Specificity for the Hairy/Enhancer of split Basic Helix-Loop-Helix (bHLH) Proteins Maps outside the bHLH Domain and Suggests Two Separable Modes of Transcriptional Repression†

STEPHANIE R. DAWSON, DAVID L. TURNER, HAROLD WEINTRAUB, AND SUSAN M. PARKHURST*

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

The Hairy/Enhancer of split/Deadpan family of basic helix-loop-helix (bHLH) proteins function as transcriptional repressors. We have examined the mechanisms of repression used by the Hairy and E(SPL) proteins by assaying the antagonism between wild-type or altered Hairy/E(SPL) and Scute bHLH proteins during sex determination in *Drosophila melanogaster*. Domain swapping and mutagenesis of the Hairy and E(SPL) proteins show that three evolutionarily conserved domains are required for their function: the bHLH, Orange, and WRPW domains. However, the suppression of Scute activity by Hairy does not require the WRPW domain. We show that the Orange domain is an important functional domain that confers specificity among members of the Hairy/E(SPL) family. In addition, we show that a *Xenopus* Hairy homology conserves not only Hairy's structure but also its biological activity in our assays. We propose that transcriptional repression by the Hairy/E(SPL) family of bHLH proteins involves two separable mechanisms: repression of specific transcriptional activators, such as Scute, through the bHLH and Orange domains and repression of other activators via interaction of the C-terminal WRPW motif with corepressors, such as the Groucho protein.

The basic helix-loop-helix (bHLH) motif is found in many proteins required during diverse developmental processes such as sex determination, segmentation, neurogenesis, and myogenesis. These proteins often appear to regulate the choice between alternate pathways and effect changes in cell fate (30, 31, 49). Biochemical and structural studies have shown that the HLH domain mediates protein homodimerization or heterodimerization, while the basic region is required for the HLH dimers to make specific contacts with DNA (3, 15, 30). Different classes of bHLH proteins act as either positive or negative regulators of transcription (1, 30, 31). For example, the *Drosophila melanogaster achaete-scute* complex (AS-C) bHLH proteins function as positive regulators: they are thought to heterodimerize with the ubiquitously expressed Daughterless (Da) protein (homologous to mammalian E proteins) and bind to specific DNA sequences (the E box), thereby activating transcription of target genes to promote neurogenesis (6, 31).

Transcriptional repression is an important feature of developmental processes (for review see references 19 and 28). bHLH proteins functioning as negative regulators or "repressor" proteins appear to fall into two different classes. One class, including the *Drosophila* Emc and mammalian Id family proteins, lacks the conserved basic residues adjacent to the HLH domain required for binding DNA. These proteins have been proposed to negatively regulate transcription by forming heterodimers with ubiquitous or tissue-specific activator bHLH proteins and sequestering them as nonfunctional heterodimers which are unable to bind DNA (1). For example, the *Drosophila* Extramacrochaetae (Emc) protein has been shown to prevent Da–AS-C heterodimers from binding DNA in vitro

(7, 10, 17, 29, 46, 47). The specificity of these proteins is presumably reflected in their choice of dimerization partner mediated by the HLH domain. Consistent with this idea, Emc is able to dimerize with either Da or Scute (7, 46, 47).

The second repressor bHLH class includes structurally related *Drosophila* proteins encoded by *hairy* (*h*), *deadpan* (*dpn*), and the *Enhancer of split* complex [*E(SPL)-C*] (2, 9, 26, 27, 38, 50), as well as several vertebrate homologs (13, 14, 23, 40, 41, 45a). Unlike the Emc/Id HLH repressors, members of this Hairy/E(SPL) class have a conserved basic domain, including a characteristic proline residue, suggesting that they do more than just passively sequester activator bHLH proteins to bring about their negative regulatory function. While the presence of Hairy or E(SPL)-C proteins does not inhibit the binding of Da–AS-C heterodimers, an intact basic region is required for Hairy function during segmentation (47, 48) and in our sex determination assays (this study). In addition, bacterially expressed fly Hairy protein, fly E(SPL)m8 protein, and mammalian HES proteins have been shown to bind N-box sequences as homodimers in vitro (32, 40, 43, 46). Recently, *Drosophila* Hairy protein was demonstrated to mediate transcriptional repression of the *achaete* bHLH gene in vivo (32, 46). Hairy/E(SPL)-class proteins have also been shown to have homology at the C terminus: all proteins end with the tetrapeptide WRPW. This tetrapeptide motif is disrupted in two independent Hairy missense mutants (48) and has been shown recently to mediate interaction with the Groucho protein (37).

A role for bHLH proteins in the regulation of *Drosophila* sex determination and dosage compensation has been revealed (8, 33, 45). X chromosomes are counted, in part, through the dosage of the X-linked *scute/AS-C T4* gene, whose protein is thought to heterodimerize with Da to initiate expression of a master regulatory gene, *Sex-lethal* (*Sxl*), whose activity must be on in females and off in males (8, 12, 33, 45; reviewed in reference 36). Increased expression of the Scute numerator protein, under the control of the *hunchback* (*hb*) gap-gene promoter (*hb-scute*), activates *Sxl* expression inappropriately, leading to male lethality (33, 35). Although Hairy does not

* Corresponding author. Mailing address: Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104. Phone: (206) 667-6466. Fax: (206) 667-6497. Electronic mail address: susanp@fred.fhcrc.org.
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normally function in sex determination, it appears to mimic the action of the structurally similar Dpn and E(SPL)m3 bHLH autosomal counting proteins when expressed prematurely at the time of sex determination (12, 33a, 50). Denominator proteins are proposed to act by reducing the effective concentration or function of numerator proteins (e.g., Scute). Consistent with this view, ectopic expression of Hairy or E(SPL)m3, driven by the *hb* promoter (*hb-h* and *hb-Espl-m3*, respectively), interferes with X chromosome counting, resulting in the inhibition of *Sxl* expression and thereby in female lethality (33; 33a). The simplest model to explain ectopic Hairy action during sex determination is one in which Hairy blocks the activation of *Sxl* by Scute. Consistent with this model, *hb*-driven Scute rescues the sex-specific lethality associated with ectopic Hairy and vice versa (33, 35).

We have used the effects of ectopic bHLH protein expression on sex determination as a convenient assay system to determine which parts of the Hairy/E(SPL) proteins are needed for rescue of *hb-scute* male lethality and to define interactions among different bHLH proteins in vivo. From this assay, we find that three evolutionarily conserved domains—the bHLH, Orange, and WRPW domains—are required for complete Hairy function. However, suppression of Scute activity by Hairy requires the Hairy bHLH and Orange domains but not the WRPW domain. In addition, we find that the Orange domain mediates functional specificity among Hairy/E(SPL) proteins. We propose that the Hairy/E(SPL) family of bHLH proteins function through two separable repression mechanisms: repression of specific activator proteins through the bHLH and Orange domains and repression of other activator proteins by a process that requires the C-terminal WRPW motif for interaction with general transcriptional corepressors, such as the Groucho protein.

MATERIALS AND METHODS

Fly stocks. Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract-agar medium at 25°C unless otherwise stated.

