitro* translated/[^{35}S]met labeled alone (*lanes 1* and *3*) or together with Da and Sc (*lanes 2* and *4*) and incubated with DNA (E_A oligonucleotide)-coated magnetic beads. *i*, input proteins (1/5); *p*, proteins retained on the beads (4/5). Appreciable amounts of E(spl)m7 are bound to E_A only in the presence of Da-Sc.

TABLE I
Yeast two-hybrid interactions

The interactions were qualitatively measured by detection of blue color on X-gal plates 24 h after plating. ++, strong blue color; +, moderate blue; −, white color; NT, not tested. Gro was used as a positive control, because it is known to interact with the C-terminal WRPW tetrapeptide of E(spl) and related proteins (17). It was fused to the VP16 activation domain, rather than B42 (9). Similarly, the functionality of the Sc fusions bearing the bHLH domain was confirmed by a LexA-$Da_{551–710}$ fusion, which carries the Da bHLH domain known to interact with Sc (30).

B42 LexA	Vector alone	m7 1–186 (full-length)	m7 1–137	m7 1–80[a]	m7 61–186	m7 81–186	m7 121–186	Da 551–710
Vector alone	NT	−	−	−	−	−	−	−
Sc 1–345 (FL)	−	++	++	++	−	−	−	++
Sc 1–320	−	+	−	−	+	−	−	++
Sc 1–260	−	+	−	−	+	+	−	++
Sc 164–345	−	++	++	+	−	−	−	−
Sc 261–345	−	++	++	+	−	−	−	−
Sc 291–345	−	++	++	+	−	−	−	−
Sc 321–345[a]	−	+	++	−[b]	−	−	−	−
Gro-VP16	−	++	−	−	++	++	++	NT

[a] B42-$Sc_{321–345}$ and LexA-E(spl)$m7_{1–80}$ were both weak constructs giving somewhat lower X-gal intensities, perhaps due to lower expression level/stability of the products.
[b] The low activity of these constructs may explain the absence of detectable interaction in their combination.

the Sc C terminus is present, namely in the full-length Sc protein (Table I). Because of the weak and inconsistent incidence of this interaction, it was not studied further. The Sc-E(spl)m7 interaction was confirmed by *in vitro* GST pull-downs; GST fusions carrying the N-terminal domain of E(spl)m7 were able to effectively pull down *in vitro* translated Sc protein (Fig. 1*B*).

In the experiments described so far the Sc protein was treated in isolation, whereas it is known that *in vivo* it forms heterodimers with the E-protein Da. To assay recruitment of E(spl)m7 onto a DNA-bound Da-Sc complex, we immobilized a biotinylated E_A oligonucleotide onto streptavidin-coated magnetic beads and incubated it with various *in vitro* translated proteins. E(spl)m7 alone was unable to bind the E_A beads, in agreement with the known target preferences of E(spl) proteins. However, addition of Da and Sc proteins quantitatively pulled down E(spl)m7 (as well as Da and Sc), consistent with formation of a DNA-Da-Sc-E(spl)m7 complex on the E_A DNA target (Fig. 1*C*).

The ability to reproduce the Sc-E(spl)m7 interaction *in vitro*, by both GST and DNA affinity pull-down, suggests that it is likely to be direct. It is noteworthy that this interaction does not involve the bHLH region of Sc. This suggests that a protein-protein contact other than the well characterized amphipathic HLH dimerization is at play here. It is also worth noting that the E(spl)m7 Orange domain, a domain found in all HES family bHLH proteins (16), is dispensable for its interaction with Sc.

The C-terminal Domain of Sc Is a Transcription Activation Domain—The interacting domain of Sc contains a C-terminal stretch of 17 amino acids that is conserved among three members of the achaete-scute complex (Sc, Ac, L'sc) and also conserved in their homologues from other species (Fig. 2*A*). The presence of six acidic residues within this stretch prompted us to investigate the possibility that this region acts as a transcriptional activation domain (TAD). We fused different fragments of Sc to a Gal4 fragment containing the DNA-binding region (Gal4D) and asked whether the resultant chimeras could activate a luciferase reporter driven by five Gal4 consensus binding sites (construct UAS-tk-luc). The assays were done in transiently transfected *Drosophila* Schneider S2 cells. All Gal4D fusions containing the C-terminal 25 amino acids of Sc activated the reporter gene, with the most active fragment being $Sc_{321–345}$, which gave up to 68× activation above basal (Fig. 2*B*). For comparison, Gal4D fusion to the extremely potent viral transactivation domain VP16 gave up to 1270× activation. $Sc_{1–320}$, $Sc_{164–320}$, and $Sc_{261–320}$ fusions did not activate transcription, even though all three were produced at sufficiently high amounts, as judged by the ability of transfected cell extracts to bind a UAS_{Gal4} containing oligonucleotide by EMSA (Supplemental Fig. 1). Thus, the C terminus of Sc is a transcription activation domain, most likely the only TAD within Sc.

