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A synthetic homeodomain binding site acts as a cell type specific, promoter specific enhancer in *Drosophila* embryos

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A DNA sequence initially defined as a consensus binding site for the Engrailed protein is also recognized by several other homeodomain proteins and mediates the transcriptional action of these regulators in transfected tissue culture cells. Here we show that these synthetic binding sites have a more restricted and specific ability to enhance transcription when assayed in transformed embryos. Several constructs with the homeodomain binding sites linked to the *fushi tarazu* or *engrailed* promoters are silent in transformed embryos. However, when linked to the *hsp70* promoter, the sites specifically activate transcription in glial cells. The effect of single base pair mutations in the binding sites suggests that activation is mediated by homeodomain protein(s). We suggest that this specific pattern of expression results from combined action at sequences within the *hsp70* promoter fragment and the homeodomain binding sites. Since the tissue culture transfection assay does not show such rigid constraints on promoter activation by homeodomain proteins, it appears that subsidiary phenomena apparent in the transgenic embryos contribute importantly to the specificity of action of functionally homologous homeodomain regulators.

Key words: Drosophila development/enhancer/homeodomain

Introduction

DNA binding proteins can be classified into families according to their levels of homology, and it has recently been recognized that closely homologous DNA binding proteins have similar DNA binding specificities. How do these related proteins fulfil their specific biological function? Some closely related regulators, such as Oct-1 and Oct-2, bind the same sites but nevertheless achieve unique regulatory roles due to differences in their ability to interact with other proteins and activate transcription (Stern *et al.*, 1989). In contrast, the closely related members of the steroid receptor family, though very similar in binding specificity, appear to distinguish among target genes by subtle differences in affinity for related sites (Glass *et al.*, 1988). Finally, a yeast homeodomain containing protein, $\alpha 2$ acts in conjunction with an accessory protein GRM by binding cooperatively to juxtaposed sites (Keleher *et al.*, 1988). Another yeast homeodomain protein, BAS1 works similarly in conjunction with BAS2 (Arndt *et al.*, 1987). In these instances, dual recognition can distinguish between related binding sites. Thus, there are at least three types of molecular interactions that contribute to the specialization of the regulatory roles of closely related DNA binding proteins.

The homeodomain proteins comprise one of the largest recognized families of DNA binding proteins. Although related by sequence, each of the genetically defined *Drosophila* homeodomain proteins plays a distinct role in embryogenesis. What is the origin of the distinct specificities? While some of the more diverged members of this family have distinct sequence specificity (Sauer *et al.*, 1988; Driever and Nüsslein-Volhard, 1989; Treissman *et al.*, 1989), several of the more closely related *Drosophila* homeotic and segmentation genes products have similar DNA binding specificities (Desplan *et al.*, 1988; Hoey and Levine, 1988). Indeed, a consensus sequence for Engrailed protein binding to DNA (TCAATTAAT) is an *in vitro* binding site for several homeodomain proteins (Desplan *et al.*, 1988; Hoey and Levine, 1988). We would like to understand how these related regulators achieve distinct regulatory roles.

Results of transient transfection assays in tissue culture cells have given us one view of the function these closely related homeodomain proteins can assume. Several homeodomain proteins act at a synthetic site (TCAATTAAATGA, NP sequence) to alter expression of a linked promoter in transfected tissue culture cells (Jaynes and O'Farrell, 1988). In this transfection assay, different homeodomain proteins influence transcription differently. Some activate (e.g. Fushi tarazu is a strong activator while Ubx is weak), others repress (e.g. Engrailed) and particular combinations function synergistically to activate transcription (Jaynes and O'Farrell, 1988; Han *et al.*, 1989; Jaynes and O'Farrell, unpublished observations). Furthermore, there are subtle differences in the binding affinities of some of these homeodomain proteins to different, but related, sites (Desplan *et al.*, 1988; Hoey and Levine, 1988; Jaynes and O'Farrell, 1988). In the transient assay, the different regulators compete for binding so that site occupancy is determined by the relative concentrations of the different proteins and by the precise nature of the binding sites (Jaynes and O'Farrell, 1988). This competition does not appear to be influenced by changes in the sequence context around the sites or by changes in the promoter. The observations suggest that different homeodomain proteins could achieve specificity by differences in their action (transcriptional activation or repression) and by their ability to distinguish among subtly different sites in target genes.