Fusion constructs. Standard molecular techniques were performed as described previously (39). Construction and germ line transformation of the *hb-h* and *hb-scute* fusion genes, as well as the *hb-neo* vector, were previously described (33, 34). To simplify future constructions, we began by removing the introns from the *hb-h* fusion gene and assaying its function. A 2,115-nucleotide (nt) *Xba*I-*Not*I genomic fragment of *hb-h* was replaced with the homologous 958-nt fragment from a *hairy* cDNA (designated *hb-h*Δint). Transformants ectopically expressing the *hb-h*Δint fusion gene are indistinguishable from those carrying the original genomic *hb-h* fusion gene: all lead to 100% female lethality. The term "*hb-h*-containing flies" refers in this paper to flies carrying this *hairy* genomic-cDNA fusion gene. All transgenic lines in this paper are referred to by the gene fusions which they harbor. The full nomenclature for such transformants is P{neo⁺, **}, where ** is the fusion gene name (i.e., *hb-h* should be expressed as P{neo⁺, hb:h}).

For all fusion gene constructs described below, the portions of the fusions made by PCR were sequenced to ensure that additional mutations were not incorporated during the PCR or subsequent subcloning. The orientation of all subclones was checked by restriction analysis. Exact primer sequences are available on request. Standard PCR conditions used were as follows: 94°C for 1 min, 58°C for 90 s, and 72°C for 2 min for 30 cycles.

hb-HLH. A 1.1-kb *Xho*I DNA fragment containing the *Xenopus hairy1* cDNA was treated with Klenow fragment to fill in 2 nt and then subcloned into a modified *hb-neo* vector containing the fly *hsp27* polyadenylation site (*hb-pA* that also had 2 nt filled in after digestion with *Bam*HI) to create *hb-Xlh1*. The GenBank accession number for the *Xenopus hairy1* sequence used here is U36194. *hb-da* and *hb-E(spl)m8* were obtained from cloned genomic DNA by PCR amplification using oligonucleotide primers with appropriate restriction sites at their 5′ termini (11). The amplified fragments were a 2.5-kb *Bam*HI-*Bgl*II fragment subcloned into the *Bam*HI site of *hb-pA* and a 1.1-kb *Bam*HI-*Sma*I fragment subcloned into the *Bam*HI-*Sma*I sites of *hb-neo*, respectively.

hb-h basic region point mutations. The *hb-h* basic region point mutations were created via PCR site-directed mutagenesis in the 958-nt Hairy *Xba*I-*Not*I fragment by using primers containing the altered nucleotide sequence. The outermost primers were just 5′ to the *Xba*I site and just 3′ to the *Not*I site in Hairy. The internal primers yielded the following nucleotide exchanges for the following

mutants: for RIP, CGG→CCC (R-33) plus CGA→CCC (R-43); for P37N (a mutant carrying a P-to-N substitution at position 37), CCC→AAC; for P37T, CCC→ACC; for M39R, ATG→CGC; and for M39L, ATG→CTG. PNMR and PNML were generated by using the MR and ML primers with *hb-h*ᴾᴺ plasmid DNA, respectively. These PCR products were cut with *Xba*I and *Not*I, purified, and then exchanged for the homologous *Xba*I-*Not*I segment in the *hb-h*Δint plasmid.

Hairy deletion mutants. The ΔOrange and ΔSPPSS constructs were made within the *hairy Xba*I-*Not*I fragment by using primers that delete a 15-bp sequence corresponding to the amino acid sequence NEVSR (amino acids [aa] 119 to 123) or SPPSS (aa 170 to 174), respectively. These PCR products were cut with *Xba*I and *Not*I, purified, and then exchanged for the homologous segment in the *hb-h*Δint plasmid. The same principle was applied to the construction of the ΔWRPW plasmid; however, the WRPW domain is outside the *Xba*I-*Not*I fragment. In this case, a new 3′ primer with an engineered *Xba*I restriction site was used.

h/bHLH swaps. All bHLH swap and Hairy/E(SPL) hybrid constructs were made by recombinant PCR (11, 20) in which PCR fragments from the unrelated sequences were combined by using complementary overlaps created by adding 5′ sequences to the primers. For example, to make h/scute(bHLH) three independent PCRs were done to create (i) the 5′ portion of the Hairy gene ending with a 10-bp overlap for the Scute bHLH, (ii) the Scute bHLH fragment beginning and ending with a 10-bp overlap to *hairy*, and (iii) the 3′ portion of *hairy* beginning with a 10-bp overlap to the Scute bHLH domain. These three PCR fragments were purified, mixed, and used as a PCR template with the most 5′ and 3′ primers from the original reactions. The resulting PCR product was cut with *Xba*I and *Not*I, purified, and then exchanged for the homologous segment in the *hb-h*Δint plasmid.

Other constructs. Construction and germ line transformation of the *hb-ftz* and *hb-eve* fusion genes were as previously described (34). The *hb-ftz*Δbox transgene was constructed by recombinant PCR resulting in the precise deletion of homeobox sequences from aa 274 to 302, corresponding to the Ftz mutant reported by Fitzpatrick et al. (16).

Germ line transformation. Germ line transformation of all fusion genes was performed as previously described (33, 34). All *neo*-resistant transformants were confirmed by PCR using primers specific to the *neo* portion of the P-element vector as previously described (35). All second-chromosome lines are marked with the recessive mutation brown. All third-chromosome lines are marked with the recessive mutation scarlet.

At least two transformant lines for each construct were analyzed in detail. All transformant lines were examined for expression of the transgene that they harbored, as well as for their effects on Sxl expression. In all cases in which protein expression could be monitored by antibody staining (Sxl, Hairy, Da, Scute, Ftz, and Eve), this was done in preference (or in addition) to in situ hybridization [*Xlh1* and *E(spl)*m8]. For transformant lines carrying hybrid proteins, both genes of the hybrid were examined. All altered Hairy proteins detected by using antibodies recognizing the Hairy protein were as stable as the wild-type Hairy protein produced by the *hb-h* transgene and showed nuclear localization.

Embryo analysis. Embryos were prepared and immunohistochemical detection of proteins was performed as previously described (33). Antibodies used were as follows: anti-Sxl antibodies from D. Bopp, Da antibodies from C. Cronmiller, anti-Scute antibodies from J. Skeath and S. Carroll, anti-Ftz antibodies from H. Krause, anti-Eve antibodies from N. Patel, anti-Hairy antibodies from J. Gates and S. Carroll, and anti-Hairy antibodies from S. Pinchin and D. Ish-Horowicz.

In situ hybridization. Immunohistochemical whole-mount in situ hybridization was performed according to the protocol of Tautz and Pfeifle (42). Digoxigenin-substituted probes were obtained by PCR amplification with primers used for the construction of the plasmids.

