

Neuronal type information encoded in the basic-helix–loop–helix domain of proneural genes

(*Drosophila melanogaster*/sensory organ/neuronal type specification/*atonal* gene)

CHENG-TING CHIEN*, CHWAN-DENG HSIAO†, LILY Y. JAN*, AND YUH NUNG JAN*‡

*Howard Hughes Medical Institute and Department of Physiology and Biochemistry, University of California, San Francisco, CA 94143-0724; and †Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan 11529

Contributed by Yuh Nung Jan, August 16, 1996

ABSTRACT The proneural genes encode basic-helix–loop–helix (bHLH) proteins and promote the formation of distinct types of sensory organs. In *Drosophila*, two sets of proneural genes, *atonal* (*ato*) and members of the *achaete–scute* complex (*ASC*), are required for the formation of chordotonal (ch) organs and external sensory (es) organs, respectively. We assayed the production of sensory organs in transgenic flies expressing chimeric genes of *ato* and *scute* (*sc*), a member of *ASC*, and found that the information that specifies ch organs resides in the bHLH domain of *ato*; chimeras containing the b domain of *ato* and the HLH domain of *sc* also induced ch organ formation, but to a lesser extent than those containing the bHLH domain of *ato*. The b domains of *ato* and *sc* differ in seven residues. Mutations of these seven residues in the b domain of *ato* suggest that most or perhaps all of these residues are required for induction of ch organs. None of these seven residues is predicted to contact DNA directly by computer simulation using the structure of the myogenic factor MyoD as a model, implying that interaction of *ato* with other cofactors is likely to be involved in neuronal type specification.

The *Drosophila* peripheral nervous system consists of four major types of sensory organs: chordotonal (ch) organs, external sensory (es) organs, multiple dendritic (md) neurons, and photoreceptors (for review, see ref. 1). Despite apparent differences among these sensory organs, the basic developmental mechanisms for their formation are quite similar. The genesis of sensory organs begins with the expression of proneural genes in small patches of the epidermal cells (proneural clusters) (2–5). This step endows each cell of a proneural cluster with the competence to become a sensory organ precursor (sop) cell. Soon after, cells within a proneural cluster compete with one another; only one or a few cells increase expression of the proneural genes and are fated to become the sop cells (6–8). The neighboring cells receive inhibitory signals from the sop cells, cease expression of the proneural genes, and adopt the epidermal cell fate. The singled-out sop cells divide to give rise to several daughter cells, which then differentiate into neurons and associated support cells of the sensory organs (reviewed in refs. 9 and 10).

Two classes of proneural genes, *atonal* (*ato*) and members of the *achaete–scute* complex (*ASC*), including *achaete* (*ac*) and *scute* (*sc*), are required for the development of almost all the sensory organs in both embryos and imaginal discs. Loss-of-function mutations of *ASC* genes abolish es organs and most of the md neurons (11–13). When overexpressed in imaginal discs, *ASC* genes are able to induce ectopic bristles (one type of es organ) at many locations, including the wings and *nota* (14–19). *ato* is responsible for the formation of ch organs and photoreceptors. In *ato*[−] mutants, ch organs and photoreceptors are almost completely missing (20–22). Overexpression of

ato induces ch organs, as expected from the *ato* mutant phenotype. It also induces bristles, even though no defects in es organs have been observed in *ato* mutants. This might arise from artefactual activation of endogenous *ASC* as a result of overexpression of *ato*.

While mutations in *ato* or *ASC* result in the loss of different subset of sensory organs, removal of the *daughterless* (*da*) gene product deletes the entire embryonic sensory peripheral nervous system. The gene products of *ato* and *ASC* belong to the basic-helix–loop–helix (bHLH) class of transcription factors, as does the product of *da* (20, 23–26). They form heterodimers with *da* and bind to E boxes (20, 27, 28), the cis-acting elements found in the promoters of downstream genes [e.g., *scabrous* and *Enhancer of split* (27)], thereby regulating gene transcription during neurogenesis. While the bHLH domains of the members of *ASC* share about 70% identity (not including the loop region) with one another, they share only 50% with the bHLH domain of *ato*. Outside the bHLH domains, no significant homology between *ato* and *ASC* was found. Given that only *ato* is capable of promoting ectopic ch organ formation, we tested chimeric genes of *ato* and *sc* for their ability to induce ch organs in transgenic flies, to identify structural elements responsible for neuronal specificity conferred by *ato*. On the basis of the structure of the myogenic factor MyoD, we have generated a model for the structure of the DNA-binding domain of the *da/ato* heterodimer, in an attempt to understand the possible roles of these structural elements in the specification of neuronal type.

MATERIALS AND METHODS

Chimeric *ato/sc* Genes and Transgenic Flies. We dissected *ato* and *sc* into three separate domains: b domain, HLH domain, and the rest of the proteins (the boundaries of the domains are according to ref. 19 and see Fig. 3). Oligonucleotides were designed to anneal to the junction of those domains and have proper mutations at the 5′ end of these oligonucleotides [for example, with the *sc-b(ato)* construct, two oligonucleotides, in combination, have sequences on their 5′ ends annealing to the b domain of *ato* and sequences at their 3′ end annealing to the flanking regions from either side of the *sc* b domain]. Each of these oligonucleotides was used with another primer either from most 5′ (carrying an *Asp718* site) or from 3′ (carrying an *XbaI* site) of *ato* or *sc* [for example, *sc* for *sc-b(ato)* construct] for doing the polymerase chain reaction (PCR). The PCR products, containing separate domains for a particular chimeric gene, were ligated into pUAST cut with *Asp718* and *XbaI*. The same method was used for creating point mutations in the b domain of *ato* or *sc*. Those chimeric constructs were sequenced and then were injected into fly

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: bHLH, basic-helix–loop–helix; ch, chordotonal; es, external sensory; md, multiple dendritic; sop, sensory organ precursor. ‡To whom reprint requests should be addressed. e-mail: ynjan@itsa.ucsf.edu.

embryos to create transgenic flies (29). The transformants were balanced with either *CyO* or *TM3 sb* according to the chromosomal locations of the transgenes.

