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Transcriptional activation by heterodimers of the *achaete-scute* and *daughterless* gene products of *Drosophila*

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The *achaete-scute* complex (AS-C) and the *daughterless* (*da*) genes encode helix-loop-helix proteins which have been shown to interact *in vivo* and to be required for neurogenesis. We show *in vitro* that heterodimers of three AS-C products with *DA* bind DNA strongly, whereas *DA* homodimers bind weakly and homo or hetero-combinations of AS-C products not at all. Proteins unable to dimerize did not bind DNA. Target sequences for the heterodimers were found in the promoters of the *hunchback* and the *achaete* genes. Using sequences of the former we show that the DNA binding results obtained *in vitro* fully correlate with the ability of different combinations to activate the expression of a reporter gene in yeast. Embryos deficient for the *lethal of scute* gene fail to activate *hunchback* in some neural lineages in a pattern consistent with the lack of a member of a multigene family.

Key words: achaete-scute/daughterless/Drosophila/helix-loop-helix/neurogenesis

Introduction

An important aspect of neurogenesis is the production of a large variety of neurons from the undifferentiated ectodermal layer. Work with the grasshopper embryo revealed that neurons of the central nervous system (CNS) are generated according to an orderly and consistent scheme by the unfolding of an invariant cell lineage from precursor neuroblasts. The family of neurons that each precursor produces is unique and correlates with the position on the ectoderm where the neuroblast originates (see Doe *et al.*, 1985).

The comparative anatomy of neural development of various insect embryos strongly suggests that the same building principle applies to *Drosophila* (Thomas *et al.*, 1984). This view is supported by the limited amount of lineage analysis undertaken (Doe *et al.*, 1988a) and by the observation that *in vitro* cultured neuroblasts, plated from dissociated embryos, also give rise to clonal clusters of neurons (Furst and Mahowald, 1985). It has been shown also that the proportion of neuronal cell markers produced *in vitro* is in good agreement with the *in vivo* estimate, thus suggesting that the neuroblast lineage develops autonomously, at least for the markers scored and within the resolution of the estimate (Doe *et al.*, 1988a; Huff *et al.*, 1989). The generation of neuronal diversity, therefore, can be seen as the deployment of a genetic programme to individual neuroblasts each of which then develops in an autonomous fashion. What is the nature of this programme and how is it implemented?

It now seems clear that at least part of the genetic complexity required to generate the observed neuronal diversity could be provided by segmentation and homeotic genes (reviewed in Doe and Scott, 1988). Many of these genes encode transcriptional regulators (reviewed in Gehring, 1987; Rhodes and Klug, 1988), potentially capable of promoting specific pathways of cell differentiation in a cell autonomous fashion. Of particular interest, however, are the the gap and pair rule genes (see Nüsslein-Volhard and Wieschaus, 1980) because, in contrast to other segmentation and homeotic genes, their expression in the CNS occurs in patterns totally unrelated to their previous boundaries of expression (reviewed in Ingham, 1988). This is important because, with the exception of the correspondence of the segment specific differences in neuronal progeny (Bate, 1976) with the distribution of the homeotic gene products, the mosaic of neuroblast fates does not correlate with the boundaries of blastoderm expression of any of these genes. Indeed, redeployment of the gap and pair rule genes takes place in subsets of neuroblasts, and in their progeny in all the metameres, in intricate patterns that portray the uniqueness of each neuroblast (Doe *et al.*, 1988a).

Phenotypic analysis with two pair rule genes, *fushi tarazu* and *evenskipped*, established that these genes were required for the diversification of binary neuronal pathways in some lineages of the CNS (Doe *et al.*, 1988a,b). In the case of the peripheral nervous system (PNS) two genes (*cut* and *numb*), encoding similar regulatory functions, were also shown to be required for the differentiation of particular sensory organ types (Bodner *et al.*, 1987; Blochlinger *et al.*, 1988; Uemura *et al.*, 1989). *cut* is expressed *de novo* by PNS precursor cells (Blochlinger *et al.*, 1990).

It appears, therefore, that some components of the genetic programme deployed on the neuroblasts have been identified. However, as the expression of these genes occurs after neuroblasts have segregated from the ectoderm, the programme must be implemented by other factors. We have proposed that this step is mediated by the *achaete-scute* gene complex (AS-C) (Alonso and Cabrera, 1988). This proposal was based on three aspects of the biology of the AS-C. Firstly, the AS-C is required for neurogenesis to proceed in both the CNS and the PNS (Jiménez and Campos-Ortega, 1979; White, 1980; Dambly-Chaudière and Ghysen, 1987; Cabrera *et al.*, 1987). Secondly, the AS-C encodes four homologous genes (Villares and Cabrera, 1987; Alonso and Cabrera, 1988), which appear to be a subset of a larger family of qualitatively similar genetic functions (Jiménez and Campos-Ortega, 1979). Finally, expression of the AS-C precedes and parallels neuroblast segregation, precisely recapitulating all its cellular aspects and revealing the influence of positional information on this process (Cabrera *et al.*, 1987; Cabrera, 1990 and unpublished; ectodermal patterning reviewed in Nüsslein-Volhard and Roth, 1989).

In addition, different members of the AS-C display partially overlapping patterns, suggesting that they could form a combinatorial system capable of orchestrating the complex mosaic of gene expression displayed by neural precursors (Cabrera *et al.*, 1987; Cabrera, 1990, and unpublished results).

It has been recently shown that the AS-C genes belong to the helix−loop−helix (HLH) family (Murre *et al.*, 1989a). The HLH motif denotes a hypothetical structure of two amphipathic helices connected by a flexible loop; proteins bearing this motif have the ability to dimerize (Murre *et al.*, 1989a). Sequence comparisons have uncovered the presence of this motif in many genes (see Benezra *et al.*, 1990) and, recently, structural searches based on pattern matching strategies have yielded an even larger collection of previously unrecognized cases (Gibson *et al.*, 1990). Particularly interesting amongst the latter is the similarity of the HLH motif with the functional domain of the Rop protein of *E.coli*. Because the crystal structure of Rop has been determined and shown to consist of a four helix bundle made of two antiparallel amphipathic helices (Banner *et al.*, 1987), these similarities lend strong support to the HLH model (Gibson *et al.*, 1990).