To explore the origins of the regulatory specificity of homeodomain proteins further, we sought to extend to the embryo observations made in transfected tissue culture cells. The embryo contains a multitude of homeodomain proteins (some positively and some negatively acting) each expressed in an intricate pattern. Where their distribution overlaps, related homeodomain proteins ought to compete for site

occupancy. Such competitive interactions might determine the pattern of expression of artificial constructs composed of a synthetic binding site upstream of a neutral promoter driving a reporter gene. For example, the NP sequence might elicit expression in the subset of *fushi tarazu* (*ftz*) expressing cells, where Ftz binding to the site is stronger than that of any potential negative regulator.

We have tested the activity in the embryo of constructs composed of homeodomain binding sites inserted upstream of a heat shock promoter driving the *lacZ* gene. These constructs were introduced into the fly genome by P-element mediated transformation. We find that a heat shock promoter with homeodomain binding sites is activated in specific cells at late embryonic stages. Thus, homeodomain binding sites can act as a cell type specific enhancer in the embryo. However, this activity of the homeodomain response elements is promoter specific, a feature that was not observed in the cell transfection experiments. Moreover, changes in the homeodomain response element suggest that the specificity of the expression pattern is not due to subtle differences in site recognition by different homeodomain proteins. Thus, in the context of the embryo, we find that interactions not evident in the tissue culture assays contribute to the specificity of action of homeodomain regulators.

Results

Synthetic homeodomain binding sites act as cell type specific enhancers

We have constructed simple artificial genes composed of homeodomain binding sites inserted upstream of heat shock promoter (*hsp70*) fragments tagged with the *lacZ* gene (Figure 1). One such gene contains six copies of the NP sequence upstream of a truncated heat shock promoter (sequences from −50 to +271). It is referred to as NP6−HZ50. This construct was introduced in the fly genome by P-element mediated transformation and embryonic expression of β-galactosidase was examined. Head involuted embryos exhibit an intricate pattern of β-galactosidase expression in a reproducible group of cells in the central and peripheral nervous systems (Figure 2). Most neurons of the peripheral nervous system (PNS) have been identified. They occupy stereotypical positions beneath the epidermis. For example, a subset of the chordotonal neurons forms a cluster of five cells located at a lateral position in each abdominal segment (Ghysen *et al.*, 1986; Bodmer and Jan, 1987). Mab 44C11 stains all neuronal nuclei and therefore illustrates directly the pattern of peripheral neurons (Bodmer *et al.*, 1987). Double staining with anti-β-gal and Mab 44C11 showed that the β-galactosidase expressing cells in the PNS are not neuronal but are closely associated with neurons (data not shown). For example, we find a cluster of five expressing cells in close apposition with the group of five chordotonal neurons. These cells are a specialized type of neuroglial cell, ligament cells (e.g. Bodmer *et al.*, 1989). The complexity of the CNS made it harder to identify specific β-galactosidase expressing cells in this region but the pattern of double staining, the morphology and position of the β-galactosidase expressing cells in the CNS are consistent with their being of glial type.

Expression is dependent on the NP6 sequence since no expression is detected in embryos carrying the construct lacking sites (HZ50 embryos). Thus, the NP6 sequence, a simple synthetic oligonucleotide, elicits cell type specific expression. It is noteworthy that no β-galactosidase expression is detected until germ band retracted stages. Thus, the artificial gene remains silent during the period of development when *ftz* is expressed. The lack of activation by Ftz is in contrast to findings made in transiently transfected tissue culture cells (see Discussion).

As expected for a transcriptional enhancer, the same pattern of expression is induced in NP6−HZ194 embryos which carry a construct (Figure 1) in which NP6 is inserted further away from the transcription start (data not shown). Additionally, decreasing the number of NP repeats alters expression quantitatively (Figure 2). Expression of β-galactosidase in NP4−HZ50 embryos (four NP repeats) is less intense than in NP6−HZ50 transformants. In NP2−HZ50 embryos (two NP repeats), intensity of expression is further reduced such that expression in the PNS can no longer be detected; weak expression is still detectable in the CNS and the brain lobes.