Assay of ch Organs and Bristle Formation on the Wings with *hs-GAL4*. Balanced male flies carrying *UAS-ato/sc* chimeras were crossed to *hs-GAL4/CyO* females at room temperature. White pupae (0–6 hr after puparium formation) from the crosses were collected and heat shocked at 39°C for 15 min. The heat-induced GAL4 then activated the expression of *ato/sc* proteins in various tissues, including the wing discs. The wings of adult flies were dissected and mounted with 80% glycerol. Ectopic bristles and ch organs were examined under the microscope. We scored the proximal quarter of the wings for the ch and es organs.

Assay for the Rescue of ch Neurons in *ato*¹ Mutant Embryos. *UAS-ato/sc ato*¹/*TM3* males were crossed to *h-GAL4 ato*¹/*TM3* females. The GAL4 is expressed in the odd-numbered segments (T2, A1, A3, A5, and A7) through the promoter of the pair-rule gene, *hairy* (*h*) (30). Embryos from these crosses were collected for an interval of 4 hr and aged for another 11 hr at 25°C. They were then fixed in 5% formaldehyde for 20–25 min, stained with monoclonal antibody 22C10 (31), and mounted in Permount (Fisher). One-quarter of the embryos with *UAS-ato/sc ato*¹/*h-GAL4 ato*¹ genotype were easily recognized due to lack of ch neurons in even-numbered abdominal segments. These embryos were scored for the number of ch neurons in the first four abdominal segments.

Computer Modeling. To build a model for the *ato/da* bHLH domain heterodimer–DNA complex, the bHLH sequences of *ato*, *da*, and MyoD were aligned by a multiple-sequence alignment (32) to locate sequence-conserved regions under the QUANTA (Polygen, Waltham, MA) environment on a Silicon Graphics IRIS 4D/25 workstation. The protein models constructed are based on the x-ray structure of MyoD bHLH domain–DNA complex at 2.8-Å resolution. The different residues in *ato* and *da* were introduced by using the “mutate” technique in QUANTA. The program aligned the replacement residues to the backbone of the original residues and maintained the dihedral angles for the side chains which are in common between the two residues. The gap regions were determined by a fragment-search process. The “regularization modeling” technique was employed to build and relax the side-chain coordinates. The complete structure was obtained by the CHARMM energy minimization process with the CHARMM/QUANTA program package by 100 cycles of steepest descent, 200 cycles of Powell conjugate gradient, and 300 cycles of conjugate gradient minimization.

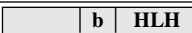

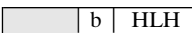





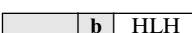
Statistics. The data in Tables 1–3 were analyzed with the SIGMASTAT software (Jandel) in which the program of descriptive statistics was used to calculate the mean and SEM and the Mann–Whitney rank sum test was used to calculate the *P* values described in the text. The rank sum test tests the hypothesis that two samples were not drawn from populations with different medians. We also did unpaired *t* tests on those comparisons, and the *P* values (not shown) from these tests gave the same conclusions as the rank sum test.

RESULTS

Phenotypes Due to Overexpression of *ato* and *sc*. A targeted gene expression system (30) was used to express chimeras of proneural genes *ato* and *sc* (*ato/sc*) (see Table 1) in transgenic flies. These chimeric genes were placed under the control of *UAS_G*, the binding site for the yeast transcription factor GAL4. We first expressed *ato* and *sc* proteins *in vivo* by *hs-GAL4* during wing development (see *Materials and Methods*). The wings from adult flies were then examined for the appearance of ectopic bristles (es organs) and ch organs. In wild-type wings, clusters of ch organs are normally found only in the ventral radius and tegula (Fig. 1*B*). Ectopic expression of *sc* did not increase the number of ch organs (1.7 ± 0.7 per wing; Table 1, line 2), but it did produce an abundance of ectopic bristles (57.0 ± 4.3 per wing; Table 1, line 2 and Fig. 1*C*). These ectopic bristles were solitary and evenly distributed on the wing. Ectopic expression of *ato*, on the other hand, resulted in numerous ch organs (31.6 ± 4.6 per wing; Table 1, line 1), which could be observed directly by using Nomarski optics, as shown in Fig. 1*A*. Most of these ectopic ch organs were distributed along the wing veins of the proximal quarter of the wing (Fig. 1*B*), and about 64% of them formed clusters, ranging from two to five in a group. Overexpression of *ato* also induced the formation of bristles (31.9 ± 2.2 per wing). Similar to those induced by overexpression of *sc*, they were solitary and evenly distributed, but not as numerous (Table 1, lines 1 and 2). The total numbers of ectopic sensory organs (es + ch organs) produced by overexpression of *ato* and *sc* are very similar (63.5 and 58.7 per wing).