RESULTS

Ectopic expression of a frog Hairy homolog, but not of the related fly E(SPL)m8 protein, rescues *hb-scute* male lethality. *hb*-driven Scute expression (*hb-scute*) leads to male lethality, whereas *hb*-driven Hairy expression (*hb-h*) leads to female lethality. The fact that these effects are mutually antagonistic led us to propose that the ectopically expressed Hairy protein inhibits *Sxl* transcription by forming inactive heterodimers with Scute protein (33, 35). To explore the specificity of Hairy rescue, we examined two other HLH repressor proteins for their ability to alleviate the unconditional male lethality associated with two copies of the *hb-scute* transgene: a frog Hairy homolog and the structurally related fly E(SPL)m8 protein. In both cases, the genes examined were placed under the control of the *Drosophila hb* promoter and then introduced into the germ line (Fig. 1B; see Materials and Methods). The effects of

A

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                  basic       helix 1   loop    helix 2                        Orange domain                      WRPW
            aa32  P37  aa47                              aa89  aa106                              aa143            aa337
             ↓    ↓    ↓                                 ↓     ↓                                  ↓                ↓
D.m. hairy       ...RRSNKPIMEKRRRAR INNCLNELKTLILDATKKDPARHSKLEKADILEKTVKHLQELQ...   ...KFKAGFADCVNEVSRF----PGIEPAQRRRLLQHLSNCINGV...   ...PWRPW
Xen. hairy1      ...RKSSKPIMEKRRRAR INESLGQLKTLILDALKKDSSRHSKLEKADILEMTVKHLRNLQ...   ...KYRAGFSECMNEVTRFLSTCEGVNTDVRTRLLGHLANCMNQI...   ...VWRPW
rat  hairy1/HES1 ...RKSSKPIMEKRRRAR INESLSQLKTLILDALKKDSSRHSKLEKADILEMTVNHLRNLQ...   ...KYRAGFSECMNEVTRFLSTCEGVNTEVRTRLLGHLANCMTQI...   ...MWRPWRN

D.m. deadpan     ...RKTNKPIMEKRRRAR INHCLNELKSLILEAMKKDPARHTKLEKADILEMTVKHLQSVQ...   ...KFKTGFVECAEEVNRYVSQMDGIDTGVRQRLSAHLNQCANSL...   ...AWRPW

D.m. E(spl) m8   ...QKVKKPMLERQRRAR MNKCLDNLKTLVAELRGDDG--ILRMDKAEMLESAVIFMRQQK...   ...SFKNGYMNAVNEVSRVMASTPGMSVDLGKSVMTHLGRVYKNL...   ...LWRPW
D.m. E(spl) m5   ...LKVKKPLLERQRRAR MNKCLDTLKTLVAEFQGDDA--ILRMDKAEMLEAALVFMRKQV...   ...SFKNGYMNAVSEISRVMACTPAMSVDVGKTVMTHLGVEFQRM...   ...MWRPW
D.m. E(spl) m7   ...RKVMKPLLERKRRAR INKCLDELKDLMAECVAQTGD--AKFEKADILEVTVQHLRKLK...   ...SFRAGYIRAANEVSRALASLPRVDVAFGTTLMTHLGMRLNQL...   ...VWRPW
D.m. E(spl) mβ/mA ...RKVMKPMLERKRRAR INKCLDELKDIMVECLTQEGEHITRLEKADILELTVEHMKKLR...   ...SFRAGYVHAANEVSKTLAAVPGVSVDLGTQLMSHLGHRLNYL...   ...MWRPW
D.m. E(spl) mγ/mB ...RKVMKPMLERKRRAR INKCLDELKDLMVATLESEGEHVTRLEKADILELTVTHLQKMK...   ...GFRSGYIHAVNEVSRSLSQLPGMNVSLGTQLMTHLGQRLNQI...   ...VWRPW
D.m. E(spl) m3   ...RKVMKPLLERKRRAR INKCLDDLKDLMVECLQQEGEHVTRLEKADILELTVDHMRKLK...   ...SFRSGYVHAADQITQVLLQ-TQQTDEIGRKIMKFLSTRLIEL...   ...VWRPW
D.m. E(spl) mδ/mC ...RKVTKPLLERKRRAR MNLYLDELKDLIVDTMDAQGEQVSKLEKADILELTVNYLKAQQ...   ...KFRAGYTQAAYEVSHIFSTVPGLDLKFGTHLMKQLGHQLKDM...   ...VWRPW

rat  HES2        ...RKSLKPLLEKRRRAR INESLSQLKGLVLPLLGAETSRYSKLEKADILEMTVRFLREQP...   ...SYLEGYRACLARLARVLPACSVLEPAVSARLLEHLRQRTVSG...   ...LWRPW
rat  HES5        ...NRLRKPVVEKMRRDR INSSIEQLKLLLEQEFARH-QPNSKLEKADILEMAVSYLKHSK...   ...DYSEGYSWCLQEAVQFLT-LHAAS-DTQMKLLYHPQRPPAPA...   ...LWRPW

                consensus:  ±FK  GF   ø EøSRøø    Gø    ø    øø HL    ø   ø
                            YR   Y          T
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B

Rescues hb-scute ?   ♀-lethal ?

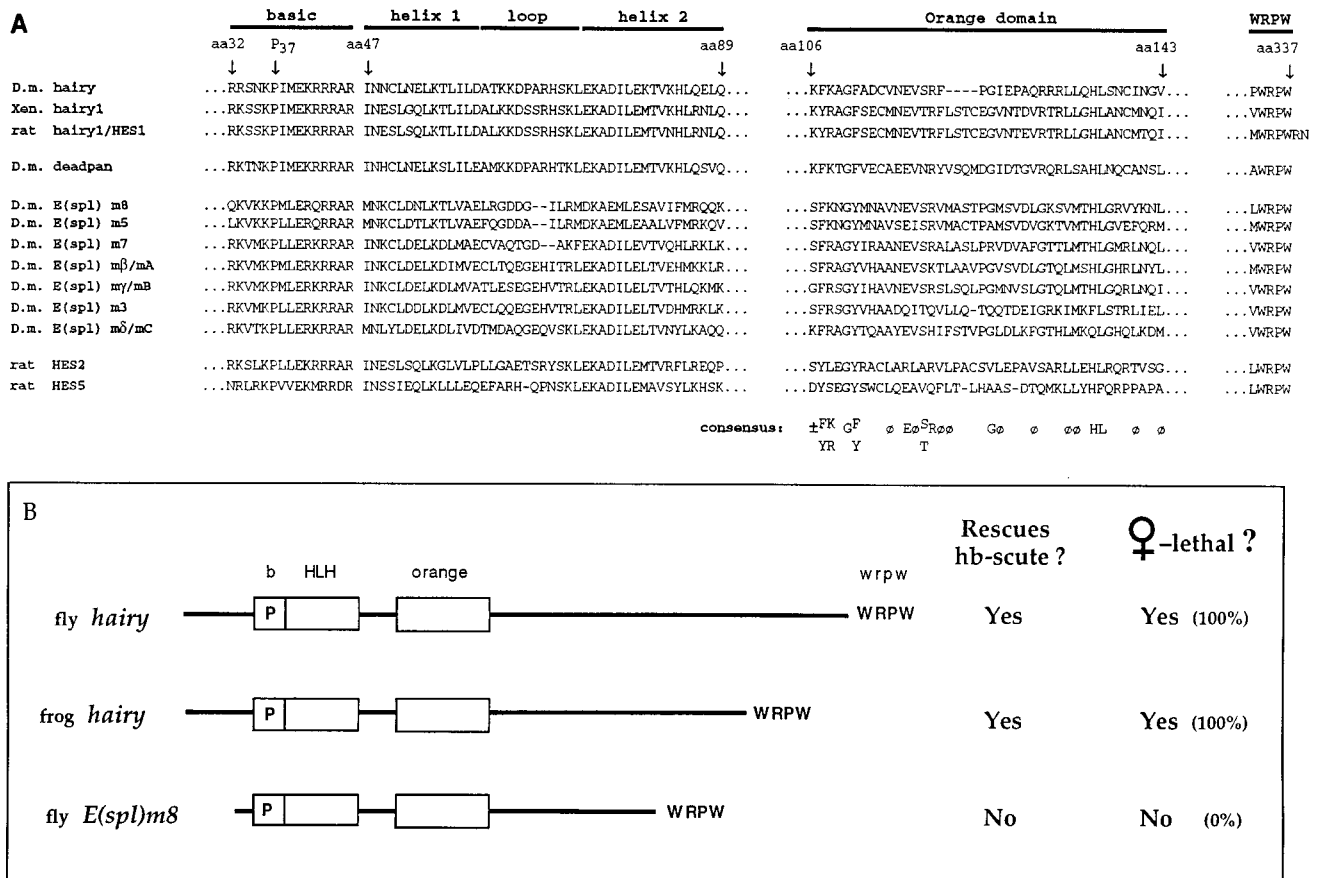|		wrpw		Rescues hb-scute ?	♀-lethal ?
fly *hairy*	b HLH orange	WRPW		Yes	Yes (100%)
frog *hairy*		WRPW		Yes	Yes (100%)
fly *E(spl)m8*		WRPW		No	No (0%)