The Sc TAD Is Inhibited by E(spl) Proteins—Because the domain of Sc responsible for interacting with E(spl)m7 is identical to its TAD, we reasoned that inhibition of Sc activity *in vivo* by E(spl)m7 may be a result of inhibition of this TAD by the Sc-E(spl)m7 interaction. To test this, we used the same transient transfection approach, where we co-expressed E(spl) proteins along with the Gal4D-Sc activators. Both Gal4D-$Sc_{1–345}$ and Gal4D-$Sc_{321–345}$ gradually lost their activity, all the way to basal levels, upon co-expression with increasing amounts of E(spl)m7 (Fig. 3*A*). This effect was specific for the Sc TAD, because Gal4D-VP16 was not repressible by E(spl)m7. The unlikely possibility that E(spl) proteins inhibited the ability of Gal4D-Sc fusions to bind DNA was eliminated by testing transfected cell extracts for their ability to bind to a UAS_{Gal4}-containing oligonucleotide by EMSA (Supplemental Fig. 1). Another possibility that we eliminated was that repression was due to spurious binding of E(spl)m7 onto the UAS-tk-luc reporter. For this we made use of E(spl)m7KNEQ, a double point mutation in the basic domain, which abolishes DNA binding *in vitro* and *in vivo* but does not affect binding to Sc (1, 14). This mutant E(spl)m7 represses Gal4D-ScTAD-driven transcription as effectively as wild type E(spl)m7 (Fig. 3*A*). In contrast, wild type E(spl)mδ, an E(spl) protein with intact basic domain but unable to interact with Sc (9), gave very weak repression of transcription driven by Gal4D-Sc-TAD (Fig. 3*A*).

We asked whether the remaining five E(spl) proteins were capable of repressing Gal4D-Sc-driven activation. The three Sc interactors, E(spl)mγ, E(spl)mβ, and E(spl)m3, gave strong repression, similar to E(spl)m7 (Fig. 3*B*). On the other hand, E(spl)m5 and E(spl)m8, which, like E(spl)mδ, did not exhibit any interaction with Sc in the yeast two-hybrid assay (9), gave weak or no repression of Gal4D-Sc (Fig. 3*B*). All E(spl) were detectable in transfected cell extracts by Western blot (Supplemental Fig. 2), eliminating the trivial possibility that some tested negative because of lack of expression. We have also used the same constructs in S2 transfection assays in the past (1),[2] where all were equally effective at repressing a luciferase reporter driven by the 5′-proximal *ac cis*-regulatory region, which contains an E(spl)-binding site.

The effect observed here is consistent with a mechanism whereby DNA-bound Gal4D-Sc recruits an E(spl) repressor via protein-protein interaction. To confirm this, we transfected S2

[2] P. Alifragis and C. Delidakis, unpublished data.

