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Functional specificity of the Antennapedia homeodomain

(Sex comb reduced/heat shock/segmental identity/gene expression/homeotic transformation)

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ABSTRACT **The segmental identity in animal development is determined by a set of homeotic selector genes clustered in the invertebrate *HOM* or vertebrate *Hox* homeo box complexes. These genes encode proteins with very similar homeodomains and highly diverged N- and C-terminal sequences. The Antennapedia (Antp) homeodomain, for instance, differs at only five amino acid positions from that of Sex combs reduced (Scr) protein. Using a heat shock assay in which chimeric Antp–Scr proteins are expressed ectopically in *Drosophila*, we have shown that the functional specificity of the Antp protein is determined by the four specific amino acids located in the flexible N-terminal arm of the homeodomain. The three-dimensional structure of the Antp homeodomain–DNA complex shows that this N-terminal arm is located in the minor groove of the DNA, suggesting that the functional specificity is determined either by slight differences in DNA binding and/or by selective interactions with other transcription factor(s).**

The body of the fruitfly is composed of a series of consecutive segments arranged along the anteroposterior axis, each of which shows a distinct identity. The genetic and molecular studies of *Drosophila* development have identified a set of homeotic selector genes that specify the morphological and functional identity of each of these metameric segments (reviewed in ref. 1). The homeotic genes are clustered in the Antennapedia Complex (ANTC) and Bithorax Complex (*BXC*) on the third chromosome encoding nuclear transcription factors that contain the homeodomain, a 60-amino acid DNA-binding domain. The protein sequences encoded by the members of the ANTC and BXC genes are largely different from each other except for their homeodomains, which show strong amino acid sequence conservation including almost identical recognition helices (2). Previous studies have indicated that the functional specificity of these homeotic selector proteins is determined by the C-terminal parts of the proteins that include their homeodomains (3–5). In this paper, we show that the four amino acid residues at positions 1, 4, 6, and 7 in the flexible N-terminal arm of the homeodomain determine the functional specificity of the Antp protein in transgenic flies. In the three-dimensional structures of the homeodomain–DNA complexes (6–8), this N-terminal arm is located in the minor groove of DNA. Our results suggest that the functional specificity of the homeotic selector proteins is determined either by a slight difference in DNA binding and/or by selective association with other specific transcription factors.

MATERIALS AND METHODS

Gene Construction. The four amino acid changes were introduced into the plasmids pHSS-AfSL and pHSS-SA26 (5) by substituting the *Bsm* I–*Xmn* I fragments with the double-stranded oligonucleotide AAT GGC GAG CGC AAA CGC GGA AGG CAG ACA TAC ACC CGG TAC CAG ACT CTA GAG CTA GAG AAG G. The mutated gene was subsequently subcloned into the unique *Not* I site of pNHT4 (5). During the confirmation of the mutant sequence, we found that six residues are missing from the reported *Scr* (Sex combs reduced) sequence 1460–1600 (9). The corrections have been submitted to the EMBO data base.

***P*-Element Transformation and Maintenance of Flies.** Transgenic flies were generated by *P*-element transformation and maintained as described (5). The rosy eye ry^{506} allele was used as recipient of the transposon. Only two or three lines were recovered from 1000 injections probably because of the toxicity of the encoded proteins expressed by leaky transcription of the heat shock gene *Hsp70* promoter.

Heat Shocks. Embryonic and larval heat shocks were applied as described (5). Briefly, embryos were heat-shocked for 20 min at 37°C 6–7 hr after egg laying and were allowed to develop at 25°C for 36 hr. For larvae, six consecutive heat shocks at 37°C for 60 min were applied at 4-hr intervals from 69 ± 0.5 hr to 88 ± 0.5 hr after egg laying.

