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Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity

Wenlin Zeng, Deborah J. Andrew, Laura D. Mathies, Michael A. Horner and Matthew P. Scott*

Departments of Developmental Biology and Genetics, Stanford University School of Medicine, Stanford, California 94305-5427, USA

*Author for correspondence

SUMMARY

The transcription factors encoded by homeotic genes determine cell fates during development. Each homeotic protein causes cells to follow a distinct pathway, presumably by differentially regulating downstream 'target' genes. The homeodomain, the DNA-binding part of homeotic proteins, is necessary for conferring the specificity of each homeotic protein's action. The two *Drosophila* homeotic proteins encoded by *Antennapedia* and *Sex combs reduced* determine cell fates in the epidermis and internal tissues of the posterior head and thorax. Genes encoding chimeric Antp/Scr proteins were introduced into flies and their effects on morphology and target gene regulation observed. We find that the N terminus of the homeodomain is critical for determining the specific effects of these homeotic proteins in vivo, but other parts of the proteins have some influence as well. The N-terminal part of the homeodomain has been observed, in crystal structures and in NMR studies in solution, to contact the minor groove of the DNA. The different effects of Antennapedia and Sex combs reduced proteins in vivo may depend on differences in DNA binding, protein-protein interactions, or both.

Key words: homeodomian, *Antp*, *Scr*, chimeric protein, functional specificity

INTRODUCTION

In insects and vertebrates, homeotic mutations cause one part of an animal to develop as a copy of another part, due to altered control of cell fates (Akam, 1987). In *Drosophila*, many of the most dramatic homeotic mutations map to two gene clusters called the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C) (Duncan, 1987; Kaufman et al., 1990). There are five ANT-C homeotic genes, all controlling head or thoracic development, including *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*). Each gene is expressed in different, though sometimes overlapping, regions of the body. The protein products are transcription factors that direct cells into the proper developmental pathways by coordinating the activities of downstream 'target' genes (Andrew and Scott, 1992).

During embryogenesis and metamorphosis, several head segments, three thoracic segments and eight abdominal body segments are formed (Campos-Ortega and Hartenstein, 1985). Each segment develops a characteristic set of structures, first when the larva develops and again when metamorphosis occurs and adult structures are produced. Products of the *Antp* gene accumulate in the thoracic segment primordia and in lower amounts in the abdominal primordia (Levine et al., 1983; Carroll et al., 1986). The *Sex combs reduced* (*Scr*) gene is expressed and required in the labium, a posterior head segment, and in the anterior portion of the first thoracic segment just anterior to the domain of *Antp* action (Kuroiwa et al., 1985; Mahaffey and Kaufman, 1987; Riley et al., 1987).

The roles of *Antp* and *Scr* are revealed by loss-of-function and gain-of-function mutations (Wakimoto and Kaufman, 1981; Struhl, 1982; Wakimoto et al., 1984; Sato et al., 1985; Schneuwly and Gehring, 1985; Martinez-Arias, 1986). Loss-of-function mutations show the locations where the gene is normally needed. The absence of *Antp* function results in the death of developing larvae, and the transformation of their second and third thoracic segments (T2 and T3) into more anterior structures with some characteristics of the first thoracic segment (T1) and some of head segments. In addition, *Antp* is required in the midgut mesoderm to direct the formation of a transverse constriction of the gut tube (Tremml and Bienz, 1989; Reuter and Scott, 1990), and for proper peripheral nervous system development (Heuer and Kaufman, 1992). The loss of *Scr* function, which is lethal to the animal, causes a transformation of T1 into T2 and of the labial segment into a copy of the next most anterior head segment, the maxillary segment. *Scr* is also required for the proper formation of the gastric caeca in the developing midgut (Reuter and Scott, 1990), the salivary glands (Panzer et al., 1992) and the peripheral nervous system (Heuer and Kaufman, 1992). In *Scr* mutants, the sali-

vary gland placodes, precursors to the invagination that forms the gland, and the gastric caeca, four tubes that evaginate from the anterior midgut, do not form.

Gain-of-function alleles of both *Antp* and *Scr* activate the homeotic gene in new places, thus revealing the capacity of the gene to predominate or interfere in tissues where it is not normally active. The best known gain-of-function *Antp* alleles cause the development of adult legs in lieu of antennae, due to the activation of *Antp* in the head where it is normally repressed (Denell et al., 1981; Hazelrigg and Kaufman, 1983; Frischer et al., 1986; Schneuwly et al., 1987b). In addition to 'spontaneous' gain-of-function mutations, engineered genes have been introduced into flies to produce a high level of a homeotic protein in all cells. Like dominant alleles, heat-shock promoter-driven *Antp* (HS-*Antp*) causes the development of antennae in place of legs (Schneuwly et al., 1987a). In embryos, HS-*Antp* causes the transformation of head segments into thoracic segments and T1 into T2, but has no effect in the regions posterior to the normal thoracic domain of *Antp* expression (Gibson and Gehring, 1988).

Gain-of-function *Scr* alleles have parts of the adult T2 and T3 segments transformed into T1 (Tokunaga and Stern, 1965; Tokunaga, 1966), due to expression of Scr protein in T2 and T3 in addition to its normal site of expression in T1 (Pattatucci and Kaufman, 1991). The corresponding HS-*Scr* construct transforms T2 and T3 into copies of T1 in the embryo, just like the dominant gain-of-function *Scr* alleles (Gibson et. al., 1990; this paper). HS-*Scr* does not induce sex combs, a marker of T1 legs, on the second and third legs of adults, in contrast to gain-of-function *Scr* mutants, for reasons unknown. HS-*Scr* also leads to the ectopic induction of a gene normally expressed in the developing embryonic salivary gland (Panzer et al., 1992).

Ectopic expression assays using a heat-inducible promoter were done because it is not yet possible to do gene replacement experiments with these large genes. The distinct effects of ubiquitous expression of *Antp* and *Scr* afford an opportunity to determine which parts of the proteins are most important for determining their specific actions. Each protein contains a region called the homeodomain, an approximately 60 amino acid DNA-binding domain composed of three alpha helical regions similar in structure to a class of bacterial DNA-binding proteins and to two products of the yeast mating type locus MAT (Steitz, 1990; Pabo and Sauer, 1992). Previous work revealed the homeodomain to be a critical determinant of specificity of homeotic protein action in vivo (Gibson et al., 1990; Mann and Hogness, 1990; Lin and McGinnis, 1992). Crystal structure studies of a fly segmentation protein homeodomain-DNA complex (Kissinger et al., 1990) and of a yeast MAT homeodomain-DNA complex (Wolberger et al., 1991), and NMR studies of an Antp homeodomain-DNA complex (Otting et al., 1990) indicate that the third helical region sits in the major groove of the DNA while the other two helices lie over it. The N-terminal arm of the homeodomain contacts the minor groove of the DNA. Little is known about the structure or function of other parts of homeotic proteins.

In this study, chimeric proteins were used to define more precisely which parts of the Antp and Scr proteins determine their specific actions. The morphological effects of ectopic expression of Antp/Scr chimeric proteins and the regulation of downstream genes by the proteins were examined. We find that the N terminus of the homeodomain is critical for determining the specific effects of Antp and Scr proteins, in agreement with studies of Dfd/Ubx homeotic proteins (Lin and McGinnis, 1992).

MATERIALS AND METHODS

Design and construction of chimeric genes

To prepare the HS-*Scr* construct, a 3.2 kb *Dra*I fragment from the *Scr* cDNA containing the entire open reading frame was ligated to *Eco*RI linkers and subcloned into the *Eco*RI site of the P-element hsp70 vector pABAL. The HS-*Antp* construct was made by Gary Winslow. A 1.7 kb *Bal*I and *Bgl*II fragment from the G1100 *Antp* cDNA (Laughon et al., 1986) which contains the entire open reading frame was ligated to *Bgl*II linkers and subcloned into the *Bam*HI site of pABAL.

Chimeric genes of *Antp* and *Scr* were constructed by PCR amplification of specific regions from the two genes, ligation of these fragments and subsequent cloning of the ligated fragments into the vector pABAL. Five oligodeoxynucleotides were synthesized as primers for PCR amplifications. Their strand specificities and positions are indicated in Fig. 1A. Since there is no convenient restriction site available, we introduced a *Bsp*MI site to the 5′ ends of the primers so that after cleavage with *Bsp*MI the two ends of the fragments that need to be ligated have unique complementary cohesive ends.

