hairy function as a DNA-binding helix-loop-helix repressor of *Drosophila* sensory organ formation

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Sensory organ formation in *Drosophila* is activated by proneural genes that encode basic-helix-loop-helix (bHLH) transcription factors. These genes are antagonized by hairy and other proline-bHLH proteins. hairy has not been shown to bind to DNA and has been proposed to form inactive heterodimers with proneural activator proteins. Here, we show that hairy does bind to DNA and has novel DNA-binding activity: hairy prefers a noncanonical site, CACGCG, although it also binds to related sites. Mutation of a single CACGCG site in the achaete (ac) proneural gene blocks hairy-mediated repression of ac transcription in cultured *Drosophila* cells. Moreover, the same CACGCG mutation in an ac minigene transformed into *Drosophila* creates ectopic sensory hair organs like those seen in hairy mutants. Together these results indicate that hairy represses sensory organ formation by directly repressing transcription of the ac proneural gene.

[Key Words: Helix-loop-helix; DNA binding; transcription; repression; neurogenesis; *Drosophila*]

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Sensory organs in insects derive from sensory organ precursors (SOPs) that divide to produce both the sensory organ, such as a sensory hair, and the sensory neuron that innervates that organ (for review, see Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1993a). Genetic analysis in *Drosophila* has shown that SOPs are formed in a precise pattern that is controlled by both positive and negative regulatory genes. The positive regulators include daughterless (da), the proneural genes of the achaete-scute complex [AS-C: achaete (ac), scute (sc), lethal of scute (l’sc), asense (ase)] and the proneural gene atonal (for review, see Campuzano and Modolell 1992, Jan and Jan 1993b). Recessive loss-of-function mutations in da lead to the loss of all SOPs and the sense organs (SOs) to which they give rise, whereas loss-of-function mutations in atonal and AS-C genes result in the loss of specific subsets of SOPs and SOs. In contrast, dominant gain-of-function mutations in the AS-C genes result in the formation of ectopic SOPs and SOs. Together, these dominant and recessive phenotypes indicate that the proneural genes act as genetic “switches” for activating SOP formation during development (Campuzano and Modolell 1992, Jan and Jan 1993b).

Negative regulation of proneural gene expression, and therefore of SOP and SO formation, occurs at two distinct levels. AS-C genes are initially expressed in proneural clusters in which many or all cells are competent to become an SOP (Ghysen and Dambly-Chaudiere 1989, Romani et al. 1989). Genes such as hairy [gene; hairy protein] and extramacrochaetae (emc) control the pattern in which proneural clusters form (Cubas et al. 1991; Skeath and Carroll 1991). That pattern is subsequently refined by the restriction of proneural gene expression to the single cell that will become the SOP. This restriction depends on the neurogenic genes such as Notch, Delta, and Enhancer of split [E(spl)], which mediate lateral inhibition of SOPs by cell–cell interactions (Artavanis-Tsakonas and Simpson 1991; Campos-Ortega 1993). In neurogenic mutants proneural clusters form at the normal positions but multiple SOPs and SOs are formed within each cluster, whereas in hairy and emc mutants proneural clusters form at ectopic positions but only one or a few SOPs and SOs are formed within each cluster.

Molecular analysis has shown that the majority of the activator and repressor genes that regulate SOP formation encode helix-loop-helix (HLH) transcription factors (Jan and Jan 1993b). The HLH domain is a dimerization domain [Murre et al. 1989a], and many HLH proteins contain an adjacent basic region that mediates DNA binding. Heterodimer formation between basic–HLH [bHLH] proteins is an important mechanism for regulating DNA-binding activity of bHLH proteins (for review, see Jones 1990). For example, the AS-C proteins do not bind DNA as homodimers, but da/AS-C heterodimers bind with high affinity to specific "E-box" sequences present in the promoter of the ac proneural gene, and heterodimer binding to these sites activates ac transcription.
Heterodimer formation is also an important mechanism for mediating repression by HLH proteins. The emc repressor protein also contains an HLH domain, but it lacks a basic region and, therefore, cannot bind to DNA. emc protein forms non-DNA-binding heterodimers with the da and AS-C activator proteins, thereby repressing their DNA-binding activity in vitro and repressing ac transcription activation by da/AS-C heterodimers in vivo [Van Doren et al. 1991, 1992]. In contrast with emc, the proline–bHLH repressor proteins all have basic regions, but unlike other bHLH proteins they each contain a proline in the basic region. The proline has been proposed to disrupt the DNA-binding activity of the basic region so that these proteins would function biochemically like emc and its mammalian homolog, Id [Benezra et al. 1990; Jones 1990]. However, at least some proline–bHLH proteins can bind to DNA: The E(spl) m8 protein and mammalian HES-1 protein have been shown to bind to noncanonical sequences (N boxes: CACGGAG and CACAAG) that are present in the m8 promoter region [Sasai et al. 1992; Tietze et al. 1992].

hairy has not been shown previously to bind to DNA, and several models of hairy repression invoke heterodimer formation between hairy and other bHLH proteins. Genetic analysis has shown that hairy specifically represses the activity of the ac gene in vivo [Moscoso del Prado and Garcia-Bellido 1984]. One model for this repression is that hairy forms inactive heterodimers with specific AS-C target proteins such as ac. These inactive heterodimers may be unable to bind to DNA, or they may bind DNA but be transcriptionally inactive [Jones 1990; Parkhurst et al. 1990]. hairy also functions in segmentation as a repressor of fushi tarazu [ftz], and one model for this repressor function is that hairy forms DNA-binding heterodimers with a bHLH corepressor protein [Wainwright and Ish-Horowicz 1992].