RESULTS

Transgenic Flies Carrying the Fusion Genes with the N-Terminal Homeodomain Arm of Antp. To test the importance of the amino acid residues in the homeodomain for functional identity, the Antp-specific four amino acids (Arg-1, Gly-4, Gln-6, and Thr-7; see Fig. 1) were introduced into the hybrid homeotic proteins G17 and G26 [encoded by the Antp–Scr (Sex combs reduced) hybrid constructs], which show strong and weak Scr phenotype, respectively, in heat-shocked transgenic flies (5). Mutated hybrid constructs, designated K17 and K26, were subcloned into the *P*-element vector pNHT4 and inserted into the *Drosophila* genome by *P*-element transformation. Two or three independent lines, which showed strong embryonic lethality upon heat shock, were obtained for each construct (Table 1). The phenotypic effects described below were qualitatively confirmed by using independent transformant lines with slightly different penetrance.

Embryonic Phenotype Induced by Expression of hs-K17 and hs-K26. The N-terminal part of the Antp and Scr proteins contains only a general potentiation domain that can be mutually exchanged without affecting the functional specificity. We chose the chimeric hybrid gene constructs G17 and G26 (5), both of which encode the general potentiation domain of the Antp protein and the Scr homeodomain (Fig. 1*B*), which differ only at the C terminus outside of the homeodomain that originates from Scr in G17 protein and from Antp in G26 protein, respectively. As has been described (5), G17 and G26 proteins show strong and weak Scr phenotype in transgenic flies respectively, which was recon-

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Abbreviations: *ANTC*, Antennapedia complex; *BXC*, Bithorax complex; hs-K17, hs-K26, hs-G17, hs-G26, heat shock products of hybrid gene constructs K17, K26, G17, and G26; YPWM, Tyr-Pro-Trp-Met.

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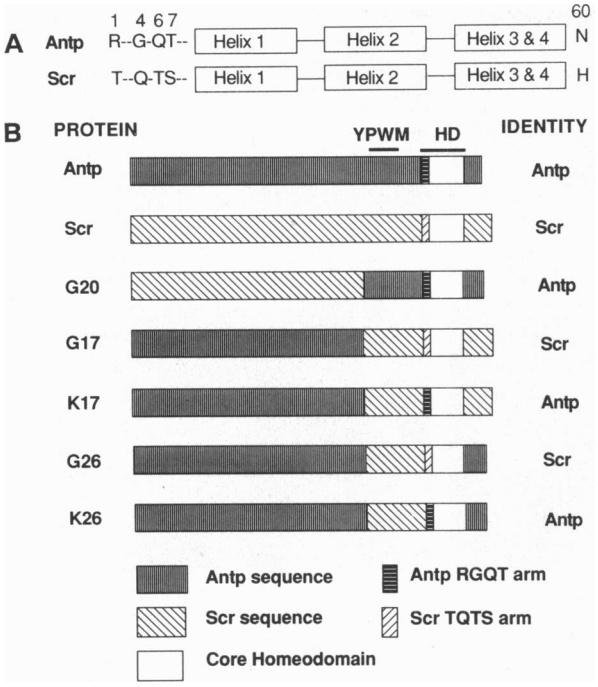

FIG. 1. (*A*) Amino acid sequences of the Antp and Scr homeodomains. The Antp and the Scr homeodomains are different at 5 of 60 positions, 4 of which are located in the flexible N-terminal arm of the homeodomain. Only the amino acids that are diverged are shown. (*B*) Structure of the Antp–Scr hybrid proteins. The functional identities of hybrid proteins G17, G20, and G26 have been described (5). As evidenced by G17 and G20, the N-terminal two-thirds of the Antp and Scr proteins are interchangeable without affecting the functional identity, while the specificity is determined by the C-terminal region that contains the homeodomain. Changing the four amino acid at positions 1, 4, 6, and 7 in the N-terminal arm of the homeodomain switches the functional identity between Scr and Antp. The hybrid proteins are ubiquitously expressed in flies by heat shock protein gene *Hsp70* promoter upon heat shock. The difference at position 60 is not indicated. The core homeodomain represents the amino acids 10–59 spanning from the beginning of the helix 1 to the end of the helix 4 (6). The location of the Tyr-Pro-Trp-Met (YPWM in single-letter code) peptide is also indicated.