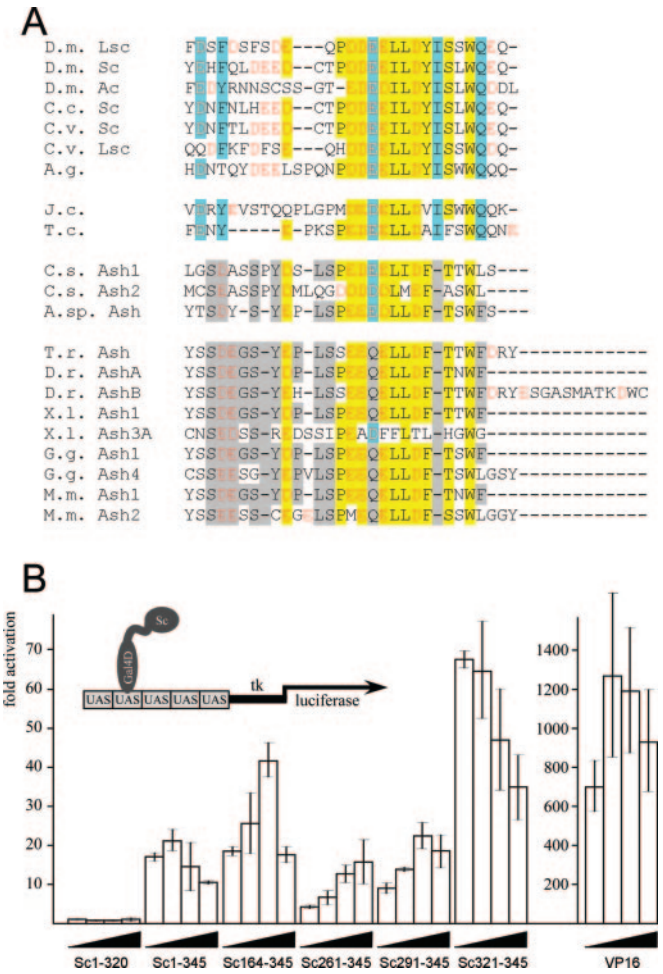

FIG. 2. **The conserved Sc C terminus is a TAD.** *A*, multiple alignment (using ClustalW 1.5) of the C termini from achaete-scute proteins from the following diptera: *Drosophila melanogaster* (*D.m.*; accession numbers P10084, P09774, and P10083), *Calliphora vicina* (*C.v.*; accession numbers AAL32067 and AAL32066), *Ceratitis capitata* (*C.c.*; accession number AAF66944), *Anopheles gambiae* (*A.g.*; accession numbers AAK97461); some other insects: *Junonia coenia* (*J.c.*; accession number AAC24714), *Tribolium castaneum* (*T.c.*; accession number AAQ23386), spider *Cupiennius salei* (*C.s.*; accession numbers CSA309490 and CAC27517); myriapod *Archispirostreptus*. *sp*. (*A.sp.*; accession number CAD60436); also from the following vertebrates: *Takifugu rubripes* (*T.r.*; accession number AAB88278), *Danio rerio* (*D.r.*; accession numbers NP_571294 and NP_571306), *Xenopus laevis* (*X.l.*; accession numbers Q06234, and AAK14425), *Gallus gallus* (*G.g.*; accession numbers I51382 and AAC60096) and *Mus musculus* (*M.m.*; accession numbers BC055748 and O35885). *Yellow highlighting*, residues similar across all species; *blue highlighting*, residues similar across insects; *gray highlighting*, residues similar across vertebrates. *Red*, acidic residues. *B*, luciferase activity (fold activation) of reporter UAS-tk-luc with increasing amounts (20, 50, 100, and 200 ng/transfection) of the indicated Gal4D fusions. All of the fusions tested, with the exception of Sc_{1-320}, activate transcription. The drop in activation levels observed upon increasing the amounts of transfected expression plasmid is probably due to the phenomenon of squelching, where high levels of a transcriptional activator cause a global drop in transcription levels.