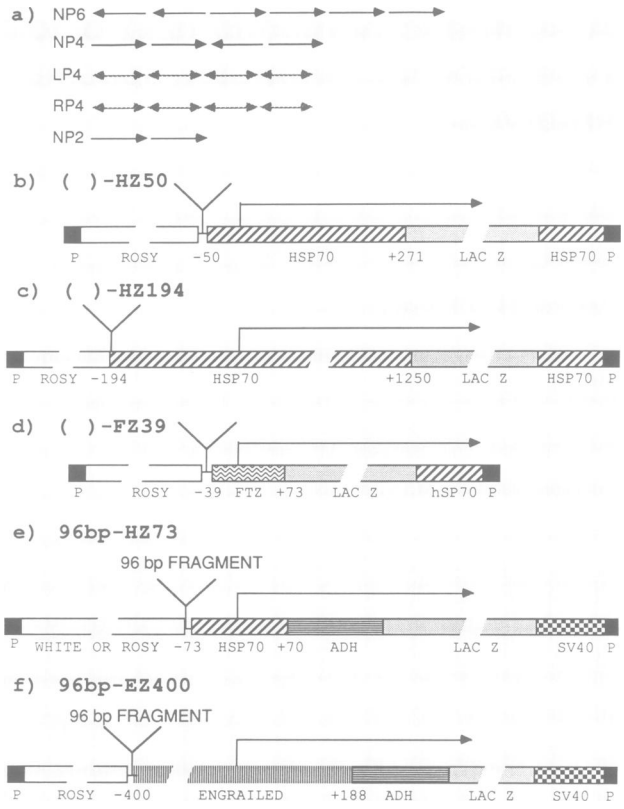

Fig. 1. (**a**) Schematic representation of the arrays of synthetic oligonucleotides incorporated in our artificial promoters (adapted from Jaynes and O'Farrell, 1988). The orientation of the NP sequence (5-TCAATTAAATGA-3′) is represented by the arrow pointing from 5′ to 3′. Since LP (TCAATTAATTGA) and RP (TCATTTAAATGA) are palindromic, they are represented by double headed arrows. Repeated arrays of synthetic oligonucleotides were inserted in transformation vectors upstream of various promoters driving *lacZ*. Two vectors contain heat shock promoter sequences: HZ50 (**b**) and HZ194 (**c**) and one contains *ftz* promoter sequences: FZ39 (**d**). An *engrailed* genomic fragment (96 bp *StuI*−*ClaI* fragment containing binding sites en1, en1′, en1″, en2 and en2′ from Desplan *et al.*, 1988; see Kassis *et al.*, 1989 for sequence) was inserted in another heat shock promoter-based construct, HZ73 (**e**). The same fragment was also inserted upstream of the *engrailed* promoter driving an Adh−β-galactosidase fusion (96bp−EZ400, **f**).