To construct HS-*ASH* three DNA fragments, the *Antp* N-terminal fragment, the *Scr* homeobox fragment and the *Antp* C-terminal fragment, were generated. The DNA template for amplifying the two *Antp* DNA fragments was pGBB, a plasmid containing the same 1.7 kb *Bal*I to *Bgl*II fragment of *Antp* cDNA cloned into the *Bgl*II site of the vector pGEM-7Zf. Two commercial primers corresponding to the T7 and SP6 promoter regions of the vector were used in addition to primers Antp 1 and Antp 2 to amplify the two fragments. The DNA template for generating the *Scr* homeobox fragment was pGEM-*Scr*, a plasmid with a 3.6 kb *Eco*RI fragment of the *Scr* cDNA cloned at the *Eco*RI site of the vector pGEM-7Zf. The two primers used were Scr 1 and Scr 2 (Fig. 1A). The three DNA fragments were digested with the restriction enzyme *Bsp*MI to generate cohesive ends. Digested fragments were gel purified and ligated. The ligated fragment was digested with *Mst*II and *Bam*HI. *Mst*II cleaves once in the coding region of the *Antp* N-terminal fragment and *Bam*HI cleaves near the 5′ end of the *Antp* C-terminal fragment. This *Mst*II and *Bam*HI fragment was isolated and used to replace the region between the *Mst*II and *Bam*HI sites in the HS-*Antp* construct.

HS-*A*^{m}*SC* was constructed using a similar strategy. Primers T7 and Scr 2 were used to generate the fragment containing the *Antp* N-terminal and homeobox regions. There is a one nucleotide mismatch between the sequences of the Scr 2 primer and the *Antp* homeobox; however, this nucleotide difference does not alter the amino acid sequence. The *Scr* C-terminal fragment was generated by PCR with the primers T7 and Scr 3.

HS-*ASHC* was constructed as follows: HS-*ASH* was digested with *Bam*HI, which cleaves in the region downstream of the homeobox. The resulting *Bam*HI end was filled in and the plasmid digested again with *Bgl*II, which cleaves within the homeobox. The larger fragment containing the vector and part of the *Antp* and *Scr* sequences was purified. Similarly, plasmid pGEM-*Scr* was digested with *Xba*I which cleaves at the position 1.6 kb downstream of the translation stop codon. This *Xba*I end was filled in and the plasmid digested again with *Bgl*II. The *Bgl*II/*Xba*I (filled-in) fragment containing the *Scr* homeobox and C-terminal region was isolated and subcloned into the purified vector fragment.

Sequences from the regions of the constructs that had been derived from PCR amplification were determined using the dideoxynucleotide sequencing method (Sanger et al., 1977) to ensure that no nucleotide changes had been introduced.

All constructs were co-injected with pD2-3 transposase DNA as helper (D. Rio, unpublished) into *Df(1)w*^{1118} embryos as previously described (Rubin and Spradling, 1982). At least three independent transformants of each construct were obtained.

Heat shocks

To examine the cuticle phenotype, embryos were collected for 1 or 2 hours and aged at 25°C for 6.5 hours after the midpoint of the collection. The embryos were transferred to a mesh screen and placed in a 37°C water bath for 20 to 60 minutes. After heat shocking, the embryos were returned to 25°C and allowed to develop for approximately 30-36 hours. Embryos were dechorionated with 50% bleach for 2 minutes, rinsed with water, and fixed in 1× PBS, 3.7% formaldehyde, 50% heptane by rotating end over end for 20 minutes. The vitelline membrane was removed by replacing the aqueous phase of the fix solution with methanol and vortexing for 1 minute. The embryos were cleared in acetic acid/glycerol and subsequently mounted in Hoyer's mountant (van der Meer, 1977). All cuticle preparations were examined and photographed using dark-field optics.

To examine induction of salivary glands, embryos were collected for 4 hours, aged for 1 hour, and heat shocked at 37°C for either 20, 30 or 45 minutes. The embryos were then aged at room temperature for approximately 12 hours, fixed and stained with antibodies.

To examine *teashirt* induction in the midgut, embryos were collected for 12 hours and heat shocked three times for 30 minutes at 37°C with a recovery at 18°C for 30 minutes between heat shocks. The embryos were allowed to recover at 25°C for 3 hours, fixed and stained for teashirt protein expression.

Heat-shock experiments to observe the adult transformation were done as follows: 1 to 2 hour collections of embryos were taken on agar plates and were allowed to develop for 24 hours at 25°C. The emerged first instar larvae were then transferred to fresh food vials. The embryos were allowed to develop at 25°C for 68 hours after the midpoint of the collection. The food vial containing the larvae were placed in a 37°C water bath and heat shocked for 10 minutes to 1 hour four times with minimum 4 hours recovery at 25°C between heat shocks. Heads of the adult flies were dissected, treated in acetic acid/glycerol and mounted in Hoyer's mountant.

For HS-*Antp*, HS-*Scr* and HS-*A*^{m}*SC*, the strongest transformations were obtained by heat shocking embryos at 6 to 7 hours AEL for 60 minutes at 37°C. Except for the mutant phenotypes induced by the ectopically expressed proteins, most cuticles were otherwise morphologically normal, even when regimens with longer heat-shock times were used. However, HS-*ASH* and HS-*ASHC* require much less heat-shock times, with 30 minutes and 20 minutes heat shocks, respectively, giving optimum effects. Longer heat inductions result in severe defects in the morphology of the cuticles including a general reduction in the number of denticles (data not shown).

Antibody staining

Fixation and hybridizaton were performed as described by Reuter et al. (1990). The primary antibody was a rat polyclonal antisera made aganst a *tsh*-trpE fusion protein produced in *E. coli*. The 2.5 kb *tsh* cDNA described by Röder et al. (1992), encoding the C-terminal 770 amino acids of the tsh protein was cloned into the *Eco*RI site of the pATH11 vector (Koerner et al., 1991). The tsh-trpE fusion protein was expressed in TG1 cells, isolated by gel purification and antibodies were produced in rats. Diluted whole serum was used for staining. The antibody was shown to be specific for teashirt protein by the absence of staining observed in embryos completely lacking *teashirt* function (data not shown). A biotin-conjugated secondary antibody (Jackson Labs) and the Vectastain elite peroxidase detection kit (Vector Labs) were used for immunolocalization.

Salivary gland induction was assayed by the ectopic expression of a salivary gland-specific enhancer trap line called B204, detected using a monoclonal anti-β-galactosidase antibody (Promega).

Scr protein levels were assayed using a monoclonal antibody (Glicksman and Brower, 1988a). Antp and Antp/Scr chimeric protein levels were assayed using monoclonal antibody 4C3 (Glicksman and Brower, 1988b), which is directed against the N-terminal portion of the Antp protein (S. Hayashi and M.P.S., unpublished).

Fly culture and crosses

Fly stocks are described in Lindsley and Zimm (1992) and in referenced papers. All fly stocks were raised at 25°C on cornmeal and molasses media.

RESULTS

In order to determine the contributions of different parts of the Antp and Scr proteins to their specific actions, the effects of normal and chimeric proteins were tested in several different assays in vivo.

Construction and expression of *Antp/Scr* chimeric genes

The Antp protein is composed of 378 amino acids of which the homeodomain is amino acids 296 through 355. The Scr protein contains 417 amino acids and has its homeodomain in positions 323 through 382. (D. J. A. and M. P. S., manuscript in preparation; our sequence differs from that previously reported). Lines of flies expressing each protein under the control of a heat-inducible promoter were established and the effects of the introduced genes were monitored in embryos and in adults.

The Antp and Scr homeodomains differ by only four amino acid residues, all located in the N terminus of the homeodomain (Fig. 1B). The remaining sequences of the two homeodomains, including the so-called 'recognition' helix, are identical. The sequences outside the homeodomains of the Antp and Scr proteins have little resemblance to each other except for the sequence YPWM located a short distance N-terminal to the homeodomain. Previous studies (Gibson et. al., 1990) showed that a chimeric protein with a Scr homeodomain and Scr sequences N-terminal (79 aa) and C-terminal (35 aa) to the homeodomain, but otherwise composed of Antp sequence, behaves like Scr protein in ectopic expression tests in the epidermis. It was not determined whether the behavior of the chimeric protein was due to the homeodomain or to the flanking sequences.