In this study we combine biochemical and genetic approaches to directly determine whether hairy is a DNA-binding protein and the mechanism by which it acts to repress SO formation. Our analysis has been guided by previous structure/function analyses of bHLH proteins, which have shown that many bHLH proteins can be divided into two classes, depending on whether they bind to class A sites or class B sites [see Fig. 1; Dang et al. 1992]. Although both class A and class B sites fit the canonical HLH-binding sequence, CACGTG, class A proteins do not bind to class B sites, and vice versa. These differences in DNA-binding specificity result from differences in the amino acid sequence of the basic region [Dang et al. 1992; Blackwell et al. 1993].

Here, we show that the Drosophila activator and proline–bHLH repressor proteins bind to class A and class B sites, respectively. In addition, although hairy binds the class B canonical sites, it prefers a noncanonical site, CACGGC, and also binds other noncanonical sites. These noncanonical sites are closely related to class B sites and are defined as class C sites. We show that the same sites are recognized by other proline–bHLH proteins, and we define the structural features of sites recognized by that family of proteins. We then show that a CACGGC class C site present in the promoter of the ac proneural gene mediates repression of ac by hairy in vivo: Mutation of that site blocks repression of ac transcription by hairy in cultured cells. Moreover, in Drosophila the same ac promoter mutation creates ectopic sensory hairs like those seen in hairy mutants. These results indicate that hairy represses sensory organ formation by binding to a specific repressor site in ac and directly repressing transcription of the ac proneural gene.

Results

hairy has the key structural feature of a class B protein and binds to class B sites

Figure 1 shows an alignment of the basic regions of several class A and class B proteins with those of the da and AS-C activator proteins and the proline–bHLH repressor proteins. Previous analysis has shown that the Arg residue at position 13 (R13) present in all class B proteins is the key structural criterion for defining class B-binding specificity to the class B basic regions [see Fig. 1 legend; Dang et al. 1992; Van Antwerp et al. 1992; Blackwell et al. 1993]. As shown in Figure 1, hairy and all of the other proline–bHLH proteins have R13 residues in their basic domains. This led us to predict that hairy would bind to class B sites but not to class A sites. Similarly, the lack of R13 in the da and AS-C proteins suggested that they would not bind to class B sites. Figure 2A shows that da/l'sc and da/sc heterodimers and da/da homodimers bind to the class A site CACCTG [Fig. 2A]. However, those protein complexes do not bind to the class B CACGTG site. Furthermore, binding of those complexes to the labeled class A site is competed by an excess of cold [unlabeled] A site, but it is not competed by an equivalent 1000-fold excess of cold B site. Thus, the da and AS-C proneural proteins bind with high specificity to class A sites but not to class B sites, as predicted.

In contrast, Figure 2B shows that both full-length hairy protein and a truncated, bHLH version of hairy [containing only the bHLH domain] bind to the class B site, CACGTG. However, neither full-length nor bHLH hairy binds to the class A site. Moreover, binding by hairy to the class B site is not competed by a 1000-fold excess of class A sites, showing that hairy also is highly specific for the class B site. The class B protein Max shows the same general pattern of binding as hairy: Max binds to the class B site with high affinity, although it does cross-react somewhat with the class A site.

We next used polymerase chain reaction [PCR] site selection [Blackwell and Weintraub 1990; Sun and Baltimore 1991] to determine whether hairy would select
fined class A proteins, and Myc, Max, USF, TFE3, and TFEB are recognized by those proteins. AP-4 (Dang et al. 1992) and MyoD (Van Antwerp et al. 1992, Blackwell et al. 1993) are previously defined class A proteins, and Myc, Max, USF, TFE3, and TFEB are previously defined class B proteins (Dang et al. 1992). The classes are operationally defined in that class A proteins bind to class A sites but not to class B sites, etc. da/AS-C heterodimers can bind to sites that fit the class A consensus (Murre et al. 1989; Delidakis and Artavanis-Tsakonas 1992; Knust et al. 1992), HES-1 (Sasai et al. 1992), and HES-5 (Akazawa et al. 1992, Sasai et al. 1992) has shown that those proteins can bind to a DNA sequence CCACGAGCGACAGG present in the m8 promoter. This sequence contains two related hexamer sequences, CACGAG and CACAAG, that are called N-box sites (Sasai et al. 1992). The N boxes do not fit the HLH canonical sequence but are closely related to it. Gel retardation analysis has shown that hairy also can bind to the m8 double hexamer sequence (data not shown). Therefore, we wished to determine whether hairy would select the N-box hexamer sequences from CNNNNNG oligonucleotides (Fig. 3A).

