Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of *achaete*

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***hairy* (*h*) acts as a negative regulator in both embryonic segmentation and adult peripheral nervous system (PNS) development in *Drosophila*. Here, we demonstrate that *h*, a basic-helix-loop-helix (bHLH) protein, is a sequence-specific DNA-binding protein and transcriptional repressor. We identify the proneural gene *achaete* (*ac*) as a direct downstream target of *h* regulation in vivo. Mutation of a single, evolutionarily conserved, high-affinity *h* binding site in the upstream region of *ac* results in the appearance of ectopic sensory organs in adult flies, in a pattern that strongly resembles the phenotype of *h* mutants. This indicates that direct repression of *ac* by *h* plays an essential role in pattern formation in the PNS. Our results demonstrate that HLH proteins negatively regulate *ac* transcription by at least two distinct mechanisms.**

[Key Words: *Drosophila*; neurogenesis; peripheral nervous system; transcriptional repression; helix-loop-helix; negative regulation]

Received August 2, 1994; revised version accepted September 21, 1994.

The sensory organs that comprise the adult peripheral nervous system (PNS) of *Drosophila* offer an excellent system for the study of pattern formation during development. These sensory organs, which include the mechanosensory bristles, are distributed over the body surface of the fly in a highly reproducible pattern. Each sensory organ arises from a single precursor cell, the sensory organ precursor (SOP) (Hartenstein and Posakony 1989). SOPs are determined in a precise spatial and temporal pattern within the developing imaginal discs and histoblast nests (Bang et al. 1991; Huang et al. 1991), the tissues that will give rise to the epidermis of the adult. The establishment of the SOP cell fate has been studied extensively at the genetic and molecular levels and can be divided into two steps. In the first step, the competence to become a SOP is conferred on groups of cells called proneural clusters by the localized activities of the proneural genes *achaete* (*ac*), *scute* (*sc*), and *daughterless* (*da*) (Cubas et al. 1991; Skeath and Carroll 1991), which encode basic-helix-loop-helix (bHLH) transcriptional activators (Villares and Cabrera 1987; Caudy et al. 1988; Cronmiller et al. 1988; Cabrera and Alonso 1991; Van Doren et al. 1992). Whereas *da* is expressed ubiquitously (Cronmiller and Cummings 1993), *ac* and *sc* are expressed in a spatially restricted manner (Cubas et al. 1991; Skeath and Carroll 1991). Mutation of genes such

as *hairy* (*h*), *extramacrochaetae* (*emc*), and *pannier* (*pnr*) leads to ectopic *ac-sc* expression and ectopic sensory organs (Mohr 1918, as cited in Lindsley and Zimm 1992; Botas et al. 1982; Cubas et al. 1991; Skeath and Carroll 1991; Cubas and Modolell 1992; Van Doren et al. 1992; Martinez et al. 1993; Orenic et al. 1993; Romain et al. 1993), indicating that negative regulation plays an important role in proneural cluster patterning. In the second step of the process, proneural potential is further restricted to a single cell within the proneural cluster, the SOP. This involves local inhibitory cell-cell interactions (lateral inhibition) mediated by the neurogenic group of genes, which includes *Notch* (*N*), *Delta* (*DI*), *Suppressor of Hairless* [*Su(H)*], and the *Enhancer of split* complex [*E(spl)-C*] (Knust et al. 1987; Hartenstein and Posakony 1990; Heitzler and Simpson 1991, 1993; Schweisguth and Posakony 1992; Parks and Muskavitch 1993). Thus, negative regulation is essential in this second step of SOP specification as well.

h was first identified by the ectopic bristle phenotype conferred by loss-of-function mutations (Mohr 1918, as cited in Lindsley and Zimm 1992) and was later shown to function also as a pair-rule gene during embryonic segmentation (Nüsslein-Volhard and Wieschaus 1980). The ectopic sensory organs observed in *h* mutants are dependent on *ac* gene activity (Sturtevant 1970; Moscoso del Prado and García-Bellido 1984), and *ac* is expressed ectopically in these mutants (Skeath and Carroll 1991; Orenic et al. 1993). These findings indicate that *h* acts as a negative regulator of *ac* during adult PNS development,

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either directly or indirectly. However, a molecular basis for *h* function in either sensory organ development or embryonic segmentation has not been determined.

The *h* protein (Rushlow et al. 1989) is a member of a structurally distinct group of bHLH proteins, first identified in *Drosophila*, that also includes the products of seven genes of the E(spl)-C (Delidakis and Artavanis-Tsakonas 1992; Knust et al. 1992). We refer to these genes collectively as the h/E(spl) family. Homologous genes have been identified in mammals (Sasai et al. 1992; Feder et al. 1993), and there is evidence that at least one h/E(spl) family member in the rat may also act as a negative regulator in nervous system development (Ishibashi et al. 1994), suggesting that this family has been conserved functionally as well as structurally during evolution. h/E(spl) family members share a characteristic basic domain adjacent to their HLH domain, which suggested that these proteins might be capable of binding DNA (Van Doren et al. 1991; Wainwright and Ish-Horowicz 1992). In vitro DNA-binding activity has been observed for some family members, including the E(spl)m8 protein (Tietze et al. 1992) and the rat HES-1, HES-2, and HES-5 proteins (Akazawa et al. 1992; Sasai et al. 1992; Ishibashi et al. 1993). Moreover, HES-1, -2, and -5 are capable of acting as transcriptional repressors in tissue culture cotransfection assays (Akazawa et al. 1992; Sasai et al. 1992; Ishibashi et al. 1993; Takebayashi et al. 1994), suggesting that the h/E(spl) family functions differently from other well-studied bHLH proteins, which act as transcriptional activators. Recent evidence indicates that both rat (Takebayashi et al. 1994) and *Drosophila* (Kramatschek and Campos-Ortega 1994) h/E(spl) proteins function in negative autoregulation.

In this report we demonstrate that *h* is a sequence-specific DNA-binding protein and binding site-dependent transcriptional repressor. We also provide in vivo evidence that the proneural gene *ac* is a direct downstream target of *h* repression. These results establish a molecular mechanism of action for *h* in regulating nervous system development and are directly relevant to the functions of other h/E(spl) family members from *Drosophila* as well as other species.