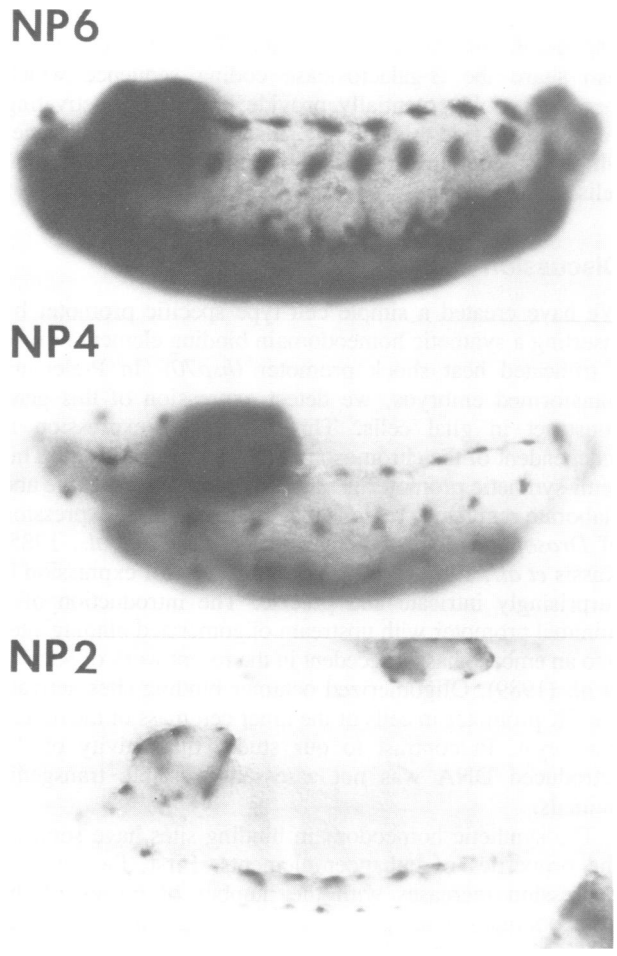

Fig. 2. Cytochemical detection of β-galactosidase activity in NP6−HZ50, NP4−HZ50 and NP2−HZ50 transformants. Decreasing the number of NP copies results in lower amounts of β-galactosidase produced while leaving the pattern of expression essentially unchanged. Embryos were aged 12−15 h at 25°C before fixation and staining. Staining of the three different lines was allowed to occur for a common time period such that differences in enzyme activity could be readily visualized.

Glial cell expression requires a homeodomain response element

Is this *cis*-acting function of homeodomain binding sites in embryos based on interaction with homeodomain-containing factors? The activities of two variant versions of the consensus were examined in embryos and compared with their characterized binding activities *in vitro* and their ability to mediate Ftz induction in tissue culture cells. Both mutant versions are derived from NP by a single base pair change. One version, TCAATTAATTGA (LP) resembles NP in that it is footprinted *in vitro* by homeodomain-containing proteins and mediates Ftz dependent activation in tissue culture cells (Desplan *et al.*, 1988; Jaynes and O'Farrell, 1988). For the other version, TCATTTAAATGA (RP), binding and activation are greatly depressed. We have generated LP4−HZ50 (four copies of LP) and RP4−HZ50 (four copies of RP) transformants. The expression patterns in LP4−HZ50 and NP4−HZ50 embryos are the same. On the other hand, the single base pair change from NP to RP results in complete loss of expression; no β-galactosidase can be detected in RP4−HZ50 transformants (four independent transformants). The parallels in the efficacy of variant sites suggests that *in vivo* site function involves interactions homologous to those characterized in tissue culture and *in vitro*. Since no known homeodomain protein is uniquely expressed in glial cells, we propose that activation is mediated by interaction with an unknown homeodomain protein or with a combination of homeodomain proteins.

A cluster of natural binding sites elicits activation in the same glial cells as the synthetic sites

The NP sequence was derived as a consensus of several sites found in the *engrailed* regulatory region. We asked what pattern of expression a 'natural' cluster of sites might elicit in the same assay. We generated transformants that carry a cluster of five binding sites from the *engrailed* upstream region (96 bp; fragment k; Desplan *et al.*, 1985; see Kassis *et al.*, 1989 for sequence) inserted upstream of the heat shock promoter driving the *lacZ* gene. Despite the fact that the spacing and sequences of the sites in the 96 bp are different from the synthetic consensus, the times and positions of β-galactosidase expression in 96 bp−HZ73 transformants share striking similarities with the ones exhibited by NP6−HZ50 transformants (Figure 3). Thus, the consensus and the authentic sites from which it was derived may interact with the same regulators in the embryo.

The pattern of expression in NP6−HZ50 transformants is dependent on, but is not specified by, heat shock promoter sequences

The *engrailed* gene is not normally expressed in the glial cells. This implies that the 96 bp fragment, when in its normal location within the *engrailed* regulatory region, does not activate the *engrailed* promoter in the glial cells. In fact, we detected no β-galactosidase in the glial cells of embryos carrying the 96 bp fragment upstream of the *engrailed* promoter (with 400 bp of *engrailed* upstream sequences) driving the *lacZ* gene (in 96bp−EZ400 transformed embryos). It is therefore possible that the homeodomain binding sites activate transcription in the glial cells in a promoter specific fashion, activating the *hsp70* promoter, but not the *engrailed* promoter. As a further test for promoter specificity we generated transformants carrying the *ftz* promoter linked to four copies of the NP sequence and found no detectable activity (as in NP4−FZ39 transformants, data not shown).