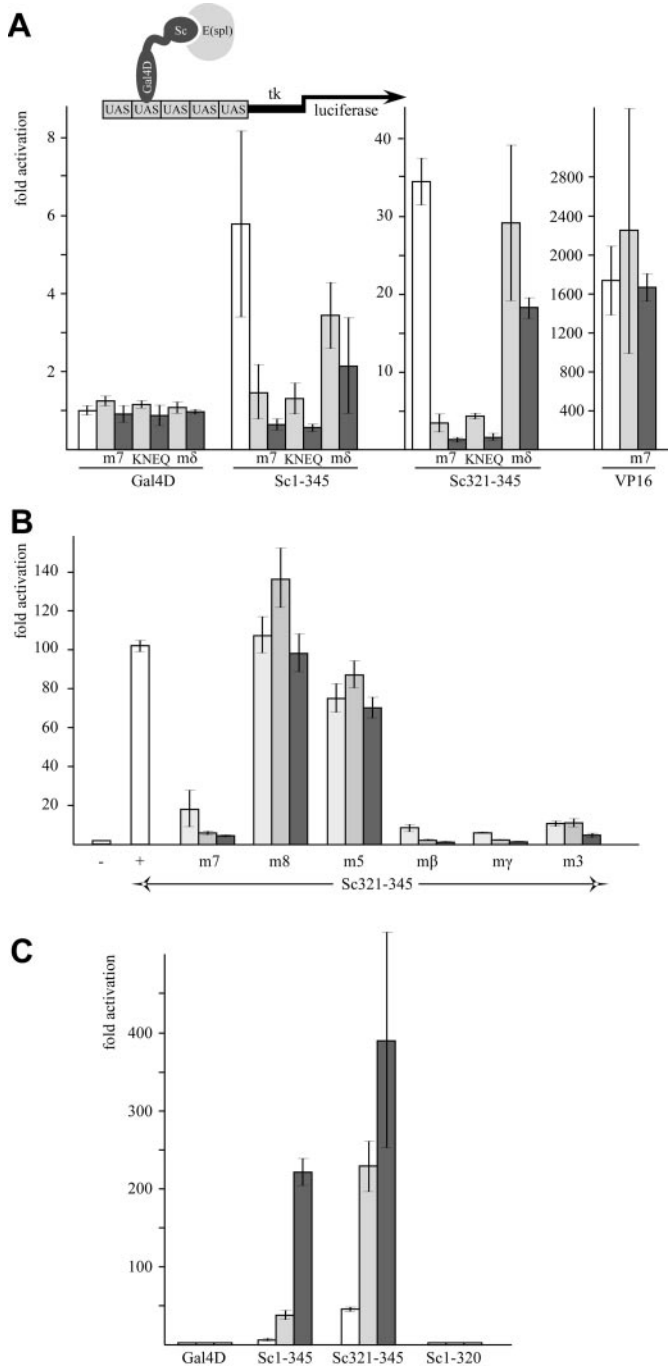

FIG. 3. **The Sc TAD is inhibited by E(spl) proteins.** *A*, fold activation of the UAS-tk-luc reporter after co-transfection with Gal4D fusions alone (50 ng, *white bars*) or in the presence of increasing amounts of E(spl) constructs as indicated (KNEQ: E(spl)m7KNEQ). *Light gray*, 20 ng; *dark gray*, 100 ng. *B*, fold activation of the UAS-tk-luc reporter co-transfected with (−) 50 ng Gal4D (basal activity defined as 1×), (+) 50 ng Gal4D-$Sc_{321-345}$. Remaining *bars*: 50 ng Gal4D-$Sc_{321-345}$ co-transfected with increasing amounts (20, 60, and 100 ng, *light* to *dark gray*) of different E(spl) expression plasmids, as indicated. *C*, fold activation of the UAS-tk-luc reporter by Gal4D alone (basal) or by three Gal4D-Sc fusions (50 ng; *white bars*). ScTAD-dependent, but not basal, activity was boosted by co-transfection of plasmids expressing E(spl)m7-VP16 (20 and 100 ng, *light* and *dark gray*, respectively).

cells with a chimeric E(spl)m7-VP16 construct, in which the C-terminal repression domain of E(spl)m7 is replaced with the strong VP16 TAD, thereby converting E(spl)m7 into an activator (14). We obtained UAS-tk-luc reporter gene activation in a fashion strictly dependent on the presence of a Gal4D-Sc fusion with an intact C-terminal TAD (Fig. 3*C*), consistent with E(spl)m7-VP16 becoming recruited onto the reporter via protein-protein interaction with the Sc TAD.

E(spl) proteins recruit the co-repressor Groucho (Gro) via their C-terminal domain, a process that has been shown to be necessary for their functions *in vivo* (17, 18). We asked whether recruitment of Gro is necessary for the observed repression of the Sc TAD by E(spl) proteins in the present S2 assay. Truncation of the C-terminal tryptophan residue of E(spl)m7 to generate E(spl)m7ΔW abolishes its ability to interact with Groucho (9). We have previously shown that this mutant is