Since the number of ectopic sensory organs induced by different chimeras is likely to vary with their expression levels, we also included the percentage of ectopic ch organs among the total ectopic sensory organs [$\text{ch \%} = 100 \times \text{ch}/(\text{ch} + \text{es})$] as an index for the ability to induce ch organs. The ch % was 3% for *sc* and 50% for *ato* (Table 1, lines 1 and 2), indicating that *ato*, but not *sc*, induces a significant number of ectopic ch organs.

Table 1. Ectopic ch and es organ induction by *ato/sc* chimeras in transgenic flies.

Line	Chimera	Structure	No. of organs per wing		ch/(ch + es), %	No. of wings	No. of lines
			Ectopic ch	Ectopic es			
1	<i>ato</i>		31.6 ± 4.6	31.9 ± 2.2	50	16	5
2	<i>sc</i>		1.7 ± 0.7	57.0 ± 4.3	3	11	3
3	<i>ato-bHLH</i> (<i>sc</i>)		0.5 ± 0.2	26.3 ± 1.8	2	34	7
4	<i>sc-bHLH</i> (<i>ato</i>)		23.5 ± 4.5	39 ± 2.1	37	18	4
5	<i>bHLH</i> (<i>sc</i>)		0 ± 0	13.5 ± 1.6	0	10	3
6	<i>bHLH</i> (<i>ato</i>)		0 ± 0	0 ± 0		10	3
7	<i>sc-b</i> (<i>ato</i>)		8.4 ± 1.6	52.3 ± 3.2	14	17	6
8	<i>sc-HLH</i> (<i>ato</i>)		0 ± 0	12.3 ± 16.5	0	8	4
9	<i>ato-HLH</i> (<i>sc</i>)		3.3 ± 0.5	31.0 ± 1.3	10	17	4

Shading in the chimeras indicates the *ato* domains. The numbers of ectopic ch (column 4) and es (column 5) organs in the basal one-quarter of the wing are given as mean \pm SEM. The numbers of ectopic ch organs divided by the total number of ectopic sensory organs are given as percentages in column 6. The total number of wings and independent lines assayed for each construct are given in columns 7 and 8, respectively.

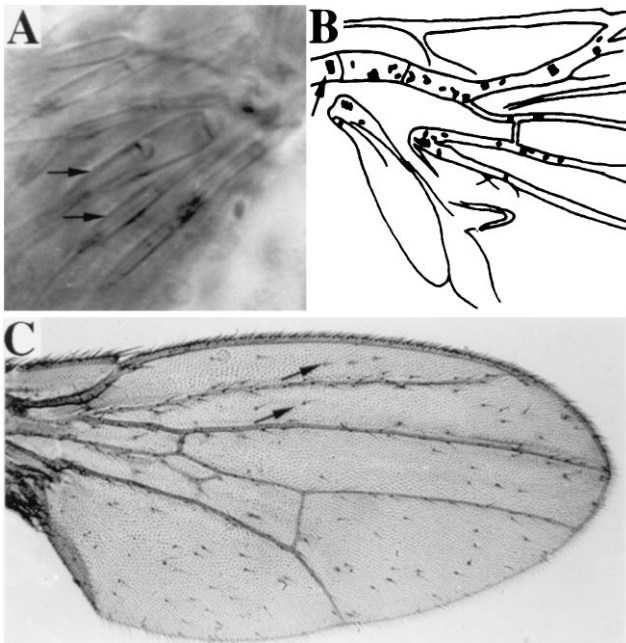


FIG. 1. (A) In the scutellum of *GAL4(109-68); UAS-ato* fly, several scolopales, each formed as a part of an ectopic ch organ by its support cells (arrows mark two of the scolopales), are visible under Nomarski optics. (B) Camera lucida representation of the basal quarter region of the wing of an *hs-GAL4; UAS-ato* fly, examined directly with Nomarski optics. Each oval represents a ch organ. Wild-type ch organs on the ventral radius vein are indicated with an arrow; the rest of the ch organs are ectopic. (C) Numerous bristles are formed on the wing of an *hs-GAL4; UAS-sc* fly. Almost all the bristles, except for those on the wing margin, are ectopic ones (two of the ectopic bristles are marked with arrows).

bHLH Domain of *ato* Specifies ch Organs. To test whether the bHLH domain of *ato* is responsible for specification of ch organs, we replaced the bHLH domain of *ato* with that of *sc* [*ato-bHLH(sc)*], and we found that the ability to promote ch organ formation is essentially abolished (average number of ch organs per wing is 0.5, or 2% of ectopic sensory organs; Table 1, line 3). Conversely, transplanting the *ato* bHLH domain into *sc* [*sc-bHLH(ato)*] conferred the ability to induce ch organs (23.5 and 37%; Table 1, line 4). Thus the bHLH domain of *ato* accounts for the ability to specify precursors for ch organs.

To test if the bHLH domain of *sc* or *ato* alone is sufficient for inducing and specifying sop cells, we examined flies expressing bHLH(*sc*) or bHLH(*ato*) without the flanking sequences of *ato* and *sc*. We found that bHLH(*sc*) was able to promote es, but not ch, organ formation (Table 1, line 5). This is consistent with the observation that the bHLH domain of lethal of scute (*l'sc*), a member of *ASC*, is sufficient for the formation of es organs (18). However, bHLH(*ato*) did not induce either type of sensory organ (Table 1, line 6). Comparison of this result with the results due to ectopic expression of *ato* and *sc-bHLH(ato)* suggests that other sequences in *ato*

or *sc* are necessary for the function or stability of bHLH(*ato*) in transgenic flies. Since the function of *ato* bHLH domain could be analyzed in the context of the full-length proteins of *ato* or *sc*, we subdivided the bHLH domain into smaller regions and generated additional chimeras, in an attempt to identify minimal structural elements that confer the specificity for the induction of ch organ precursors.