Despite the promoter specificity seen in P-element transformed embryos, no significant promoter specificity is seen in transient assays in tissue culture cells. In such assays, five promoters were activated to comparable levels by Ftz when juxtaposed to homeodomain binding sites. For example, in one experiment, the NP4−HZ194 and NP4−FZ39 genes were activated 7- and 8-fold respectively by Ftz in transient assays (Jaynes and O'Farrell, unpublished observations). Since these two promoters also have comparable basal activities, the ability to detect their activation should be comparable. These results indicate that the enhancement activity is not intrinsically limited to the heat shock promoter in the transient assay. Thus, additional or different types of interactions occur in P-element transformed embryos and these are promoter specific.

All the constructs that are activated in glial cells (NP6−HZ50, NP6−HZ194, 96bp−HZ73) contain variable

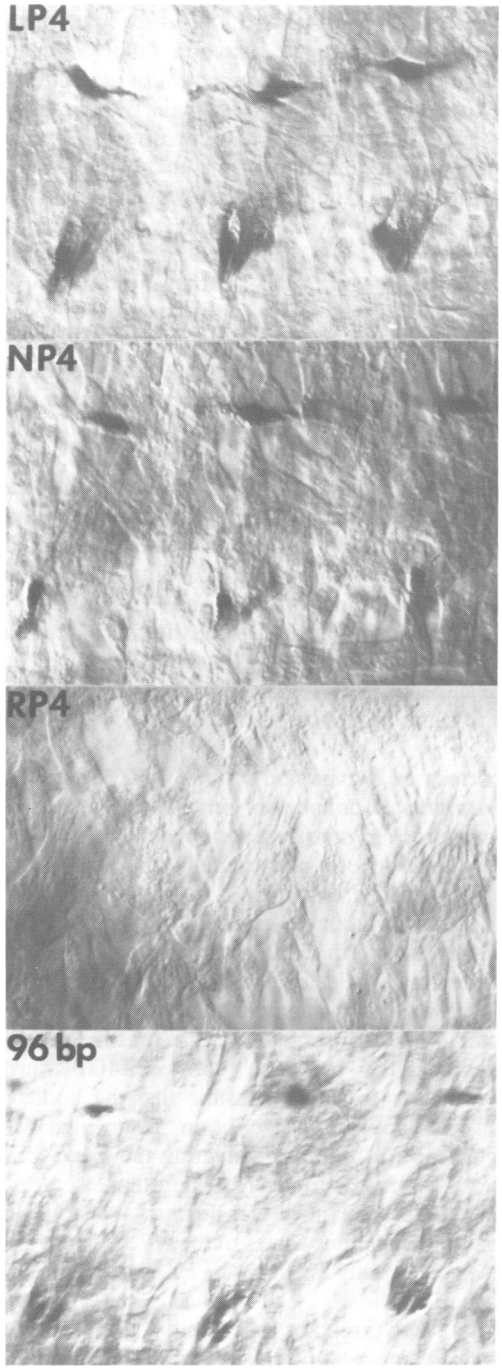

Fig. 3. Immunocytological detection of β-galactosidase in LP4−HZ50, NP4−HZ50, RP4−HZ50 and 96bp−HZ73 transformants. β-galactosidase is present in the CNS and PNS of LP4−HZ50, NP4−HZ50 and 96bp−HZ73 transformants while it is completely absent in RP4−HZ50 transformants. Here, we compare the patterns of β-galactosidase expression in the PNS of the four different transformants. All NP4−HZ50 and LP4−HZ50 lines express β-galactosidase in the groups of cells shown: the ligament cells that link the chordotonal organs to the epidermis and the support cell associated with the bipolar cell of the dorsal cluster (Ghysen *et al.*, 1986; Bodmer *et al.*, 1987). The level of expression is lower in NP4−HZ50 than in LP4−HZ50 transformants (this is not the case in the CNS where expression levels are similar for both transformants). The expression level in 96bp−HZ73 transformants is lower than in LP4−HZ50 transformants in both the CNS and PNS with some line to line variation of the relative levels of expression in different regions. Embryos are approximately the same ages as the ones shown in Figure 2.

amounts of heat shock sequence. The sequences they share extend from −50 to +70. Other sequences appear to be dispensable for glial cell expression. The active constructs also share the β-galactosidase coding sequence which therefore could potentially provide part of the activating function. We find this unlikely since the NP4−FZ39 gene, which also contains the *lacZ* gene, is not activated in glial cells.