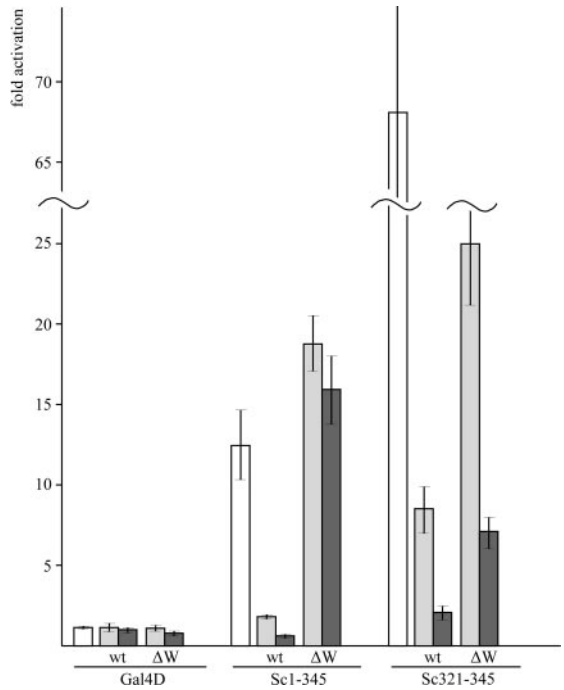

FIG. 4. **Role of the Groucho co-repressor in the inhibition of Sc transactivation by E(spl).** Fold activation of the UAS-tk-luc reporter by Gal4D fusions alone (50 ng, *white bars*) or in combination with increasing amounts (20 and 100 ng, *light* and *dark gray*, respectively) of E(spl)m7 wild type or the ΔW variant, which cannot recruit the co-repressor Groucho. Note that although E(spl)m7ΔW does not inhibit the activity of Gal4-$Sc_{1–345}$ (it even shows slight activation), it significantly inhibits the activity of Gal4D-$Sc_{321–345}$ ($p < 0.01$, using Student's unpaired *t* test).

inactive in transgenic flies, because it was unable to suppress neurogenesis and also unable to repress a Sc-driven reporter gene (1). Similarly, when tested against Gal4D-$Sc_{1–345}$, E(spl)m7ΔW was unable to repress the UAS-tk-luc reporter (Fig. 4). However, in the present minimal system E(spl)m7ΔW partially repressed Gal4D-$Sc_{321–345}$, a fact suggesting that the interaction between the Sc TAD and E(spl)m7 can in itself partially mask the activity of the former. Even in this case, recruitment of Gro enhances repression significantly (*cf.* E(spl)wt *versus* E(spl)ΔW on Gal4D-$Sc_{321–345}$ in Fig. 4).

TAD-less Versions of Sc Are Defective in Recruiting E(spl)m7 in Vivo—The domain dissection experiments described above established that the C-terminal TAD of Sc is targeted by E(spl) proteins and thereby inhibited from activating transcription. However, *in vivo* Sc does not act in isolation, it rather heterodimerizes with Da, which could provide an alternative TAD (19) as well as an alternative E(spl) interaction domain (9). To address the role of the Sc TAD in an *in vivo* context, we used a transgenic reporter system (1). *EE4-lacZ* is an artificial reporter driving expression off a tandem multimer of E_A boxes (8). Both endogenously expressed Sc and that provided by a *UAS-sc* transgene can heterodimerize with ubiquitous endogenous Da to activate *EE4-lacZ*. We constructed two C-terminal truncations of a *UAS-sc* transgene; these terminate at amino acid 260 or 290, and thus lack the TAD, but have an intact bHLH domain. When expressed in the thorax, both $Sc_{1–260}$ and $Sc_{1–290}$ elicited significantly weaker activation of *EE4-lacZ* than Sc (full-length) (Fig. 5, *A–C*; ectopic expression within the region *boxed* in Fig. 5*B*), likely because of the removal of the Sc TAD. The fact that weak activation was observed is consistent with residual TAD activity from the Da molecules recruited onto the reporter via truncated Sc.

Recruitment of E(spl)m7 was assayed in the same system by co-expressing a *UAS-sc* transgene with *UAS-E(spl)m7KNEQ-VP16* (1, 14). The latter is an *E(spl)m7* transgene bearing two modifications: 1) a replacement of the C-terminal repression