FIG. 1. Three domains are evolutionarily conserved among the Hairy, E(SPL), Dpn proteins and are required for their proper function. (A) Sequence alignment of the conserved bHLH, Orange, and WRPW domains of the Hairy, Dpn, E(SPL), and HES bHLH proteins. Amino acid numbers at the top refer to the *D. melanogaster* (D.m.) Hairy protein. References for the sequences are given in the text. The conserved proline residue in the basic region is marked. The consensus sequence for the Orange domain is given. ø, hydrophobic residues. Xen., *Xenopus*. (B) Ectopic expression of a frog Hairy homolog, but not the related fly E(SPL)m8 protein, causes female lethality and rescues *hb-scute* male lethality. The coding regions of the genes used for ectopic expression are shown to scale (see Materials and Methods for the details of their construction). The relative positions and sizes of the conserved basic, HLH, Orange, and WRPW domains are indicated. ♀, female.

their ectopic expression during sex determination were assayed by their ability to rescue *hb-scute* males and by their associated sex-specific lethality.

A *Xenopus* homolog of *hairy* (*Xlh1*) that is 79% identical in the conserved bHLH domain to fly Hairy (Fig. 1A) has been isolated (45a). Like *Drosophila* Hairy expression, *hb*-driven Xlh1 expression (*hb-Xlh1*) rescues *hb-scute* male lethality (Table 1). Surprisingly, *hb*-driven E(SPL)m8 expression was not able to rescue *hb-scute* male lethality (*hb-Espl-m8*; Table 1). The integrity of these and all other constructs was tested by DNA sequencing. In addition, *hb*-driven expression of all constructs was confirmed by antibody staining or in situ hybridization (see Materials and Methods). Thus, rescue of *hb-scute* male lethality by frog Hairy but not by fly E(SPL)m8 indicates that different specificities exist among the Hairy/E(SPL) repressor proteins.

Suppression of *hb-scute* male lethality by Hairy requires the bHLH and Orange domains, but not the WRPW domain. Sequence comparison of the fly and frog Hairy and E(SPL) bHLH proteins, fly Dpn, and the mammalian HES proteins highlights four evolutionarily conserved domains that are presumably conserved because they are required functionally (Fig. 1A). All have a basic domain with the signature proline residue, as well as the HLH and C-terminal WRPW domains. Members of this class also have a conserved region downstream of the bHLH,

suggested to form two amphipathic helices (helix III and helix IV [9, 27]). However, the ability of this region to form helices is less apparent now that sequence comparisons include recently isolated Hairy/E(SPL) homologs in other species (13, 14, 23, 40, 41, 45a). We refer to this conserved motif as the Orange domain (Fig. 1A).

To determine which Hairy domains are required to rescue *hb-scute* male lethality, we tested *hb*-driven expression of mutant Hairy proteins. Changing two conserved basic region arginine residues (R-33 and R-43) to prolines (*hb-h*[RIP]; see Materials and Methods; Fig. 2A) completely abolishes Hairy rescue of *hb-scute* male lethality (Table 1). We also swapped the basic, HLH, and bHLH domains of Hairy with the corresponding regions of other bHLH proteins, including the widely expressed Da protein and the tissue-specific Scute protein (Fig. 2B). In both cases, rescue of *hb-scute* male lethality was completely disrupted (Table 1), suggesting that the bHLH domain is required for Hairy to suppress Scute activity. These constructs are expressed (see Materials and Methods), and so their failure to affect sex determination is indeed due to the altered proteins.

To determine if each of the non-bHLH evolutionarily conserved domains is required for Hairy repression of Scute activity, we made small deletions in each conserved region and in one nonconserved region. A 5-aa deletion in the Orange do-

TABLE 1. The Hairy bHLH and Orange domains are required for rescue of *hb-scute* male lethality

Genotype of:		No. of offspring with the following no. of copies of *hb-scute*:			
		Female		Male	
Female parent	Male parent[a]	1	2	1	2
hb-scute/hb-scute	*CyO/+; hb-scute/+*	97	91	69	0
hb-scute/hb-scute	*hb-h/+; hb-scute/+*	94	85	48	33
hb-scute/hb-scute	*hb-Xlh1/+; hb-scute/+*	106	109	72	40
hb-scute/hb-scute	*hb-Espl-m8/+; hb-scute/+*	112	89	56	0
hb-scute/hb-scute	*hb-da/+; hb-scute/+*	106	95	63	0
hb-scute/hb-scute	*ΔWRPW/+; hb-scute/+*	108	99	58	48
hb-scute/hb-scute	*ΔOrange/+; hb-scute/+*	107	100	78	0
hb-scute/hb-scute	*ΔSPPSS/+; hb-scute/+*	118	105	52	31
hb-scute/hb-scute	*RIP/+; hb-scute/+*	104	88	37	0
hb-scute/hb-scute	*MR/+; hb-scute/+*	86	82	30	0
hb-scute/hb-scute	*ML/+; hb-scute/+*	119	113	61	0
hb-scute/hb-scute	*PNMR/+; hb-scute/+*	93	79	35	0
hb-scute/hb-scute	*h/espl(bHLH)/+; hb-scute/+*	103	88	55	49
hb-scute/hb-scute	*h/espl(b)/+; hb-scute/+*	101	87	60	33
hb-scute/hb-scute	*h/espl(HLH)/+; hb-scute/+*	112	109	72	19
hb-scute/hb-scute	*h/da(bHLH)/+; hb-scute/+*	114	101	68	0
hb-scute/hb-scute	*h/scute(bHLH)/+; hb-scute/+*	95	87	56	0
hb-scute/hb-scute	*h/espl(OrW)/+; hb-scute/+*	99	91	52	0
hb-scute/hb-scute	*h/espl(W)/+; hb-scute/+*	92	87	44	12
hb-scute/hb-scute	*espl/h(OrW)/+; hb-scute/+*	107	81	69	16
hb-scute/hb-scute	*espl/h(Or)/+; hb-scute/+*	95	90	67	9