Many HLH proteins have been shown to bind to DNA, a property conferred by an adjacent conserved basic region and requiring previous dimerization of the protein (Davis *et al.*, 1990; Voronova and Baltimore, 1990). Although most of the DNA-binding HLH proteins function as transcription factors (Murre *et al.*, 1989a; Davis *et al.*, 1990; Henthorn *et al.*, 1990; Beckmann *et al.*, 1990; Hu *et al.*, 1990; Braun *et al*, 1990; Williams and Tjian, 1991; see review by Weintraub *et al.*, 1991) some bind to yeast centromeres (Cai and Davis, 1990).

Here we report on the DNA-binding properties of the AS-C products, on their own and in combination with the product of the *daughterless* (*da*) gene. The zygotic activity of *da* is also required for neurogenesis (Caudy *et al.*, 1988a) and it encodes a HLH protein as well (Murre *et al.*, 1989a; Caudy *et al.*, 1988b). In addition, we test the functionality of the AS-C and da proteins in a heterologous yeast system and identify genes which may be potential targets for the regulatory activity of the AS-C.

Results

The products encoded by the transcripts T3, T4 and T5 [which most likely correspond to the genetically defined functions *lethal of scute (lsc), scute (sc) and achaete (ac)*] are the three members of the AS-C used in this study for the following reasons. Firstly, they share two conserved domains: an N-terminus HLH motif and a C-terminus acidic domain, whereas the fourth member (T8 or *asense*) lacks the latter (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Murre *et al.*, 1989a). Secondly, the onset of the expression of *ac*, *sc* and *lsc* occurs on the ectoderm, before neuroblast segregation, whilst asense is activated only after neuroblasts have segregated (Cabrera *et al.*, 1987, Romaní *et al.*, 1987; Alonso and Cabrera, 1988; Cabrera, 1990).

Formation of DNA binding heterodimers

The polypeptides encoded by AS-C and *da* (henceforth denoted by capital characters *AC*, *SC*, *LSC* and *DA*) were prepared by *in vitro* translation in rabbit reticulocyte lysates.

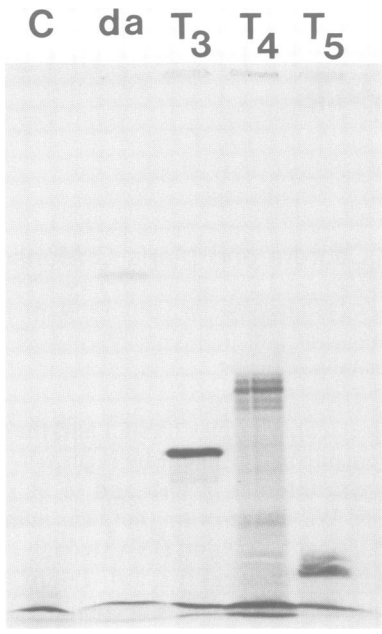

Fig. 1. Production of the AS-C and *da* polypeptides by *in vitro* translation. T3 and T4 were translated from the amount of RNA produced from transcription reactions containing 1 μg of DNA and T5 and *da* from 2.5 μg of DNA. Aliquots of the reticulocyte lysate programmed with the RNAs indicated above each lane were analysed by SDS−PAGE. The autoradiogram shown was loaded with the same amounts of lysate used for the DNA-binding assays: 3 μl of *da*; 0.5 μl of T3; T4 and T5, 1 μl. Lane C contains 3 μl of unprogrammed lysate.

The efficiency of translation of each RNA was examined by titration in the reticulocyte system and consequently adjusted so that comparable amounts of each protein were used in the DNA-binding reactions (Figure 1).

It has been shown that the *LSC* product belongs to class B HLH proteins, which form heterodimers with class A HLH proteins, like *DA*, and that these complexes recognize the core DNA sequence CAGGTG, the *x*E2 site (Murre *et al.*, 1989b). Based on this evidence we searched for putative target sequences by homology in the promoters of genes likely to be subjected to AS-C regulation (see Introduction). Two cases were found: (i) between positions 3836−3842 of the *hunchback (hb)* gene, corresponding to the zygotic promoter (Tautz *et al.*, 1987); and (ii) between positions 816−822 of the *ac* gene promoter (Villares and Cabrera, 1987). We therefore prepared probes from these sequences (see Materials and methods) to test the DNA binding activity of the aforementioned protein combinations.

The results of such experiments are presented in Figure 2, showing that (i) none of the three AS-C products tested binds to the probes, in neither homo nor heterodimer combinations; (ii) *DA* elicits a weak homodimer binding; and (iii) *AC, SC, LSC/DA* heterodimers bind tightly. These data confirm previous findings for *LSC* and *DA* (Murre *et al.*, 1989b), extending them to the other AS-C proteins and showing that the three possible combinations of these latter products do not bind DNA, as provided by the probes used. In addition, these experiments reveal a differential affinity of the three *AC, SC, LSC/DA* heterodimers for the two probes. Figure 2a shows that with the *hb* probe these relative affinity values are $SC > LSC > AC$, whereas for the *ac* probe we observe $LSC \geq AC > SC$ (Figure 2c). These differences must be