We constructed a chimeric *Antp/Scr* gene, HS-*ASH*, which has the homeobox of the *Antp* gene precisely replaced by the homeobox of *Scr* (Fig. 2). The sequence of the ASH protein (Antp protein with a Scr homeodomain, hence ASH) is identical to that of Antp except for the four amino acid residues in the N-terminal region of the homeodomain, which have been changed to the corresponding Scr residues. To determine whether sequences in the C terminus are also required to distinguish the specific activities of the two pro-

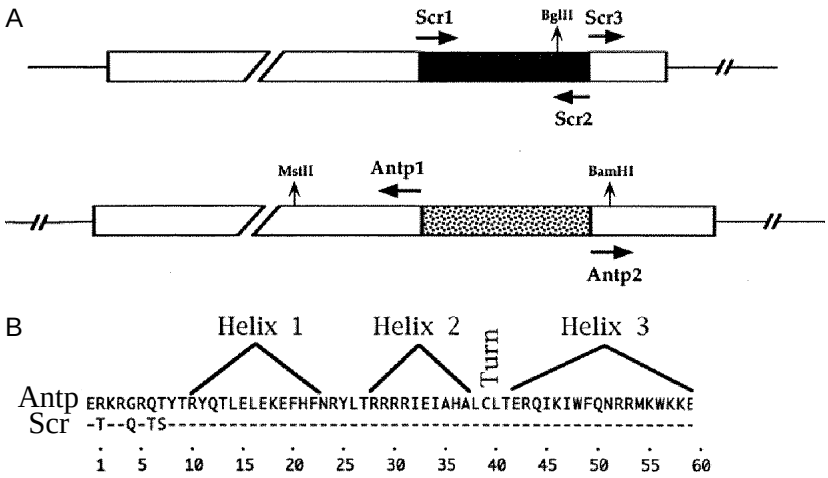

Fig. 1. Design and construction of chimeric proteins. (A) Diagrams of the *Antp* and *Scr* cDNAs. The coding regions are represented by the bar and the homeodomains are indicated by the shaded area. The five primers, Antp 1, Antp 2, Scr 1, Scr 2 and Scr 3, are indicated by arrows underlining or overlining their corresponding sequences in the diagrams. The arrows indicate the 5′ to 3′ direction. Three of the restriction enzyme sites used in cloning the PCR fragments are indicated by the arrows. (B) Amino acid sequences of the Antp and Scr homeodomains. Dashes represent residues that are identical in both sequences. The positions of the three alpha helices are overlined.

teins, the chimeric gene HS-*ASHC* was also constructed. HS-*ASHC* has the *Antp* homeobox and C-terminal coding sequence replaced with the corresponding *Scr* sequence. A third chimeric gene, HS-*A^{m}SC*, has only the coding sequence downstream of the homeobox replaced by the corresponding *Scr* sequence. A 12 amino acid epitope tag from the mouse *c-myc* gene was inserted between residues 135 and 136 in the N-terminal half of the A^{m}SC protein. This was done for reasons unrelated to the present study to distinguish chimeric protein expression from the endogenous Antp protein with antibodies against the c-*myc* epitope. All chimeric genes were fused to the heat-inducible promoter from the *Drosophila hsp70* gene in the pABAL vector and introduced into the fly genome using P-element transformation (Spradling and Rubin, 1982). Further details of the construction of these chimeric genes are provided in Materials and Methods. Three independent lines of flies carrying each of the three chimeric genes were generated and analyzed.

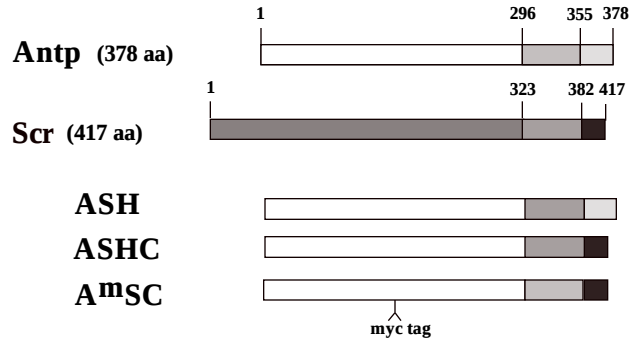

Fig. 2. Diagrams of Antp/Scr chimeric proteins. Specific regions of the Antp protein were replaced with the corresponding regions of the Scr protein. The proteins, have just the homeodomain precisely replaced (ASH), the homeodomain plus the sequence C-terminal to the homeodomain (ASHC), or just the sequence C-terminal to the homeodomain (A^{m}SC). A^{m}SC also has a myc epitope tag inserted into the glutamine-rich region. Open bar, crossed bar and the bar with a back slash represent the sequences N-terminal to the homeodomain, the homeodomain and the sequence C-terminal to the homeodomain of Antp protein. The light-shaded bar, the slashed bar and the dark-shaded bar represent the corresponding sequences of the Scr protein.

The ectopic expression of the different heat-inducible genes was examined by immunostaining whole embryos with a monoclonal α-*Antp* antibody that recognizes an epitope N-terminal to the Antp homeodomain (S. Hayashi and M.P.S., unpublished data). The three proteins are identical in the region where the antibody reacts. Embryos carrying the HS-*Antp* gene or one of the chimeric genes were stained with the antibody. Without heat induction, only the wild-type expression pattern of the endogenous Antp protein was observed. If the embryos were heat shocked for 1 hour at 37°C and allowed to recover for one hour at 25°C, the nuclei of all cells were found to be strongly stained (data not shown). No discernible variations in staining intensities were observed either within a set of three lines carrying the same transgene or between lines carrying different transgenes. Protein levels were found to remain high 3 hours after heat induction suggesting that the proteins are quite stable, in contrast to some other heat-shock-induced regulatory proteins, which decay rapidly (eg Noordermeer et al., 1992; Lin and McGinnis, 1992). High-level, stable expression of Scr protein from the HS-*Scr* gene was also observed, using a monoclonal antibody against the Scr protein(data not shown). Because two different antibodies were used, it is difficult to compare the expression levels of the Antp and chimeric proteins with the levels of the Scr protein.

The N-terminal region of the homeodomain is sufficient to distinguish the specificity of the Scr protein from that of Antp

We used four different assays to determine whether the Antp/Scr chimeric proteins behave like one or the other parental protein: (1) development of the larval cuticle, which is different for HS-*Scr* and HS-*Antp*, (2) induction of ectopic anterior *teashirt* expression in the embryonic midgut and head epidermis, an *Antp* property, (3) induction of ectopic larval salivary glands, an *Scr* property, and (4) transformations of the adult head, which are different for HS-*Scr* and HS-*Antp*. The results of all four assays are summarized in Table 1.

Assay one: larval cuticle transformations caused by the ectopic expression of the chimeric proteins

The cuticle from a heat-shocked *Df(1)w^{1118}* animal, the host

strain for our injections, is morphologically indistinguishable from a wild-type animal (Fig. 3A). At this stage of development, most of the head structures of the embryo have involuted and are located inside the anterior end. The first thoracic segment can be readily distinguished from the others by the shape and the size of its denticle belt on the

Table 1. Summary of the effects of HS-*Antp*, HS-*Scr*, HS-*ASH*, HS-*ASHC* and HS-*A^{m}SC* in four different assays

	HS-*Antp*	HS-*A^{m}SC*	HS-*Scr*	HS-*ASH*	HS-*ASHC*
Assay one - effects on cuticle:					
Failure of head involution	+++	+++	+++	+++	+++
Ectopic denticles in the head	+++	+++	–	–	–
Loss of T1 beard	+++	++	–	–	–
Ectopic beards in T2, T3 segments	–	–	+++	+	++
Thickening of T2 and T3 denticle belts	–	–	++	–	–
Assay two - regulation of *tsh*:					
Ectopic induction of *tsh* in the head	+++	++	–	–	–
Ectopic induction of *tsh* in midgut	+++	++	–	–	–
Assay three - effects on salivary gland					
Induction of ectopic salivary gland	–	–	+++	+	++
Inhibition of endogenous salivary gland	+++	–	–	–	–
Assay four - effects on adult head:					
Antenna-to-leg transformation	(+++) Antenna to T2 leg	(++) Antenna to T2 leg	Aristae (only) thickened	Aristae thickened and second antennal segment transformed into femur	
Cephalothorax transformation	(+) Denticles on the head	(++) Novel, head to thorax	–	–	(+++) Novel, head to thorax
Eye size reduction	+++	+++	+	+	+++

+++, strong effect; ++, medium effect; +, weak effect; –, no effect.