Results

h binds to a specific site in the upstream region of *achaete*

Genetic evidence indicating that *h* is a regulator of *ac*, as well as structural features of the *h* protein suggesting a possible DNA-binding activity, prompted us to look for specific h-binding sites in the *ac* upstream region (Fig. 1A). As shown in Figure 1B, a single 110-bp restriction fragment from the 5'-flanking sequence of *ac* is preferentially precipitated by a purified protein consisting of glutathione S-transferase (GST) fused to a truncated form of *h* (GST-htr). This activity is not an artifact of deleting part of the *h* protein or fusing *h* to GST, as we have found that a full-length GST-h fusion protein binds with similar specific activity and that *h* synthesized in vitro in

Figure 1. Identification of a h-binding site in the *ac* upstream region. (A) Diagram of the *ac* region immediately 5' to the transcription start site. Indicated are the three E-box binding sites (T5E1-3) that mediate *ac-sc*-dependent auto- and cross-activation of *ac*, the 110-bp *Hae*III restriction fragment that interacts specifically with *h*, and the newly identified h/E(spl)-binding site (h/E-1). Numbers are in base pairs relative to the start of transcription (+1). (B) Precipitation of *Hae*III restriction fragments from 0.9 kb of *ac* upstream sequence by GST-htr protein. (Input) Untreated radiolabeled *Hae*III fragments (10% of the amount precipitated in Ppt lane) separated on a nondenaturing polyacrylamide gel. (Ppt) The same *Hae*III fragments after incubation with GST-htr protein and precipitation with glutathione-Sepharose beads. A single 110-bp fragment is precipitated by GST-htr. (C) Methylation protection assay of thrombin-cleaved htr binding to the 110-bp *Hae*III fragment. (F) Cleavage pattern of DMS-treated free DNA (outside lanes); (B) cleavage pattern of DNA bound by htr (middle lane). The brackets indicate the region protected from methylation by htr. The labeled DNA strand corresponds to the upper strand in D. (D) Summary of methylation protection results from both DNA strands in the vicinity of the h/E-1 site. (●) Nucleotides protected from methylation by DMS.

wheat germ translation lysates also binds efficiently (not shown; see Fig. 2B,C). Dimethyl-sulfate (DMS) methylation protection assays identify within this fragment a single 10-bp protected sequence, GGCACGCGAC, beginning at position -302 upstream of the *ac* transcription start site (Fig. 1A,C-D). We refer to this site as the

Figure 2. DNA-binding characteristics of the h protein. (A) Consensus GST-htr-binding site determined by random binding site selection. The frequency with which a given nucleotide was observed at each position in the aligned sequences is shown. Percentages are rounded to the nearest integer, so totals do not always equal 100%. Twenty-nine independent clones were sequenced, but the *n* for each position varies, because whenever nonrandom portions of the random-1 oligonucleotide overlapped the aligned sequence, these base pairs were excluded from the analysis. The consensus site shown was derived by selecting any position where a nucleotide was present at >50% frequency (lowercase letters) or >70% frequency (uppercase letters). Also shown are the consensus E-box sequence and the *ac* h/E-1 sequences from both *D. melanogaster* and *D. virilis*. (B) EMSA with full-length GST-h protein (~40 fmoles) and the *D. melanogaster ac* h/E-1 site oligonucleotide (40 fmoles) as a probe. (Lane 1) No specific competitor; (lanes 2–5) various specific competitor oligonucleotides, as indicated, in a 20-fold molar excess over the probe. Sequence differences between wild-type (wt) and mutant (m1, m2, m3) competitors are indicated. (C) EMSA combining different forms of h protein [full-length GST-h fusion (GST-h) and truncated, in vitro-translated h (htr)] as an assay for h multimerization. Lanes 1 and 5, respectively, show the pattern of shifted complexes observed with htr or GST-h alone. The predominant complex formed with each protein is labeled (A for GST-h and C for htr). Lanes 2–4 show combinations of both proteins in the same binding reaction. As more GST-h is included with a constant amount of htr, a single new binding complex is formed (complex B) with an electrophoretic mobility intermediate between the major complexes observed with either GST-h or htr alone.

ac h/E-1 site, because of its ability to bind both h and at least one bHLH protein encoded by the E(spl)-C (see below).

We determined an optimal consensus sequence for GST-htr binding using a random binding site selection protocol (S. Erdman and K. Burtis, pers. comm.). A GST-htr protein column was used to fractionate a double-stranded random oligonucleotide pool in which each oligonucleotide contained 14 bp of random sequence at its center. After two rounds of selection, GST-htr exhibited clear binding preferences over a 10-bp region, with sequences in the center of the region being selected for more strongly than those at either end (Fig. 2A). Interestingly, the *ac* h/E-1 site matches this consensus at 10 of 10 positions (Fig. 2A). Moreover, the upstream region of the *ac* gene from a distantly related species, *Drosophila virilis*, also contains a 10 of 10 match to the h consensus (Fig. 2A). This 10 of 10 match is part of a 15-bp

stretch of sequence that is identical between the *ac* genes of *D. melanogaster* and *D. virilis*, though the two upstream regions are, for the most part, highly diverged (not shown).

The random selection experiments suggest that the peripheral nucleotides of the h-binding site contribute to the specificity of the interaction but not as much as nucleotides in the center of the site (Fig. 2A). We tested this directly with a full-length GST-h fusion protein in an electrophoretic mobility shift assay (EMSA), with a double-stranded oligonucleotide *ac* h/E-1 probe. GST-h binds efficiently to the *ac* h/E-1 site in the absence of specific competitor (Fig. 2B, lane 1), but a 20-fold molar excess of unlabeled wild-type oligonucleotide effectively competes for this binding activity (lane 2). When the G in position 1 of the *ac* h/E-1 site is changed to a T (m1, Fig. 2B), we find that the ability of the mutant oligonucleotide to act as a competitor is slightly but reproduc-

ibly diminished from that of the wild-type oligonucleotide (lane 3), whereas mutation of the C at position 3 of the *ac* h/E-1 site (m2, Fig. 2B) has a much more drastic effect (lane 4). These results are consistent with the random selection data (Fig. 2A). Changing the *ac* h/E-1 site to fit the E-box (CANNTG) consensus (position 7 C → T; m3, Fig. 2B) yields a sequence that is still an excellent competitor for GST-h binding (lane 5). However, when this E-box version of the h-binding site is compared with the wild-type *ac* h/E-1 site in binding to and eluting from a GST-htr column, we find that the E-box version (m3) elutes at a lower salt concentration than does the wild-type site (data not shown).

Finally, we investigated whether, by analogy to certain other bHLH proteins (Murre et al. 1989), h binds DNA as a homo-oligomer. Figure 2C shows the results of an EMSA experiment combining full-length GST-h with a truncated h protein synthesized by in vitro translation in a wheat germ lysate (htr). As expected from their relative molecular masses (64 kD for GST-h and 12 kD for htr), these two forms of h individually yield protein-DNA complexes of very different electrophoretic mobilities (lanes 1 and 5, complexes A and C). As the amount of GST-h is increased in the presence of a constant amount of htr, a single complex of intermediate mobility appears (complex B) and increases in abundance at the expense of complex C (lanes 2–4). This indicates that htr and GST-h can both be present in the same oligomeric, probably dimeric, DNA-binding complex.

h acts as a transcriptional repressor

We used cotransfection assays in *Drosophila* Schneider (S2) cells to address what effect on gene expression, if any, h exhibits when bound to DNA. As illustrated in Figure 3A, we constructed a reporter gene containing four tandem copies of the *ac* h/E-1 site immediately upstream of an alcohol dehydrogenase (ADH) minimal promoter directing expression of a bacterial chloramphenicol acetyltransferase (CAT)-coding sequence. We also generated an identical reporter construct in which each of the four *ac* h/E-1 sites contains a 1-bp mutation that severely reduces h binding in vitro (m2, Fig. 2B). As shown in Figure 3B, full-length h clearly acts to repress expression from the ADH minimal promoter, reproducibly showing a 5- to 10-fold reduction in CAT activity. Furthermore, repression by h in this assay strictly requires the integrity of the *ac* h/E-1 sites, as demonstrated by the elimination of h's effect on reporter gene expression when these sites have been altered by a single base pair. This experiment establishes that a short oligonucleotide containing the *ac* h/E-1 site can confer h-dependent transcriptional repression on a heterologous promoter.