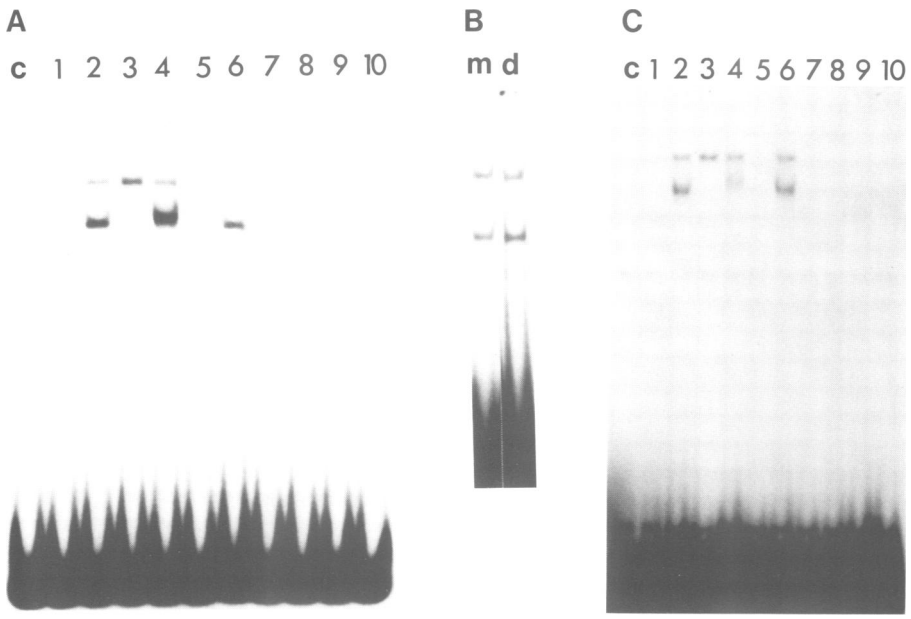

Fig. 2. Analysis of DNA-binding complexes produced by the *AC, SC, LSC* and *DA*. *In vitro* translated T3, T4, T5 and *da* products were assayed for DNA binding in the gel retardation experiments with: (**A**) the *hb* site as a monomer; or (**B**) comparison of *LSC/DA* binding affinities to the *hb* probe as monomer (m) and dimer (d); and (**C**) the *ac* site. Lane c is a control containing unprogrammed lysate. Other lanes contained lysates programmed with the RNAs or mixtures thereof as follows: 1. T3; 2. T3/*da*; 3. *da*; 4. T4/*da*; 5. T4; 6. T5/*da*; 7. T5; 8. T3/T4; 9. T3/T5; 10. T4/T5.

contributed by sequences other than the core CAGGTG, which is identical in both probes (see Materials and methods).

We also found that the proportion of DNA bound by *DA* homodimers varied in relation to that bound by the heterodimers (see Figure 2a, c). We investigated whether this behaviour was the result of a higher stability of the *DA* homodimer or to uneven production in the *in vitro* system of any of the products involved. To test these alternatives we titrated the DNA complexes bound by *DA* homodimers with increasing amounts of the *LSC* product. Figure 3 depicts the result of such experiment, showing that *LSC* can fully compete *DA* homodimers, demonstrating that the heterodimer is the preferred combination and proving that small differences in the ratio of the two components can explain the observed variability. This experiment also lends supports to the differential affinities of the three heterodimeric combinations described above. Those reactions have a slight excess, but constant amount of *DA*, which limits the proportion of heterodimer binding and makes the three combinations directly comparable.

The specificity of the binding reactions was tested by addition of either anti-*LSC*, *SC* and *AC* antibodies to the corresponding DNA binding reactions and monitoring the production of an additional shift on the protein–DNA complexes. Figure 4 shows that the antibodies elicit two effects: they partially and specifically inhibit binding of the corresponding heterodimer, as well as producing the additional shift. The specificity of the antibodies in recognizing the different proteins made in the reticulocyte lysates is shown in Figure 5.

Specificity of heterodimer formation

We have shown that combinations of the *AC*, *SC* and *LSC* lack the ability to bind DNA, as provided by the two probes used here. This could be due to insufficient affinity of the resulting homo- or heterodimers for these sequences or to failure of the proteins to form dimers at all. These alternatives were tested in a scheme in which heterodimers allowed to form in the absence of DNA were immunoprecipitated with antibodies against one of the components of the mixture and co-immunoprecipitation of the other component assayed.

Fig. 3. Titration of *DA* homodimer binding by the *LSC*. Binding reactions containing the same amount of *DA* and increasing amounts of the *LSC* were set up with the *hb* probe and analysed. Lanes are: C. control as in Figure 2; other lanes contained lysates programmed with the RNAs or mixtures 1. 1 μl of T3; 2. 3 μl of *da*; 3. 3 μl of *da* and 1 μl of T3; 4. 3 μl of *da* and 2 μl of T3; 5. 3 μl of *da* and 4 μl of T3.

In a first series of experiments we show that, in line with the DNA binding results *AC, SC, LSC/DA* heterodimers

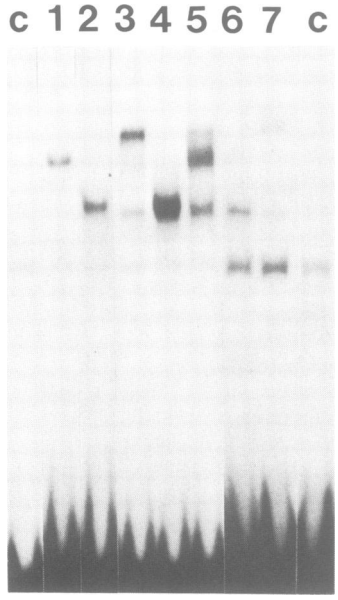

Fig. 4. Binding specificity of the *AC, SC, LSC/DA* heterodimers. Binding reactions set up with the *hb* probe as in Figure 2 were analysed with or without incubation with affinity purified antibodies as indicated. For this experiment DTT was excluded from the binding buffer. Lanes are: c. control as in Figure 2; other lanes contained lysates programmed with the RNAs or mixtures: 1. *da*; 2. T3/*da*; 3. T3/*da* plus *LSC* antibody; 4. T4/*da*; 5. T4/*da* plus *SC* antibody; 6. T5/*da*; 7. T5/*da* plus *AC* antibody. Analysis of the complexes reveals a further shift of the specific heterodimer band due to complexing of the antibody as well as an enhancement of *DA* homodimer in lane 5. The latter appears to result from a partial inhibition of the heterodimers by the antibodies. Note that the band produced by lysate without added RNA (c) does not change position. Lanes 1–5 were obtained in a different experiment than 6 and 7.

form in the absence of DNA, since immunoprecipitation of the AS-C product brings down *DA* (Figure 5A). In addition these experiments reveal that the stability of the three heterodimers with *DA* runs as follows: *LSC>AC>SC*.

We similarly tested combinations of *AC, SC, LSC*; however, the sequence similarities of the C-terminal peptides used as immunogens led to cross-reactions which precluded the resolution of *LSC/SC* and *SC/AC* mixtures. To overcome this problem we used constructs of the *lsc* and *ac* products lacking the C-terminal region (T3-C and T5-C) to immunoprecipitate the above mentioned combinations. In Figure 5B we present the results of these experiments showing that combinations of the three AS-C products fail to co-immunoprecipitate. In certain combinations, however, we observed that the lack of the C-terminus domain led to co-immunoprecipitation (Figure 5B, lanes 9 and 13). Nonetheless, we show that this is not consistent with the behaviour of the full length products (lane 11), nor does it occur in every instance in which one of the components lacks the C-terminus domain (lane 7). We therefore believe that the overall structure of the AS-C products prevents them from maintaining stable heterodimers and that an alteration of this structure is produced in some cases by removal of the C-terminus region. It is in this latter situation that heterodimers can form.