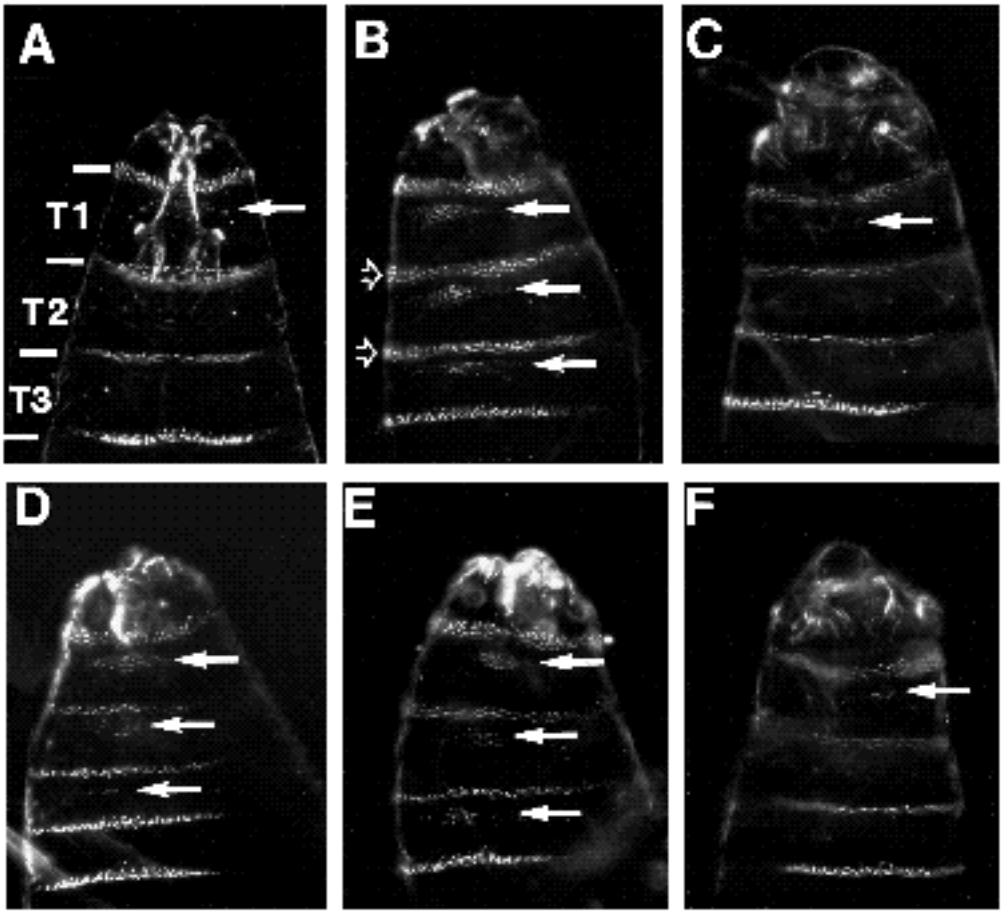

Fig. 3. Cuticle transformations caused by the ectopic expression of Antp, Scr and the Antp/Scr chimeric proteins. Embryos were heat shocked at 37°C for 60 minutes (A-D), or 30 minutes (E,F) at 7 hours AEL, and were allowed to develop into first-instar larvae at 25°C. The anterior halves of the larval cuticles were photographed and shown. (A) *Df(1)w* cuticle, the host strain for the P-element transformation. T1, T2 and T3 indicate the three thoracic segments. Arrow points to the 'beard' that is characteristic of T1. (B) Effect of HS-*Scr*. The ectopic T1 'beards' in the T2 and T3 segments are indicated by the arrows. (C) Effect of HS-*Antp*. T1 segment was transformed into T2 by the disappearance of the 'beard' in the most anterior thoracic segment (arrow). (D) Effect of HS-*ASH*. ASH protein acts like the Scr protein by inducing the formation of ectopic T1 'beards' in the two posterior segments (arrows). (E) Effect of HS-*ASHC*. The ASHC protein acts like the Scr protein. The two ectopic T1 'beards' are indicated by the arrows. Notice that the two ectopic 'beards' in E are bigger than those in D. (F) Effect of HS-*A^{m}SC*. The A^{m}SC protein acts like the Antp protein. Arrow indicates that the T1 'beard' is missing in the most anterior thoracic segment. Ectopic expression of each of the proteins blocks the proper involution of head structures.

ventral surface of the cuticle. The prothoracic segment (T1) has an extra patch of denticles called the 'beard' located posterior to the main T1 denticle belt (Fig. 3A, arrow). The anterior T1 denticle belt is also broader than those of the mesothoracic and metathoracic segments (T2 and T3).

After a one hour of treatment at 37°C, ectopically expressed Antp protein in the embryo causes the transformation of T1 into a T2 segment, as can be seen by the disappearance of the 'beard' (the small posterior patch of denticles in the first thoracic segment) from T1 and the decreased width of the anterior T1 denticle belt (Fig. 3C). In addition, the involution of head structures was completely blocked. Ectopic denticles were also observed on the dorsal surface of the head (data not shown).

Ectopic expression of the Scr protein has a very different effect: it causes ectopic T1 'beards' to appear in T2 and T3, indicating that both the T2 and T3 segments have been transformed into T1 (Fig. 3B). The denticle belts of the two transformed segments are thicker, also indicating a T1 identity. Head involution is blocked by the ectopic Scr protein. However, the head defects are distinct from those caused by Antp : Antp protein causes a complete failure of head involution whereas in HS-*Scr* embryos the head structures gather at the anterior of the embryo and start to involute but fail to complete the process (compare Fig. 3B,C). In addition, no ectopic denticles were seen on the heads transformed by Scr protein. Our observations are consistent with previous reports on the effects of these proteins (Gibson and Gehring, 1988; Gibson et al., 1990). The distinct phenotypes caused by the ectopic expressions of the Antp and Scr proteins allowed us to determine the functional specificities of Antp/Scr chimeric proteins.

The transformation resulting from the ectopic expression of the chimeric protein ASH is clearly *Scr*-like (Fig. 3D). Ectopic T1 'beards' were induced in both of the two posterior thoracic segments indicating that both have been at least partially transformed to a T1 identity. The head defects are identical to the defects caused by the Scr protein. An *Scr*-like transformation is also induced by the chimeric protein ASHC (Fig. 3E). Notice the larger size of the ectopic 'beards' in both of the transformed thoracic segments compared to the corresponding ectopic 'beards' induced by the ASH protein (compare Fig. 3D,E). This phenotype is consistently observed, so the ASHC protein has a stronger transformation effect than the ASH. Thus although the Scr homeodomain is sufficient to cause the chimeric protein to behave like Scr, the sequence C-terminal to the homeodomain contributes to the full activity of the wild-type Scr protein. The greater activity of the ASHC protein is also demonstrated by the frequency of transformations. After heat induction of ASH expression, an average of 18.5% of the embryos have both T2 and T3 segments transformed into T1 segments, while about 50% of the embryos have only the T2 segment transformed into the T1. In contrast, ectopic expression of the ASHC protein causes 78% of the heat-shocked embryos to have both the T2 and T3 segments transformed; 16% of the embryos have only the T2 to T1 transformation. In neither the HS-*ASH* nor the HS-*ASHC* cuticles was there an increase in the size of the denticle belts like that observed with HS-*Scr*. Scr sequences other than the homeodomain and the C terminus may be needed for this activity.

The *Scr*-like transformation of the HS-*ASH* and HS-*ASHC* cuticles is not due to activation of the endogenous *Scr* gene. Embryos containing the HS-*ASH* or HS-*ASHC* construct were heat shocked for 1 hour at 37°C and allowed to recover at 25°C for either 1 or 3 hours. The embryos were stained with a monoclonal antibody against the Scr protein. The antibody, which does not recognize either the ASH or the ASHC chimeric proteins, revealed only wild-type expression patterns of the Scr protein (data not shown). Therefore no ectopic Scr protein was induced from the endogenous *Scr* gene. The transformations caused by the HS-*ASH* and HS-*ASHC* constructs were therefore due to ectopically expressed ASH and ASHC proteins.

Replacing only the C terminus of the Antp protein with that of Scr protein did not switch the protein's specificity. The chimeric protein $A^{m}SC$ still functions like Antp protein in this assay (Fig. 3F). Ectopic expression results in a strong reduction in the size of the T1 denticle 'beard' and a complete failure of head involution. As in the HS-*Antp* cuticles, ectopic denticles were observed on the dorsal surface of the head (data not shown).

Assay two: regulation of *teashirt* expression in the midgut by the chimeric proteins

An additional assay of the functional specificity of Antp/Scr chimeric proteins is provided by the *Antp* target gene, *teashirt (tsh)*. *tsh* is a gene expressed in the trunk of the animal where it prevents head structures from forming (Fasano et al., 1991; Röder et al., 1992), thus acting as a homeotic gene that distinguishes head from trunk. It encodes a putative zinc finger protein and is activated by homeotic genes, including *Antp*, in the epidermis (Röder et al., 1992) and midgut (L. Mathies, S. Kerridge, and M.P.S., in preparation). The expression pattern of *tsh* in heat-shocked control embryos is the same as in untreated wild-type embryos (Fig. 4A). The embryo shown, from the $Df(1)w^{1118}$ stock used as a recipient for P element injections, was heat shocked three times for 30 minutes at 37°C with a recovery of 30 minutes at 18°C between heat shocks. The tsh protein is present in parasegments 3 to 13 in the epidermis. It is also expressed in two regions of the visceral mesoderm in the midgut.

No change in *tsh* expression is seen in HS-*Scr* embryos (Fig. 4B). In contrast, ectopic expression of Antp protein, caused by heat shocking three times for 30 minutes at 37°C results in ectopic expression of tsh protein in the head, anterior to the normal limit of expression at parasegment 3 (Fig. 4C,E). The anterior expression of tsh protein in the epidermis is consistent with the RNA expression of *tsh* in HS-*Antp* embryos reported by Röder et al. (1992). In addition, the anterior domain of *tsh* expression in the midgut is expanded anteriorly in heat-shocked HS-*Antp* embryos.