Involvement of b Domain and HLH Domain of *ato* in the Specification of ch Organ Precursors. The bHLH domain of *ato* or *sc* is composed of a b domain, which is involved in DNA binding, and an HLH domain, which is involved in dimerization (33, 34). We have further tested some chimeras with either the b domain or the HLH domain swapped between *ato* and *sc*. Replacement of the b domain of *sc* with that of *ato* [*sc-b(ato)*] allowed this chimera to induce ch organs (Table 1, line 7). This is reflected by a statistically significant increase in the absolute number of ectopic ch organs (8.4 per wing) and the percentage of ch organs (ch % = 14%), as compared with the corresponding numbers of *sc* (1.7 and 3%; $P = 0.001$, see *Materials and Methods*). The only difference between *sc* and *sc-b(ato)* resides in the b domain. These results indicate that the b domain of *ato* contains the ability to induce ectopic ch organs. Consistently, *ato-HLH(sc)*, consisting of the b domain of *ato* and HLH domain of *sc*, retained the ability to promote ectopic ch organ formation (3.3 and 10%; Table 1, line 9), in contrast to *ato-bHLH(sc)* [0.5 and 2%; $P < 0.0001$ for *ato-HLH(sc)* versus *ato-bHLH(sc)*; Table 1, line 3], which contains both b and HLH domains of *sc*. On the other hand, the HLH domain appears insufficient for the specificity; transplanting the *ato* HLH domain into *sc* [*sc-HLH(ato)*] did not allow ch organ induction (ch % = 0%; Table 1, line 8). Thus the abilities of *sc-b(ato)* and *ato-HLH(sc)* in promoting ectopic ch organ formation indicate that the b domain of *ato* is able to confer the specificity for ch organ formation. However, these abilities of *sc-b(ato)* and *ato-HLH(sc)* are not as strong as *sc-bHLH(ato)* and *ato*, respectively [Table 1, $P = 0.001$ for *sc-b(ato)* versus *sc-bHLH(ato)* and $P < 0.0001$ for *ato-HLH(sc)* versus *ato*]. This indicates that, in the presence of *ato* b domain, the HLH domain of *ato* is more effective than the HLH domain of *sc* in promoting ch organ formation in this assay.

Rescue of ch Neurons in *ato*¹ Embryos by *sc-bHLH(ato)* and *sc-b(ato)*. We further tested whether the b domain of *ato* also suffices to specify ch neurons in embryos lacking endogenous *ato* activity. In *ato*¹ mutant embryos, almost all the ch neurons are eliminated (22), including the lateral five ch neurons (*lch5*) in each abdominal hemisegment (Fig. 2 B and G, compared with the wild-type embryos in Fig. 2 A and F and Table 2, lines 1 and 2). We used *h-GAL4* (30) to express chimeras of *ato* and *sc* in alternate segments (T2, A1, A3, A5, and A7) of the embryos. As expected, segmental expression of *ato* in *ato*¹ mutants restored most of the *lch5* neurons in the odd-numbered abdominal segments (e.g., 82% in A1 and 100% in A3; Table 2, line 3, and Fig. 2 C and H) but not in even-numbered abdominal segments (such as A2 and A4). The rescue was also observed in T2, but not T3 segments (data not shown). By contrast, *sc* was incapable of rescuing the *ato*¹

Table 2. Rescue of *lch5* neurons in *ato*¹ mutant embryos by *ato/sc* chimeras

Line	Embryo	No. of ch organs in hemisegment				No. of embryos	No. of lines
		A1	A2	A3	A4		
1	Wild type	5	5	5	5		
2	<i>ato</i> ¹ / <i>ato</i> ¹	0.4 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	14	2
3	<i>ato</i>	4.1 ± 0.6	0.6 ± 0.2	5.0 ± 0.2	1.6 ± 0.4	12	2
4	<i>sc-bHLH(ato)</i>	4.2 ± 0.6	0.5 ± 0.2	4.2 ± 0.5	0.8 ± 0.3	6	3
5	<i>sc-b(ato)</i>	2.6 ± 0.3	1.4 ± 0.4	3.4 ± 0.4	1.1 ± 0.3	14	2
6	<i>sc</i>	0.7 ± 0.2	0.3 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	15	3

The number (mean ± SEM) of ch organs in *ato*¹ mutant and *ato*¹ mutants expressing *ato/sc* chimeras is given for each of the first four abdominal hemisegments. The last columns indicate the number of embryos and the number of lines scored.