Further support for this view was obtained by gel retardation assays in which a construct consisting of a HLH domain and adjacent basic region (bHLH) of the *lsc* gene was used in DNA-binding assays. Figure 6 shows that this polypeptide is, in fact, capable of binding DNA as a homodimer and in heterodimers with *DA*, but not in mixtures with the full length product. We have been unable, however, to obtain DNA-binding with *LSC* bHLH homodimers to sequences other than the κE2 site. The cause of this

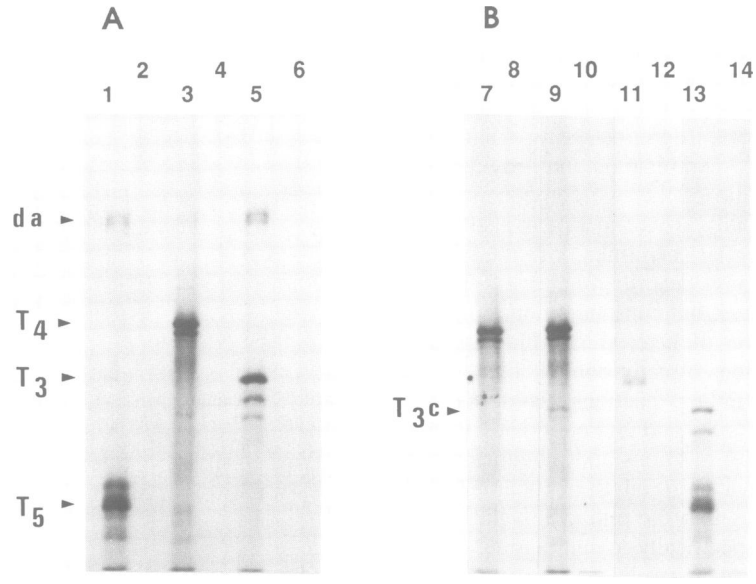

Fig. 5. Partner selection specificity of the *AC, SC* and *LSC*. Immunoprecipitation of AS-C products in **A.** *AC, SC, LSC/DA* mixtures and **B.** *AC, SC, LSC* mixtures. Analysis of the immunoprecipitated products was carried out by SDS–PAGE and two lanes are shown for each case, a control lane containing only the product whose co-immunoprecipitation is being assessed and a experimental lane containing the mixture. Lanes contained lysates programmed with the RNAs or mixtures thereof as follows: 1. T5/*da* plus *AC* antibody; 2. *da* plus *AC* antibody; 3. T4/*da* plus *SC* antibody; 4. *da* plus *SC* antibody; 5. T3/*da* plus *LSC* antibody; 6. *da* plus *LSC* antibody; 7. T4/T5-C plus *SC* antibody; 8. T5-C plus *SC* antibody; 9. T4/T3-C plus *SC* antibody; 10. T3-C plus *SC* antibody; 11. T3/T5 plus *LSC* antibody; 12. T5 plus *LSC* antibody; 13. T5/T3-C plus *AC* antibody; 14. T3-C plus *AC* antibody. The different protein bands were identified by running samples in parallel and are indicated on the left margin.

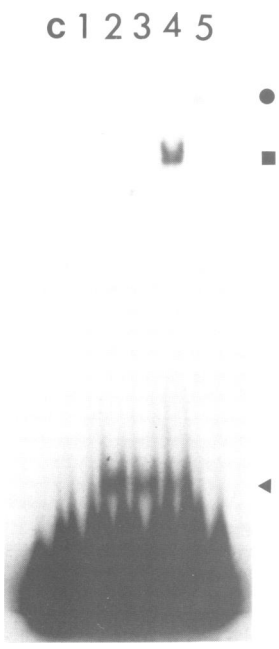

Fig. 6. A bHLH fragment of the *LSC* binds DNA. Binding reactions were set up with the kE2 probe (Murre *et al.*, 1989a) and analysed. Lanes are: C) control as in Figure 2; other lanes contained lysates programmed with the RNAs or mixtures thereof as follows: 1. 1μl T3; 2. 3 μl T3-C and 1μl T3; 3. 3 μl T3-C; 4. 2.5 μl T3-C and 2.5 μl of *da*; 5. 2.5 μl of *da*. Symbols to the right of the figure denote the different protein–DNA complexes ◀ *LSC* bHLH homodimer, ● *LSC* bHLH/*DA* heterodimer and ■ *DA* homodimer.

restriction on sequence recognition by the *LSC* bHLH domain remains unclear.

LSC/DA heterodimers activate transcription in yeast

The role of the AS-C products is best understood as that of transcriptional activators (Alonso and Cabrera, 1988), but there is no formal proof for this view, nor for any of the other *Drosophila* HLH proteins. We therefore set out to test whether the AS-C and *da* proteins could activate transcription of a lacZ reporter gene in a yeast assay system (Guarente, 1983). For this purpose the *lsc* and *da* genes were cloned in single copy yeast plasmids, with different selection markers, under the control of the Gal4 promoter. The reporter lacZ gene was constructed with or without an UAS, consisting of two copies of the *hb* probe described above (the affinity of the *LSC/DA* heterodimers for this sequence is comparable to that of the *hb* monomer; see Figure 1b).

Table I shows that only upon induction with galactose and exclusively in the presence of the *hb* UAS (i) the *lsc* gene does not induce β-galactosidase activity; (ii) *da* produces a slight activation and (iii) the presence of both *lsc* and *da* leads to a strong activation of the reporter gene. These data exactly correlate with the *in vitro* DNA-binding results, demonstrating that the *LSC/DA* heterodimers can function as transcriptional activators in direct proportion to their DNA-binding affinities. Given the sequence conservation amongst the AS-C gene products this conclusion is likely to apply to all of them.

Using this yeast assay system we next asked whether the conserved C-terminus domain of *AC, SC, LSC* could act as an activation domain, a possibility suggested by its acidic nature (Ptashne, 1988). For this purpose we devised a

Table I. Transcriptional activity of *lsc/da* in yeast

Construct	β-galactosidase units	
	Gal	Glu
T3	< 10.0	< 10.0
da	217.0	< 10.0
T3 + *da*	3635.7	< 10.0
T3 + *da* (no UAS)	< 10.0	< 10.0
T3-Cterm + *da*	2357.1	< 10.0

construct of the *lsc* gene with an ochre termination codon engineered six nucleotides upstream of the C-terminus domain (thus allowing for reversion of the phenotype in a mutant suppressor background). The result of this experiment is presented in Table I, where the absence of this conserved domain is shown to have very little effect on the level of activation of the reporter gene. The DNA-binding properties of the truncated *LSC* were found to be indistinguishable from that of the full length product when assayed in the gel retardation system (not shown).