The chimeric proteins ASH and ASHC do not change *tsh* expression (data not shown), which is consistent with the cuticle transformation results. Thus, a change of four amino acids in the N terminus of the Antp homeodomain (HS-*ASH*) abolishes the ectopic activation of the *tsh* gene observed with HS-*Antp*. The HS-*ASH* and HS-*ASHC* embryos were stained to detect the chimeric proteins and it was verified that they were produced at a level comparable

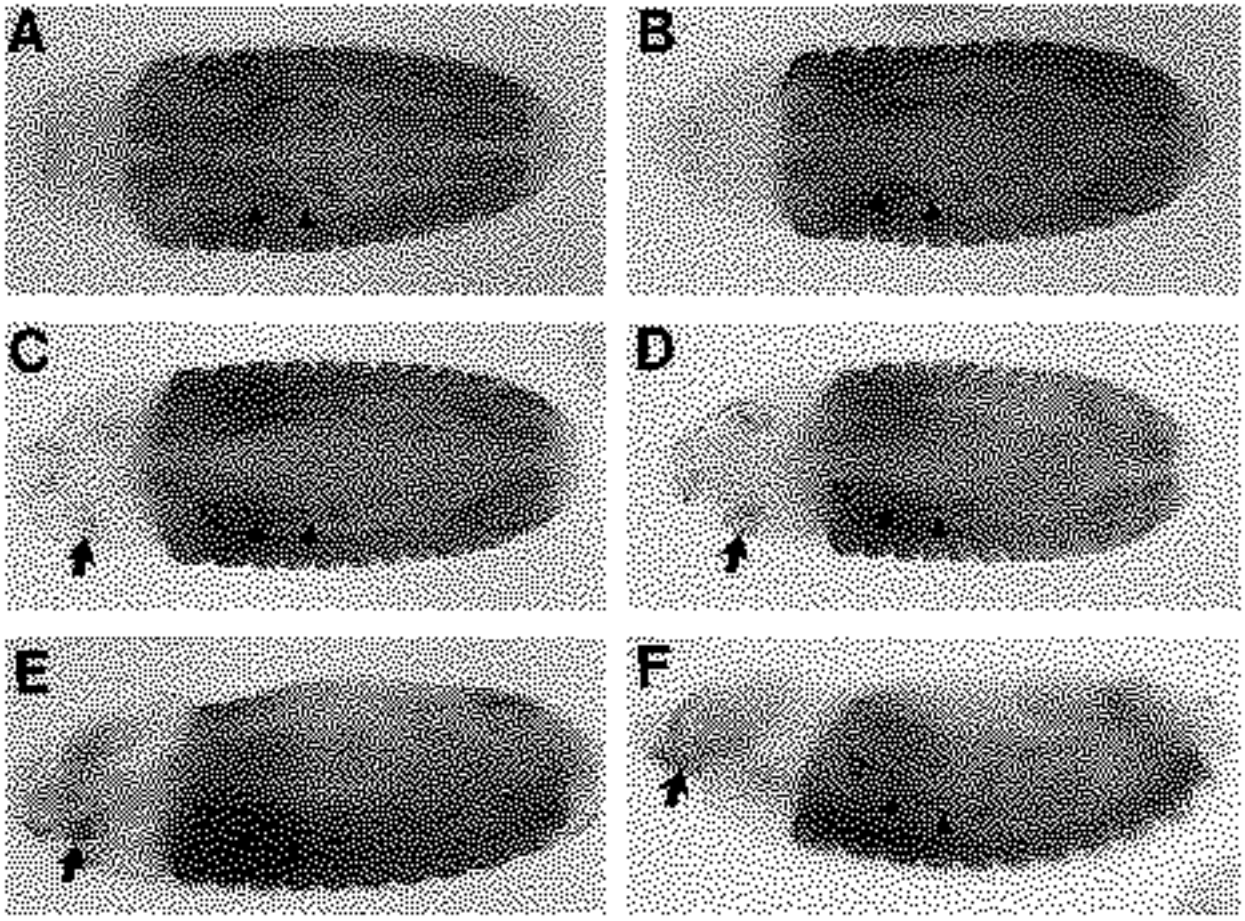

Fig. 4. Effects of ectopic expression of *Scr*, *Antp*, and *Antp*/*Scr* chimeric proteins on *teashirt* expression. Embryos were heat shocked three times for 30 minutes at 37°C with a recovery at 18°C for 30 minutes between heat shocks. Embryos were then stained with antibody to *tsh* protein 3 hours after the heat shock regimen. (A) *Df(1)w* embryos, showing the wild-type expression of *tsh* in the epidermis from PS3 to PS13 and in the visceral mesoderm. The normal limits of *tsh* expression in the anterior visceral mesoderm are denoted by arrowheads. (B) HS-*Scr* transformant embryos have no change in *tsh* expression pattern. Parasagittal (C) and horizontal (E) views of HS-*Antp* transformant embryos demonstrating ectopic expression of *tsh* protein in the head (solid arrows) and in the anterior visceral mesoderm (open arrow). Parasagittal (D) and horizontal (F) views of HS-A^mSC transformant embryos. HS-A^mSC acts as HS-*Antp* to induce ectopic *tsh* protein in the head (solid arrows) and visceral mesoderm (open arrows).

to *Antp* expression due to HS-*Antp* (data not shown). Changing the amino acids C-terminal of the homeodomain did not alter the specificity of the Antp protein with respect to the regulation of *tsh*: ectopic expression of the A^mSC protein results in ectopic *tsh* expression in the epidermis and the visceral mesoderm (Fig. 4D,F).

Assay three: ectopic induction of salivary glands

The larval salivary gland requires *Scr* for its formation. Panzer et al. (1992) demonstrated that loss of *Scr* function results in a complete loss of the salivary glands, and the induced expression of Scr protein in other segments leads to the appearance of ectopic glands, as assayed by the accumulation of a salivary gland marker protein, fork head (*fkh*; Weigel et al., 1989). In contrast, we find that ectopic expression of Antp protein not only fails to induce additional salivary glands (Table 2; Fig. 5C) but inhibits the formation of the endogenous glands. The differential effect of misexpressing either the Scr or Antp proteins on salivary gland formation provides an assay for the specificity of chimeric protein function.

To assay salivary gland formation, we used an enhancer trap line called B204 that is expressed in the salivary gland primordia and continues to be expressed in the salivary glands throughout embryogenesis. Heat-shock treatments and staining are described in Materials and Methods. We find that embryos induced to express any of the genes that encode an Scr homeodomain, i.e. HS-*Scr*, HS-*ASHC*, HS-*ASH*, as well as a chimeric protein construct with the Scr homeodomain and next six amino acids, HS-*ASH6*, form ectopic salivary glands (Table 2; Fig. 5D,E,F and H). Additional salivary glands are detected only in parasegments 0 and 1, anterior to the normal salivary glands in parasegment 2. Ectopic glands are not found in embryos expressing genes containing *Antp* homeoboxes, i.e. HS-*Antp* and HS-A^mSC (Table 2; Fig. 5C,G).

The frequency and quality of ectopic salivary gland formation increases with increasing heat-shock time. With

Table 2. Ectopic salivary gland induction with HS-*SCR*, HS-*ANTP* and HS-*ANTP/SCR* chimeric proteins

% of embryos with ectopic salivary gland(s)	Heat shock-induced protein						
	None	HS-SCR	HS-ASHC	HS-ASH6	HS-ASH	HS-ASC	HS-ANTP
No heat shock	0.0% (*n*=75)	1.0% (*n*=100)	2.2% (*n*=91)	1.3% (*n*=157)	0.0% (*n*=105)	0.0% (*n*=82)	0.0% (*n*=75)
20 min. heat shock	0.0% (*n*=94)	27.2% (*n*=114)	30.0% (*n*=122)	4.3% (*n*=254)	4.0% (*n*=123)	0.0% (*n*=103)	0.0% (*n*=94)
30 min. heat shock	0.0% (*n*=128)	46.8% (*n*=139)	29.6% (*n*=81)	4.7% (*n*=170)	0.0% (*n*=119)	0.0% (*n*=75)	0.0% (*n*=110)
45 min. heat shock	0.8% (*n*=121)	79.0% (*n*=167)	36.2% (*n*=112)	13.9% (*n*=115)	4.7% (*n*=191)	0.0% (*n*=66)	0.0% (*n*=80)

Heat shock treatments of embryos were carried out between 1 and 5 hours of development for 0, 20, 30 or 45 minutes at 37°C. Embryos were collected and aged at room temperature prior to fixation.

long heat-shock treatments of 45 minutes, 79% of HS-*Scr* animals have extra glands in anterior segments. The extra glands develop quite far: they invaginate like wild-type glands and often form a lumen. Although chimeric proteins with the Scr homeodomain induce extra salivary glands, the extra glands occur at a reduced frequency compared to HS-*Scr* (Table 2), and are less fully developed.

Severe head defects resulting from extensive cell death occur in many animals misexpressing any of the four chimeric proteins, but not in animals expressing intact Scr or Antp proteins. The anterior ends of the embryos are most often affected, and in many cases some or all of the cells which would normally give rise to salivary glands are absent. The occurence of cell death in the chimeric protein-expressing animals reduces the frequency with which ectopic salivary glands can be observed, thus leading to a systematic underestimate of the frequency of ectopic gland formation. Head defects are less severe in embryos expressing HS-*ASH* and HS-*A^{m}SC* compared to HS-*ASH6* and HS-*ASHC*, suggesting that the Scr homeodomain and adjacent C-terminal amino acids in combination with the Antp N-terminal sequencec are important in causing cell death.