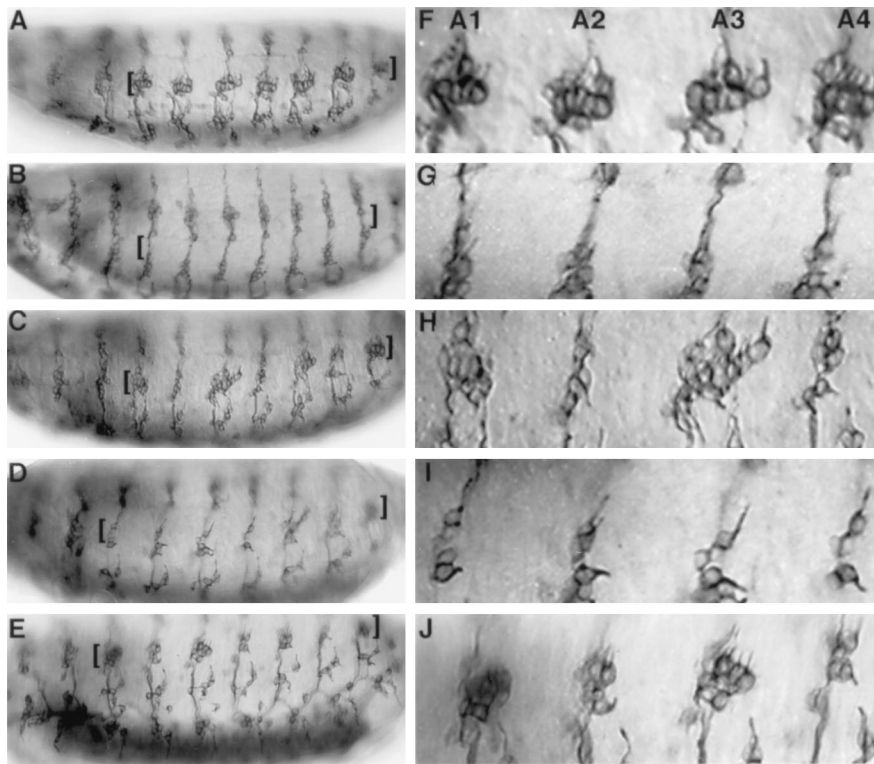


FIG. 2. Rescue of ch organs in *ato*¹ mutant embryos by *UAS-ato/sc* chimeric genes. (A and F) Wild-type embryo contains five ch neurons in the lateral region (lch5, marked by the brackets in A–E) in each abdominal hemisegment. Most of the ch neurons are eliminated in *ato*¹ mutant embryo (B and G). The lch5 of *ato*¹ embryos in alternate segments were rescued by *h-GAL4; UAS-ato* (C and H) and by *h-GAL4; UAS-sc-b(ato)* (E and J) combinations, but not by *h-GAL4; UAS-sc* (D and I). In F–J, the first four abdominal hemisegments of the embryos in A–E are shown, respectively.

mutant phenotype in any of the segments (Fig. 2 D and I and Table 2, line 6). Replacing the bHLH domain of *sc* with that from *ato* [*sc-bHLH(ato)*] allowed the mutant *sc* protein to functionally substitute for *ato* in inducing ch neurons at their normal locations. The extent of rescue was about 84% (both A1 and A3 segments; Table 2, line 4), which is comparable to that by wild-type *ato*. The *sc-b(ato)*, with only the b domain replaced by that of *ato*, also rescued *ato*¹ mutants to a high degree (52% in A1 segments and 68% in A3 segments; *P* < 0.0001 for A1 and *P* = 0.0001 for A3 when compared with *sc*; Table 2, line 5 and Fig. 2 E and J). This is consistent with the results of overexpression of the *sc-b(ato)* in the wing discs and it further confirms that the b domain of *ato* is sufficient for the specification of ch organ precursors.

Mutagenesis Analysis of the *ato* b Domain. To test whether specific residues of the b domain could endow the specificity

for induction of ch organ precursors, we examined the phenotypes caused by point mutations in the b domains of *ato* or *sc*. The b domains of *ato* and *sc* differ in 7 of 15 residues (Table 3, lines 1 and 2). Of these 7 residues, 4 represent amino acid substitutions at homologous positions, and another 3 residues (Leu, Ala, and Ala) from the *ato* b domain correspond to a gap in the *sc* b domain. Of the various point mutations (Table 3, lines 3–7) tested, the substitution of Asn in *sc* with Arg, the corresponding residue in *ato*, increased the number of ch organs (compare lines 3–5 with lines 6 and 7 of Table 3). But none of these changes in the number of ectopic ch organs are statistically significant (*P* > 0.05 for every chimera compared with *sc*). On the other hand, substitution of the Arg in wild-type *ato* with Asn from *sc*, which is conserved among members of ASC, reduced the number of ectopic ch organs [17.1 organs per wing and 31%; Table 3, line 9, *P* = 0.01 for

Table 3. Ectopic ch and es organs induced by various *ato* or *sc* proteins with mutations in the b domains

Line	Protein	b domain sequence	No. of organs per wing		ch/(ch + es), %	No. of wings	No. of lines
			Ectopic ch	Ectopic es			
1	<i>ato</i> (WT)	KRKRRLAANARERRR	31.6 ± 4.6	31.9 ± 2.2	50	16	5
2	<i>sc</i> (WT)	SVQRR---NARERNR	1.7 ± 0.7	57.0 ± 4.3	3	11	3
3	<i>sc</i> -m14	KRKR R---NARERRR	2.9 ± 0.9	21.1 ± 1.0	12	8	4
4	<i>sc</i> -m24	SRQRR---NARERRR	5.2 ± 1.9	24.7 ± 1.1	17	8	4
5	<i>sc</i> -m34	SVQRR---NARERRR	4.0 ± 1.4	23.8 ± 1.3	14	10	4
6	<i>sc</i> -m15	KRKR R---NARERNR	0.3 ± 0.2	20.8 ± 1.7	1	8	4
7	<i>sc</i> -m25	SRQRR---NARERNR	0.5 ± 0.3	18.1 ± 1.9	3	6	2
8	<i>sc</i> -in	SVQRR LA ANARERNR	2.1 ± 0.6	43.6 ± 2.2	5	21	8
9	<i>ato</i> -(R-N)	KRKRRLAANARERNR	17.1 ± 4.8	38.7 ± 1.7	31	16	4
10	<i>ato</i> -del	KRKR R---NARERRR	13.5 ± 2.6	31.8 ± 3.2	30	13	2