Surprisingly, hairy preferentially selected neither class B nor N-box sites (Fig. 3A). Rather, it preferentially selected a different noncanonical sequence, CACGC, with the highest frequency (22/35). The class B CACGTG site was also selected with high frequency (9/35), and the CACGAG N-box site was selected with lower frequency (2/35). Given that hairy selects noncanonical sites, we asked whether the class B protein Max was also capable of selecting noncanonical sites. In contrast with hairy, Max exclusively selected the class B canonical sites, CACGTG and CATGTG (Fig. 3B). Like hairy, Max always selected sites with a G nucleotide at position 4, as predicted by the R13 residue present in both class B and proline−bHLH proteins. However, Max showed distinctly different selection than hairy at position 5 in the hexamer. Whereas hairy selected sequences with various nucleotides at position 5, Max exclusively selected sequences with T, which therefore are canonical class B sites.

The three classes of sites are closely related to one another, and in some cases, sites of distinct classes differ by only a single nucleotide within the core hexamer. The three classes are

| Class A sites: | CACCTG | CAGCTG |
|---------------|--------|--------|
| Class B sites: | CACGTG | CATGTG |
| Class C sites: | CACGCC | CACGAG |

where the CACGCC and CACGAG class C sites shown are the two noncanonical sites selected most frequently by hairy (Fig. 3A). These sites differ from the class B CACGTG site only by a single nucleotide change at position 5.

Figure 1. Basic region similarities between class A, class B, and proline−bHLH proteins, and the class A and class B sites recognized by those proteins. AP-4 (Dang et al. 1992) and MyoD (Van Antwerp et al. 1992, Blackwell et al. 1993) are previously defined class A proteins, and Myc, Max, USF, TFE3, and TFEB are previously defined class B proteins (Dang et al. 1992). The classes are operationally defined in that class A proteins bind to class A sites but not to class B sites, etc. da/AS-C heterodimers can bind to sites that fit the class A consensus (Murre et al. 1989b; Cabrera and Alonso 1991; Van Doren et al. 1991), and their basic regions are similar to those of MyoD and AP-4. The proline−bHLH proteins have been aligned in a third group. The P [Pro] residues at position 6 in the proline−bHLH proteins, which is the defining feature of that class of bHLH proteins, are boxed. The R at position 13 (R13) in the class B and proline−bHLH proteins also is boxed. Basic amino acids R [Arg] and K (Lys) are shaded. Crystal structure analysis of protein−DNA octamers of the class B bHLH protein Max has shown that the R13 in Max makes direct contact with the G nucleotide at position 4 (G4) in the class B site, CACGTG (Ferre-D’Amare et al. 1993). The G4 nucleotide is the distinguishing feature of class B sites (Fig. 1, bottom), and by binding to the G4 nucleotide the R13 amino acid provides the key specificity determinant for class B bHLH proteins. The presence of an R13 residue in all of the proline−bHLH proteins suggests that they will bind to class B sites. The sequences are taken from AP-4 (Hu et al. 1990), MyoD (Davis et al. 1987), da (Caudy et al. 1988), ac, sc (Villares and Cabrera 1987), lsc, ase (Alonso and Cabrera 1988, Gonzalez et al. 1989), c−, N−, and L-Myc (Batterly et al. 1988; Kohl et al. 1986; DePinho et al. 1987), Myn/Max (Blackwood and Eisenman 1991, Prendergast et al. 1991), USF (Gregor et al. 1990), TFE3 (Beckmann et al. 1990), TFEB (Carr and Sharp 1990), hairy (Rushlow et al. 1989, E(spl) m8, m5, m7, m3 [Klamp et al. 1989; Delidakis and Arvanitis-Tsakonas 1992, Knust et al. 1992], HES-1 (Sasai et al. 1992), and HES-5 (Akazawa et al. 1992).
We then performed gel retardation assays to further examine the relative affinities of hairy and Max for the class B [CACCTG] and class C [CACGCG] sites. Figure 3C shows that Max binds very strongly to the class B site but only weakly to the class C site. In addition, a 1000-fold excess of class C site cannot compete the class B site away from Max. In contrast, full-length hairy and bHLH hairy bind to both of the class B and class C sites, and binding to the class B site can be competed by an excess of either class B or class C site.

Thus, PCR site selection and gel retardation assays both show that the class B protein Max does not have the same sequence specificity as the proline–bHLH protein hairy. Rather, hairy appears to be a class B protein with an expanded repertoire of binding sites, which include the class C CACGCG and the CACGAG sequences, and possibly other related noncanonical sites.