firmed in this study (Table 2, Fig. 2 *B* and *E*). In heat-shocked embryos, ectopic expression of Scr produces ectopic beards of denticles in the posterior thoracic segments (T2 and T3) by transforming T2 and T3 toward T1 (in the anterior direction). This transformation is exactly the opposite of that of heat shock-induced expression of Antp, which suppresses the formation of the T1 beard and generates ectopic denticle bands in the head by transforming T1 and the head segments towards T2 (in the posterior direction). Ectopic expression of both Antp and Scr causes defects in head involution with stronger values for Antp. The embryonic phenotypes of hs-K17 and hs-K26 were scored for these cuticular patterns (Table 2). As evidenced by the strong suppression of the T1 beard, ectopic denticles in the head, and stronger head involution defects, hs-K17 showed a strong Antp phenotype in the heat-shocked embryos (Fig. 2 *C* and *D*). The phenotypic effect of hs-K17 is as strong as for heat-shock wild-type *Antp* constructs in transgenic lines such as H22 and H45, but weaker than in H4, the strongest heat-shock Antp-expressing line (see ref. 10). The expression of hs-K26 produced a similar effect, which can be classified as a moderate Antp phenotype (Fig. 2*F*).

Table 2. Effects of the Antp and Scr chimeric proteins on the embryonic segmental identity

Protein*	Head involution defect†			T1 beard‡			Ectopic denticles§		
	+/−	+	++	Normal	Reduced	Missing	Head	T2	T3
G17	19	25	56	97	3	0	0	66	35
G26	47	36	17	94	6	0	0	14	0
K17	19	10	71	12	50	38	56	1	0
K26	22	41	37	18	70	12	11	0	0

Data are the number of embryos that showed the indicated phenotype by heat induction of the chimeric protein. For each protein, 100 embryos are screened.
*Lines used were G17A, G26A, 6.45, and B21.
†Based on the values defined in ref. 5. +/−, Values 1 and 2; +, values 3 and 4; ++, values 5 and 6.
‡Normal, more than five rows; missing, less than 20 denticles (see ref. 10).
§Embryos that show more than five denticles in the indicated segment are counted.

Adult Head Transformation by Expression of hs-K17 and hs-K26. One of the most striking phenotypic effects caused by the ectopic expression of the Antp protein is the transformation of the antennae on the head of the fly into the mesothoracic (T2) legs. This phenotype was originally observed in the Antp gain-of-function mutants (11). This antenna to leg transformation can be phenocopied by overexpressing the Antp protein under a heat shock promoter during the early third larval stage when the determination of the eye–antennal imaginal disc is still labile (12). To examine whether the substitution of the four amino acids in the N-terminal arm of the homeodomain can also lead to the transformation of the antennae into T2 legs, we induced the expression of the mutant proteins during the early third larval stage by multiple heat shocks. Since many of the flies of hs-G17- and hs-K17-expressing lines failed to eclose from the pupal case after heat shock (the pupal survival rates are 10% with G17, 50% with K17, 100% with G26, 100% with K26), we examined the head structures of both eclosed and uneclosed mature adult flies for these cases.

The phenotypic effects of hs-G17 and hs-G26 are consistent with the previous observation (ref. 5; Table 3). The expression of hs-G17 causes a partial transformation of the arista into tarsal structures (Fig. 3*C*), strong reduction of eyes, and a Cephalothorax-like ($Antp^{Ctx}$; ref. 11) transformation of the occiput (Table 4). The phenotypic transformations induced

Table 1. Fly stocks used in this study

Stock	Chromosome	Balancing	Protein	% hatched after heat shock	Source or ref.
G17A	III	TM3	G17	0 (0/100)	5
G26A	II	Homozygous	G26	22 (14/64)	5
5.32	II	CyO	K17	0 (0/100)	This work
6.37	II	CyO	K17	3 (3/100)	This work
6.45	III	TM3	K17	4 (4/104)	This work
B21	II	CyO	K26	8 (5/60)	This work
C11	II	CyO	K26	10 (7/67)	This work

All the lines were generated by using ry^{506} as a host for *P*-element transformation.