Mutation of the *ac* h/E-1 site results in a h mutant phenotype in vivo

Because the *ac* h/E-1 site is an optimal h-binding site in vitro and is conserved in distantly related species of

Figure 3. Binding site-dependent transcriptional repression by h. (A) Diagram of the reporter construct used in cotransfection assays. *ac* h/E-1 sites upstream of the ADH minimal promoter are either wild-type in sequence or mutated at a single base pair. Changes in ADH promoter activity are assayed by measurement of levels of CAT enzyme activity. (B) Effects of h on expression from the wild-type or mutant *ac* h/E-1 ADH-CAT construct. Experiments indicated as + hairy included an expression construct that expresses h from the *Drosophila* actin 5C promoter. Control experiments indicated as - hairy included the parental expression vector without insert. Fold repression was calculated relative to the - hairy control. Results shown are an average of five independent experiments.

Drosophila, we were interested in investigating the importance of this site for the regulation of *ac* by h in vivo.

Male flies carrying the *sc*¹⁰⁻¹ mutation, which inactivates both *ac* and *sc*, completely lack mechanosensory bristles on the head and thorax. We have shown previously that a 2.2-kb genomic DNA fragment containing the *ac* transcription unit, as well as 0.9 kb of 5'-flanking sequence (Fig. 1A), is capable of providing partial *ac* function in transgenic flies that carry this mutation (referred to hereafter as *ac*⁻ *sc*⁻). Thus, a wild-type *ac* transgene can rescue numerous mechanosensory bristles in normal positions on the body surface of *ac*⁻ *sc*⁻ males (Van Doren et al. 1992).

Figure 4 demonstrates that like the endogenous *ac* gene, the activity of the wild-type *ac* transgene is subject to negative regulation by h and that this regulation is independent of endogenous *ac*-*sc* function. A represen-

Figure 4. A wild-type *ac* transgene is subject to regulation by *h*. Mounted wing blade preparations are shown, focusing on the proximal portion of wing vein L5. (A) Wild-type *ac* transgene insertion C3-2 in an *ac*⁻ *sc*⁻ genetic background [genotype *sc*¹⁰⁻¹ *w*¹¹¹⁸/Y; C3-2]. No ectopic bristles are observed. (B) Wild-type *ac* transgene insertion C3-2 in an *ac*⁻ *sc*⁻ *h*⁻ genetic background [genotype *sc*¹⁰⁻¹ *w*¹¹¹⁸/Y; C3-2 *se h*¹]. Note the four ectopic bristles along wing vein L5 (arrowheads).

tative wild-type transgene insertion produces no ectopic wing bristles in *ac*⁻ *sc*⁻ males ($n=20$ wing blades; Fig. 4A). However, the same transgene in a genetic background that is also mutant for *h* (*ac*⁻ *sc*⁻ *h*⁻; Fig. 4B) gives a clear ectopic bristle phenotype (mean = 6.8 ± 3.1 ectopic bristles per wing blade, $n=4$).

We then constructed a mutant *ac* transgene that is identical to the wild-type transgene except for mutation of 3 bp within the *h*/E-1 site (core hexamer CACGCG changed to CCCTCT), which severely reduces or eliminates *h* binding to this site (not shown). We compared the bristle phenotypes of 6 independent transformant lines carrying the wild-type *ac* transgene with the phenotypes of 10 independent lines carrying the mutant transgene. As shown in Figure 5A and summarized in Table 1, four of the six insertions of the wild-type *ac* transgene produce no ectopic wing bristles in wild-type females, although they can restore many normal microchaete bristles to *ac*⁻ *sc*⁻ males (Van Doren et al. 1992). In the other two lines, a single ectopic bristle is observed on wing vein L5 in a small fraction of individuals (Table 1). In contrast, 9 of the 10 insertions of the *h*/E-1 mutant *ac* transgene show an ectopic bristle phenotype in wild-type females (Fig. 5C,D; Table 1). The single *h*/E-1 mutant *ac* transgene insertion that does not exhibit an ectopic bristle phenotype in wild-type flies (line I4-1) also

has a severely reduced ability to rescue normal microchaetes in the *ac*⁻ *sc*⁻ background (not shown), indicating that it is located in a chromosomal position that strongly represses its activity. The other nine *h*/E-1 mutant *ac* transgenic lines display a range of phenotypes, with the weakest (Fig. 5C; Table 1, line I3-4) consisting of one to four bristles on wing vein L5 in 75% of the animals ($n=20$). This effect, however, is still clearly stronger than the strongest phenotype observed among the lines carrying the wild-type *ac* transgene (Table 1, cf. lines C5-1 and I3-4). The stronger *h*/E-1 mutant *ac* transgene phenotypes observed (Table 1) consist of numerous ectopic bristles on the wing (Fig. 5D) and pleura (Fig. 5I), ectopic campaniform sensilla on the wing blades, and one or a few ectopic bristles on the scutellum and legs. Strikingly, these phenotypes are also observed in non-transgenic *h* mutant flies (Fig. 5, cf. C and D to B; cf. I to H; see also Table 1), though, as expected for a transgene that supplies only partial *ac* activity, none of the transgenic lines exhibit a complete *h* mutant phenotype.

Because of the known auto- and cross-regulatory activities of *ac* and *sc* in the imaginal disc (Martinez and Modolell 1991; Van Doren et al. 1991, 1992; Martinez et al. 1993), we anticipated that the *ac* transgenes would be subject to activation by the endogenous *ac* and *sc* genes, and vice versa. This prompted us to test the independent

Figure 5. (See facing page for legend.)