Another proline–bHLH protein, E(spl) m5, also binds to class B and class C sites in vitro

We wished to determine whether other proline–bHLH proteins also recognize the class B and class C sites. As shown in Figure 1, the hairy and E(spl) m5 proteins represent relatively divergent members of the proline–bHLH family. Together, they span the range of sequence variations seen within the basic regions of proline–bHLH proteins. Figure 4 shows that m5 binds specifically to the class B and class C sites but not to the class A site, and binding to the class B and C sites cannot be...
hairy represses transcription of the achaete gene through a CACGCG site in the achaete promoter

Having identified the general set of binding sites recognized by hairy in vitro, we asked whether hairy binds to those sites to repress the ac proneural gene in vivo. Previous analysis of the ac promoter region has shown that there is a cluster of three sites to which da and AS-C heterodimer complexes bind to activate ac transcription in vivo [Fig. 5A; Van Doren et al. 1991, 1992; Martinez et al. 1993]. These are sites that we now recognize as class A sites. Having identified CACGCG as a high-affinity binding site for hairy in vitro, we observed that there is a CACGCG site ~50 bp upstream of the class A cluster in the ac promoter [Fig. 5A]. Previous work using cultured Drosophila cells has shown that cotransfection of an ac reporter gene with da and AS-C expression constructs results in activation of ac transcription by da/AS-C heterodimers, but not if the class A sites are mutated [Van Doren et al. 1992]. Therefore, we asked whether this activation of ac could be repressed by simultaneous transfection with a hairy expression construct, and if so, could that repression be negated by mutation of the distal CACGCG site. Figure 5B shows that cotransfection of da and sc expression constructs activates ac transcription but that additional cotransfection of a hairy expression construct represses that activation. Repression also was produced by cotransfection with an emc expression construct, as has been shown previously [Van Doren et al. 1992]. emc forms non-DNA-binding heterodimers with the da and/or AS-C proteins, thereby repressing their ability to bind and activate ac.

If the CACGCG site is mutated [Fig. 5C], da and AS-C still activate ac transcription to the same level, and addition of emc still can repress that activation. However, hairy no longer represses ac transcription. Thus, hairy requires the CACGCG site for repression. Given that this is the preferred binding site for hairy in vitro and that the presence of the binding site does not mediate
hairs along the L2 wing vein (Fig. 6, top) or other non-margin veins. In hairy \(^4\) mutants there are many ectopic hairs on the L2 vein (Fig. 6, middle). Figure 6, bottom, shows that a similar phenotype is produced in transformants in which the single CACGCG site present in the ac promoter region has been mutated. In 10 of 13 homozygous transformant lines generated with a mutated CACGCG site there were large numbers of ectopic hairs on the wings and body and these hairs were in similar

hairy represses sensory organ formation
in Drosophila through the CACGCG site
in the achaete promoter

To test for hairy binding to the CACGCG site in Drosophila, we mutated the site in an ac minigene and introduced it into Drosophila by P-element transformation (Rubin and Spradling 1982). Previous work has shown that an ac minigene can rescue many of the sensory hairs that are missing in achaete null \(\text{ac}^-\) mutants (G. Panganiban and S. Carroll, unpubl.). Therefore, this provides a favorable in vivo system for directly testing the function of the CACGCG-binding site in the repression of sensory organ formation, not just in the repression of ac transcription.

Analysis of adult wings from transformants revealed that disruption of the CACGCG-binding site resulted in the presence of ectopic hairs [bristles] along the veins of the wing [Fig. 6]. In wild-type flies there are no sensory

Figure 4. Binding by E(spl) m5 to class B site and class C sites. m5 protein binds to the class B and the class C sites but not to the class A site. m5 is competed away from the class B site and partially competed from the class C site by an excess of class C DNA but not by an excess of class A DNA. \(\text{m5} 300\text{ ng of full-length m5 protein was used per reaction in this experiment.}

Figure 5. hairy-mediated repression of achaete transcription via a CACGCG-binding site in the ac promoter region. (A) Schematic representation of the ac proximal promoter region [Vil-lares and Cabrera 1987]. A cluster of three class A sites is upstream of the TATA box in the ac promoter. A CACGCG [class C] hairy-binding site is located immediately upstream of the three class A sites. (B) Effect of hairy and emc on the transcriptional activation of the native ac promoter by da/AS-C activator proteins. Cotransfection of Drosophila Schneider cells with da and sc expression constructs results in activation of ac transcription. Additional cotransfection with either hairy or emc results in repression of ac transcription. The reporter construct contains 0.9 kb of the wild-type ac 5' promoter fused to a luciferase reporter gene [see Materials and methods]. Values are the mean of at least four samples and error bars represent the standard error. (C) Effect of mutating the CACGCG site on repression of ac transcription by hairy and emc. Mutation of the CACGCG-binding site does not affect activation by da/sc proteins. In addition, emc still represses ac transcription by da/sc activator complexes in the absence of the CACGCG site. However, in contrast, hairy does not repress in the absence of the site. Thus, the CACGCG site is essential for hairy-mediated repression of ac transcription in cultured Drosophila cells.
DNA binding by hairy HLH repressor protein