The following controls were undertaken to ensure the performance of the yeast system. Firstly, we ascertained that all recombinant strains used in this work were wild type for the suppressor gene by plating in low adenosine (10 mg l^{-1}) medium and monitoring the accumulation of red pigment. Secondly, we performed inter-strain conversion (*lsc* to *lsc/da*; *da* to *lsc/da* and to *lsc-C/da*), by transformation with the appropriate plasmids, and determined their β-galactosidase phenotypes by either the suspension or the filter assays (up to 48 different strains were analysed by the filter method). And thirdly, we ensured that the strains contained the plasmids in their original state by probing blots containing digests of total yeast DNA with the appropriate labelled fragments (not shown).

The expression of HB in AS-C mutants

Like many other segmentation genes *hb* is re-expressed in the nervous system (Tautz *et al.*, 1987; Jiménez and Campos-Ortega, 1990). The presence of a target sequence for *AC, SC, LSC/DA* binding on the *hb* promoter and the ability of this sequence to elicit the expression of a reporter gene in the yeast assay system prompted us to ask whether the expression of *HB* is impaired in AS-C mutant embryos. For this purpose we chose to test the absence of the *lsc* gene, for it produces the strongest effect of any single AS-C gene deletion (Jiménez and Campos-Ortega, 1987) without a major effect on neuroblast segregation (Cabrera *et al.*, 1987 and unpublished; Martín-Bermudo *et al.*, 1991). In this way we were able to assay for expression of *HB* rather than the absence of *hb* expressing cells.

During wild type CNS development *HB* is detected associating with mature neuroblasts and their neuronal progeny. The first *HB* staining cells appear in two rows of ~11–15 cells per thoracic hemineuromere (Figure 7), additional cells arising subsequently in the same as well as intermediate positions. This arrangement is a faithful reproduction of the first wave of neuroblasts segregation (Campos-Ortega and Hartenstein, 1985) and therefore we conclude that *HB* is expressed in all of these precursors at this stage.

Embryos lacking the *lsc* gene were recognized by the absence of the β-galactosidase marker (see legend to

Figure 7). In these embryos *HB* expression is normal in the blastoderm (not shown). However, in the CNS the number of *HB* positive cells in the thoracic hemineuromeres is only 5−10 in comparison to 11−15 in wild type embryos (Figure 7). In the absence of the β-galactosidase marker we can not be certain of which cells of the normal pattern fail to express *HB* in the mutant. In addition to the decrease in their number, *HB* expressing cells appear to have a more regular arrangement than in the wild type, suggesting that the *lsc* gene may have a more pervasive effect than just regulating the expression of *HB* in some lineages.

Discussion

We have shown that the AS-C products are potential DNA-binding proteins, a property conferred to them by their bHLH domains. This activity, however, is manifested exclusively when the AS-C products are juxtaposed by the *da* protein. The latter binds DNA as a homodimer, although weakly, but the heterodimers display a much increased affinity (see also Murre *et al.*, 1989b). At equilibrium this affinity differs in the various combinations and probes. Given the degree of sequence conservation amongst the AS-C HLH domains (Alonso and Cabrera, 1988) this differential affinity might reflect the influence of the overall structure of these products.

It has been shown that MYOD/E12 heterodimers can form in the absence of DNA (Murre *et al.*, 1989b). We have obtained similar results for *AC, SC, LSC/DA* mixtures. In addition, these experiments revealed that *SC/DA* heterodimers are the least stable of the three combinations tested. This correlates inversely with the DNA-binding affinities observed with the *hb* probe, thus suggesting that two factors, the affinity of partner selection and the avidity of the heterodimers for DNA, contribute to the formation of these protein−DNA complexes. Indeed, DNA-binding with MYOD, E12 and E47 requires previous dimerization (Davis *et al.*, 1990; Voronova and Baltimore, 1990) and kinetic measurements have proven that a stable protein−DNA complex can equally form with either low