[a] *CyO*, balancer chromosome.

main (ΔOrange; Fig. 2A) renders Hairy unable to rescue *hb-scute* male lethality (Table 1). In contrast, removal of the four conserved terminal amino acids (ΔWRPW) or deletion of five amino acids (ΔSPPSS) in a nonconserved region immediately C terminal to the Orange domain did not affect the ability of Hairy to alleviate *hb-scute* male lethality (Fig. 2A; Table 1). Thus, the conserved bHLH and Orange domains, but surprisingly not the WRPW domain, are required for Hairy repression of Scute activity.

Functional differences between the Hairy and E(SPL) proteins map to the Orange domain. Despite the clear requirement for the conserved basic, HLH, and Orange domains for Hairy rescue of *hb-scute* male lethality, there remains a paradox, since E(SPL)m8 has all of these domains and yet its ectopic expression does not rescue *hb-scute* males. We examined the role of the bHLH domain by replacing the Hairy basic, HLH, and bHLH regions with those of E(SPL)m8 [*h/espl(b)*, *h/espl(HLH)*, and *h/espl(bHLH)*, respectively; Fig. 2C]. The resulting altered Hairy proteins rescued *hb-scute* male lethality (Table 1), suggesting that structurally similar family members can partially substitute for one another and that the differences in their action must map outside the bHLH domain.

To determine if the Hairy Orange domain provides the specificity for inhibition of Scute, we made hybrids of the Hairy and E(SPL)m8 proteins. One chimeric protein containing the E(SPL)m8 Orange domain [encoded by *h/espl(OrW)*] and three different hybrid proteins containing the Hairy Orange domain [encoded by *h/espl(W)*, *espl/h(OrW)*, and *espl/h(Or)*] were examined (Fig. 2D). Only those hybrid proteins with the Hairy Orange domain rescued *hb-scute* males (Table 1). Taken together, our results suggest that while both the basic and HLH domains are required for Hairy rescue of *hb-scute* male lethality, these domains are substitutable by E(SPL), whereas the significant differences between Hairy and E(SPL) suppression of Scute activity map to the Orange domain.

The Hairy bHLH, Orange, and WRPW domains are required for *hb-h* female-specific lethality. We have shown previously that ectopic Hairy expression from the *hb* promoter causes female-specific lethality by inhibiting initiation of *Sxl* expression (33). *hb-h* is unconditionally female lethal in one dose and results in female embryos with a distinct Sxl staining pattern: ubiquitous Sxl expression in the posterior and no staining detected in the anterior, where Hairy is ectopically expressed (4, 33). If Hairy works by inhibiting Scute activation of *Sxl*, then we would expect the same Hairy/E(SPL) domain requirements and Orange domain differences that were identified for *hb-scute* male rescue to be required for Hairy female-specific lethality. Thus, we examined all the transgenes described above to determine if their expression during sex determination resulted in female lethality (Tables 2 and 3).

Similar to *Drosophila* Hairy expression, *hb*-driven *Xenopus* Hairy1 expression resulted in 100% female lethality (Table 2), with female embryos exhibiting altered Sxl expression patterns identical to those seen with *hb-h* (data not shown). Disruption of the basic region (*hb-h*[RIP]), bHLH domain swaps of Hairy with Da or Scute, and small deletions in the Orange (ΔOrange) or WRPW (ΔWRPW) domain, but not in the nonconserved region (ΔSPPSS), eliminated Hairy's ability to cause female lethality (Table 2). These results agree with those obtained in the *hb-scute* male rescue assay, with one exception: the ΔWRPW deletion protein rescued *hb-scute* males but did not cause female lethality.

Ectopic expression of E(SPL)m8 did not confer female-specific lethality (Table 2), consistent with its failure to rescue *hb-scute* male lethality. We examined embryos from flies containing two, three, or four copies of the *hb-Espl-m8* transgene and could not detect any changes in the Sxl expression pattern (data not shown). Analysis of the Hairy/E(SPL)m8 basic, HLH, or bHLH swaps showed that all of these altered Hairy proteins still caused female lethality, albeit less efficiently (Table 3), suggesting that the difference in the abilities of Hairy and E(SPL)m8 to confer female lethality does not map to the bHLH region. Analysis of the Hairy/E(SPL)m8 chimeric proteins showed that the Orange domain was again responsible for the
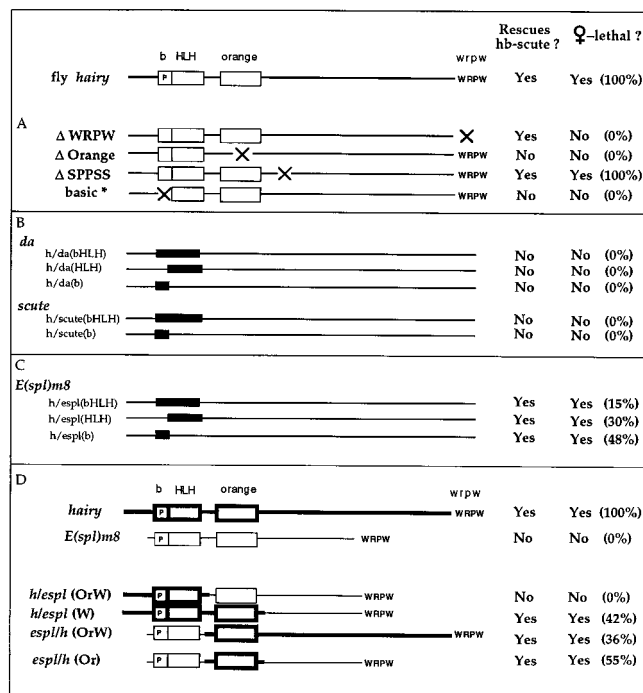
FIG. 2. The basic, HLH, Orange, and WRPW domains are all required for Hairy function, whereas differences in action between the Hairy and E(spl)m8 proteins map to the Orange domain. (A) Constructs that delete or disrupt the conserved domains of Hairy (indicated by Xs) were made. The four terminal amino acids (aa 334 to 337) of the WRPW motif were deleted to generate ΔWRPW. Five amino acids (NEVSR; aa 119 to 123) were deleted to create ΔOrange. Five amino acids were also deleted from a nonconserved region (SPPSS; aa 170 to 174) to create ΔSPPSS. The basic region was disrupted by simultaneously exchanging two conserved arginine residues (R-33 and R-43) for proline residues (basic*; see Materials and Methods on these constructs). Wild-type Hairy coding sequences are shown for comparison. ♀, female. (B) The basic, HLH, and bHLH regions of Hairy were swapped with the corresponding regions from the Da or AS-C Scute proteins. Solid boxes represent the regions of Da or AS-C Scute proteins present in the otherwise Hairy proteins. See Materials and Methods for the details of fusion gene construction. (C) The basic, HLH, or bHLH region of Hairy was swapped with the corresponding region from E(spl)m8 to determine the relative contributions of these domains. Dark boxes represent the regions of E(spl)m8 present in the otherwise Hairy proteins. (D) Chimeric proteins were made between Hairy (thick lines) and E(spl)m8 (thin lines) to determine which portions of the Hairy protein were needed to induce female lethality when ectopically expressed. The wild-type Hairy and E(spl)m8 regions are shown for comparison. See Materials and Methods for the details of fusion gene construction.