Figure 6. Derepression of sensory hair formation in Droso-
phila by mutation of the CACGCG site in an ac minigene. (Top) Wild-type wings have sensory hairs (bristles) only along the wing margins; the L2 veins and other nonmargin veins (not shown) are normally completely devoid of hairs. (Middle) In hairy mutants loss of hairy repressor function results in the formation of ectopic sensory hairs along the L2 veins and other veins. Previous work has shown that ac expression along L2 is derepressed in hairy mutants [Skeath and Carroll 1991] and that hairy functions genetically as a specific repressor of the ac proneural gene [Moscoso del Prado and Garcia-Bellido 1984]. (Bottom) Disruption of the CACGCG site in an ac minigene results in the formation of ectopic sensory hairs along the L2 vein. This line [M4] has an intermediate phenotype relative to the strongest transformant line [M1, see Table 1] but it is comparable to the phenotype of hairy mutants. In contrast, none of the control lines had ectopic hairs on L2, and the controls at most had two hairs on L5 [Table 1]. The wing photographs are 175× magnifications of the anterior wing margin and the L2 vein.

positions as in hairy mutants [Table 1A]. In contrast, the control lines were essentially wild type, with no more than two hairs on any wing (one or less, on average). Similar ectopic hair phenotypes were seen in both the presence and absence of the endogenous, wild-type ac gene [Table 1B], indicating that the ectopic hairs result from derepression of the ac minigene, not from an increase in overall expression of ac product in the presence of additional copies. Similar phenotypes were also seen in hairy mutants [data not shown], indicating that hairy represses ac primarily by acting through the CACGCG site. Both mutant and control transformants rescued most of the hairs on the notum that are missing in ac- mutant flies; thus, mutation of the CACGCG site does not affect activation of the ac minigene [data not shown].

The phenotype observed in the CACGCG- mutants further mimics the hairy phenotype in that ectopic hairs also are present at other locations like those seen in hairy mutants, for example, on the mesopleurae and head [data not shown]. This phenotype is distinct from that seen in clones deficient for neurogenic genes such as E(spl), in which clusters of hairs are seen at the normal positions [P. Heitzler and P. Simpson, pers. comm.] rather than single hairs at many ectopic positions, as in hairy. Thus, it appears that the ectopic sensory hairs created in the ac binding site mutants results from the lack of hairy protein binding to that site rather than the lack of E(spl) protein binding to the ac promoter.

Discussion

Mechanism of action of hairy as a proline–bHLH repressor of sensory organ formation

We have combined biochemical and genetic analyses to determine the mechanism by which the proline–bHLH protein hairy specifically represses the ac proneural gene. We have shown that despite the presence of the proline residue in the basic region, hairy is a sequence-specific DNA-binding protein with novel DNA-binding specificity. hairy binds as a homomer to class B canonical HLH sites, as predicted by the presence of the key amino acid residue for class B binding in its basic region [Fig. 1]. However, unlike the class B protein Max, hairy binds preferentially to a site, CACGCG, that does not fit the HLH canonical sequence [Fig. 3]. Determination of hairy-binding sites in vitro has allowed us to identify a single CACGCG site present in the ac promoter region [Fig. 5A]. In a cell culture assay, hairy represses transcriptional activation of the native ac promoter by da/AS-C proteins. However, mutation of the CACGCG promoter site results in blocking repression of ac transcription by hairy, although repression by emc is not affected. Thus, the CACGCG site is essential for repression of ac by hairy. Moreover, mutating that site in a ac minigene transformed into Drosophila creates ectopic sensory organs like those seen in hairy mutants [Fig. 6].

The above results provide strong evidence that hairy represses sensory organ formation by directly repressing transcription of the ac proneural gene, and similar results have been found by another group [van Doren et al.
M1, M2, etc., are transformant lines with the CACGCG site mutated; C1, C2, etc., are control lines with identical ac mini-genes, except that the CACGCG site has not been mutated. The numbers given under the L2, L5, and TOT headings are the genes, except that the CACGCG site has not been mutated. The transformant lines rescued most of the hairs missing from the notum and ac and/or a repressor-binding site, and/or a region very sensitive to low levels of ectopic ac protein expression.

Table 1. Ectopic hair formation in achaete transformants with hairy-binding site mutations

| A | L2 | L5 | TOT | wild-type | M1 | 48 | 27 | 135 | C1 | 0 | 1 | 1 |
| M2 | 31 | 27 | 96 | C2 | 0 | 1 | 1 |
| M3 | 36 | 23 | 90 | C3 | 0.8 | 0.8 |
| M4 | 28 | 23 | 86 | C4 | 0.7 | 0.7 |
| M5 | 50 | 20 | 68 | C5 | 0.7 | 0.7 |
| M6 | 25 | 18 | 65 | C6 | 0.6 | 0.6 |
| M7 | 25 | 20 | 65 | C7 | 0.5 | 0.5 |
| M8 | 24 | 17 | 62 | C8 | 0.3 | 0.3 |
| M9 | 13 | 13 | 60 | C9 | 0.3 | 0.3 |
| M10 | 19 | 17 | 55 | C10 | 0 | 0.2 | 0.2 |
| M11 | 0 | 4.6 | 5 | C11 | 0 | 0 |
| M12 | 0 | 0 | 0 | C12 | 0 | 0 |
| M13 | 0 | 0 | 0 | C13 | 0 | 0 |

| B | ac- (Df[ac]) | ac+ |
| females | males | females | males |
| L2 | L5 | TOT | L2 | L5 | TOT | L2 | L5 | TOT | L2 | L5 | TOT |
| M1 | 53 | 30 | 140 | 48 | 35 | 155 | 59 | 32 | 145 | 48 | 27 | 135 |
| M4 | 26 | 25 | 86 | 26 | 23 | 86 | 28 | 21 | 75 | 28 | 23 | 87 |
| M3 | 43 | 31 | 74 | 93 | 29 | 122 | 78 | 38 | 124 | 36 | 23 | 90 |