The amino acid sequences of the b domains start at Lys-253 of *ato* or Ser-100 of *sc*. WT, wild type. The *ato* sequence is in boldface type. The conserved residues are marked with *. Other columns are described in the legend of Table 1.

ato versus ato-(R-N)]. Insertion of the Leu, Ala, and Ala residues of ato into sc (sc-in; Table 3, line 8) did not increase the ability to induce ch organs (2.1 and 5%; $P > 0.05$ for sc versus sc-in). But the deletion of these three residues from ato (ato-del; Table 3, line 10) partially reduced ch organ induction in a statistically significant way (13.5 and 30%; $P = 0.002$ for ato versus ato-del). Therefore, our mutagenesis analysis indicates that the specificity for ch organ induction could arise from a combination of most or all of the 7 residues of the ato b domain that differ from their counterpart in sc.

Model for the DNA-Binding Domain of ato/da Heterodimer.

To better understand the role of the b domain of ato in inducing ch organ precursors, we used the crystal structure of the MyoD bHLH domain (35) to predict the structure of ato/da bHLH heterodimer–DNA complex. In the model that we generated (Fig. 3), the b domain of ato forms an α -helical structure and is embedded in the major groove of DNA. Seven residues (Arg-256, Arg-257, Asn-261, Arg-263, Glu-264, Arg-265, and Arg-267, colored in magenta in Fig. 3) in the b domain of ato are predicted to contact DNA directly through hydrogen bonding (data not shown). Interestingly, these residues are conserved between ato and sc (Table 3, lines 1 and 2). Some of the nonconserved residues (Arg-254, Lys-255, Leu-258, Ala-259, and Arg-266, colored in green) and one of the conserved residues (Ala-262, in green) are on the other side of the α -helix. Their side chains are predicted to protrude away from DNA and are available for contact with other proteins. Thus, in this model, the residues that contact DNA are conserved between the b domains of ato and sc. In contrast, the seven nonconserved residues in the b domain of ato do not contact DNA and could be involved in interaction with other protein(s) that are also involved in the specification of ch organ precursors.

DISCUSSION

bHLH proteins play crucial roles in controlling cell fate in a variety of tissue types. In ectopic expression experiments, closely related bHLH proteins can often substitute for each other in promoting certain cell fates. For instance, each of the myogenic factors (MyoD, Myf-5, myogenin, and MRF4) is capable of transforming fibroblasts into myoblasts in a tissue culture system (for review, see refs. 35–37). Likewise, each member of the *ASC* (*ac*, *sc*, *ase*, and *l'sc*) can promote ectopic es organ development in wing discs (14–19). Those bHLH factors have a high degree of sequence homology in the bHLH domains ($\approx 80\%$ identity among members of myogenic factors

and $\approx 70\%$ among members of *ASC*) and are interchangeable in gain-of-function experiments, making it difficult to identify domains of these bHLH proteins required for specifying a particular cell type. ato is more distantly related to members of *ASC* (50% identity in bHLH domain) and is unique in its ability to promote ch organ formation. This has provided us with an opportunity to identify the structural elements that confer the neuronal type information. Previous work on myogenic factors has indicated that residues in the b domain contain the information needed to promote nonmuscle cell to differentiate into muscle cells (38, 39). From overexpression of chimeric proteins in the wing discs and in *ato*¹ mutant embryos, our results indicate that the bHLH domain, particularly the b domain, of ato not only is able to promote neuronal cell fate but also confers the neuronal type information (in this case, ch neuron). The possible contributions of the b and HLH domains to neuronal type specification are discussed below.

Specificity of Sensory Organ Induction by ato and sc. Each member of the *ASC*, when overexpressed, promotes ectopic es, but not ch, organ formation. On the other hand, overexpression of ato induces ch as well as es organs, even though normal es organ formation requires *ASC* genes, but not *ato*. This apparent discrepancy could be accounted for by one of the following possibilities. First, it is possible that overexpression of ato leads to fortuitous transcriptional activation of *ASC* genes. If overexpression of ato could cross-activate *ac* or *sc* in the regions where they are not normally expressed, it would consequently induce ectopic es organs. To test this possibility, one could examine the phenotypes of overexpressing ato in the *ASC*[−] mutants.

Second, ato could induce either type of sensory organs; it induces ch organs in the presence of a cofactor(s) and es organs in the absence of this cofactor(s). We have observed that most of the ectopic ch organs induced by ato were near the wing veins at the proximal regions, in contrast to the evenly distributed ectopic bristles induced by either *sc* or *ato*. This could result from the postulated cofactor(s) being restricted to the wing veins at the proximal regions. This hypothesis also presumes that *sc*, or any other member of *ASC*, is not able to interact with the cofactor(s) to direct ch organ formation.

Although we cannot distinguish among these possibilities at the moment, the unique ability of ato to induce ch organs supports the idea that the neuronal type information of ch organs arises from certain features of ato that are not present in *sc* or any other *ASC* proteins.