difference in action of the Hairy and E(spl) proteins: only the hybrid proteins containing the Hairy Orange domain conferred female-specific lethality (Table 3). We also examined the various *hb*-HLH, as well as altered *hb-h* and *hb-Espl-m8*, transgenes for their ability to interfere with the ectopically expressed wild-type Hairy but found no dominant negative effects (Table 4).

Interaction of Hairy with the Ftz homeodomain protein. We looked at the effect of another transgene, *hb-ftz*, which ectopically expresses the pair-rule segmentation gene *ftz* (34), on *hb-h*-dependent female lethality. Our attention was drawn to these transgenic flies by the fact that *hairy* behaves genetically as a negative regulator of *ftz* during embryonic segmentation and by the observation that reduced activity of the Hairy-like bHLH protein Dpn in males resulted in a loss of genitalia (50) similar to that induced by ectopic Ftz expression in *hb-ftz* flies (34). A weak posterior stripe of ectopic Ftz expression driven by the *hb* promoter overlaps temporally and spatially with the

most posterior (eighth) stripe of wild-type Dpn expression and the genital anlagen (2, 24, 42). Given that Hairy's normal role during segmentation is to repress *ftz* transcription, and the antagonistic relationship between Hairy and the AS-C bHLH proteins (5), we were intrigued by the observation that gain-of-function (ectopic) Ftz showed a phenotype similar to that of loss-of-function Dpn. This suggested that the ectopic Ftz protein might be titrating Dpn activity in the genital anlagen, perhaps reflecting an analogous antagonistic interaction between Ftz and Hairy during segmentation. If Ftz expression was indeed interfering with Dpn activity, then *hb-ftz* would be expected to interfere with Hairy activity and prevent *hb-h* female lethality. Indeed, *hb-ftz/+*; *hb-h/+* females are viable (Table 4), indicating that ectopic Ftz blocks the effects of ectopic Hairy. Fitzpatrick et al. (16) have shown that a homeo-domain-deleted version of the Ftz protein under the control of the heat shock promoter leads to the same anti-*ftz* phenotype as the wild-type Ftz protein and suggested that this phenotype may result from protein-protein interactions. We examined an *hb-ftz* transgene containing an identical deletion of the homeodomain and found that it no longer induced loss of genital structures (data not shown) or rescued *hb-h* female-specific lethality (Table 4), suggesting that the Ftz homeodomain is required for this interaction. Ectopic Ftz also overcomes *hb-Xlh1*-associated female lethality: *hb-ftz/+*; *hb-Xlh1/+* females are viable (Table 4), suggesting that the evolutionarily conserved domains of Hairy mediate this interaction. This *hb-h* rescuing activity is specific to Ftz, since ectopic expression of Eve, a distinct pair-rule homeodomain protein, from an *hb-eve* transgene had no effect on the survival of *hb-h* females (Table 4).

DISCUSSION

Using an in vivo assay, we have shown that the conserved Hairy bHLH, Orange, and WRPW domains are required for the female lethality associated with *hb*-driven Hairy expression. We also have determined that the ability of chimeric Hairy/E(spl) proteins to interfere with sex determination depends on the source of the Orange domain and not on that of the bHLH domain. Thus, we define the Orange domain as an important new functional domain for the Hairy/E(spl) proteins that mediates the specificity of their biological action in vivo. The requirement for the Orange domain is more stringent than that for the bHLH domain: altered Hairy proteins in which the Hairy basic, HLH, or bHLH domains were swapped for those of E(spl)m8 rescued *hb-scute* male lethality, whereas swaps with Da or Scute did not. The *Xenopus* Hairy1 homolog was also examined and was shown to conserve not only Hairy's structure but also its biological activity in vivo. In addition, we showed that suppression of Scute activity by Hairy requires the bHLH and Orange domains; the WRPW domain is required for Hairy-induced female lethality, but it is dispensable for suppression of *hb-scute* male lethality.

Our results are consistent with studies of *Drosophila* E(spl)-C bHLH proteins showing that the basic domain is required for their function (43, 44), as well as with studies of Hairy missense mutations showing changes mapping to the bHLH and WRPW domains but not to the Orange domain (48). This absence of Hairy missense mutations in the Orange domain could be due to the small number of *hairy* missense mutants examined (only four exist) or, less likely, to the Orange domain's not being required for Hairy function during segmentation. Examination of the effect of altered Hairy proteins under the control of the *hairy* promoter during segmentation is hampered by the extensive *cis*-regulatory region required for proper Hairy expression,

TABLE 2. The Hairy bHLH, Orange, and WRPW domains are required for *hb-h* female lethality[a]

Mutation	Genotype of[b]:		No. of offspring with the following no. of transgene copies:						~% female lethality
	Female parent	Male parent	Female			Male			
			2	1	0	2	1	0	
hb-HLH transgenes	+/+	*hb-h/bal*		0	150		162	125	100
	+/+	*hb-Xlh1/bal*		0	134		153	136	100
	hb-Esplm8/hb-Esplm8	*hb-Esplm8/hb-Esplm8*	154			149			0
	hb-da/hb-da	*hb-da/hb-da*	194			185			0
	hb-scute/hb-scute	*hb-scute/bal*	169	184		0	107		0
	hb-h deletions								
hb-h deletions	Δ*WRPW*/Δ*WRPW*	Δ*WRPW*/Δ*WRPW*	173			179			0
	Δ*Orange*/Δ*Orange*	Δ*Orange*/Δ*Orange*	211			196			0
	+/+	Δ*SPPSS/bal*		0	72		81	77	100
hb-h basic region point mutations	*RIP/RIP*	*RIP/RIP*	188			172			0
	+/+	*PT/bal*		0	111		126	99	100
	+/+	*PN/bal*		0	92		137	86	100
	MR/bal	*MR/bal*	5	72		119	116		67
	ML/ML	*ML/ML*	121			133			0
	PNMR/bal	*PNMR/bal*	38	78		190	122		63
	+/+	*PNML/bal*		2	97		118	89	99

[a] While the results for only one transformant line are shown in each case, similar results were obtained with at least two independent lines.
[b] *bal*, balancer chromosome. The chromosome containing the transgene is marked with the recessive eye color marker brown or scarlet (see Materials and Methods).

complicating both the construction of the transgenes and the ability to isolate transgenic animals. However, the regulatory mechanisms in all three situations are likely to be similar, since the same Hairy protein that is active in segmentation and bristle patterning hinders *Sxl* activation.