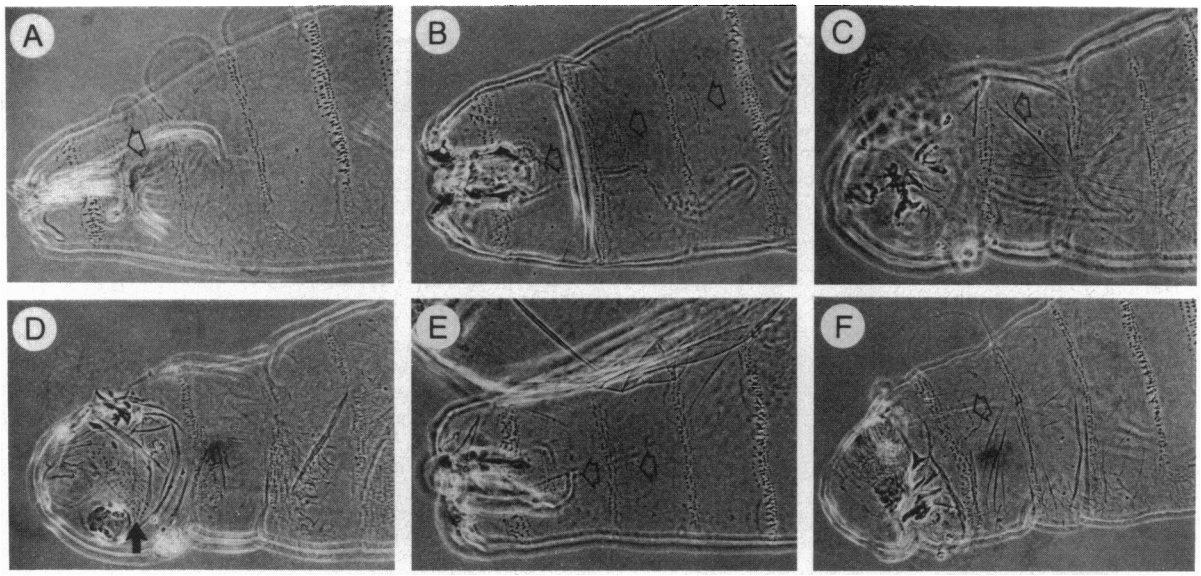

FIG. 2. The embryonic transformation induced by the chimeric protein. (*A*) Wild type. Open arrow indicates the prothoracic beard. (*B*) G17 (line G17A). The head involution defect is moderate; however, note the ectopic beards in T2 and T3. (*C*) K17 (line 6.45). The T1 beard is reduced to only seven denticles in this embryo. Head involution is strongly suppressed. (*D*) Dorsal side of the same embryo as in *C*. An ectopic denticle belt, indicated by a solid arrow, is developed in the head. (*E*) G26 (line G26A). An ectopic beard is induced in T2. The head involution defect is moderate. (*F*) K26 (line B21). Note the strong suppression of the T1 beard and the strong head involution defect. Embryos were heat-shocked for 20 min at 37°C 6–7 hr after egg laying.

by hs-G26 are weak but qualitatively similar to those of hs-G17 (Fig. 3*E*). Since the transformation of the eyes and the occiput is caused by both Antp and Scr proteins, we took the transformations of the second and third antennal segments into the mesothoracic leg structures as the most significant indications for Antp specificity.