In the larval cuticle and *tsh* activation assays, HS-*A^{m}SC* behaves like HS-*Antp*. In contrast, HS-*A^{m}SC* does not behave like HS-*Antp* in its effects on salivary gland development. Ectopic expression of Antp protein causes up to 81.3% of embryos to have severely reduced or absent salivary glands, even though extensive cell death does not occur in these animals. Ectopic expression of HS-*A^{m}SC*, which has all *Antp* sequence except for the C-terminal region, never detectably represses the formation of the endogenous salivary glands. Therefore the repression of salivary gland formation requires not only an Antp homeodomain but also the C terminus of the protein.

Assay four: effects of ectopic production of chimeric proteins on adult morphogenesis

As previously shown (Gibson and Gehring, 1988; Gibson et al., 1990), ectopically expressed Antp and Scr proteins cause distinct transformations in the adult fly (Fig. 6B,C). The chimeric A^{m}SC protein causes an *Antp*-like transformation in adults as well as in embryos. Third instar larvae containing HS-*A^{m}SC* were subjected to 37°C for 60 minutes four times during the period 70 to 94 hours after egg laying and then allowed to develop at 25°C. The antennae of the adults (or pharate adults) are substantially transformed into the mesothoracic legs just as in HS-*Antp* flies (compare Fig. 6C,F). The eyes of the flies are severely reduced in both cases. However, Antp and the A^{m}SC chimeric protein differ in that ectopic production of the A^{m}SC protein in larvae results in an additional head to thorax transformation. In about 30% of the A^{m}SC pharate adults, two patches of thorax-like cuticle with bristles that slightly protrude from the posterior ends of the dorsal head were observed (arrows in Fig. 6F). In contrast, ectopic expression of Antp protein causes only random ectopic bristles on the head (Fig. 6C). The distinct effect of A^{m}SC is not due to the insertion of the *c-myc* tag into the protein because Antp protein with a myc tag inserted at the same position does not have such an effect; it behaves exactly like unaltered Antp protein (data not shown). This 'cephalothorax' transformation is also induced by a similar *Antp*/*Scr* chimera (Gibson et al. 1990), although the transformation observed in the present experiments is stronger.

Expression of the ASH protein in third instar larvae causes adult head transformations similar to those caused by Scr, but the Antp sequence in the chimeric protein appears to confer some *Antp*-like activity (compare Fig. 6B,D). ASH protein has a stronger effect on the antenna than Scr protein does. In addition to thickening the arista as Scr protein does, ASH transforms the second segment of the antennae into femur (arrow in Fig. 6D). However, ASH and Scr proteins reduce the eyes less than Antp protein does. The ectopic bristles on the head caused by HS-*Antp* are not seen with ASH or Scr. ASHC, like ASH, causes the second antennal segment-to-femur transformation (Fig. 6E). Ectopic expression of ASHC also causes a dramatic head-to-thorax transformation much stronger than that observed in the HS-*A^{m}SC* flies (Fig. 6F). The two mirror-image thorax-like structures were observed in about 80% of the pharate and eclosed adult flies. The sequence contributing *Antp*-like activity to ASH and ASHC is very likely N-terminal to the homeodomain.

DISCUSSION

The critical importance of the homeodomain N terminus

The functional specificity of Antp/Scr chimeric proteins fur-

ther emphasizes the importance of the homeodomain for determining the character of the protein. A change of only four of the 378 amino acids in the Antp sequence to the corresponding Scr residues causes the protein to behave like the Scr protein in four assays in vivo: adult head development, formation of bristle patterns in the embryonic cuticle, regulation of *teashirt* in midgut morphogenesis and induction of salivary gland placodes. The switch from *Antp* to *Scr*-like action is not due to activation of the endogenous *Scr* gene, and therefore most of the Antp protein sequence is compatible with multiple activities of Scr.

Protein activity can be influenced by sequences outside the homeodomain

Although the functional specificity of the Antp/Scr chimeric proteins is largely determined by which homeodomain they have, in some respects chimeric proteins retain activities characteristic of their non-homeodomain parts. For example, the $A^{m}SC$ protein acts like Antp in cuticle development, antenna-to-leg transformation and regulation of *tsh*. However, it differs from Antp in not blocking formation of the endogenous salivary glands. *Antp* does not interfere with gland formation by repressing *Scr*, as Scr protein levels are little affected, if at all, in embryos expressing ubiquitous Antp protein (Gibson and Gehring, 1988). Whatever the mechanism, either Antp sequence C-terminal to the homeodomain is required to inhibit gland formation or the Scr C terminus is incompatible with the inhibitory activity of other parts of Antp.

Similarly, chimeric proteins ASH and ASHC act like Scr in most assays but lack some of its activity. HS-*Scr* thickens the denticle belts of the two posterior thoracic segments (Fig. 3B). In contrast, ASH and ASHC proteins do not cause the thickening of the T2 or T3 denticle belts. Also the size of the ectopic 'beards' is smaller and the frequency of 'beard' induction in T3 is lower compared to the changes induced by HS-*Scr*. The ectopic salivary glands induced by the chimeric proteins are less fully developed and occur at a reduced frequency. The Antp protein sequence N-terminal to the homeodomain may not fully substitute for the function of the corresponding Scr sequence, either in the potency of the protein or in the ability of the protein to regulate specific target genes.

Possible mechanisms of specificity control by the homeodomain N terminus

One important question about homeotic genes is whether the different actions of homeotic proteins are due to the different target genes that they affect, or distinct effects upon the same targets. Crystallographic studies of two homeodomains with extremely divergent primary sequences (Kissinger et al., 1990, Wolberger et al., 1991) and NMR analyses of the Antp homeodomain (Otting et al., 1990) are largely in agreement on the backbone structure. Therefore, it is reasonable to extrapolate those structures to other homeodomains. The tri-α helical part of the homeodomain binds to DNA with the 'recognition' helix, helix 3, lying in the major groove. Additional contacts are made between the non-α helical N-terminal part of the homeodomain and two nucleotides, through the minor groove of the DNA. The N-terminal contacts are primarily made through two arginine residues, Arg-3 and Arg-5. Arg-3 and Arg-5, and all of helices 2 and 3, are common to the Antp and Scr proteins. It is the Arg-1, Gly-4, Gln-6 and Thr-7 residues of the Antp homeodomain that differ in the Scr sequence. If the two proteins have different DNA-binding specificities, it is likely that the sequence differences may affect the orientation, and therefore affinity, of one or both arginines for sites in the minor groove.

In a study of heat-shock-induced chimeric proteins consisting partly of Ubx sequence and partly of Dfd sequence, it was found that the N-terminal homeodomain residues are important for the determination of specificity in those proteins as well (Lin and McGinnis, 1992). Their key result is that a protein almost entirely Dfd in sequence, but with six residues from the Ubx sequence at the N terminus of the homeodomain, acts like Ubx in its ability to regulate *Antp* transcription and in morphological transformations of segments. The residues known to contact DNA bases, Arg-3 and Arg-5, are common to Dfd and Ubx as they are to Scr and Antp. Therefore again it is difficult to explain the results as a simple change in DNA-binding specificity. Lin and McGinnis (1992) note the orientation of N-terminal side chains away from the DNA, based on modeling the structures from the crystal structures, and suggest the possible importance of protein-protein interactions.

In this view, altered associations with other proteins would affect the ability of each homeotic protein to act upon downstream target genes, perhaps in combination with differential DNA binding. No cofactors have been identified as yet for the proteins encoded by any of the ANT-C or BX-C genes. The best candidate for such a cofactor is the product of the gene *extradenticle*. Mutations in *extradenti - cle* cause homeotic transformations without changing the spatial or temporal patterns of expression of ANT-C or BX-C genes (Peifer and Wieschaus, 1990).

There are several well-documented cases of associations of other homeodomain proteins with cofactors. The OCT1 homeodomain proteins associates with the powerful viral transcriptional activator VP16 and a third protein to form a potent transcription complex (Kristie et al., 1989; Stern et al., 1989; Stern and Herr, 1991). Similarly, the yeast mating type protein MATα2 forms a complex with MAT **a**1 or with MCM1, and some of the protein-protein contacts may occur very close to the homeodomain (Goutte and Johnson, 1988; Keleher et al., 1988; Smith and Johnson, 1992). The hepatocyte nuclear factor 1 (HNF1) protein dimerizes and forms a complex with two copies of a smaller protein, DCoH, which appears to stabilize the HNF dimer (Mendel and Crabtree, 1991; Mendel et al., 1991). The HNF association is mediated by an N-terminal region outside the homeodomain. Thus it is plausible that associated proteins may affect the differential actions of Scr and Antp proteins.