The WRPW domain is required for Hairy to inhibit SXL **expression but not for it to suppress Scute activity.** The requirement of the WRPW domain for *hb-h* to inhibit SXL expression, resulting in female lethality, but not for Hairy rescue of *hb-scute* male lethality implies that *hb*-driven Hairy protein

likely does more than inhibit Scute to block SXL expression. The WRPW domain of the fly Hairy/E(SPL) proteins has recently been shown to interact in vitro and in vivo with the Groucho protein (37). Groucho has been proposed to act as a corepressor of transcription, i.e., as a protein that is not able to bind DNA on its own but is capable of repressing transcription when brought to the DNA by its interactions with another protein (37). We suggest that Groucho may be recruited by Hairy, not to interfere with Scute activity but to interfere with the action of other proteins involved in SXL activation, perhaps

TABLE 3. The Orange domain confers functional specificity among the Hairy/E(SPL) bHLH proteins[a]

Genotype	No. of offspring with the following transgene or chromosome[b]:								~% female lethal[c]
	Female				Male				
	trf/trf (2)	trf/bal (1)	trf/mkr (1)	mkr/bal (0)	trf/trf (2)	trf/bal (1)	trf/mkr (1)	mkr/bal (0)	
Hairy/bHLH swaps									
h/da(bHLH)	103	99	107	90	98	103	95	87	0
h/da(HLH)	112	108	91	94	114	117	86	57	0
h/da(b)	163	138	137	165	165	146	152	141	0
h/scute(bHLH)	109	116	120	117	86	103	115	107	0
h/scute(b)	222	192	196	152	177	186	207	142	0
Hairy/E(SPL)m8 bHLH swaps									
h/espl(bHLH)	16	89	97	83	74	88	76	81	15
h/espl(HLH)	8	140	162	138	143	140	162	144	30
h/espl(b)	4	178	143	136	238	220	172	148	48
Hairy/E(SPL)m8 hybrid proteins									
h/espl(OrW)	180	154	77	107	144	106	78	106	0
h/espl(W)	14	86	81	129	107	109	98	86	42
espl/h(OrW)	2	100	136	144	128	111	134	123	36
espl/h(Or)	5	104	86	126	155	160	124	133	55

[a] While the results for only one transformant line are shown in each case, similar results were obtained with a minimum of two independent lines.
[b] Numbers in parentheses are numbers of transgene copies. All crosses were done as follows: female transformant (trf)/balancer (bal) × male transformant/dominant marker (mkr). The chromosome containing the transgene is marked with the recessive eye color marker brown or scarlet (see Materials and Methods).
[c] Percent female lethal equals the number of females with one or two copies of the transgene divided by the number of males with one or two copies of the transgene.

TABLE 4. Rescue of *hb-h*-induced female lethality

| Genotype of[a]: | | No. of offspring with the following gene or chromosome: | | | |
| | | Female | | Male | |
Female parent	Male parent[b]	hb-h	CyO	hb-h	CyO
bw; st	hb-h/CyO	0	108	98	104
hb-scute	hb-h/CyO	67	106	82	57
hb-ftz	hb-h/CyO	54	186	236	199
hb-ftz	hb-Xlh1/CyO	78	165	187	138
hb-ftzΔbox	hb-h/CyO	0	113	125	107
hb-eve	hb-h/CyO	0	226	215	221
hb-Espl-m8	hb-h/CyO	0	162	145	152
hb-da	hb-h/CyO	0	128	115	111
ΔWRPW	hb-h/CyO	0	167	153	129
ΔOrange	hb-h/CyO	0	154	147	101
RIP	hb-h/CyO	0	109	110	94
MR/bal	hb-h/CyO	0	123	89	85
ML	hb-h/CyO	0	126	93	81
PNMR/bal	hb-h/CyO	0	113	101	76

[a] Unless a balancer chromosome is indicated (*bal*), females were homozygous (i.e., had two copies) of the transgene.

[b] *CyO*, balancer chromosome.

other numerators such as Sis-a or Runt. Interestingly, Runt and its mammalian homologs terminate with the tetrapeptide motif WRPY (25). Mutually dependent genetic interactions between *hairy* and *runt* have been demonstrated to occur during embryogenesis for proper segmentation (22). It seems unlikely that the presence and conservation of the C-terminal WRPY motif in Runt and a WRPW motif in Hairy/E(SPL) is a mere coincidence. With the possibility of differential regulation of activity afforded by phosphorylation at tyrosine (Y), it is possible that these proteins compete directly with each other.

The Orange domain confers specificity to members of the Hairy/E(SPL) family. We have revealed a new functional domain, the Orange domain, that is largely responsible for the differences in behavior among the Hairy/E(SPL) class of bHLH proteins. *hb*-driven expression of Hairy/E(SPL)m8 chimeric proteins with the Hairy Orange domain causes female lethality and rescues *hb-scute* males, whereas expression of those with the E(SPL)m8 Orange domain does not. There are several possible roles for the Orange domain. The Hairy Orange domain could be required for the WRPW domain to bind Groucho (and/or other proteins), although this possibility seems unlikely since E(SPL)m8 binds Groucho in vitro (37). The Hairy Orange domain may regulate other Hairy functions (e.g., dimerization or DNA binding through intramolecular interactions), or the Orange domain might be necessary to stabilize Hairy binding to DNA, perhaps via protein-protein interactions with other proteins such as Scute. The fact that Hairy and E(SPL)m8 do not differ in either their DNA binding or their ability to bind Groucho in vitro suggests that the difference in their function may reflect differences in the proteins with which each can interact.