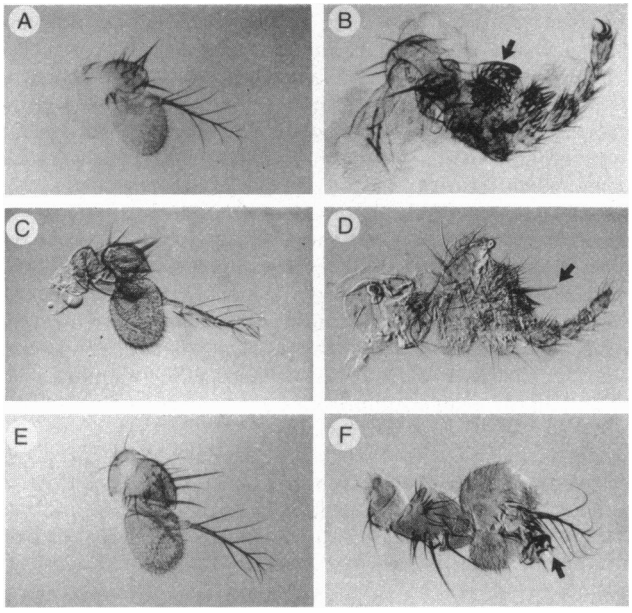

FIG. 3. The antenna transformation induced by the ectopic expression of the chimeric protein. (*A*) Wild-type antenna. (*B*) Transformed antenna by heat-induced wild-type Antp protein (line G1A, see ref. 5). The solid arrow indicates the ectopically induced apical bristle that is T2 leg specific. (*C*) Transformation by hs-G17 (line G17A). Only thickening of arista is induced. (*D*) Transformation by hs-K17 (line 5.32). The entire antenna is transformed into T2 leg structures with ectopically induced bract-bristles and an apical bristle (solid arrow). (*E*) Transformation by hs-G26 (line G26A). The antenna is unaffected except for the slightly thickened basal cylinder of the arista. (*F*) Transformation by hs-K26 protein (line C11). The entire antenna is transformed. Ectopic bract-bristles and an apical bristle (solid arrow) are developed.

As shown in Fig. 3*D*, the expression of hs-K17 induced the transformation of the entire antenna into mesothoracic leg structures carrying leg-specific bristles with bracts (13). Moreover, 54% of the ectopically produced legs formed the apical bristles at the distal end of the tibia that are characteristic for the T2 legs, confirming that the ectopically developed legs are indeed of mesothoracic identity. Similar phenotypic transformation of the antennae was caused by the ectopic expression of hs-K26 (Fig. 3*F*). The penetrance of hs-K26 was lower than that of hs-K17 (Table 3), but the mesothoracic leg identity was again confirmed by the development of bristles with bracts in all of the induced leg structures and by the presence of the apical bristle on the ectopic legs in three of the cases. None of the mutant proteins

Table 3. Transformation of the antenna segments into leg-like structures by the chimeric proteins

	% transformation				Total antennae examined†
Protein	Arista*	Ant III	Ant II	Ectopic apical bristle	
G17	56 (9)	0	0	0	16
G26	6 (4)	0	0	0	68
K17	99 (66)	82 (55)	16 (11)	54 (36)	67
K26	55 (43)	54 (42)	0	4 (3)	78

Lines used are G17A, G26A, 5.32, and C11. Ant III, the third antenna segment; Ant II, the second antenna segment. Numbers in parentheses signify actual numbers of antennae counted.

*The arista transformation is qualitatively different between the proteins. G17 produced a thickened arista including the basal cylinder, but the size of the arista was like that in wild type, while K17 transformed the whole arista including the basal cylinder into two to five tarsus-like segments with a short residual arista structure attached at the tip. The phenotypic effect of G26 is limited to thickening of the basal cylinder at very low penetrance, while K26 transformed the basal cylinder to tarsus like structure with bristles, and the length of the arista was shortened (see Fig. 3).

†For G17, only about 10% of the heat-shocked larvae metamorphosed into pharate adults.

Table 4. Summary of the homeotic transformations caused by the hybrid (G17 and G26) and mutant hybrid (K17 and K26) proteins

Transformation	wt Scr	wt Antp	G17	K17	G26	K26
Embryonic						
Head involution defect	++	+++	++	+++	+	++
Ectopic anterior denticles	−	+++	−	+++	−	+
T1 beard suppression	−	+++	−	+++	−	++
Ectopic T2/T3 beard	+++	−	+++	−	+	−
Adult head						
Arista to tarsus*	++	+++	++	++	+/−	++
Ant III/II to T2 leg structures†	−	+++	−	+++	−	+
Reduction of eyes	++	+++	++	++	+	+
Cephalothorax	++	+++	++	++	−	−

wt, Wild type.
*Including the thickening of arista.
†Transformation of the second and the third antenna segments to the mesothoracic leg structures with bract and apical bristles.

caused transformation of other parts of the body including the thoracic and abdominal segments.