Table 1. Phenotypes of wild-type and h/E-1 mutant ac transgenic lines

Transgene	Line or mutation	n	Number of wing bristles ^a	Phenotype ^b				
				1	2	3	4	5
Wild-type ac transgene	C3-1	20	0					
	C4-1	20	0					
	C6-2	20	0					
	C7-4	20	0					
	C3-2	25	0.16	X				
	C5-1	25	0.24	X				
h/E-1 mutant ac transgene	I4-1 ^c	15	0 ^c					
	I3-4	20	1.3	X	X			
	I7-1	15	1.5	X	X			
	I6-3	25	1.8	X	X			
	I7-2	10	2.2	X	X			
	I4-2	10	4.6		X	X		
	I1-1	10	7.3		X	X	X	
	I5-1	5	40		X	X	X	X
	I3-2	5	47		X	X	X	X
	I6-2	5	72		X	X	X	
None	h ¹	5	90		X	X	X	X

^aMean number of ectopic nonmargin bristles per wing in females homozygous for each insertion or for the h¹ mutation. The mean numbers of ectopic bristles observed in the h/E-1 mutant ac transgenic lines are significantly different from the mean numbers of bristles observed in the wild-type ac transgenic lines (Mann-Whitney U test; $P = 0.0042$).

^bFemales from each line were scored for the following phenotypes: (1) Single ectopic bristle only on wing vein L5; (2) multiple ectopic bristles on wing vein L5; (3) ectopic bristles on other nonmargin regions of the wing; (4) ectopic bristles on the pleura; (5) ectopic bristles on the scutellum.

^cLine I4-1 is also extremely weak in rescuing wild-type bristles in a *sc*¹⁰⁻¹ genetic background, indicating that the transgene is inserted in a chromosomal position that represses its activity.

activity of the h/E-1 mutant ac transgene insertions by introducing them into a genetic background lacking endogenous ac-sc function. Besides rescuing many normal microchaete bristles, three of three independent insertions of the mutant transgene still produce ectopic bristles in ac⁻ sc⁻ flies (Fig. 5E,F), although some reduction in the total number of ectopic bristles is observed. Thus,

the ability of the mutant transgene to promote bristle development in ectopic locations does not require the activity of the endogenous ac and sc genes.

In summary, a similar in vivo phenotype is obtained either by mutation of the cis-regulatory site in ac to which h binds in vitro or by mutation of the gene encoding the h trans-regulator.

The E(spl)m7 protein can repress transcription through the ac h/E-1 site

Genetic evidence that the E(spl)-C is required for negative regulation of ac expression during lateral inhibition (Skeath and Carroll 1992), along with the strong sequence similarities in the basic regions of h and the E(spl) bHLH proteins, suggested that E(spl) bHLH proteins might interact directly with regulatory sequences in the ac gene, specifically the h/E-1 site. We found that a GST-E(spl)m7 fusion protein, containing the first 127 amino acids of E(spl)m7 (GST-m7tr), binds to the ac h/E-1 site (Fig. 6A, lane 1) with a specific activity similar to that of GST-h (not shown). Moreover, the sequence specificity of GST-m7tr mimics that of GST-h in a competition assay, as shown by the very similar response of GST-m7tr binding to single base pair changes in the ac h/E-1 site (cf. Fig. 2B, lanes 2–5 with Fig. 6A, lanes 2–5). Thus, ac h/E-1 appears to be a specific binding site for the E(spl)m7 protein in vitro.

By use of the cotransfection assay described above (see Fig. 3A), we tested the ability of E(spl)m7 to act as a transcriptional repressor through interaction with the h/E-1 site from ac. As shown in Figure 6B, full-length E(spl)m7 inhibits the activity of the ADH minimal promoter by ~10-fold when wild-type h/E-1 sites are present upstream, whereas little or no repression is observed when these sites are mutated at a single position (m2 of Fig. 2B; see Fig. 6A, lane 4). We conclude that E(spl)m7 can function as a DNA-binding protein and transcriptional repressor and that, as with h, the ac h/E-1 site can confer E(spl)m7-dependent repression on a heterologous promoter.

Discussion

The *Drosophila* gene h encodes a bHLH protein that

Figure 5. Mutation of the ac h/E-1 site leads to the appearance of ectopic sensory organs. (A) Wing blade of a w¹¹¹⁸ female homozygous for the wild-type ac transgene (line C3-2). The majority of wing blades of this genotype have no ectopic bristles, even on the proximal region of wing vein L5 (inset). (B) Wing blade of a homozygous h¹ female. Note the ectopic bristles along all longitudinal wing veins and in some intervein regions. (C) Wing blade of a w¹¹¹⁸ female homozygous for an insertion of the h/E-1 mutant ac transgene that gives a weak effect (line I3-4). Note the ectopic bristles on the proximal region of wing vein L5 (arrowheads and inset). (D) Wing blade of a w¹¹¹⁸ female homozygous for an insertion of the h/E-1 mutant ac transgene that gives a strong effect (line I6-2). Note the numerous ectopic bristles along wing veins L2, L3, and L5, as well as in some intervein regions. (E–F) Wing blades of ac⁻ sc⁻ males homozygous for h/E-1 mutant ac transgene insertions. (E) I3-4 (genotype sc¹⁰⁻¹ w¹¹¹⁸/Y; I3-4); (F) I6-2 (genotype sc¹⁰⁻¹ w¹¹¹⁸/Y; I6-2). Ectopic bristles (indicated by arrowheads in E) are still observed with the h/E-1 mutant ac transgene in an ac⁻ sc⁻ genetic background. (G–J) Lateral view of adult male thoraces, showing the pleural region. Dorsal is at the top; anterior is to the right. (G) Wild-type (w¹¹¹⁸/Y). No bristles appear on the pleura of these animals. (H) h¹ homozygous male. Note the ectopic microchaete bristles on the pleura. (I) w¹¹¹⁸/Y; I6-2. In the stronger h/E-1 mutant ac transgenic lines, ectopic bristles are observed on the pleura. (J) sc¹⁰⁻¹ w¹¹¹⁸/Y; I6-2. Ectopic bristles are still observed on the pleura in an ac⁻ sc⁻ background. Note the missing macrochaetae and reduced numbers of microchaetae resulting from the loss of endogenous ac and sc activity.

Figure 6. E(spl)m7 can bind to and act as a transcriptional repressor through the *ac h/E-1* sequence. (A) EMSA with GST-m7tr protein (~40 fmoles) with the *ac h/E-1* site oligonucleotide (40 fmoles) as a probe. (Lane 1) No specific competitor; (lanes 2–5) specific competitor oligonucleotides, as indicated, at a 20-fold molar excess over the probe. Sequence differences between wild-type (wt) and mutant (m1, m2, m3) competitors are as shown in Fig. 2B. (B) Cotransfection assays measuring the effects of E(spl)m7 on expression of the *ac h/E-1* ADH-CAT reporter constructs diagramed in Fig. 3A. Experiments indicated as + E(spl)m7 included an expression construct that expresses E(spl)m7 from the *Drosophila* actin 5C promoter. Control experiments indicated as – E(spl)m7 included the parental expression vector without insert. Fold repression was calculated relative to the – E(spl)m7 control. Results shown are the average of four independent experiments.

functions as a negative regulator in both embryonic segmentation and adult PNS development. In this report we show that *h* is a sequence-specific DNA-binding protein and transcriptional repressor, and we identify the proneural gene *ac* as a direct downstream target of *h* regulation. In combination with previous findings concerning the action of *emc*, these results demonstrate that HLH proteins utilize at least two distinct mechanisms to negatively regulate *ac* transcription and thus the pattern of adult sensory organs.