Discussion

We have created a simple cell type specific promoter by inserting a synthetic homeodomain binding element next to a truncated heat shock promoter (*hsp70*). In P-element transformed embryos, we detect expression of this gene construct in glial cells. This pattern of expression is independent of the chromosomal location of the insert. This semi-synthetic promoter is much simpler than the large and elaborate regulatory regions that guide localized expression of *Drosophila* developmental genes (Karch *et al.*, 1985; Kassis *et al.*, 1985, 1989). Yet, its pattern of expression is surprisingly intricate and precise. The introduction of a minimal promoter with upstream oligomerized binding sites into an embryo has a precedent in the recent work of Schöler *et al.* (1989). Oligomerized octamer binding sites activate the TK promoter in cells of the inner cell mass of the mouse blastocyst. In contrast to our study, the activity of the introduced DNA was not assessed in stable transgenic animals.

The synthetic homeodomain binding sites have some of the properties of enhancer elements. First, the level of expression increases with the number of copies of the homeodomain binding sites while the spatial or temporal specificity of expression remains unchanged. This quantitative effect, in the absence of a qualitative change in expression is also seen with changes in the number of bicoid binding sites in constructs assayed in the early embryo (Driever *et al.*, 1989) or with changes in the number of a variety of enhancer sites in constructs assayed in tissue culture transfection. (Ondek *et al.*, 1987; Schirm *et al.*, 1987; Jaynes and O'Farrell, 1988). Second, we obtain the same pattern of expression whether or not the homeodomain binding sites are placed 50 or 194 bp upstream of the start of transcription showing some independence of distance from the start of transcription.

Activation of transcription by the homeodomain response element in embryos is promoter specific since the synthetic sites do not activate expression of the *ftz* promoter or the *engrailed* promoter in P-element transformed embryos while they clearly activate the *hsp70* promoter. Several characterized enhancers can activate different promoters (Atchison, 1988 and references therein) but not necessarily with the same efficiency. In cell culture, the octamer binding site appears to activate at least two promoters (TK and SV40; Schöler *et al.*, 1989). However, substantial quantitative differences (10-fold) are seen. In tissue culture cell transient assays, homeodomain response elements can similarly activate several promoters including the *ftz* promoter and the *engrailed* promoter. It might be argued that the *hsp70* promoter is simply more effective and is unique in giving detectable expression in the embryo. However, NP4−HZ194 gives easily detectable expression in embryonic glial cells while in the tissue culture transfection assays, it has

an activity slightly lower than that of NP4−FZ39, even though the latter has no detectable expression in the embryo. Somehow, our assay of enhancer action in the embryo uncovers a promoter specificity that is not apparent in tissue culture. Comparison of the various constructions shows that *hsp70* sequences between −50 and +70 are responsible for this activation. Despite the apparent promoter specificity, it is clear that the pattern of activation of our construct is not inherent to the heat shock promoter. Activation of the heat shock promoter in NP6−HZ50 transformants results from the presence of the NP sites since no expression is detected in HZ50 transformants (no site). Furthermore, the HZ50 gene has previously been used as a test gene for assays of enhancer activity of DNA segments and in such experiments, the HZ50 gene is induced in patterns unrelated to that reported here (Hiromi and Gehring, 1987).