C1 0 1 0.7 0 0 0.4 0 0.3 0 1 1
C5 0 0 0 0 0 1 0.8 0 1.0 0 1 0.7
C4 0 0 0.2 0 0 0.2 0 0 1 0 0 1 0.7

Figure 7. Model for active repression of the achaete proneural gene by hairy. Hairy protein binds to the CACGCG class C site that lies distal to the three class A sites that are bound by da/AS-C heterodimers. Binding of the activator proteins to the class A sites facilitates [arrows] the activation of transcription of the ac gene. Hairy does not bind to the class A sites [Fig. 3] and therefore does not passively repress by competing with activator proteins for those sites. Rather, hairy bound to the distally located CACGCG repressor-binding site, and mutation of that site results to derepression of ac both in Schneider cells and in Drosophila. This indicates that hairy functions as an “active repressor” [Fig. 7], that interferes with ac transcription presumably through an activator-binding site in vitro. This region appears to be a “hot spot” for expression of the truncated ac minigene present in the transposon, and/or a region very sensitive to low levels of ectopic ac protein expression.
intrinsic repression domain [Jaynes and O’Farrell 1991; Cowell 1994]. Although an intrinsic repression domain has not been identified in hairy, it does have a polyglutamine/polyalanine domain similar to the repression domains observed in other active repressor proteins [Cowell 1994]. hairy also has a carboxy-terminal WRPW amino acid domain that is present in all of the proline–bHLH repressor proteins and may function as a repression domain.

Our results do not exclude the possibility that hairy, or other proline–bHLH proteins, may form non-DNA-binding heterodimers with da or AS-C proteins in other contexts. There is evidence that the mammalian HES-1 proline–bHLH protein can repress transcriptional activation of an artificial target gene in cultured mammalian cells both by binding to N-box sequences and by directly interfering with activator protein function [Sasai et al. 1992], and similar repression events may occur in Drosophila. In addition, although our results clearly show that hairy can bind to DNA as a homomer in vitro, we cannot exclude the possibility that hairy binds as a heterodimer with other bHLH proteins in Schneider cells or in Drosophila.

The proline–bHLH proteins form a third class of bHLH proteins

Our results extend the class A versus class B scheme proposed by Dang and co-workers [Dang et al. 1992] to include a third class, class C, which are the proline–bHLH proteins. These proteins have distinct basic region sequences and other distinct domains not found in the class A and class B proteins. None of the class A or class B proteins have the WRPW amino acid domains that are present in all proline–bHLH proteins. In addition, whereas all class B proteins have a leucine zipper dimerization domain adjacent to their bHLH domain, none of the proline–bHLH proteins have a leucine zipper. Thus, the class C proteins are structurally distinct overall from the class A and class B proteins.

The class C basic regions have DNA-binding properties that are clearly distinct from the class A basic regions. The class C repressor proteins bind to both class B and class C sites as we have shown for hairy and the E(spl) m5 protein. The binding to class B sites is presumably attributable to the presence of the R13 residue in all class C proteins, as R13 residues in class B proteins directly contact the G4 nucleotide that distinguishes class B sites from class A sites [Ferre-D’Amare et al. 1993]. All of the class C sites also contain a G4 nucleotide, but they vary from the class B sites at position 5 in the core hexamer. Class C sites include the CACGAG N-box site that is recognized by the E(spl) m8 protein [Tietze et al. 1992] and by mammalian HES homologs of the hairy and E[spl] proteins [Akazawa et al. 1992; Sasai et al. 1992]. Together, the results for the hairy, m5, m8, and HES proteins indicate that many or all proline–bHLH proteins will bind to class B and class C sites.

The class C basic regions are clearly distinct from the class B regions, and this may reflect different DNA-binding properties. Another group has found that Myc–Max heterodimers occasionally (2/59) select CACGCG sites in PCR site selection assays [Blackwell et al. 1993]. However, in our studies, Max exclusively selected class B sites under the same conditions in which hairy selected both class B and class C sites. Fusion of the hairy basic region onto the Max HLH–zipper domain produces a chimeric protein that binds as well as a homodimer to class C sites [T. White and M. Caudy, unpubl.], confirming that some feature of the hairy basic region confers high-affinity binding to class C sites.

Structure/function relationships within bHLH protein classes

Our results suggest that precise structure/function relationships may hold for the various classes of bHLH proteins. For the Drosophila proline–bHLH proteins and class A proteins, there are distinct biological functions associated with the different protein classes. The hairy and the E(spl) proline–bHLH proteins all function as repressors of sensory precursor formation during nervous system development. In contrast, the class A da and AS-C proteins clearly function genetically as activators of neuronal [sensory] precursor formation.