Analysis of Hairy/E(SPL) function suggests two separable repression mechanisms. The Hairy Orange domain is required for ectopic Hairy to interfere with sex determination and mediates the differences between the Hairy and E(SPL)m8 proteins in our assay. The requirement of the WRPW domain for *hb-h* female lethality, but not for rescue of *hb-scute* males, suggests that the Hairy/E(SPL) proteins may utilize two separable forms of transcriptional repression: the bHLH and Orange domains may provide a mechanism for inhibiting specific
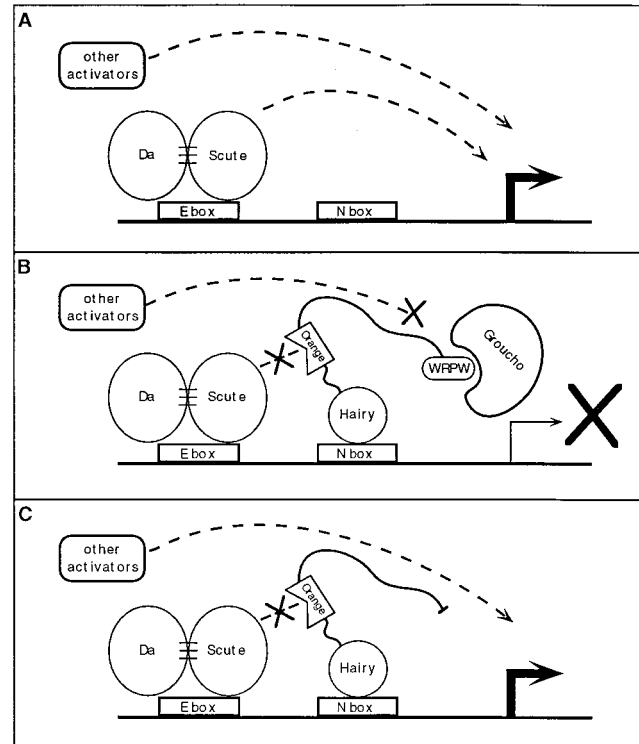

FIG. 3. Model for Hairy repressor function. (A) bHLH heterodimers such as Da-Scute bind to E-box DNA sequences and, along with other activator proteins, activate transcription of downstream target genes. Other activators are not shown in detail, but in the case of the *Sxl* target gene, they are likely to include DNA-binding proteins such as Sis-a and Runt. (B) When a downstream target gene includes N-box sequences, Hairy can bind DNA via its basic region and prevent activation of the downstream target gene via interactions through the Orange domain and by recruiting a corepressor protein, such as Groucho, with the C-terminal WRPW motif. (C) Hairy repression of downstream target gene transcription involves two separable repression mechanisms. Hairy repression of transcriptional activation by Da-Scute heterodimers requires the presence of Hairy DNA binding sequences (N box) that bring Hairy near the Da-Scute heterodimers; Hairy inhibits the activity of Scute through specific interaction requiring its Orange domain. Transcriptional inhibition of other activators requires an intact WRPW domain and, presumably, the presence of transcriptional corepressors. Thus, Hairy proteins lacking a WRPW domain, and thereby unable to recruit corepressors such as Groucho, can still inhibit Da-Scute activation of downstream targets, but they are unable to abolish transcription of the downstream gene, presumably because of the effects of other activators.

transcriptional activator proteins (such as Scute), while the WRPW domain mediates a more general repression mechanism. However, the possibility that the differing responses of the ΔWRPW construct in the two assays reflect differing sensitivities of the assays cannot be ruled out.

Hairy is likely to interfere with sex determination by inhibiting the activity of Scute numerator protein. Whereas both the C-terminal and bHLH domains of Scute are required for *hb-scute* male lethality, the Scute C-terminal domain alone can rescue *hb-h* female lethality (when fused to the C terminus of a heterologous protein) (40a). Although no biochemical proof exists at present, the requirement for the Hairy Orange domain and the Scute C-terminal domain for their mutual suppression suggests that Hairy may inhibit sex determination by suppressing Scute activity directly through protein-protein interactions (Fig. 3B). We envision this interaction occurring on the DNA, since both the Hairy and Scute bHLH domains are required to induce sex-specific lethality. The Scute C-terminal domain is rich in acidic residues, characteristic of transcriptional activa-

tion domains. If the C terminus is an activation domain, its ability to activate may be masked by interaction with the Hairy Orange domain. Alternatively, the Hairy Orange domain could bind DNA and prevent Scute DNA binding; however, the requirement for only the C-terminal domain of Scute makes this unlikely.

In the case of sex determination, Hairy may inhibit Scute by binding to an N-box site located near an E-box site bound by Da-Scute heterodimers. The Hairy Orange domain would then block the ability of the Scute C-terminal domain to activate *Sxl* expression. The E and N boxes would not necessarily need to be physically next to each other if DNA looping in the promoter is considered; however, in the promoters of two known in vivo targets, *achaete* and *E(spl)m8*, closely linked E and N boxes have been detected (32, 46). Also recent analysis of the *Sxl* early promoter detected the presence of several E-box sites (12). The specificity of this repression would depend on the presence of E- and N-box DNA sequences in the downstream target gene (e.g., *Sxl*), the DNA binding specificity of the Hairy/E(SPL) protein, and possibly the choice of the Hairy/E(SPL) dimerization partner. The WRPW domain is likely to be less specific, since all Hairy/E(SPL) family members contain this motif and should be able to recruit corepressors (such as Groucho or other related proteins) to the DNA. Analysis of a non-bHLH repressor protein, the *snail* product, has suggested a similar repression strategy involving both protein-protein interactions and DNA binding (18).

The failure of Hairy proteins with a mutation in the Orange domain to repress Sxl leads us to speculate that Orange domain function may be necessary for Hairy to establish a stable repressing complex on the *Sxl* promoter. This could allow precise control of Hairy-mediated repression: Hairy might be recruited to repress promoters only if both an appropriate DNA binding site and a suitable target activator protein are present (i.e., an activator protein that the Orange domain can interact with, such as Scute). Under these conditions, Hairy could then form a stable complex on the DNA, repress the target activator protein via the Orange domain, and also recruit a corepressor via the WRPW domain to repress other activators on the same promoter.

Interaction between Hairy and non-bHLH proteins. During segmentation, *hairy* behaves genetically as a repressor of *ftz*. The ability of ectopic Ftz to rescue *hb-h* female lethality suggests that Ftz interferes either directly with Hairy or with some other aspect of *Sxl* initiation. The latter possibility seems unlikely, since ectopic Ftz mimics the loss-of-function Dpn genitalia phenotype and *Sxl* is unlikely to be the target of Dpn in this case. It has been shown that *ftz* enhances its own expression during segmentation via autoregulatory enhancer elements upstream of the *ftz* transcription start site (21). Hairy could interfere with Ftz autoactivation via protein-protein interactions similar to those described for Hairy and Scute above. A role for protein-protein interactions in *ftz* regulation has also been suggested on the basis of the function of a variant of Ftz from which the homeobox was deleted (16). While the interaction between Hairy and Ftz requires the Ftz homeodomain, we are unable to determine which part of the Hairy protein is required, since this would require detecting a decrease in the already low ($\sim$20%) penetrance of *hb-ftz*-induced loss of genitalia (34). However, since ectopic Ftz also overcomes *hb-Xlh1*-associated female lethality, one of Hairy's evolutionarily conserved domains likely mediates this interaction. Alternatively (or perhaps in combination), Hairy's ability to affect *ftz-lacZ* transgenes lacking the *ftz* autoregulatory domain (21) favors the possibility that Hairy can repress Ftz activity indirectly, by repressing the activity of a Ftz regulatory protein.

Our results are consistent with the idea that multiple protein domains and DNA binding are necessary for Hairy/E(SPL) bHLH proteins to assemble a functional repressing complex. The requirement for multiple domains may account for the difficulty in showing interactions in vitro by using the isolated components of this system. Identification of additional proteins interacting with the conserved domains of the Hairy/E(SPL) bHLH proteins should help to unravel their repression mechanisms.

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