How is this remarkable cell specificity of our glial specific promoter achieved? Since a single base pair change that drastically reduces *in vitro* binding eliminates glial specific enhancer action, it is likely that activation in glial cells is mediated by interaction with a homeodomain protein. Given the promiscuous action of many homeodomain proteins on these sites in transient transfection assays and the multitude of homeodomain proteins expressed in the embryo, we had not expected such a restricted pattern of expression. One could *a priori* consider two models for how glia specific expression arises. First, that there is a homeodomain protein expressed in glial cells that binds with uniquely high affinity to the NP sequence and that in the context of the embryo, only this homeodomain protein is capable of inducing expression. This would be the simplest view. Second, that the pattern of expression derives from the competitive interactions of negatively and positively acting homeodomain proteins such that the NP sites are occupied by activator only in glial cells. This model follows directly from observations of competitive interactions of homeodomain proteins in transient transfection assays (Jaynes and O'Farrell, 1988). Neither of these models accounts for the observation reported here that the embryonic expression pattern depends on the heat shock promoter. One could argue that this is not a sufficient reason to discount the two models since it is possible that our *ftz* and *engrailed* promoter fragments are inactive (or poorly active) in the embryo or in the glial cells for some unrelated reason. However, the *ftz* promoter can be influenced by a variety of enhancers in diverse patterns in enhancer trap assays. In fact, in a fraction of the transformant lines generated in such assays, the *ftz* promoter is activated in a different subset of glial cells (longitudinal gliablasts; Jacobs *et al.*, 1989).

A second experimental finding weakens the two models further. According to either model, the specification of the spatial pattern of expression resides entirely in the specificities of homeodomain proteins for the NP sites. If the sequence of the site is altered, the relative affinities of different homeodomain proteins for the site change (Desplan *et al.*, 1988; Kalionis and O'Farrell, unpublished). It follows, in the context of either model, that alterations of the sites should alter interactions with the multitude of homeodomain regulators in the embryo and should alter the spatial and temporal pattern of the construct. As described here, a change in the sequence of the site (NP4 to LP4) does not change the pattern of heat shock promoter activity. Furthermore, a natural DNA fragment having five sites related to the NP sequence elicits a similar pattern of heat shock promoter activation even though the spacing and the exact sequence of the sites differ from those in the NP constructs.

We propose an alternative view, namely that there is a second factor that binds to sequences associated with the heat shock promoter fragment and contributes to the specificity of our promoter. Such a view offers possible explanations for two observations: first that glial cell specific expression requires the heat shock promoter (*hsp70*) and second that the same specific pattern is elicited by a variety of related homeodomain binding sites. Thus, a requirement for an accessory protein interacting with sequences in the heat shock promoter fragment can explain why other promoter constructs fail to express. Furthermore, in this model, there are two distinct contributors to the specificity of expression: the homeodomain binding sites and the site for the accessory interaction. Accordingly, homeodomain binding sites might work to give glial expression only when associated with the particular accessory site in the *hsp70* promoter and variants of the NP sequence might maintain this specificity when associated with this accessory site. Note that the immediate sequence flanking the homeodomain binding site does not matter since two constructs (NP6−HZ 50 and NP6−HZ194) for which the immediate sequence context where NP6 is inserted differs, are similarly active. Thus, a fairly limited portion of the *hsp70* promoter along with a homeodomain binding site are sufficient to make a glia specific promoter. The proposed requirement for an accessory protein for homeodomain protein function is not without precedent. The octamer motif is a binding site for Oct-1 and Oct-2, two homeodomain proteins. The *cis*-action of the octamer motif is promoter specific. In the context of the β-globin promoter, the octamer motif is a B cell specific enhancer element whereas it is a ubiquitous enhancer for the U2 snRNA promoter (Tanaka *et al.*, 1988). To explain why Oct-1, a ubiquitous factor does not activate a β-globin promoter with octamer oligomers, it was proposed that Oct-1 requires cooperation with a factor associated with the U2 promoter for its action. (Schaffner, 1988; Stern *et al.*, 1989.) In fact, the heat shock promoter and *ftz* promoter fragments that we have used in our experiments are very small. To our knowledge, these fragments do not contain recognized protein binding sites other than a TATA element. This suggests the possibility that the accessory activity of our model might be a component of a specialized transcription complex.

In contrast to the glial cell expression in the P-element transformed lines, the activity of the NP sequences in transfection experiments is independent of the promoter and is responsive to a diversity of homeodomain proteins (in the context of our model, the activity of the NP sites in transient assays is independent of an accessory activity). For example, in transient cotransfections, Ftz strongly activates five promoters when they are linked to NP sites. Yet, when introduced into embryos by P-element transformation, none of the constructs we tested respond to the endogenously expressed Ftz. Thus, DNA that is integrated in the *Drosophila* genome appears to be under more stringent control than DNA transiently introduced in tissue culture cells. To understand the exquisite specificity of developmental regulators, it is going to be important to define the complex regulatory interactions that produce specificity in the context of the embryo.