The clearest case of protein associations involving the N terminus of the homeodomain comes from studies of several POU-class homeodomain proteins. At the N terminus of the homeodomain, the POU proteins have several basic residues followed by threonine and serine. One of the POU proteins, I-POU, lacks two of the basic residues found in the other POU proteins, including the lysine that corre-

sponds to Arg-3 of Antp and Scr proteins. An alternatively spliced form of I-POU, tI-POU, contains the two basic residues. I-POU fails to bind DNA but can hetero-oligomerize specifically with the POU protein Cf1a (Johnson and Hirsh, 1990), thus inactivating Cf1a, while tI-POU does bind to DNA and acts as a transcription activator and does not associate with Cf1a (Treacy et al., 1991, 1992). Thus the N-terminal homeodomain, along with helix 1 and 2, is involved in protein-protein interactions of I-POU, and in protein-DNA interactions of tI-POU, which also involve helix 3. A sequence similar to that of the POU domain N terminus has been implicated in protein-protein interaction between molecules of bacteriophage T4 gene 32 protein (Casas-Finet et al., 1992).

These precedents suggest possible involvement of the N terminus of homeodomains in protein associations. We note also that the alternative splicing of *proboscipedia*, *Antp* and *Ubx* RNAs affects the protein sequence near the N terminus of the homeodomain (Bermingham and Scott, 1988; O'Connor et al., 1988; Stroeher et al., 1988; Kornfeld et al., 1989; Cribbs et al., 1992). The short alternative and optional protein sequences in this region may affect contacts with other proteins, conceivably together with the homeodomain N terminus.

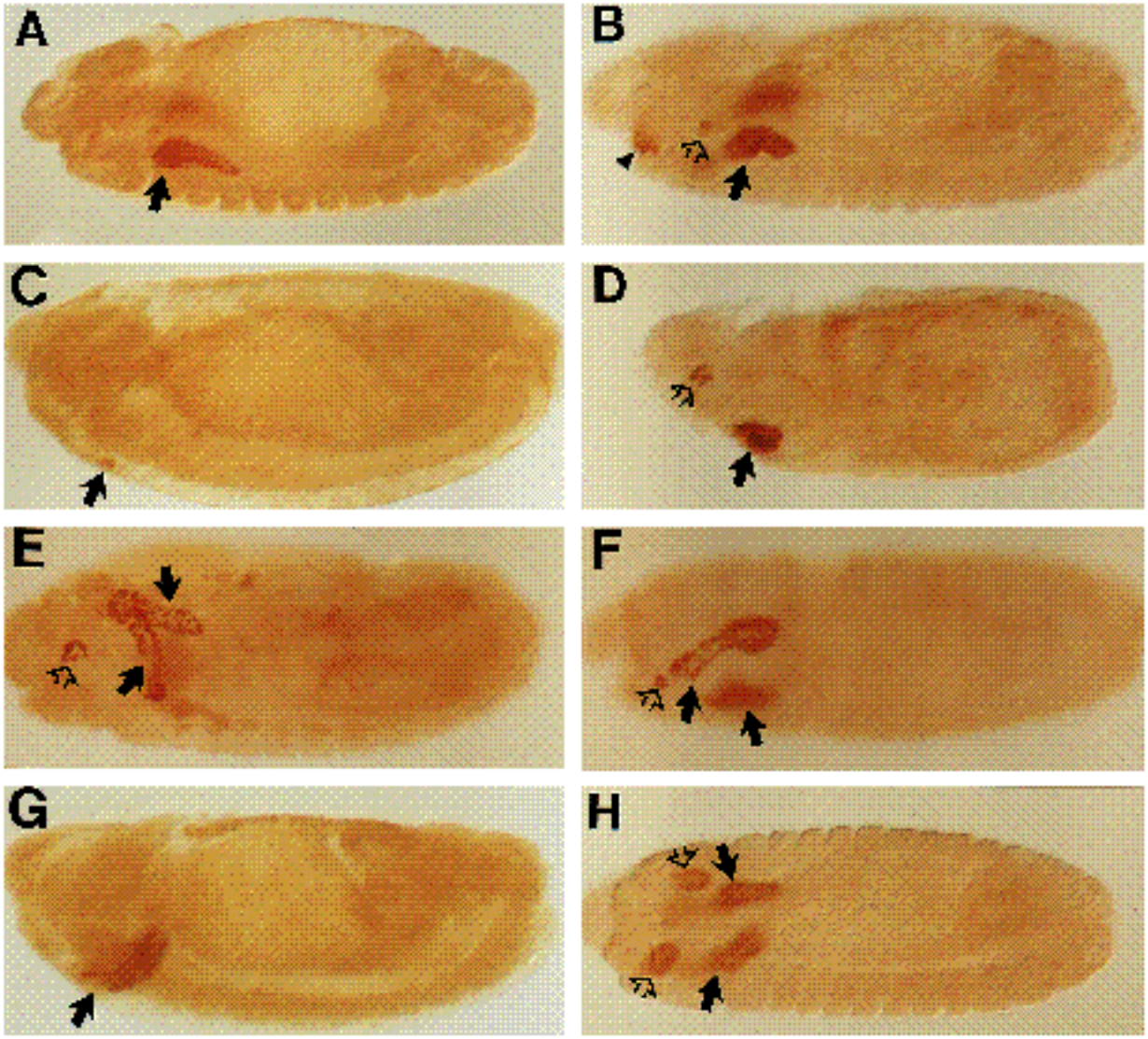

Fig. 5. Ectopic salivary gland induction by Scr, Antp and Antp/Scr chimeric proteins. An enhancer trap line which expresses β-galactosidase at high levels in the secretory epithelial cells of the embryonic salivary gland and at low levels in the amnioserosa was used in this experiment. Embryos were heat shocked as described and stained with an antibody against β-galactosidase. (A) β-galactosidase staining in a normal embryo of the enhancer trap line. (B,C) β-galactosidase staining in HS-*Scr* and HS-*Antp* transformant embryos, respectively. (D-G) β-galactosidase staining in embryos carrying various *Antp/Scr* chimeric constructs: (D) HS-*ASHC*; (E) HS-*ASH6*; (F) HS-*ASH* and (G) HS-A^mSC. (H) Ventral view of HS-*Scr* embryo showing the ectopic salivary glands in the approximately the dorsal/ventral position in the embryo as the endogenous salivary glands. In each panel, the dark heavy arrow demarcates the salivary glands arising in the normal position in the embryo (parasegment 2). The open arrows indicate glands derived from the next anterior segment (parasegment 1) and the arrowheads indicate glands derived from the more anterior segment (parasegment 0).

Scr and salivary gland formation: separable induction of different downstream genes

Ectopic Scr protein and derivative chimeric proteins leads to ectopic expression of two salivary gland-specific markers, indicating partial formation of extra glands. The ectopic glands appear to be bona fide salivary glands for several reasons. (1) Chromosomes of the salivary gland become polytene very early in the morphogenesis of this tissue and