h is a sequence-specific DNA-binding protein

We have shown that purified *h* fusion proteins exhibit sequence-specific DNA-binding activity in vitro and can recognize a 10-bp sequence in the upstream region of the *ac* gene, the *ac h/E-1* site. Determination of a consensus *h*-binding site by random oligonucleotide selection indicates that *ac h/E-1* is an optimal site for *h* binding. Several characteristics of the DNA-binding activity of *h* are worthy of note. The first is that the amino-terminal 109 amino acids of *h* (of 337 in the full-length protein) are sufficient to mediate sequence-specific DNA binding. This truncated form of *h* includes only ~50 amino acids in addition to the complete bHLH domain and lacks other conserved domains of the *h/E(spl)* family of proteins, such as the two postulated helical regions that lie carboxy-terminal to the bHLH sequence (Knust et al. 1992), and the extreme carboxy-terminal Trp-Arg-Pro-Trp (WRPW) motif (Klambt et al. 1989; Rushlow et al. 1989; Delidakis and Artavanis-Tsakonas 1992; Knust et al. 1992). Thus, these regions are not required for DNA binding by *h*. Second, both the random binding site selection experiments (Fig. 2A) and the DNA-binding competition assays (Fig. 2B) indicate a strong preference by *h* for specific nucleotides in the core of its binding site (especially the hexamer CACGCG), as well as weaker selectivity in the flanking nucleotides. The stringent selection for CG at the center of the *h*-binding site is consistent with the presence of an arginine residue at amino acid 44 in the basic region of the protein. An arginine residue in the analogous position in other bHLH or bHLH/ZIP proteins that bind sites with a CG center has been shown to be critical for recognition of these bases (Dang et al. 1992; Ferre-D'Amare et al. 1993). Third, the optimal *h*-binding site is not a typical E-box (CANNTG; Fig. 2A), whereas the binding sites of almost all bHLH proteins besides those in the *h/E(spl)* family fit this consensus. We have shown that *h* binds well to an E-box version of the *ac h/E-1* site (GGCACGTTGAC; Fig. 2B), though this complex is not as salt resistant as *h* complexes with *ac h/E-1*. In addition, the *h* consensus site is related to, but does not match, the N-box sequence (CACNAG), first identified in the upstream region of the *E(spl)m8* gene (Tietze et al. 1992). N box-containing sequences have been shown to be in vitro binding sites for the E(spl)m8, HES-1, HES-2, and HES-5 proteins (Akazawa et al. 1992; Sasai et al. 1992; Tietze et al. 1992; Ishibashi et al. 1993), though it has not been determined whether these are optimal binding sites for any of these

proteins. We find that *h* can bind to the N-box sequence from *E(spl)m8* but more weakly than to the *ac* h/E-1 site (not shown). Finally, we note that although we have analyzed the DNA-binding properties of *h* alone (homooligomeric complexes) in this study, it is possible that *h* may also interact with other proteins in hetero-oligomeric DNA-binding complexes. In either case, our data indicate that, at least in part, *h*'s mechanism of action involves sequence-specific DNA binding.

h and *E(spl)m7* are transcriptional repressors

We have shown here that the *h* and *E(spl)m7* proteins can act as binding site-dependent transcriptional repressors through the same *ac* h/E-1 sequence. It is significant that an oligonucleotide containing the h/E-1 site is sufficient to confer transcriptional repression by *h* and *E(spl)m7* on a heterologous promoter. This indicates that the activity of these proteins as repressors does not depend on the specific context of the *ac* promoter. For example, *ac* is subject in vivo to auto- and cross-activation by proneural bHLH protein complexes that bind to three E-box sequences in the *ac* promoter (Martinez and Modolell 1991; Skeath and Carroll 1991; Van Doren et al. 1991, 1992; Martinez et al. 1993), and one possible model for *h* regulation of *ac* is that *h* specifically interferes with this activation. However, our data indicate that *h* and *E(spl)m7* can act as repressors independently of the function of bHLH activators.

In addition to their activity as binding site-dependent transcriptional repressors, the rat HES-1 and HES-5 proteins have been reported to antagonize the function of bHLH activator proteins by forming inactive hetero-oligomeric complexes with them (Akazawa et al. 1992; Sasai et al. 1992; Takebayashi et al. 1994). Thus far we have not observed that *h* interferes with the DNA-binding activity of *da/ac-sc* protein complexes in vitro (Van Doren et al. 1991) or with their ability to act as transcriptional activators in cotransfection assays (M. Van Doren, unpubl.). Nevertheless, it remains possible that *h* may have regulatory functions in vivo that do not involve interacting with DNA.

Genetic data indicate that *h* plays an important role in embryonic segmentation, in part by negatively regulating the expression of *fushi tarazu (ftz)* (Carroll and Scott 1986; Howard and Ingham 1986; Ish-Horowicz and Pinchin 1987). The product of the *deadpan (dpn)* gene is very closely related to *h* (Bier et al. 1992) and acts as a negative regulator in sex determination in the early fly embryo (Younger-Shepherd et al. 1992). Our finding that *h* acts as a DNA-binding transcriptional repressor in adult sensory organ development suggests that the same molecular mechanism may contribute to *h* and/or *dpn* function in these other developmental processes.

The functional evidence to date is consistent with the generalization that both the fly and mammalian members of the h/E(spl) family of bHLH proteins act as transcriptional repressors, either in negative auto-regulation (Kramatschek and Campos-Ortega 1994; Takebayashi et al. 1994) or in repression of downstream genes (this pa-

per). In contrast, the regulatory activities that have been described so far for other DNA-binding bHLH proteins (not including bHLH/ZIP proteins) generally involve transcriptional activation. Thus, activation and repression appear to be functions characteristic of structurally distinct subgroups of bHLH transcriptional regulators.

ac is a direct downstream target of *h* repression

The observations reported here strongly support the conclusion that *h*, or a complex including *h*, functions as a direct transcriptional repressor of *ac* through the *ac* h/E-1 site in vivo (Fig. 7A) and that such regulation plays an essential role in the patterning of adult sensory organs in the fly. Ohsako et al. (this issue) have independently come to a similar conclusion.

The identification of a proneural gene as a direct downstream target of regulation by a h/E(spl) family member may suggest at least one general function for these proteins in both vertebrate and invertebrate neurogenesis. For example, the HES-1 protein has been implicated as a negative regulator of nervous system development in the rat (Ishibashi et al. 1994); downstream targets of repression by HES-1 or related proteins may include genes that are directly involved in promoting the neural cell fate.