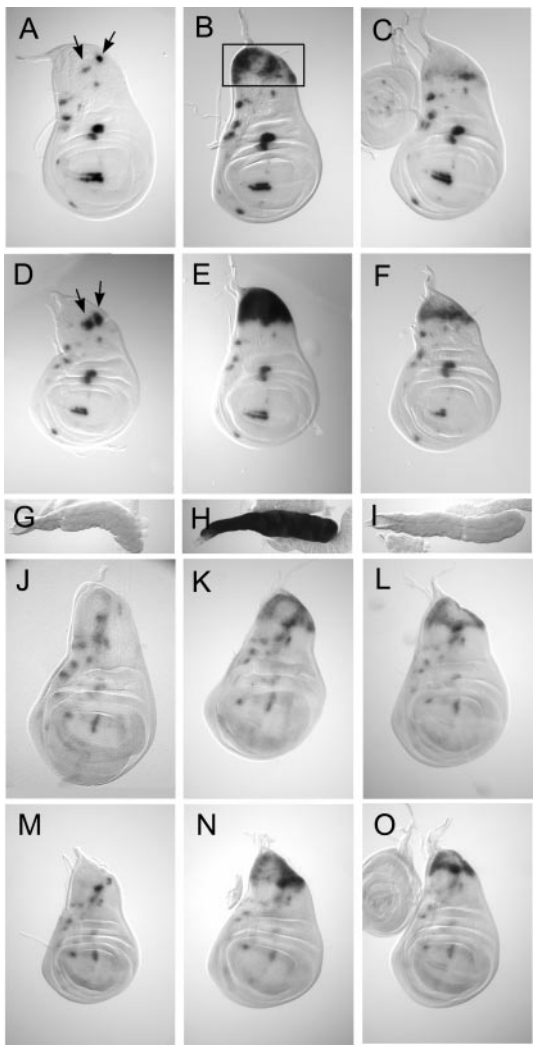

FIG. 5. **Transgenic animal assays for Sc TAD function.** Third instar wing imaginal disks (*A–F* and *J–O*) or salivary glands (*G–I*) carrying the *pnr-Gal4* driver, which expresses in the proximal-most notum (*boxed area* in *B*), as well as the whole salivary gland. *A–I* also carry *EE4-lacZ*. *J–O* also carry *ac-lacZ*. β-Galactosidase activity is visualized by X-gal histochemical staining; in each row all samples were developed under identical conditions. *A*, endogenous *EE4-lacZ* pattern (no responder gene). *B*, *UAS-sc* activates more broadly and more strongly than $Sc_{1–260}$ (*C*). *D–F* are same as *A–C*, with the co-expression of *UAS-E(spl)m7KNEQ-VP16*. *D*, activation is detected only at the two proneural clusters within the *pnr-Gal4* expression domain. These are marked with *arrows* in *A* and *D* and correspond to the sites of endogenous *sc* expression. *E*, upon *UAS-sc* co-expression, activation becomes strong throughout the *pnr-Gal4* domain. *F*, $Sc_{1–260}$ cannot recruit E(spl)m7KNEQ-VP16 to the same extent as full-length Sc, resulting in much less activation than *E*. Note that the two endogenous spots have also weakened, suggesting that ectopic $Sc_{1–260}$ likely displaces endogenous full-length Sc from the *EE4-lacZ* enhancer in these cells. *G–I* identical genotypes with *D–F*, salivary glands are shown. In this tissue the differences are more pronounced. None of the *UAS-sc* (not shown), *UAS-sc260* (not shown), or *UAS-E(spl)m7KNEQ-VP16* (*G*) transgenes produces any *EE4-lacZ* activation. The only combination that gives activation in this tissue is Sc + E(spl)m7KNEQ-VP16 (*H*). This is in sharp contrast to the same combination with $Sc_{1–260}$ (*I*), which is completely inactive. *J–O*, a different reporter, *ac-lacZ* (*J*) is activated equally well by *UAS-sc* (*K*) or *UAS-sc*$_{1–260}$ (*L*). *M–O*, E(spl)m7KNEQ-VP16 recruitment assays. *M*, *UAS-E(spl)m7KNEQ-VP16* alone activates in the two proneural clusters, as in *D*. In the presence of either form of Sc (*N* and *O*) staining increases significantly. The two samples do not differ appreciably in staining intensity. In all of the assays tested UAS-$sc_{1–290}$ behaved identically to UAS-$sc_{1–260}$ (see also Suppl. Fig. 3).