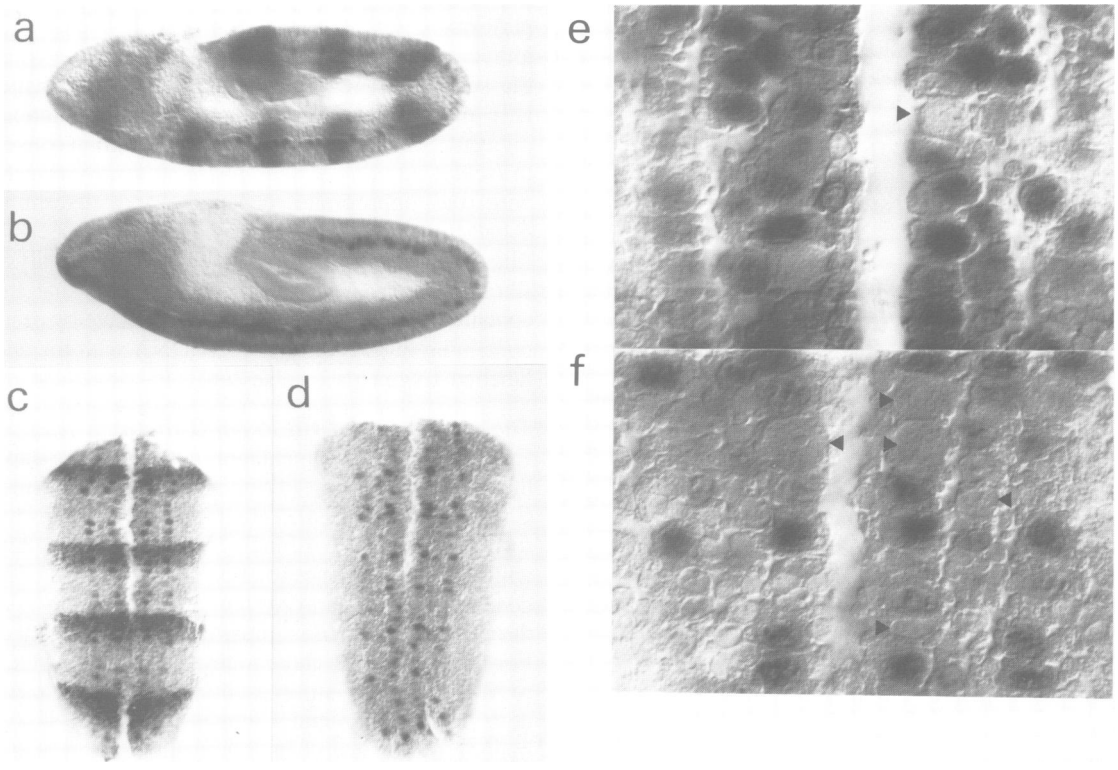

Fig. 7.- Expression of the *HB* in wild type and *lsc* deficient embryos. Embryos of the stock Df(1)sc^{4L}-sc^{9R}, balanced with a FM7 chromosome marked with *ftz*−lacZ, were double stained with antibodies against *HB* and β-galactosidase. *lsc*$^{+}$ embryos (**a**, **c** and **e**) were recognized by the *ftz*-lacZ staining and *lsc*$^{-}$ (**b**, **d** and **f**) by the lack of it. Stained embryos were mounted, staged under the microscope, photographed and then dissected and mounted flat, ventral side up. The pictures show medio-lateral views (a and b) of whole mount mid stage 9 embryos oriented anterior to the left and dorsal upwards or ventral views (c and d) of the same embryos, subsequently dissected and oriented anterior upwards. Note the decreased number of stained cells in the mutant embryo (d). The embryo with the normal *HB* pattern clearly belongs to the first wave of neuroblast segregation, given the number of neuroblasts observed and their distribution in two rows. The picture in panel b shows that the mutant embryo is at the same stage than that in a. We ascertained that the expression of *HB* in the mutant case is not the result of small differences in staging or abnormal, transient behaviour of this genotype. As mutant embryos can be readily distinguished by the lack of β-galactosidase staining we studied over four hundred embryos under the dissecting microscope. One fourth were mutant and displayed a pattern consistent with the case in d. We dissected seven of these mutant embryos and studied them flat under the microscope. This sample contained embryos of the first and second waves of neuroblast segregation and in all cases our observations were consistent with the decreased number of cells and more regular arrangement reported for d. Panels e and f display higher magnifications of second−third thoracic metameres focused subectodermally showing that the number of presumptive neuroblasts not expressing *HB* (arrowheads) is higher in the mutant case. The embryo in e is different, but of similar stage, than that in a and c and it was selected for its weaker β-galactosidase marker staining.

dimerization affinity and subsequent high avidity for DNA or vice versa (Sun and Baltimore, 1991).

We have also found that combinations of *AC, SC, LSC* do not bind to DNA, a feature that we have shown to correlate with their inability to dimerize *in vitro*. This behaviour highlights the specificity of partner selection displayed by these proteins (see also Murre *et al.*, 1989b), which appears to result from an inhibitory effect of the overall structure of these products on the maintenance of stable dimers, as opposed to an inherent inability of their HLH domains to homo or heterodimerize. A similar lack of homodimerization reported for the E12 product was shown to result from the inhibitory effect of an N-terminal domain (Sun and Baltimore, 1991). At the moment it is unclear whether the same principle applies to *AC, SC, LSC*.

In addition to the specificity of partner selection, the AS-C genes are expressed in highly elaborate temporal and spatial patterns (Cabrera *et al.*, 1987; Romaní *et al.*, 1987; Cabrera, 1990). In this respect *AC, SC, LSC* are prototype class b-HLH proteins, as opposed to class A, like *DA*, which form homodimers and whose expression is ubiquitous (Murre at al., 1989b). Class A and B proteins form DNA-binding heterodimers and, in the case of the *AC, SC, LSC* and *DA*, support for the significance of this interaction *in vivo* has been obtained by genetic analysis. Indeed, individuals heterozygote for AS-C or *da* deficiencies are normal whereas the double heterozygote lacks sensory organs (Dambly-Chaudière *et al.*, 1988). This dominant synergistic interaction strongly suggests that the *AC, SC, LSC* and *DA* are *in vivo* partners (Murre *et al.*, 1989b, Cline, 1989).

Why the AS-C needs *da* to carry out its function is unclear. However, we recognize that the properties of *AC, SC, LSC/DA* heterodimers disclosed in this work provide a much wider spectrum of regulatory modulation than would have been possible should the AS-C products function as homodimers as well. These properties might prove useful in the functional context of operation of the AS-C.

We have reported on two sites for *AC, SC, LSC/DA* binding, one in the *ac* gene and the other in *hb*; in both cases the sites occupy positions upstream of the TATA box (Villares and Cabrera, 1987; Tautz *et al*, 1987). If functional *in vivo* the former may elicit AS-C cross-regulatory and feed-back interactions, whilst the latter may produce AS-C dependent expression. We have tested the significance of the *hb* site in two experimental schemes: responsiveness in an *in vivo* yeast assay system and effect of an AS-C mutant on the expression of *HB* in the CNS.

The yeast system allowed us to show that *DA* and *LSC/DA* are capable of activating transcription of a reporter gene and that this activation is fully dependent on the presence of the *hb* site in the target promoter. The levels of reporter gene activation obtained in this assay are in full agreement with the performance of the corresponding combinations in gel retardation experiments. DNA-binding affinities *in vitro* thus translate directly into potential transcriptional efficiency *in vivo*, something which may prove extremely useful in the functional dissection of these proteins. A step in this direction was taken in this work, by testing whether the conserved acidic C-terminus domain of the AS-C genes could serve as the transcription activation moiety (Alonso and Cabrera, 1988). The results clearly indicate that this is not so. The functional importance of this domain, however, is supported by its sequence conservation and we have pointed out that it may serve as a phosphorylation site for tyrosine kinases (Villares and Cabrera, 1987). We have discussed elsewhere the regulatory potential and functional context of such a modification (Cabrera, 1990).

The fact that *DA* can activate transcription on its own suggests that the activation domain might map within this product. Its poor homodimerization affinity or low avidity for DNA, however, may prevent *DA* from being an efficient activator on its own.

We have monitored the expression of *HB* in the wild type and in a deletion of the *lsc* gene. We have dealt exclusively with the onset of *HB* expression in the CNS because at this time the pattern is simple enough to allow assessment of the differences between wild type and mutant embryos in a semi-quantitative manner. We have found that *HB* is expressed by all the neuroblasts in the wild type and that the number of these cells in mutant embryos drops significantly.