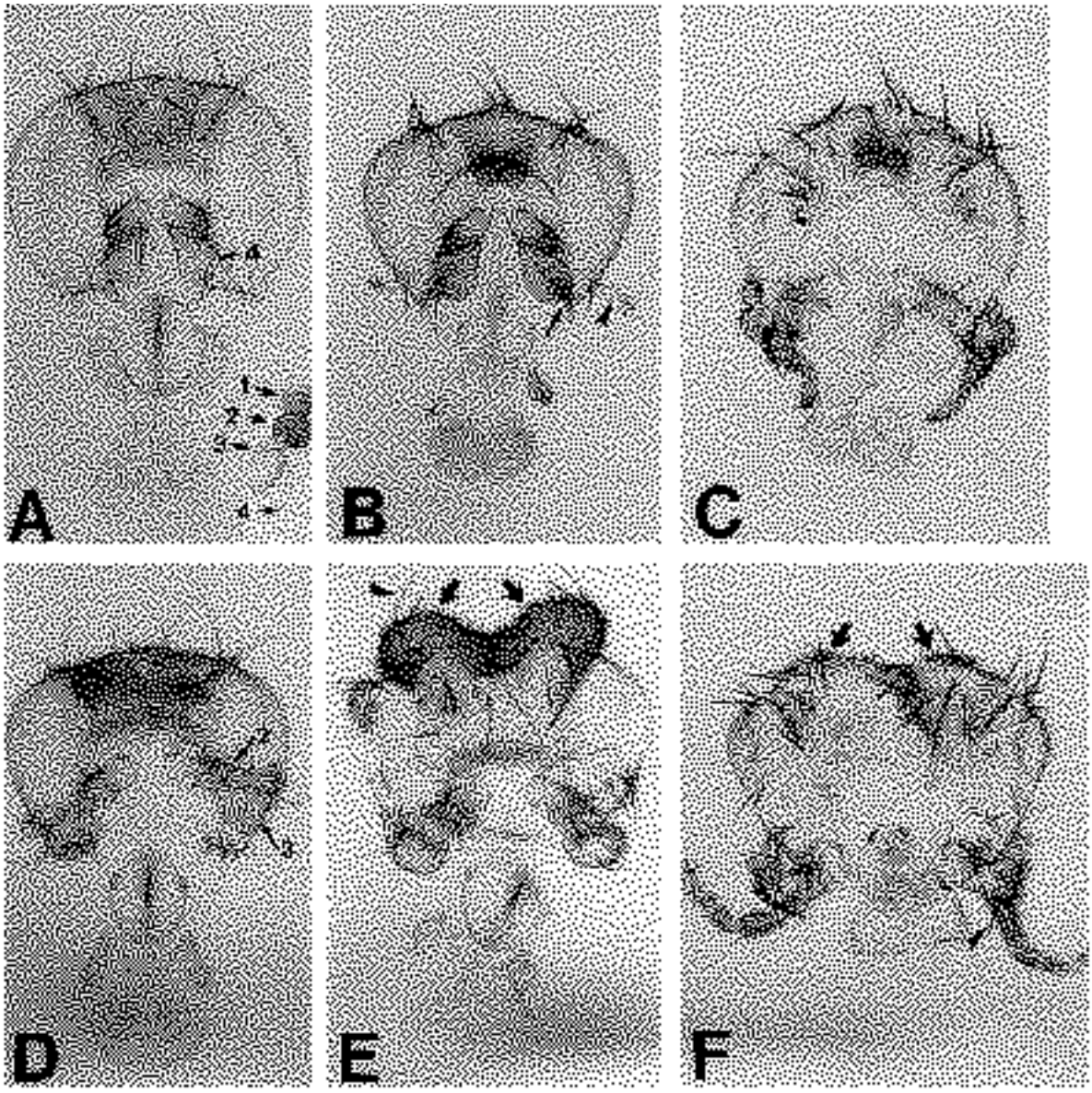

Fig. 6. Transformations of adult structures caused by ectopic expression of *Scr*, *Antp*, and chimeric proteins. Larvae were heat shocked four times at 37°C for 60 minutes (A, B, C and F), 45 minutes (D), or 10 minutes (E) beginning at 68 hours AEL. They were allowed to develop at 25°C. Heads of the eclosed or Pharate adult flies were dissected. After mounting in Hoyer's medium, they were photographed and shown. (A) Head of a *Df(1)w* adult fly. A picture showing the detailed structures of the antenna is inserted at the lower right corner. The four segments of the antenna are marked by the arrows and numbered 1 to 4. The fourth segment is the arista. (B) The effect of HS-*Scr*. The obvious changes are the thickening of the aristae and the appearance of extra bristles in segments 2 and 3 (arrows). The size of the eyes is also reduced. (C) The effect of HS-*Antp*. The antennae are transformed into the mesothoracic legs. There are ectopic thoracic like bristles on the head. (D) The effect of HS-*ASH*. The effects on the eye size and the pattern of bristles on the head are similar to that of HS-*Scr*. However, in addition to the arista, the second and third segments of the antennae are also affected (indicated by arrows and numbers). They are elongated and swollen. (E) The effect of HS-*ASHC*. The effects on the antenna are similar to that of HS-*ASH*. But the posterior-dorsal parts of the head are strongly transformed into thoracic structures by the ASHC chimeric protein as indicated by the thick arrows. (F) The effect of HS-A^mSC. The effect on the antenna development is Antp-like in that ectopic expression of the protein causes the transformation of antennae to the mesothoracic legs. Arrowhead points to the apricot bristle which is characteristic of the mesothoracic leg. But the A^mSC protein also acts like ASHC in that it causes the head to thorax transformation (arrows), although the transformation is weaker that observed in (E).

the degree of polyteny is reflected in nuclear size. The size of the nuclei in the ectopic glands is the same as in wild-type gland cells. (2) The extra glands occur in the same relative dorsal/ventral position in the segment as the normal glands, taking into account head involution cell movements. (3) The marker-expressing cells invaginate as do the normal gland cells. (4) The ectopically induced salivary glands secrete an antigen into the lumen that can be detected by the lumen-specific antibody D3 (D. J. A., unpublished result).

It is likely that *Scr*, in combination with dorsal/ventral signals, determines the number of cells that will constitute this structure (Panzer et al., 1992). Once the salivary gland cells invaginate, Scr protein can no longer be detected. Therefore it seems likely that *Scr* initiates salivary gland morphogenesis through the activation of a set of early acting regulatory molecules that relay information from *Scr* to more terminal differentiation functions. Among the best candidates for such a relay molecule are *forkhead* (*fkh*), which encodes a putative DNA-binding transcription factor (Weigel et al., 1989; Weigel and Jäckle, 1990) and the gene marked by the B204 enhancer trap line. Both *fkh* and B204 genes are expressed at an early time when Scr protein is present, continue to be expressed following invagination, and are expressed in all of the primordial salivary gland epithelial cells.

Our experiments reveal that induction of *fkh* is separable from induction of other salivary gland markers. Panzer et al. (1992) reported ectopic expression of *fkh* in placodes in almost all of the embryonic segments due to HS-*Scr* induction, whereas we observe ectopic glands in parasegments 0 and 1 only. Very similar heat-shock treatments were used, and our HS-*Scr* construct also induces ectopic *fkh* expression (Zhao et al., 1993). Thus posterior segments are permissive for induction of *fkh* by *Scr*, but not for induction of B204. In parasegments 0 and 1, both markers are induced. Recently, we have tested two other salivary gland markers and both are ectopically expressed in only parasegments 0 and 1 (data not shown). *fkh* and the gene driving the expression of the B204 enhancer-trap, as well as the genes corresponding to the two marker lines, could be activated by Scr protein but differentially regulated by other homeotic proteins. B204 could be activated by Scr and repressed by Antennapedia complex and/or Bithorax complex proteins, or region-specific gap segmentation gene products, in more posterior segments while *fkh* is activated by *Scr* but not affected by more posteriorly acting proteins.

The quantitative predominance of *Antp* or *Scr*

The heat-shock promoter experiments demonstrate that even when a homeotic protein is produced in all cells, only some cells are visibly affected. In some cases, only certain tissues are affected (Mann and Hogness, 1990). Sometimes the presence of other homeotic proteins appears to block the influence of the introduced protein (Gibson and Gehring, 1988; Kuziora and McGinnis, 1988; Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes et al., 1990; Mann and Hogness, 1990), a phenomenon known as 'phenotypic suppression'. The same principle may apply to mammalian homeotic genes (Balling et al., 1989; Lufkin et al., 1992).

With *Scr* and *Antp*, the ratio of homeotic protein concentrations may be important. Ectopic expression of *Scr* transforms T2 and T3 into T1-like segments, thus overruling *Antp* in its normal place of expression. However, ectopic expression of *Antp* in T1 will cause transformation into a T2-like segment. Thus there is no simple dominance of one gene over the other; whichever product is produced at a high level predominates over the other. In contrast, heat-shock-driven *Antp* expression has no effect in abdominal segments (where *Ubx* is expressed) but ectopically expressed *Ubx* causes thoracic segments (where *Antp* is expressed) to develop as abdominal segments. Phenotypic suppression is not merely due to the repression of *Antp* expression by *Ubx*. Rather it involves a dominating effect of Ubx protein even where both Ubx and Antp proteins are made (Gonzalez-Reyes et al., 1990).

How is phenotypic suppression and the more equal relationship between *Antp* and *Scr* related to the control of target genes? The balance between *Scr*-controlled target genes and *Antp*-controlled targets determines the phenotypic outcome. Phenotypic suppression, in contrast, could involve the domination of one homeotic protein by another if both proteins have the same effects, except one has at least one additional capability. This hypothesis predicts, for example, that *Ubx* controls all of the target genes that *Antp* affects, and additional ones as well. Alternatively, Ubx protein could predominate by causing the inactivation of Antp protein, perhaps by indirectly controlling its phosphorylation state.

Cell death caused by chimeric proteins

The effects of the $A^{m}SC$ and ASHC chimeric proteins on embryonic development are striking, with drastic deformation of head segments and highly aberrant structures in multiple regions of the embryo. The misexpression of either parent protein does not give rise to the extensive cell death seen in the chimeric protein-expressing embryos, so it is the combination of Antp and Scr sequences that causes the massive defects. Severe head defects and extensive cell death were also observed in embryos expressing a chimeric protein that has a Scr homeodomain and adjacent C-terminal 6 amino acids with the remainder sequence being Antp (data not shown). In adults, both $A^{m}SC$ and ASHC chimeric proteins have the ability to transform the head to thorax ('cephalothorax'), while HS-*Antp* and HS-*Scr* do not, indicating that the cephalothorax transformation is a novel property created by the combining parts of the Antp and Scr proteins. One allele of *Antp*, called $Antp^{Cephalothorax}$, has a similar effect, but is associated with a chromosome rearrangement that appears to fuse the normal protein-coding exons of *Antp* to a foreign promoter (Scott et al., 1983). However, the protein sequence in this strain has not been examined.

The potency of the chimeric proteins may be due to the activation rather than repression (or vice versa) of a target of *Antp* or *Scr*, new target gene specificity distinct from either parent protein, the formation of a protein that binds irreversibly to DNA or to other proteins, or the synergistic action of proteins that associate with the Antp N terminus and with the Scr C terminus. The Antp C-terminal sequence (NKTKGEPGSGGEGDEITPPNSPQ) differs from the Scr

sequence (HKMASMNIVPYHMGPYGHPYHQFDIHP-SQFAHLSA) in being shorter, relatively acidic, and having fewer tyrosines and histidines.

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