domain with the VP16 TAD, as described above, and 2) the basic domain inactivating KNEQ double point mutation (1, 14). Because of these modifications transcriptional activation by this effector transgene depends on the existence of a DNA-bound transcription factor to recruit it to the enhancer under study. Indeed, when expressed alone, E(spl)m7KNEQ-VP16 produces strong transcriptional activation of *EE4-lacZ* only in proneural clusters, where endogenous *sc* is expressed (1) (see also Fig. 5*D*, *arrows*). When co-expressed with *UAS-sc* (full-length) very strong ubiquitous activation ensues (Fig. 5, *E* and *H*), suggesting effective recruitment by the now ubiquitous Da-Sc complex. In contrast, *UAS-sc*$_{1–260}$ results in very slight (Fig. 5*F*) or no (Fig. 5*I*) activation when co-expressed with E(spl)m7KNEQ-VP16, suggesting that the latter cannot be efficiently recruited onto *EE4-lacZ* by the Da-Sc$_{1–260}$ complex. This was further confirmed by co-expressing the truncated versions of Sc with an unmodified E(spl)m7. The weak activation elicited by either Sc$_{1–260}$ or Sc$_{1–290}$ was not repressible by E(spl)m7 (Supplemental Fig. 3), in sharp contrast to the strong repression seen when wild type Sc is co-expressed with E(spl)m7 (1).

We tested the same combinations of effector transgenes on a different reporter gene, *ac-lacZ*, driven by a promoter proximal 5′ regulatory fragment of *ac*. This contains binding sites for a number of transcription factors, including Da-Sc, E(spl), and Sens (15, 20) and is expressed in a proneural cluster pattern (Fig. 5*J*). Unlike *EE4-lacZ*, both *UAS-sc* and *UAS-sc*$_{1–260}$ can substantially activate *ac-lacZ* ectopically (Fig. 5, *K* and *L*), suggesting that in this enhancer context the presence of the Sc TAD is dispensable. We then assayed E(spl)m7KNEQ-VP16 recruitment using the same strategy as the one used for *EE4-lacZ*. Fig. 5 (*M–O*) shows that this E(spl)m7 chimera produces equivalent reporter activation when co-expressed with either Sc or Sc$_{1–260}$, suggesting that also in E(spl)m7 recruitment the Sc TAD is rendered dispensable in the *ac-lacZ* reporter context.

DISCUSSION

The Sc TAD and Other Interaction Partners of E(spl) Proteins—The fact that proneural and E(spl) bHLH proteins have mutually antagonistic activities has long been accepted (4). Here we describe for the first time a molecular basis for this antagonism of the Sc-E(spl)m7 pair, which relies on the ability of the latter to interact and inhibit the activity of the TAD of the former. We have dissected Sc and E(spl)m7 and in the process have identified 1) the TAD of Sc, which resides in its 25 C-terminal amino acids, 2) the E(spl)m7 interaction domain of Sc, which is identical to or overlaps with its above mentioned TAD, and 3) the Sc interaction domain of E(spl)m7, which is contained within the N-terminal 80 amino acids. Three more of the seven E(spl) proteins, E(spl)mγ, E(spl)mβ, and E(spl)m3, share the ability E(spl)m7 to inhibit the Sc TAD, consistent with an increased structural similarity among these four E(spl) proteins.

To address a possible *in vivo* role for this interaction between Sc and E(spl) proteins, we have to take some points into consideration. Natural enhancers recruit a number of transcription factors and co-factors to assemble an enhanceosome (21), which regulates transcription initiation. For example, Da-Sc target enhancers may variably contain additional activators, such as a putative NFκB-like α-factor (8), Sens (20, 22), or Sis-a (23). While affording robustness in gene regulation, the multifactorial nature of the enhanceosome and its ability to assemble itself using multiple alternative macromolecular interactions may cause frustration to the researcher trying to dissect out the function of individual components. Artificial enhancers, on the other hand, can reveal functions of individual domains, because they rely on a small number of transcription factors because of the very simplicity of their design. Using the artificial enhancers *UAS-tk-luc* and *EE4-lacZ*, we showed that the Sc C terminus is necessary for both transcriptional activation and recruitment of E(spl) proteins. Already, when assayed on a more complex natural enhancer, *ac-lacZ* (Fig. 5, *J–O*), the role of the Sc C terminus starts becoming blurry. Equally good activation and E(spl)m7KNEQ-VP16 recruitment appears to take place whether the Sc C terminus is present or not. We attribute this to the presence of alternative TADs and alternative contact surfaces that are able to recruit E(spl)m7 onto this enhanceosome but not onto the simpler *EE4-lacZ*. Other than Sc, transcription factors that have been reported in the literature to interact with E(spl)m7 are Da (9) and Sens (20). Although the presence of Da (predicted to bind on *EE4-lacZ*) can only weakly sustain transcription and E(spl)m7 recruitment in the absence of Sc TAD (Fig. 5, *C*, *F*, and *I*), the presence of Sens or some other yet-to-be-identified E(spl) interaction surface on the *ac-lacZ* appears to render the Sc TAD dispensable in the assays of Fig. 5 (*J–O*). It is noteworthy that E(spl) use different domains to contact Sc (the N-terminal region; this work) *versus* Sens (the middle Orange region (20)). The existence of more than one protein-protein interaction domain on any given factor is likely to be advantageous in complex formation. Establishing contacts via both the N terminus and the Orange domain would likely result in cooperative recruitment, allowing an E(spl) protein to repress a Sc+Sens-containing enhanceosome more effectively. Further functional characterization of the E(spl) proteins will determine the relative contribution of each documented (or yet-to-be documented) protein-protein interaction, as well as of direct DNA binding, to recruitment onto target genes.