We believe it is significant that the *E(spl)m7* protein

Figure 7. *h* and *emc* exhibit distinct mechanisms for negative regulation of *ac* expression. (A) Model for regulation of *ac* expression by *h*. *h*, in either a homo-oligomeric complex or a hetero-oligomeric complex with an unknown factor, binds to the h/E-1 site, and acts to repress *ac* transcription. (B) Model for regulation of *ac* expression by *emc*. *ac* is subject to direct auto- and cross-activation, most likely by *ac/da* and *sc/da* (not shown) protein complexes, acting through E-box binding sites upstream of *ac*. By forming complexes with these proteins that are inactive for DNA binding, *emc* inhibits their function as transcriptional activators of *ac*.

can bind to the *ac* h/E-1 site with a sequence specificity and specific activity similar to that of h and can repress transcription through this site (Fig. 6; M. Van Doren, unpubl.). The E(spl)-C is required for the process of lateral inhibition in the proneural clusters, which acts to restrict proneural potential to the SOP. During determination of the SOP, proneural gene (including *ac*) expression is elevated in this cell and down-regulated in the remaining cells of the cluster (Cubas et al. 1991; Skeath and Carroll 1991), in a manner requiring neurogenic gene [including E(spl)-C; Skeath and Carroll 1992] function. Our results raise the possibility that one or more of the E(spl) bHLH proteins act as direct transcriptional repressors of *ac* during lateral inhibition. In this view, h acts as a global regulator in the imaginal disc to help restrict *ac* expression to the proneural clusters, whereas E(spl) bHLH proteins might act as local regulators to inhibit the expression of *ac*, and possibly other targets, in those cells of the proneural cluster that are prevented from assuming the SOP fate. Such an activity of the E(spl) genes in the proneural clusters may depend on the lateral inhibitory signal. It is clear, however, that singularization of the SOP does not occur solely through transcriptional regulation of proneural gene expression, since individual, well-spaced bristles are produced when proneural genes are expressed under the control of heterologous promoters that are not likely to be regulated by lateral inhibition (Rodriguez et al. 1990; Hinz et al. 1994). Thus, it is possible that E(spl) bHLH proteins might be direct repressors of *ac* even though we do not observe a neurogenic bristle tufting phenotype in flies carrying the *ac* h/E-1 mutant transgene. It has been shown recently that *E(spl)m7* expression in the wing imaginal disc is under the direct control of the *ac* and *sc* bHLH activators (Singson et al. 1994). This suggests that a transcriptional feedback loop may exist in which *ac* first directly activates *E(spl)m7* expression throughout the proneural cluster, and E(spl)m7, in turn, acts to repress *ac* expression in all cells of the cluster except the SOP.

Two distinct mechanisms of negative transcriptional regulation by HLH proteins

The work presented here and in previous reports reveals two distinct mechanisms by which the HLH proteins h and emc can negatively regulate the transcriptional activity of the same gene, *ac* (Fig. 7).

Here, we have described evidence that h acts as a negative regulator of *ac* by a mechanism of direct DNA binding and transcriptional repression (Fig. 7A). In contrast, previous studies have elucidated a very different mechanism of action for emc (Fig. 7B). The emc protein contains an HLH domain, but lacks the adjacent basic region that constitutes the DNA-binding domain of bHLH proteins (Ellis et al. 1990; Garrell and Modolell 1990). The *ac* and *sc* proteins can both form complexes with da that are capable of binding DNA and activating transcription (Cabrera and Alonso 1991; Van Doren et al. 1991, 1992). emc can act as a negative regulator of transcription by forming complexes with the *ac*, *sc*, and da

proteins (P.A. Powell and J.W. Posakony, in prep.) that are inactive in DNA binding (Van Doren et al. 1991) and transcriptional activation (Van Doren et al. 1992). The *ac* gene itself is a direct target of activation by *ac-sc*-containing protein complexes (Van Doren et al. 1992; Martinez et al. 1993), and emc negatively regulates *ac* expression by interfering with this auto- and cross-regulation (Fig. 7B; Van Doren et al. 1992; Martinez et al. 1993). Thus, two HLH proteins, h and emc, function in vivo as negative regulators of *ac* transcription via distinct molecular mechanisms. Given that *ac* itself encodes a bHLH transcriptional activator, it is apparent that the regulatory versatility of the HLH proteins is well illustrated by the process of adult sensory organ patterning in *Drosophila*.

Materials and methods

Drosophila stocks

Flies were raised on standard yeast/cornmeal/molasses/agar medium. Mutant strains are described in Lindsley and Zimm (1992).

General molecular biology methods

Standard techniques of molecular biology were performed as described (Ausubel et al. 1987; Sambrook et al. 1989).

Protein expression and purification

For the full-length GST-h fusion protein construct, a h cDNA (Van Doren et al. 1991) was cut at the *Bst*EII site immediately 3' of the start codon, and the ends were blunted with Klenow DNA polymerase. This DNA was then digested with *Sal*I and subcloned into *Eco*RI-cut, Klenow-blunted, and *Sal*I-cut pGEX KG (Guan and Dixon 1991), creating pGEX-h. The predicted fusion protein from this construct contains all of the h protein except for the initiator methionine. For the truncated GST-htr fusion protein, a *Bam*HI-*Xmn*I fragment from pGEX-h was subcloned into *Hind*III-cut, Klenow-blunted, and *Bam*HI-cut pGEX KG, creating pGEX-htr. The predicted fusion protein produced from this construct contains amino acids 2–110 of h (of 337 amino acids total) and also has 5 additional amino acids added to the carboxyl terminus. For the truncated GST-m7tr fusion protein, an *Msc*I-*Nco*I fragment of *E(spl)m7* genomic DNA was subcloned into *Xba*I-cut, Klenow-blunted, and *Nco*I-cut pGEX KG, creating pGEX-m7Nco. The predicted fusion protein from this construct contains amino acids 2–127 of E(spl)m7 (of 186 amino acids total), and 10 additional amino acids are added to the carboxyl terminus.

For fusion protein production, the above expression constructs were transformed into BL21 DE3 competent cells. For each construct, 500 ml of Luria broth plus 100 µg/ml of ampicillin (LB AMP) was inoculated with a 10-ml culture that had grown overnight in LB AMP at 37°C, and was incubated at 37°C to an optical density at 600 nm (OD₆₀₀) of ~0.2. The culture was transferred to 30°C until it reached an OD₆₀₀ of 0.6–0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the culture was incubated 1 hr at 30°C. Cells were harvested by centrifugation at 4000g for 5 min and washed in ~150 ml of cold phosphate-buffered saline (PBS). Cells were resuspended in 8 ml of ice-cold GST sonication buffer [GSB: 25 mM K⁺ HEPES at pH 7.5, 0.3 M KCl, 1 mM