Our aim being to assess the influence of the AS-C on the regulation of the *hb* gene, we sought to elicit the strongest possible phenotype without affecting the segregation of these precursors. Indeed, the absence of neuroblasts will score as failure to activate *hb* as well, but this may well be a secondary effect of the mutant phenotype. The deficiency of the *lsc* gene is ideal for our purpose because it produces the strongest phenotype of any single AS-C gene deletion (Jiménez and Campos-Ortega, 1987). However, this appears to occur without a major effect on neuroblast segregation. Firstly, a larger deficiency deleting the *ac* and *sc* genes, in addition to *lsc*, leads only to the unambiguous absence of the median unpaired neuroblast (Cabrera *et al.*, 1987). In fact, it has been recently shown that it takes deficiencies extending further proximally to prevent a larger population of neuroblasts from segregating (Jiménez and Campos-Ortega, 1990). Secondly, in the absence of the *lsc* gene the full complement of *AC* expressing neuroblasts arises normally (our unpublished observations). Finally, reconstruction of the map of neuroblast of *lsc* mutant embryos revealed that a maximum of two neuroblasts may be missing by the second wave of segregation (Martín-Bermudo *et al.*, 1991). We therefore conclude that the decreased number of *HB* expressing cells in the mutant is due to failure of *hb* activation rather than the absence of the *HB*-expressing cells.

The biochemical and genetic data discussed strongly suggests that the *lsc* gene controls the activation of *hb*. However, it is clear that in spite of *lsc* being expressed by all neuroblasts its absence does not lead to the total suppression of *hb* expression. This is consistent with *lsc* being a member of a multigene family, of which the AS-C is just a subset (Cabrera *et al.*, 1987; Alonso and Cabrera, 1988; Cabrera, 1990). A network of such transcriptional activators may provide the required complexity to set up the mosaic of cell fates characteristic of the neuroblasts by controlling gene expression in these precursors.

Materials and methods

In vitro transcription and translation

The cDNA A31 cloned in the Bluescribe plasmid (Vector labs) was used as a source of T3 RNA and it was obtained by screening an embryonic library (provided by M.Goldsmidt-Clermont and D.Hogness) with the corresponding genomic probe (see Alonso and Cabrera, 1988). A version of this plasmid lacking the C-terminus domain was obtained by engineering a stop codon by site-specific mutagenesis (Kunkel, 1985) six nucleotides

upstream of this conserved domain. A bHLH fragment was prepared from the original A31 clone by PCR (Saiki *et al.*, 1988), with primers extending from positions 541–555 of the coding and positions 733–741 of the non-coding strands (Alonso and Cabrera, 1988). The primers also provided cloning sites and termination codon. The resulting product was cloned into the *Nco*I site of pT7βSal (a plasmid which provides a β-globin mRNA leader and initiation codon (see Norman *et al.*, 1988).

T4 RNA was prepared from a composite clone constructed from two cDNAs C31 (Villares and Cabrera, 1987) and B11, both obtained from the library described above and cloned in the Bluescribe plasmid. The construct was necessary because C31 contains a frameshift mutation (Villares and Cabrera, 1987) which was eliminated by swapping the *Sca*I 3′-half fragment with that of clone B11. Synthesis of the appropriate protein was assessed by Western blot analysis of the *in vitro* translated product with an antipeptide antibody against the C-terminus of the polypeptide (see below).

T5 RNA was obtained from the previously described genomic DNA (Villares and Cabrera, 1987) by cloning a blunt ended 786 bp *Mae*I–*Bal*I fragment into the *Sma*I site of the Bluescribe plasmid. A version of this plasmid with the C-terminus domain was obtained by run off transcription of a *Pst*I digest after elimination of the *Pst*I site from the vector's polylinker.

The *da* cDNA NM6 (Cronmiller *et al.*, 1988) was cut with *Bsty*I, blunt ended, the restriction fragment containing the coding region purified and cloned into the *Hinc*II site of pT7βSal (see above and Norman *et al.*, 1988). The fusion protein generated in this fashion bears five additional N-terminus residues. This construction was necessary to generate comparable amounts of *in vitro* translated *da* protein relative to the other clones.

RNAs were synthesized in 50 μl reactions using linearized DNAs and T3 or T7 RNA polymerases (Pharmacia) in the presence of RNasin (Promega), m7GpppG (New England Biolabs) (Melton *et al.*, 1984). The reactions contained 1 to 2.5 μg of DNA depending on the optimum RNA requirement of the different constructs, which was assessed by titration in a standard translation assay. The RNAs were extracted with phenol and recovered by ethanol precipitation, dissolved in 20 μl of complete reticulocyte lysate (purchased from the Department of Biochemistry, University of Cambridge) and incubated at 30°C for 90 min with ^{35}S-labelled or unlabelled methionine as described (Jackson and Hunt, 1983). Protein production was monitored by SDS–PAGE (Laemmli, 1970). The synthesized proteins were used immediately.

DNA binding assay

Probes were prepared from the synthetic oligonucleotides:

hb aatTCCCGCAGGTGTAAGaatt;

ac GGTAGTCACGCAGGTGGGATCCCATCCGGG

(lower case characters represent bases added to reconstruct a restriction site and are not present in the naturally occurring sequence) by annealing 100 pmol of the two strands in 20 μl of 0.1 M PB and end-labelling 0.5 pmol with Klenow fragment in the presence of [α-^{32}P]dATP or dCTP. Probes of the *x*E2 site (Murre *et al.*, 1989a) and in some instances *hb* were also prepared from cloned material. In brief, the annealed oligonucleotides were phosphorylated and cloned in the *Eco*RI site of the Bluescribe vector and several insert bearing colonies were screened by DNA sequencing (Sanger *et al.*, 1977). The insert was released by digestion with *Eco*RI or *Ava*I and labelled as above. All probes were purified in a 10% polyacrylamide gel in 1×TBE (90 mM Tris–borate; 1 mM EDTA pH 8.0), eluted from the gel and cleaned on a Sephadex G-50 spun column.

DNA binding assays were performed at 24°C in 20 μl of 10 mM HEPES pH 8.0; 50 mM NaCl; 1 mM EDTA; 2 mM DTT; 5 mg ml^{-1}; 1 BSA; 10% glycerol; 1 μg poly d(I:C); 25 μg ml^{-1}; 1 denatured and 2.5 μg ml; 1 native calf thymus DNA. Reactions were started by addition of 0.5 to 4 μl of the various *in vitro* translated proteins; lysate without RNA was used as control and to adjust the volume of lysate to the same level in all samples. After 15 min, 5 fmol of probe were added to each tube and incubated 20 min. The reactions were then immediately loaded on a 3.5% polyacrylamide gel in 0.25×TBE and analysed by the gel retardation assay (Garner and Revzin, 1981; Fried and Crothers; 1981). The specificity of the DNA-binding complexes was tested by incubation of the the standard reactions with 1 μl of antibodies specific for the different AS-C products for 20 min at the same temperature and analysis of the complexes in the same gel system. For the antibody shift experiments DTT was left out of the binding buffer.