Addendum

While this manuscript was in review, we obtained transformants with a larger number of copies of the NP sequence (six) linked to the *ftz* promoter driving β-galactosidase (NP6−FZ39 transformants). In contrast to the *ftz* promoter constructs with fewer NP sites (e.g. NP4−FZ39), these transformants (four independent lines) express β-galactosidase. Expression is in a very restricted set of cells in a pattern that is different from the β-galactosidase expression pattern in transformants with heat shock promoter constructs (e.g. NP6−HZ50 transformants). This result reinforces our conclusion that homeodomain binding sites stimulate transcription in a promoter specific fashion. More details will be presented in a subsequent publication.

Materials and methods

Plasmid construction

Various arrays of synthetic oligonucleotides were inserted in the unique restriction site of three transformation vectors: HZ50, HZ194 and FZ39. HZ50 was obtained from Y.Hiromi (University of California, Berkeley) and contains 5′ *hsp70* sequences up to −50 from the transcription start. HZ194 is plasmid pShxLac-7 obtained from P.Wensink's lab. It has 194 bp of upstream sequences from the *hsp70* promoter. FZ39 (obtained from J.Topol, Cal Tech) contains *ftz* sequences from −39 to +73 as in plasmid 5′Δ-40 from Dearolf *et al.* (1989).

A natural cluster of binding sites (96 bp *StuI−ClaI engrailed* genomic fragment containing sites en1, en1′, en1″, en2 and en2′ from Desplan *et al.*, 1988; see Kassis *et al.*, 1989 for sequence) was inserted in heat shock *lacZ* vectors in several steps. First, a plasmid was made that contains *hsp70* promoter sequences from −73 to +70 (obtained from P.Wensink's lab) cloned in the vector pC4AUGBgal (Thummel *et al.*, 1988). In this plasmid, the untranslated leader is an *hsp70*−Adh fusion and an Adh−β galactosidase fusion protein is made. The 96 bp fragment was inserted upstream of the *hsp70* sequences. An *Eco*RI fragment which extends from the inserted *engrailed* upstream sequences to the SV40 terminator was excised from this construct and subcloned into either the carnegie 20.1 vector (Simon *et al.*, 1985; contains the *rosy* gene as a selectable marker) or the CaSpeR vector (Pirotta, 1988; contains the *white* gene). Constructions differing in their orientations of the *engrailed* fragment were introduced into the *white*-based vector. They gave equivalent results.

Plasmid 96bp−EZ400, which contains the 96 bp fragment upstream of the *engrailed* promoter, was constructed as follows. A fragment of *engrailed* DNA extending from −400 to+188 was cloned into the *Sma*I site of pC4AUGβgal (see above). In our stable assay, this promoter fragment is inactive without the insertion of additional regulatory sequences. The 96 bp fragment was cloned upstream of the *engrailed* promoter. The orientation of the 96 bp fragment in this construct is opposite to the one within the *engrailed* regulatory region.

Transgenic flies

P-element mediated transformation was performed according to standard procedures (Hiromi *et al.*, 1985). At least four independent transformants were analyzed for each construct.

Staining of embryos

Cytochemical detection of β-galactosidase activity. Embryos were dechorionated in 50% bleach, and fixed in heptane saturated with glutaraldehyde/cacodylate (Hiromi *et al.*, 1985). β-galactosidase activity was detected *in situ* by the X-Gal (Boehringer) color reaction (Hiromi *et al.*, 1985).

Immunocytological detection of β-galactosidase. Embryos were prepared for immunodetection as described in DiNardo and O'Farrell (1987). Rabbit anti-β-galactosidase was obtained from Cappell (used at 1/2500 dilution in 10% normal goat serum). We used biotinylated secondary antibodies in conjunction with streptavidin horseradish peroxidase (BRL). Peroxidase was detected with diaminobenzidine and hydrogen peroxide (Sigma).

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