Conservation of the Sc Activation Domain and Its Implications—The C terminus of Sc is conserved in other Sc family proneural proteins in *Drosophila* (Ac and L'sc), as well as homologues from other phyla (Fig. 2*A*), which in itself argues for some important function. Its role had been overlooked so far; in fact an earlier report had proposed that it is dispensable for the proneural activity of Lethal of Scute (L'sc) (24). In that work, a transgene essentially consisting of only the bHLH domain of L'sc (l'scΔNΔC) was able to promote ectopic sensory organ (bristle) production, only slightly more weakly than full-length L'sc. Because that transgene was not tested against specific reporter genes such as the ones we used here, we should be cautious in drawing conclusions about the function of the L'sc C terminus for the reasons described above. Namely, bristle production is the outcome of the activation of a (still ill-defined) number of Sc (L'sc) target genes driven by complex enhancers and multiple factors, the presence of which might compensate for the lack of the L'sc C-terminal domain. So, in a transgenic assay, the presence of the Sc (or L'sc) TAD may be dispensable, whereas its bHLH domain is sufficient to recruit Da to the bristle-promoting target genes to nucleate the assembly of complex enhanceosomes. Indeed the behavior of our C-terminally truncated transgenes *sc*$_{1–260}$ and *sc*$_{1–290}$ is entirely consistent with that of *l'sc*Δ*N*Δ*C*. Adult flies expressing the *UAS-sc* transgene have approximately the same number of ectopic bristles as those expressing *UAS-sc*$_{1–260}$ or *UAS-sc*$_{1–290}$ (data not shown). These very same genotypes, however, display a dramatic difference in the activation of the *EE4-lacZ* reporter (Fig. 5, *B* and *C*).

Does phylogenetic conservation of the C terminus imply that both functions, TAD and HES repressor recruitment, have also been conserved? We have preliminary data to suggest that at

least the TAD function has been conserved in Mash1.[3] Additionally, some evidence exists in the literature, consistent with protein interaction-mediated antagonism between Mash1 and HES1. Castella *et al.* (25) showed that Mash1 promotes and HES1 inhibits neuronal differentiation of rat hippocampal neural precursors in culture. Importantly, transfection of Mash1 together with HES1 also inhibited neuronal differentiation, suggesting that HES1 antagonizes Mash1 post-translationally. In a reporter assay (26), this ability of HES1 to antagonize Mash1 was retained by a basic region mutant of HES1 (25). This would be consistent with HES1 interacting with the TAD of Mash1 to block its activity, independently of the ability of HES1 to bind DNA. Further dissection of these and related vertebrate bHLH proteins will reveal the extent to which the present documented mechanism has been conserved through evolution.

Another interesting question raised by our work regards the remaining proneural proteins. The second subclass of proneural proteins, the Ato/Ngn subclass, is equally important in neural precursor commitment (27) but has a bHLH domain different from that of the achaete-scute proteins and, most importantly for the present discussion, lacks the conserved C-terminal TAD (28). In fact the TADs of Ato/Ngn proteins remain to be identified. It will be interesting to determine whether the Ato/Ngn TADs have also evolved to be inhibited by HES proteins. Our preliminary analysis has shown that Ato can interact with two E(spl) proteins in yeast two-hybrid (9), so an analogous mechanism for this proneural subclass is conceivable.

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[3] I. Zarifi and C. Delidakis, unpublished observations.

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