DISCUSSION

By exchanging amino acid residues 1, 4, 6, and 7 between the Antp and Scr proteins, we have shown that the functional specificity of the Antp protein is determined by these four amino acids located in the flexible arm of the homeodomain. Changing the four amino acid residues to those of the Antp homeodomain switches the functional identity of the chimeric proteins, which originally showed either strong (G17) or weak (G26) Scr phenotype, to that of Antp (see the summary in Table 4). The change in specificity between G17 and K17 strongly suggests that, when compared with Scr, the four amino acids are sufficient to confer the Antp-like identity in transgenic flies. Although we cannot rule out the possibility that the Antp-derived N-terminal general potentiation domain present in K17 and K26 proteins could also contribute to this specificity, the strong Scr activity of G17 protein with this Antp domain indicates that the contribution of this region to the Antp identity may be subtle and quantitative rather than qualitative. On the other hand, the strong suppression of Scr activity of G26 protein as compared with G17 reveals that the C terminus of the Scr protein outside of the homeodomain is required for the optimal Scr activity. Whether this part of the Scr protein contributes to the Scr activity in a qualitative way awaits further characterization. However, this C terminus seems to confer a strong Cephalothorax-like activity to G17, K17, and Scr proteins, indicating that the sequence might carry some qualitative information.

The importance of the four amino acid residues for the functional specificity of the Antp homeodomain is consistent with the functional conservation of a homologous vertebrate homeodomain protein. One of the mouse Antp homologs, Hox-2.2 (Hox B6), when ectopically expressed in developing *Drosophila* embryos by heat shock, can induce homeotic transformations that are nearly identical to those caused by the heat shock Antp protein (14). The protein sequence of the Hox B6 protein is highly diverged from that of the Antp protein except for its homeodomain and the short stretches that include the amino terminus of the protein and the Tyr-Pro-Trp-Met (YPWM) motif. We have noticed that three of the four amino acids at positions 4, 6, and 7 in the N-terminal arm of the Hox B6 homeodomain are identical to those of the Antp homeodomain (Fig. 4).

Our results are complementary to those of Zeng *et al.* (15) who showed that, by placing the Scr homeodomain in the Antp protein context, amino acids at position 1, 4, 6, and 7 in the N terminus of the Scr homeodomain confer Scr-like functional specificity. Recently, Zhao *et al.* (16) have shown that flies that carry the mouse *Hox-1.3* (*HoxA5*) gene under a *Hsp70* promoter display Scr-like homeotic transformations after heat shock. It is intriguing that the amino acids at the four positions in the N-terminus of the Hox A5 homeodomain are also diverged from those of the Antp homeodomain, while the residue at position 6 (threonine) is identical to that of the *Drosophila* Scr homeodomain (Fig. 4).

Lin and McGinnis (17) have mapped the amino acids required for the functional identity of the Deformed (Dfd) and Ultrabithorax (Ubx) proteins by similar heat shock assays in flies. They have shown that, in the context of a Dfd protein, the N-terminal amino acids of the homeodomain are sufficient to switch from Dfd to Ubx-like targeting specificity, although the resulting chimeric protein activates the *Antp* gene, which is opposite to the normal regulatory circuit in which Ubx protein represses the *Antp* gene. On the other hand, in addition to the N-terminal arm of the homeodomain, the sequence at the C terminus of the homeodomain is also required for the functional identity of the Dfd protein. The functional specificity of the Ubx homeodomain has also been investigated by Chan and Mann (18) who showed that, when compared to the Antp homeodomain, changing six out of the seven homeodomain differences and the residues immediately adjacent to the C terminus of the homeodomain switches the functional specificity from that of Antp to Ubx.

So far we know nothing about how the homeodomain N-terminal arm determines the functional identity in development. However, the three-dimensional structures of the homeodomain–DNA complexes of the Antp (6), engrailed (7) and MATα2 (8) proteins show that the N-terminal arm is located in the minor groove of the DNA. Moreover, recent data on an extended Antp homeodomain polypeptide that includes the conserved YPWM motif suggests that the homeodomain is connected to the N-terminal potentiation domain through a flexible linker that contains the YPWM motif and the homeodomain N-terminal amino acid residues (19).