Immunoprecipitations

The various *in vitro* translated proteins labelled with [^{35}S]methionine were mixed in the same buffer used for DNA binding assays, but without DTT or competitor DNAs, and incubated for 20 min at 24°C. 1 μl of affinity purified anti-*LSC*, *SC*, and *AC* antibodies (Cabrera, 1990 for the *LSC* antibody; *SC* and *AC* antibodies were obtained likewise with peptides from the same C-terminal conserved domain and will be described elsewhere) was then added and the reactions incubated a further 20 min. 20 μl of Affigel–Protein A beads were added, incubated on ice for 1 h. and washed four times with 1 ml 50 mM HEPES pH 7.0; 50 mM NaCl; 5 mM EDTA; 0.1% NP-40. The final pellets were extracted with 15 μl of SDS–PAGE sample buffer, boiled 5 min and resolved in 12.5% SDS–PAGE gels (Laemmli, 1970). As some antibodies elicited a slight cross-reaction, immunoprecipitation of *SC*/*LSC* and *SC*/*AC* combinations were performed with mixtures in which one of the components was synthesized without the C-terminus domain (see above).

LacZ assays in yeast cells

Three different plasmids were used to assay β-galactosidase expression under the control of AS-C proteins. The target plasmid was GV 256, a derivative of pLG669Z which contains the CYC promoter upstream of a CYC–lacZ fusion gene, the URA3 marker gene and the 2 μ origin of replication (Guarente and Ptashne, 1981), modified by removal of the UAS and insertion of a *Bgl*II linker (Cousens *et al.*, 1989). We introduced the sequence, 5′-gatcTCCCGCAGGTGTAAGCtTCCCGCAGGTGTAAGCactcg-3′, into the *Bgl*II site of GV 256. This sequence was derived from the *hb* promoter as detailed in Results and it was synthesized as a tandem duplication separated by a single T, to generate a diagnostic *Hin*dIII site, and it is flanked by *Bgl*II sites. The plasmid used for the experiments hereby described carried one copy of the above sequence in the orientation depicted, as ascertained by direct sequencing.

The T3 and *da* cDNAs were first cloned into the pKV701 vector (provided by D.J.Cousens and C.R.Goding), which carries the Gal1 promoter and PGK terminator, flanked by respectively *Eco*RI and *Sal*I sites and separated by an unique *Bgl*II cloning site. From these plasmids *Eco*RI–*Sal*I fragments were purified and cloned into pRS vectors, which carry the CEN6/ARSH4 and either the His3 or Trp1 marker genes (pRS313 and 314 respectively; Sikorski and Hieter, 1989). The cDNA A31 was used for constructs of the T3 gene and it was subjected to *in vitro* mutagenesis (Kunkel, 1985) to introduce *Bgl*II sites 19 nt upstream of the ATG and 3 nt downstream of the termination codon (Alonso and Cabrera, 1988), both by substitution of two nucleotides. All mutants were confirmed by sequencing.

The *da* cDNA was also subjected to *in vitro* mutagenesis to eliminate the internal *Eco*RI site, by a single nucleotide substitution which does not alter the code, and to reconstruct a *Bgl*II site upstream of the ATG, disrupted by the intron–exon junction (Cronmiller *et al.*, 1988).

Mixtures of the above plasmids were introduced in the yeast strain Y700 (MATα, ade2-1, trp1-1, can1-100, leu2-3, leu2-112, his3-11, 15, ura3, pho4:HIS3; constructed by R.Rothstein) by electroporation (Becker and Guarente, 1990) and plated on selective medium with glucose (Sherman *et al.*, 1979). For β-galactosidase assays cultures were derived from single colonies and grown in liquid selective medium with glucose. Induction was achieved by inoculation of selective medium with galactose with 1/20 of exponentially growing glucose cultures. Induced or uninduced cultures were grown to OD_{600} ~1, harvested, washed with water, resuspended in 0.1 M Tris (pH 7.5); 0.1% Triton X-100 at 50 OD/ml and cells permeabilized by freezing at −80°C and thawing on ice (Miozzari *et al.*, 1978). β-galactosidase assays were performed as described (Miller, 1972), adding 10–200 μl of the above suspension of yeast cells to 800 μl buffer Z equilibrated with 200 μl of 4 mg ml^{-1} o-nitrophenyl-β-D-galactopyranoside. Reactions were stopped with 500 μl of 1 M sodium carbonate, cells removed by centrifugation and the OD_{420} of the supernatants measured. The cell pellet was resuspended in water and the OD_{600} taken to normalize the β-galactosidase to number of cells. The numbers shown in Table I represent relative β-galactosidase units obtained from the equation $(A_{420}/A_{600})(t^{-1})(v^{-1})\times 1000$, where t is the time of the reaction in min and v the volume of extract added in millilitres. Linearity of the reaction was assessed for both variables, cell volume and time, and the numbers shown here were all obtained with 40 μl of cells, 7 min reactions for all induced cultures and 30 min for all uninduced ones. Two independent isolates of each construct were tested by this assay but larger numbers of strains were tested by a filter β-galactosidase assay as specified in the legend to Table I (Breeden and Nasmyth, 1985)

Yeast DNA blots

Total yeast DNA was prepared from overnight 5 ml cultures in selective glucose medium processed as described (Sherman *et al.*, 1979). Restriction enzyme digests were blotted (Southern, 1975) and hybridized as described before (Villares and Cabrera, 1987) with [^{32}]P-labelled random primed probes (Feinberg and Vogelstein, 1984) prepared from inserts of the T3 and *da* cDNAs.

Immunochemistry

Embryo collection, double staining and mounting was performed as described (Lawrence *et al.*, 1987). Two antibodies were used, a rabbit polyclonal

anti-*hb*, generously provided by D.Tautz (Tautz, 1988) and a mouse monoclonal anti-β-galactosidase (Sigma). The stock Df(1)sc^{4L}-sc^{9R} carries a deficiency for the *lsc* gene and it was balanced with a FM7 chromosome marked with a *fushi tarazu*-β-galactosidase gene fusion (Hiromi and Gehring, 1987) to allow identification of mutant embryos.

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