Possible Roles of the b Domain and the HLH Domain of ato in ch Organ Specification. The ability to induce ch organs can

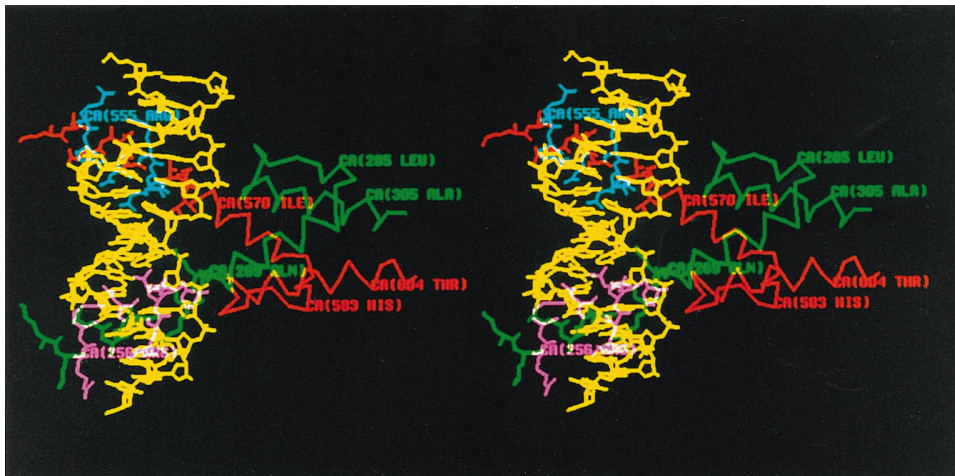


FIG. 3. Stereo diagram of the predicted structure of ato/da bHLH domain heterodimer–DNA complex by computer modeling. The sequence of the DNA-binding site in this model is TCAACAGCTGTTGA, containing an E box. DNA is colored in yellow, ato in green, and da in red. Those residues of ato and da predicted to contact DNA are colored in magenta and cyan, respectively. Only the side chains of the residues in the b domains are shown.

be transferred to the sc protein by replacing its b domain with the corresponding domain of ato [sc-b(ato); Table 1, line 7]. Also, compared with ato-bHLH(sc), ato-HLH(sc) that has the ato b domain is more effective in promoting ch organ formation (Table 1, lines 3 and 9; $P < 0.0001$). Another experiment revealed that sc-b(ato) can restore ch neurons to a high degree in *ato¹* mutant embryos (Table 2, line 5). Thus, the b domain of ato harbors to some extent the neuronal type information.

The ability to induce ch organs is further enhanced when the ato b domain is accompanied with the ato HLH domain as in sc-bHLH(ato) (Table 1, line 4), even though presence of only the ato HLH domain in sc-HLH(ato) (Table 1, line 8) is insufficient to promote ch organ formation. The importance of the ato HLH domain is further shown with ato-HLH(sc) (Table 1, line 9) which is much less effective in promoting ch organ formation compared with wild-type ato (Table 1, line 1). These observations indicate that the difference between the HLH domains of ato and sc also contributes to the specification of neuronal type. This could arise if these HLH domains interact differently with positive or negative factors in a quantitative or qualitative way. It is also conceivable that these HLH domains affect the folding or stability of the chimeric proteins. Notwithstanding the quantitative differences in ectopic ch organ induction attributable to the HLH domains, the requirement of the ato b domain for ch organ formation indicates that the neuronal type information derives partially from the seven residues in the ato b domain that are different from those in sc.

The Seven Residues in the b Domains of ato That Differ from the Corresponding Residues in sc. Strong conservation of ato b domain, including some of the seven residues that distinguish ato from sc b domain, has been observed among ato homologs from different species. For example, MATH1, a mammalian bHLH protein (40), has 12 residues that are identical to those in the b domain of ato, which contains 15 residues (Table 3, line 1). The gene product of *Caenorhabditis elegans lin-32*, which functions in an analogous manner as do the proneural genes of *Drosophila* (41), shares 11 residues with ato in the b domain. A *Xenopus* homolog of ato (M. Vetter, L.Y.J., and Y.N.J.) has 12 residues identical to those in the b domain of ato. It will be interesting to know whether the b domains of ato homologs in those species are involved in neuronal type specification.

Mutations, including the substitution of Asn of sc with Arg-266, the corresponding residue in ato, slightly increase the ability of sc to induce ch organs (Table 3, lines 3–5), but this increase is not statistically significant ($P > 0.05$). Insertion of three residues, Leu-258, Ala-259, and Ala-260 of ato, into sc does not confer on sc the ability to induce ch organ formation (Table 3, line 8). On the other hand, either deletion of the three residues Leu-258, Ala-259, and Ala-260 of ato or mutation of the Arg-266 of ato to the corresponding Asn of sc reduces the ability to induce ectopic ch organs in a statistically significant way (Table 3, lines 9 and 10, $P = 0.002$ or $P = 0.01$ compared with ato, respectively). Only the chimera sc-b(ato), in which seven mutations were introduced into the b domain of sc to convert it into the b domain of ato, acquires the ability to induce ch organs. Whereas it remains possible that fewer than seven mutations are necessary to endow sc with the ability to induce ch organs, it appears from the series of mutagenesis examined in this study (Table 3) that most, if not all, of the seven residues in the ato b domain contribute to the unique ability of ato to specify ch organ precursors.

In the computer model (Fig. 3), the b domain of ato forms α -helix. Most of the seven residues that differ from their counterparts in sc are on the opposite side of the surface that contacts DNA, suggesting that they might form an interface for protein–protein interaction. This interaction may affect the recognition and interaction of DNA by ato/da heterodimers and/or the subsequent effects on transcription, potentially

leading to transcriptional regulation of downstream genes involved in the formation of ch organs.