The close proximity of the homeodomain N-terminal residues to the DNA in the DNA–protein complexes raises the possibility that the four amino acids might determine the

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                        Helix 1                  Helix 2           Helix 3 & 4

                     10          20            30          40        50          60
                     *           *             *           *         *           *
Antp      RKRGRQTYT  RYQTLELEKEFH  FNRYLT  RRRRIEIAHAL  CLT  ERQIKIWFQNRRMKWKKE  N

Hox-2.2   GR-------  ------------  Y-----  -----------  ---  ------------------  S

Ubx       -R-------  ------------  T-H---  ------M----  ---  --------------L---  I

Scr       T--Q-TS--  ------------  ------  -----------  ---  ------------------  H

Hox-1.3   G--A-TA--  ------------  ------  -----------  --S  -----------------D  -
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FIG. 4. Comparison of the Antennapedia homeodomain with those of Hox 2.2 (Hox B6), Ubx, Scr, and Hox 1.3 (Hox A5) proteins. Dashes indicate identical amino acids.

functional specificity by regulating DNA-binding specificity although none of them directly contacts base pairs in the DNA-binding site. Only position 6 contacts the sugar-phosphate backbone of DNA in the Antp and engrailed DNA complexes (7, 20, 21). A preliminary *in vitro* DNA binding study with a purified chimeric homeodomain that introduces the Scr N-terminal four amino acids into the sequence of the Antp homeodomain suggests that changing the four amino acids results in very similar but distinct affinities for a series of synthetic binding sites (G. Halder, personal communication), suggesting that the small difference in the DNA binding caused by the four amino acids could be crucial for developmental functional specificity. It is intriguing that *Scr*, *Ubx*, and *Abd-B* genes are haplo-insufficient in flies (22), indicating that only a 2-fold difference in their gene dosages affects their phenotypes. Moreover, tiny differences in DNA-binding affinities between the λ phage repressor proteins and the operators are also known to be important for switching between the phage life cycles (23).

Genetic control through selective protein–protein interaction with other specific transcription factors is also plausible. In yeast, the homeodomain-containing protein MATα2 controls cell-type specificity by association with other proteins. In the α-haploid cells, α2 protein represses the transcription of the **a**-specific genes by associating with MCM1 protein while in **a**/α diploid cells, α2 represses another set of targets, the haploid specific genes by heterodimerization with MATa1 protein, which is another homeodomain-containing protein (ref. 24; reviewed in ref. 25). It is noteworthy that amino acids in the flexible extension of the α2 homeodomain mediate the interaction between α2 and MCM1 proteins (26). I-POU, a member of the POU domain proteins expressed in the developing *Drosophila* nervous system, forms a high-affinity heterodimer with a second POU transcription factor, Cf1-a. tI-POU, an alternatively spliced product of the I-POU gene, is incapable of dimerization with Cf1-a because of two basic amino acid residues that are absent in I-POU in the N-terminal arm of the homeodomain (27). Mutation of another *Drosophila* gene, extradenticle (*exd*), alters segmental identity without affecting the pattern of expression of homeotic selector genes possibly affecting their targeting specificity *in vivo* (28). In zygotic *exd* null mutants, the segmental identity specified by the Antp protein seems to be altered into prothoracic identity, which is specified by Scr in wild-type flies. It is noteworthy that mutations of *exd* alter the functional identities of homeotic proteins even when the proteins are ectopically expressed under the control of a heat shock promoter.

A molecular switch that recruits both specific DNA binding and selective protein–protein interaction is also conceivable. Since the flexible N-terminal arm of the Antp-class homeodomains is necessary for efficient DNA binding (29), interaction of this part of the homeodomain with other regulatory factors may block the specific target site binding of the homeodomain if the associating factors fail to bind the target DNA site either because the DNA sequences at the homeodomain binding site are not bound by the factors or because the factors are themselves non-DNA-binding ones. This kind of simple molecular switch provides selective target gene-dependent modulations by the homeodomain-containing proteins in various cell types in development.

The mechanisms that provide functional specificity with little observable difference in DNA binding are not confined to homeodomain proteins. The muscle-specific transcriptional activation by MyoD protein is mediated by the specific amino acid residues in the basic region of the basic helix–loop–helix (bHLH) protein presumably through interaction with a recognition factor that confers differential functional specificities to various bHLH proteins of almost identical DNA binding specificities (30). Biochemical and genetic characterization of this kind of developmental modulatory factors should lead to the better understanding of the regulatory mechanisms of animal development.

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