In general, class A proteins tend to function as activators of cell determination pathways in both vertebrates and invertebrates. In both Drosophila and mammals, neuronal cell determination pathways are mediated by the proneural da and AS-C bHLH genes or their homologs in other systems [Campos-Ortega 1993; Guillemot et al. 1993; Jan and Jan 1993b]. Muscle cell determination is regulated by another set of class A bHLH proteins, the myogenic bHLH factors that include MyoD, myogenin, MRF4, and myf-5 [Weintraub et al. 1991]. Like the AS-C proteins and homologs, the myogenic class A proteins bind to DNA as heterodimers with the multifunctional E12 and E47 class A proteins, which are homologs of da [Brennan and Olson 1990; Lassar et al. 1991].

The class B proteins are the least understood in terms of biological function. Although it appears that the Myc family is involved in apoptosis, cell proliferation control and/or repression of cell differentiation [Marcu et al. 1992], it seems unlikely that all of the class B proteins have similar functions during normal development. What does seem clear, though, from the biochemical analysis of the class B proteins is that they are likely to be competing for binding to the same DNA sites in target genes, and thus interacting at some level in vivo.

The classification of bHLH proteins as class A, class B, and class C proteins provides a useful framework for analyzing and understanding how those proteins function in gene regulation. Although this may be an oversimplification and some exceptions will likely be found, the utility of this framework lies in its predictive power, such as the ability to successfully predict that the proline–bHLH proteins would bind to class B sites because they have R13 residues. As illustrated here for hairy, the detailed knowledge of the binding sites recognized by
these various classes of proteins is useful in identifying
candidate in vivo targets. By combining the biochemical
analysis of hairy in vitro with molecular genetic analysis in vivo, we have
been able to identify a site present in the ac proneural
gene through which hairy acts as a specific repressor of
ac transcription and thereby represses sensory organ forma-
tion in Drosophila.

Materials and methods

Drosophila stocks

Flies were raised on the standard yeast/commene/molasses/
agar medium. The w-, yw3sc8 strain is described in Lindsley
and Zimm (1992) and is used here as an ac background for P
element-mediated transformation.

Plasmid constructions

Nomenclature for plasmids is as follows: T3 = 1sc, T4 = sc, and
T5 = ac. da, T3, T4, h, bhlH, and m5 bacterial expression vec-
tors were constructed from pET14b (Novagen) that has a
polyhistidine leader sequence and thrombin cleavage site added to a
conventional pET3 expression construct. pET14b-da: The 2.5-
kb BglII fragment of a da genomic DNA clone was cloned into
BlueScript (BS, Stratagene) and subsequently cloned into the
BamHI site of the pET3a, generating pET3a-da [generously pro-
vided by H. Vaessin, Ohio State University, Columbus]. This
construct encodes a fusion protein that contains the pET gene
10 leader sequence, 12 amino acids encoded by the BS poly-
linker, and 4 amino acids encoded by the 5' noncoding region of
da fused to the full 710-amino-acid native da protein. The
pET3a-da Ndel–EcoRI fragment was then transferred into
pET14b, generating pET14b-da. pET14b-T3: A BglII linker was
added to a PromII site (at nucleotide 43) of a T3 cDNA clone, and
the resulting BglII–BamHI [at nucleotide 631] fragment was
cloned into the BamHI site of the pET3b. The BamHI–EcoRI
fragment of the 3' portion of the T3 cDNA clone was cloned into
the BamHI–EcoRI site of the above pET3b, generating
pET3b-T3 [H. Vaessin]. This contained the region corresponding
to the T3 16–259 amino acids and was transferred into pET14b,
genrating pET14b-T3. T4, PET14b-T4: The PCR fragment (nu-
cleotides 39–754 in T4 cDNA) was cloned into the Smal site in
B5 into which a BamHI linker was added to EcoRV site and the
resultant BamHI fragment was cloned into the BamHI site of the
pET3c, generating pET3c-T4 [H. Vaessin]. This construct
encodes a protein containing the pET gene 10 leader sequence,
an additional 7 amino acids from the BS polylinker, and amino
acids 14–345 of T4. The Ndel–HindIII fragment from pET3c-T4
was shuttled into pET14b to make pET14b-T4. PET14b-h: The
hairy cDNA clone (BSD2E6, generously provided by C.
Rushlow, Columbia University, New York) was digested with
Ndel filled in with Klenow DNA polymerase to kill this site, and
then mutated at a BstEII site at the amino terminus to generate an
Ndel site at the initiation codon of the open reading frame. This construct was then shuttled into pET14b by Ndel–
EcoRI-directed cloning, generating an expression construct for
full-length hairy protein fused to the polyhistidine leader en-
coded by pET14b. pET14b-h BLHL: the region encoding the
BLHL domain of hairy was PCR amplified with primers that
added Ndel and BamHI sites at the 5' and 3' ends, respectively,
which allowed directed cloning into Ndel–BamHI-digested
pET14b to yield pET 14b-h BLHL that expressed a truncated
hairy protein consisting of amino acids 29–96 in which histi-
online was substituted for serine at residue 29. The PCR ampli-
Figur of the above DNA, generating pET14b-T3 and the poly-
linker with 4 amino acids encoded by the 5' noncoding region of
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hairy protein consisting of amino acids 29–96 in which histi-
mm CaCl₂, 0.1% Triton X-100, 10% glycerol containing 4 M urea, then 2 M urea, and then straight buffer. Next, they were cleaved by human thrombin to remove the polyhistidine leader and were dialyzed against 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM DTT, and 1 mM EDTA. The Max bHLH leucine zipper protein was kindly provided by A. Ferre-D’Amare and S. Burley (Ferre-D’Amare et al. 1993). This Max protein is identical to the protein used for X-ray crystallography analysis. Proteins were quantitated with bovine serum albumin (BSA) as the standard, and the quantitations were also confirmed by Coomassie blue staining of the proteins fractionated by SDS-PAGE with protein standards of known concentrations. In general, the proteins purified using the histidine leader were >90% pure, as estimated by Coomassie gel analysis.