We thank Dr. Sonsoles Campuzano for sc cDNA and I. Clark, D. Doherty, T. Doniach, M. Utset, M. Vetter, and W. Zhong for their comments on the manuscript. C.-t.C. is a fellow of The Jane Coffin Childs Memorial Fund for Medical Research. C.-D.H. was supported in part by a grant from the National Science Council, Taiwan. L.Y.J. and Y.N.J. are Investigators of the Howard Hughes Medical Institute.

- Jan, Y. N. & Jan, L. Y. (1993) in *The Peripheral Nervous System*, eds. Bate, M. & Martinez-Arias, A. (Cold Spring Harbor Lab. Press, Plainview, NY), Vol. II, pp. 1207–1244.
- Ghysen, A. & Dambly-Chaudiere, C. (1989) *Trends Genet.* **5**, 251–255.
- Jan, Y. N. & Jan, L. Y. (1990) *Trends Neurosci.* **13**, 493–498.
- Cabrera, C. V. (1992) *Development (Cambridge, U.K.)* **15**, 893–901.
- Campuzano, S. & Modolell, J. (1992) *Trends Genet.* **8**, 202–208.
- Campos-Ortega, J. A. (1988) *Trends Neurosci.* **11**, 400–405.
- Artavanis-Tsakonas, S. & Simpson, P. (1991) *Trends Genet.* **7**, 404–408.
- Ghysen, A., Dambly-Chaudiere, C., Jan, L. Y. & Jan, Y. N. (1993) *Genes Dev.* **7**, 723–733.
- Jan, Y. N. & Jan, L. Y. (1995) *Neuron* **14**, 1–5.
- Posakony, J. W. (1994) *Cell* **76**, 415–418.
- Garcia-Bellido, A. & Santamaria, P. (1978) *Genetics* **88**, 469–486.
- Garcia-Bellido, A. (1979) *Genetics* **91**, 491–520.
- Dambly-Chaudiere, C. & Ghysen, A. (1987) *Genes Dev.* **1**, 297–306.
- Campuzano, S., Balcells, L., Villares, R., Garcia-Alonso, L. & Modolell, J. (1986) *Cell* **44**, 303–312.
- Balcells, L., Modolell, J. & Ruiz-Gomez, M. (1988) *EMBO J.* **7**, 3899–3906.
- Rodriguez, I., Hernandez, R., Modolell, J. & Ruiz-Gomez, M. (1990) *EMBO J.* **9**, 3583–3592.
- Brand, M., Jarman, A. P., Jan, L. Y. & Jan, Y. N. (1993) *Development (Cambridge, U.K.)* **119**, 1–17.
- Hinz, U., Giebel, B. & Campos-Ortega, J. A. (1994) *Cell* **76**, 77–88.
- Dominguez, M. & Campuzano, S. (1993) *EMBO J.* **12**, 2049–2060.
- Jarman, A. P., Grau, Y., Jan, L. Y. & Jan, Y. N. (1993) *Cell* **73**, 1307–1321.
- Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. & Jan, Y. N. (1994) *Nature (London)* **369**, 398–400.
- Jarman, A. P., Sun, Y., Jan, L. Y. & Jan, Y. N. (1995) *Development (Cambridge, U.K.)* **121**, 2019–2030.
- Villares, R. & Cabrera, C. V. (1987) *Cell* **50**, 415–424.
- Alonso, M. C. & Cabrera, C. V. (1988) *EMBO J.* **7**, 2585–2591.
- Gonzalez, F., Romani, S., Cubas, P., Modolell, J. & Campuzano, S. (1989) *EMBO J.* **8**, 3553–3562.
- Caudy, M., Vaessin, H., Brand, M., Tuma, R., Jan, L. Y. & Jan, Y. N. (1988) *Cell* **55**, 1061–1067.
- Cabrera, C. V. & Alonso, M. C. (1991) *EMBO J.* **10**, 2965–2973.
- Singson, A., Leviten, M., Bang, A. G., Hua, X. H. & Posakony, J. W. (1994) *Genes Dev.* **8**, 2058–2071.
- Spradling, A. C. & Rubin, G. M. (1982) *Science* **218**, 341–347.
- Brand, A. H. & Perrimon, N. (1993) *Development (Cambridge, U.K.)* **118**, 401–415.
- Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. & Benzer, S. (1984) *Cell* **36**, 15–26.
- Feng, D. F. & Doolittle, R. F. (1987) *J. Mol. Evol.* **25**, 351–360.
- Murre, C., Schonleber-McCaw, P. & Baltimore, D. (1989) *Cell* **56**, 777–783.
- Murre, C., Schonleber-McCaw, P., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H. & Baltimore, D. (1989) *Cell* **58**, 537–544.
- Ma, P. C. M., Rould, M. A., Weintraub, H. & Pabo, C. O. (1994) *Cell* **77**, 451–459.
- Weintraub, H. (1993) *Cell* **75**, 1241–1244.
- Olson, E. N. & Klein, W. H. (1994) *Genes Dev.* **8**, 1–8.
- Brennan, T. J., Chakraborty, T. & Olson, E. N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5675–5679.
- Davis, R. L. & Weintraub, H. (1992) *Science* **256**, 1027–1030.
- Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S. & Kageyama, R. (1995) *J. Biol. Chem.* **270**, 8730–8738.
- Zhao, C. & Emmons, S. W. (1995) *Nature (London)* **373**, 74–78.