ethylenediaminetetracetic acid (EDTA), 0.1% Nonidet P40 (NP-40), 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml of aprotinin, 2 µg/ml of leupeptin, 1 µg/ml of pepstatin A, and 5 mg of lysozyme was added followed by a 15-min incubation on ice. Cells were then sonicated on ice (5 × 30 sec on/30 sec off) at 60% power using a Fisher sonicator (Dismembrator model 300). The lysate was clarified by centrifugation at 20,000g for 10 min, and the supernatant was incubated for 30 min at 4°C with 1 ml packed volume of glutathione–Sephadex beads (Pharmacia), pre-equilibrated with GSB. The beads were collected by centrifugation at 1000g for 30 sec and washed six times in 10 ml of GST wash buffer (GWB=GSB without glycerol), including a 5-min incubation with agitation during the second and third washes. Protein was eluted by washing the beads three times in a total of 2 ml of GWB plus 50 mM glutathione (reduced form), incubating 5–10 min at 4°C during each elution. The eluate was dialyzed overnight against 3 × 2 liters of 1.1× dialysis buffer (1.1× DB: 27.5 mM K⁺ HEPES at pH 7.5, 55 mM KCl, 0.11 mM EDTA, 0.11% NP-40), with 0.2 mM PMSF included in the first 2 liters. The dialyzed protein was recovered, one-tenth volume of glycerol was added, and aliquots were frozen in liquid nitrogen and stored at –80°C. The yield of full-length fusion protein was estimated by separating samples on polyacrylamide gels and comparing the Coomassie staining intensity of the appropriate band to known amounts of bovine serum albumin standard.

For the restriction fragment precipitation experiments, fusion protein production differed from the above protocol in that protein induction was done at 37°C, cells were lysed by freeze/thawing in the presence of 1% Triton X-100, 5 mM MgCl₂ was included in the buffers, the KCl concentration was only 20 mM during lysis, only the HEPES, DTT, and 10 mM glutathione were present during the elution, and NP-40 was not used in any step. For the DMS protection experiments, the purification protocol differed from the one detailed above in that 12.5 mM MgCl₂ was present during sonication, only the HEPES, DTT, and 10 mM glutathione were present during the elution, 5 mM MgCl₂ was present in the dialysis buffer, and NP-40 was not present in the dialysis buffer.

For in vitro synthesis of htr protein with wheat germ translation lysates, the *h* cDNA was cut with *Xmn*I, and RNA and protein were synthesized as described previously (Van Doren et al. 1991).

Precipitation of labeled restriction fragments with G–htr

A 0.9-kb *Eco*RI–*Eco*47III fragment of *ac* genomic DNA, including 874 bp 5' of the start of transcription, was subcloned into pKS1⁺ (Van Doren et al. 1992). The entire fragment was liberated from this subclone by digestion with *Pst*I and *Xba*I, gel purified, and digested with *Hae*III. The resulting fragments were dephosphorylated with calf intestinal alkaline phosphatase and then radioactively labeled with [γ -³²P]ATP and T4 polynucleotide kinase. The fragments were incubated in a 15-µl reaction with 1 µg of GST–htr, 1 µg of poly[d(I–C)], and reaction buffer containing 25 mM K⁺ HEPES (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT. After a 30-min incubation at room temperature, 10 µl of packed volume of glutathione–sephadex beads (pre-equilibrated in reaction buffer) was added, followed by an additional 10-min incubation at room temperature. The reaction was chilled on ice, and the beads were washed quickly twice with 0.5 ml of ice-cold reaction buffer. Complexes were eluted from the beads by washing twice with 50 µl of 25 mM K⁺ HEPES (pH 7.5) plus 10 mM glutathione at room temperature. After elution, 5 µl of 10% SDS was added to the eluate, which was then incubated for 5 min at 65°C. The eluate was extracted

with 300 µl of phenol/chloroform (1:1), and the nucleic acid was precipitated by addition of 2 µg of yeast tRNA, 10 µl of 3 M sodium acetate, and 250 µl of ethanol. The pellet was dissolved and electrophoresed on an 8% polyacrylamide/Tris-borate–EDTA (TBE) gel along with a sample of 10% of the input (untreated) labeled *Hae*III fragments.

Methylation protection assays

The 110-bp *Hae*III fragment from the 0.9-kb *ac* upstream fragment was subcloned into the *Sma*I site of pKS1⁺. An *Eco*RI–*Xba*I fragment containing the entire 110-bp sequence was excised, as was an *Ava*II–*Sac*I fragment that contains approximately one-half of this sequence. When these fragments are radioactively labeled with [α -³²P]dCTP and Klenow DNA polymerase, only one of the two DNA strands incorporates the label, and the two fragments differ as to which strand becomes labeled. htr was cleaved from GST–htr by incubation of 14 µg of GST–htr with 10 units of human thrombin (Sigma) in the presence of 2.5 mM CaCl₂ for 30 min at room temperature. Cleaved htr (1 µg) was combined with 200,000 cpm of labeled DNA and 6 µg of poly[d(I–C)] in a volume of 60 µl, including 25 mM K⁺ HEPES (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA and 6.7% glycerol. After 20 min at room temperature, the reactions were chilled on ice, and 1 µl of DMS was added, followed by an additional 1-min incubation on ice. The reactions were centrifuged for 10 sec and 1 µl of 14.4 M β -mercaptoethanol was added to 55 µl of the supernatant, which was immediately loaded onto a 4% polyacrylamide/0.5× TBE gel and electrophoresed at 7.5 V/cm for 2.5 hr. The gel was exposed to film, and regions of the gel corresponding to free probe and probe complexed with htr were excised and processed for piperidine cleavage of methylated bases and gel electrophoresis as described (Ausubel et al. 1987).

Random binding site selection

A double-stranded random oligonucleotide pool was generated by annealing the random-1 oligonucleotide, 5'-GTAAAACG-ACGGCCAGTGAAGCTTGAATTCNNNNNNNNNNNNNNNNNNNNNGGATCCTCTAGACATGGTCATAGCTGTTTCC, with the M13 reverse sequencing primer, 5'-GGAAACAGCTATGAC-CATG, and extending with Klenow DNA polymerase at 50°C. Glutathione–Sephadex beads containing bound GST–htr protein were prepared as described above, but the protein was not eluted. Instead, 200 µl of packed bead volume was used to make a column, which was blocked with 250 µg of sheared salmon sperm DNA and 20 µg of poly[d(A–T)] in 150 µl of HEGN 100 (25 mM K⁺ HEPES at pH 7.5, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 100 mM KCl; in subsequent steps, various KCl concentrations were employed, as indicated by the number, in millimolarity, following HEGN). The column was washed 2× in HEGN 500, 2× in HEGN 1000, and 3× in HEGN 100 (0.5-ml washes), and loaded with 2 µg of double-stranded random-1, 5 µg of poly[d(A–T)], and ~10⁵ cpm radiolabeled *ac* h/E-1 oligonucleotide probe (below) in a volume of 50 µl and final buffer concentration of 1× DB. The labeled *ac* h/E-1 probe was used to monitor column performance but does not contain the restriction sites necessary for the subsequent cloning steps. After incubating for 40 min at room temperature, the column was washed with 10 column volumes of HEGN 400 and 10 column volumes of HEGN 500, and was then eluted with 250-µl fractions of HEGN 800. The second HEGN 800 fraction contained a majority of the input *ac* h/E-1 oligonucleotide and was precipitated by addition of 144 µl of dH₂O, 2 µl of 1 M MgCl₂, 4 µg of yeast tRNA, and 1 ml of ethanol. After recovery, the eluted