Gel retardation assays

In all of the DNA-binding assays shown, highly purified proteins were used. In all cases, the class A site used was the CACCTG hexamer and flanking sequences from the ac promoter region (see Fig. 5A); the class B site used was the CACGTG hexamer and flanking sequences from the upstream stimulatory factor-binding site in the adenovirus major late promoter (Gregor et al. 1990); and the class C site used was the CACC GG site from the ac promoter region (Fig. 5A). The exact sequences of the DNA probes used were CACCTG (CAGGTG on the opposite strand), 5'-GATCGTCACGCAGGTGGGATC- CCTA-3' and 5'-GATCGGATCCACCTGCGTACTAC-3' for the opposite strand for the class A site; and [CACCTG], 5' primer, 5'-GATCGCAGTCACTGTCGCCGC-3' and 5'-AAT- TGCCACGTGCACGG-3' on the opposite strand for the class B site; [CACC GG] C'TAGCCGCAACCAGCAGG-3' and 5'-AATTCCTGTCGCGTGCCGGC-3' on the opposite strand for the class C site. Gel retardation assays were performed as described (Benezza et al. 1990) except that the DNA-binding reaction mixture contained 10 mM DTT instead of 1 mM DTT and 0.3 mg/ml of BSA.

PCR-assisted DNA-binding site selection

PCR-assisted DNA-binding site selection was performed essentially as described previously (Blackwell and Weintraub 1990; Sun and Baltimore 1991). Briefly, 38-bp degenerate oligonucleotides (5'-CTAGTCGTGATCTCTGTCN2ANNTGCAGAAT- TCGAGGG-3') or 36-bp degenerate oligonucleotides (5'-CTAG- TGGATCTCTGTCN2ANNTGCAGAAT- TCGAGGG-3') were converted to double-stranded DNA by Klenow DNA polymerase after priming with the 15-bp 3' primer (5'-CCTCGAAT- TCTTGCC-3'). This product was purified on a 10% acrylamide gel as described above. After three rounds of selection, oligonucleotides were cloned into pT7Blue T-Vector (Novagen). DNA sequences were determined using the AmpliTaq Cycle Sequencing kit (Perkin-Elmer Cetus).

DNA transfection and transient expression assay

DNA transfection into Drosophila Schneider L2 cells (generously provided by J. Colgan and J. Manley, Columbia University, NY) was performed as described previously (Han et al. 1989). Ten micrograms of DNA was used per 60-mm culture dish, and 2 μg of pcDNA LTR-lacZ (J. Colgan and J. Manley) was included as an internal control, 0.5 μg of either the pT5-0.9 wt/luc or pT5-0.9 mut/luc reporter plasmids, 3 μg total of expression vector DNAs (with or without cDNA insert), and 4.5 μg of BS as a carrier DNA. Following 2 days of incubation with the CaPO₄ precipitate, cells were washed with PBS, harvested by scraping with a rubber cell scraper, and lysed in 250 μl of the cell lysis buffer (25 mM Tris-Po₄, pH 7.8, 2 mM EDTA, 20 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity was determined using a Luciferase assay kit (Promega). β-Galactosidase activity was determined as described previously (Han et al. 1989). Luciferase activity was normalized to β-galactosidase activity to control for variations in transfection efficiency.

Genetic analysis of ac transgenic flies

Transgenic flies were generated using standard procedures of microinjection (germ-line transformation) and F element-mediated transposon mobilization (Rubin and Spradling 1982, Laski et al. 1986). A w, y¹pr, e¹[b (ac deficiency) line was used as the recipient strain. Flies heterozygous for the ac minigene–mutant construct [p[T5-M]] show very few ectopic hairs, whereas homozygous flies have a large number of ectopic hairs on the wing and on the mesopleura. Flies heterozygous for the ac minigene–control construct [p[T5-C]] never show any hairs, the homozygous flies show one or two ectopic hairs at a very low frequency [see Results]. Results from genetic crosses indicate that all lines are single insertions. For counts of wing hairs, wings were removed from flies and mounted in 80% glycerol in PBS.

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