DNA was used to load a second GST-htr column as above, which was washed with 10 column volumes of HEGN 400, 5 column volumes of HEGN 500, and eluted with 250- μ l fractions of HEGN 800. Again, the second HEGN 800 fraction contained the majority of the *ac* h/E-1 oligonucleotide and was precipitated as above. By taking a single column fraction in each of the two rounds of purification, 38% of the input *ac* h/E-1 oligonucleotide was recovered. The eluted DNA was then digested with *Hind*III and *Xba*I, and subcloned into pKS1⁺. Plasmids with correct random-1 insertions were then sequenced as double-stranded DNA. In the aligned sequences (Fig. 2A), bases derived from nonrandom portions of the random-1 oligonucleotide were not included as part of the final analysis.

EMSA

An *ac* h/E-1 probe was generated by annealing the following oligonucleotides: 5'-GGCAGCCGGC**ACGCGACAGGG** and 5'-CCCCCTGTCGCGTGCCGGCTG, where the region protected from methylation by GST-htr is indicated in bold. The resulting double-stranded oligonucleotide was radiolabeled by extension of the overhanging ends with [α -³²P]dCTP and Klenow DNA polymerase. Mutant competitor probes were generated by annealing oligonucleotides that are identical to the ones above except for the single base pair changes indicated in Figure 2B. The DNA-binding reactions contained, in addition to protein (~40 fmoles for the competition assays), 40 fmoles of labeled *ac* h/E-1 probe, 100 ng of poly[d(A-T)], 7% glycerol, and 1 $\times$ DB (final concentration) in a volume of 10 μ l. After a 20-min incubation at room temperature, 8 μ l of each reaction was loaded on a 4% polyacrylamide/0.5 $\times$ TBE gel and electrophoresed at 7.5 V/cm for 2.5 hr. The gel was then dried and exposed to film. For the oligomerization experiment (Fig. 2C), the proteins were preincubated in 1 $\times$ DB plus 10% glycerol in a volume of 7 μ l for 1 hr at room temperature prior to the addition of DNAs and the 20-min binding incubation.

Cloning of the *D. virilis* ac gene

A *D. virilis* genomic DNA library, a kind gift of Ron Blackman (University of Illinois, Urbana), was screened by low-stringency hybridization with a probe derived from a portion of the *D. melanogaster ac* coding sequence that lacked the region encoding the glutamine repeats (J. Esnayra, unpubl.). DNA from a bacteriophage recovered in this screen was subcloned and identified as containing the *D. virilis ac* homolog by comparison of coding region sequence data to the *D. melanogaster ac* sequence.

Cotransfection assays

Drosophila S2 cells were grown in Schneider cell medium (GIBCO) supplemented with 12.5 % fetal calf serum (Gemini), penicillin, and streptomycin.

The *ac* h/E-1 wild-type and mutant target promoters were generated by ligating annealed, double-stranded *ac* h/E-1 oligonucleotides or mutant m2 oligonucleotides with T4 DNA ligase, and gel purifying products containing four copies; the ends of these fragments were then blunted with Klenow DNA polymerase, and they were subcloned into *Sma*I-digested pKS1⁺. After the correct sequence and orientation were verified, these fragments were cloned into pD86 (England et al. 1990) by use of *Pst*I and *Xba*I. Expression constructs were based on the parental vector pAct5CPPA (Han et al. 1989), which expresses inserted DNA by use of the *Drosophila* actin 5C promoter. For *h*, a *h* cDNA was digested with *Xba*I, blunted with Klenow DNA

polymerase, and digested with *Sal*I. This fragment was subcloned into pKS1⁺ digested with *Sma*I and *Sal*I, creating pKSh. pKSh was digested with *Xho*I, partially extended with Klenow DNA polymerase adding only dCTP and dTTP, and digested with *Sac*I. This fragment was cloned into pAct5CPPA that had been digested with *Bgl*III, partially extended with Klenow DNA polymerase by use of only dATP and dGTP, and digested with *Sac*I, creating pACh; this construct is expected to express full-length *h* protein. For *E(spl)m7*, an *Xmn*I-*Bgl*III fragment of *E(spl)m7* genomic DNA was cloned into pAct5CPPA that had been digested with *Bam*HI, blunted with Klenow DNA polymerase, and digested with *Bgl*III, creating pACm7; this construct is expected to express full-length *E(spl)m7* protein.

Transfections, β -galactosidase assays, and CAT assays were carried out as described (Gorman 1985; Van Doren et al. 1992), except that 10⁶ S2 cells were plated the day before transfection, and 0.2 μ g of wild-type or mutant *ac* h/E-1-ADH-CAT DNA, 0.33 μ g of pACh or pACm7 DNA, and a total of 3 μ g of pAct5CPPA DNA (parental plus expression constructs) were used per plate of cells.

Construction, transformation, and analysis of *ac* transgenes

The construction and germ-line transformation of the wild-type *ac* transgene, as well as the generation of many of the plasmids used, has been described previously (Van Doren et al. 1992). PCR was used to generate a *Nae*I-*Bam*HI fragment of the *ac* upstream region that contained a mutated *ac* h/E-1 site (CACGCG $\rightarrow$ CCCTCT). The sequence of the entire fragment was verified and used to replace the wild-type *Nae*I-*Bam*HI fragment from pT5-2.2. The 2.2-kb *Eco*RI fragment from this h/E-1 mutant version of pT5-2.2 was then subcloned into pCaSpeR (Pirrotta 1988) in the same orientation as pCSP52.2. Transgenic flies containing this construct were then generated by standard P element-mediated germ-line transformation techniques (Rubin and Spradling 1982), using *w*¹¹⁸ as the recipient strain.

Adult tissue was dissected and mounted for light microscopy in Hoyer's medium.

Acknowledgments

We thank Ron Blackman for providing the *D. virilis* genomic DNA library, Andy Singson, Bruce England, Dave Kosman, and Steve Small for providing plasmid DNAs, Scott Erdman and Ken Burtis for providing the random binding site selection protocol prior to publication, Ingrid Ghattas for suggestions on the methylation protection assay, Leslie Kerrigan for suggestions on protein purification, Eben Masari for construction of pGEX-htr, Dahna Pasternak and Pam Nero for technical assistance in cloning the *D. virilis ac* homolog, and Geoff Cereghino, Rick Firtel, Josh Kavaler, Mike Levine, Kees Murre, and Andy Singson for critical reading of the manuscript. We also thank Michael Caudy for discussions and exchange of information prior to publication. This work was supported by National Institutes of Health (NIH) predoctoral training grants and by NIH grant GM46993.

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Genes Dev. 1994, **8**:

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