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Mechanisms regulating target gene selection by the homeodomain-containing protein Fushi tarazu

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SUMMARY

Homeodomain proteins are DNA-binding transcription factors that control major developmental patterning events. Although DNA binding is mediated by the homeodomain, interactions with other transcription factors play an unusually important role in the selection and regulation of target genes. A major question in the field is whether these cofactor interactions select target genes by modulating DNA binding site specificity (selective binding model), transcriptional activity (activity regulation model) or both. A related issue is whether the number of target genes bound and regulated is a small or large percentage of genes in the genome. In this study, we have addressed these issues using a chimeric protein that contains the strong activation domain of the viral VP16 protein fused to the *Drosophila* homeodomain-containing protein Fushi tarazu (Ftz). We find that genes previously thought not to be direct targets of Ftz remain unaffected by FtzVP16. Addition of the VP16 activation domain to Ftz does, however, allow it to regulate previously identified target genes at times and in regions that Ftz alone cannot. It also changes Ftz into an activator of two genes that it normally represses. Taken together, the results suggest that Ftz binds and regulates a relatively limited number of target genes, and that cofactors affect target gene specificity primarily by controlling binding site selection. Activity regulation then fine-tunes the temporal and spatial domains of promoter responses, the magnitude of these responses, and whether they are positive or negative.

Key words: Homeodomain, *fushi tarazu*, Target genes, Specificity, *Drosophila*

INTRODUCTION

Homeodomain proteins are arguably the most important class of transcription factors in early developmental patterning (McGinnis and Krumlauf, 1992). As with most other DNA binding transcription factors, they require interactions with other cofactors in order to recognize and regulate specific target genes (Mann and Chan, 1996; Biggin and McGinnis, 1997; Mann and Affolter, 1998). These interactions, however, are particularly important for homeodomain proteins because of the relatively low DNA binding specificity of the homeodomain. Most homeodomains bind sequences with an ATTA core motif (Gehring et al., 1994) and these motifs are abundantly distributed throughout the genomes of eukaryotic organisms (Desplan et al., 1985; Appel and Sakonju, 1993; Walter and Biggin, 1996). A classic example of an interaction that overcomes this problem of limited specificity is the interaction between the yeast homeodomain proteins MATa1 and MATα2, and their cofactor MCM1. In different combinations, these three proteins recognize unique binding sites and coordinate the differential gene expression patterns that define alternate yeast mating types (Goutte and Johnson, 1988; Keleher et al., 1988, 1989; Dranginis, 1990). An alternative strategy used by some homeodomain proteins is the incorporation of additional DNA binding domains (Bopp et al., 1986; Sturm and Herr, 1988; Karlsson et al., 1990; Treisman et al., 1991). Nevertheless, these compound homeodomain proteins also appear to require cofactor interactions in order to bind and regulate specific target genes (Kristie et al., 1989; Stern et al., 1989; Kristie and Sharp, 1990; Zwilling et al., 1995; Copeland et al., 1996).

In this study, we focus on the *Drosophila* homeodomain-containing protein Fushi tarazu (Ftz) and cofactors that are required during early embryogenesis. Ftz is a member of the Q50 class of homeodomain proteins, meaning that it has a glutamine at position 50 of the homeodomain (Treisman et al., 1992). Q50 homeodomain proteins represent the largest class of homeodomain proteins, and because the residue at position 50 is the main determinant of specificity, these proteins all bind in vitro to nearly identical sites (Desplan et al., 1988; Hoey and Levine, 1988; Laughon et al., 1988; Treisman et al., 1992). In the embryo, however, they control very different aspects of development. Ftz, for example, belongs to the pair-rule class of segmentation proteins (Nusslein-Volhard and Wieschaus, 1980; Wakimoto and Kaufman, 1981; Wakimoto et al., 1984). It controls the patterning of alternate segmental regions by binding and regulating target genes that are expressed in these regions.

In cultured cells, Ftz acts as a relatively strong activator of both synthetic and actual target gene promoters (Jaynes and O'Farrell, 1988; Han et al., 1989; Winslow et al., 1989). In the embryo, however, it appears to act as both an activator and repressor of transcription (Ingham et al., 1988; Ish-Horowicz et al., 1989; Nasiadka and Krause, 1999). Several lines of evidence emphasize the importance of cofactors in the binding and regulation of these target genes. First, when reporter genes with synthetic promoters that contain only consensus Ftz binding sites are introduced into embryos, they fail to respond to Ftz (Vincent et al., 1990). Second, homeodomain-deleted versions of Ftz that are incapable of binding to DNA are still able to regulate Ftz target genes, and can even rescue *ftz*-dependent segmentation (Fitzpatrick et al., 1992; Copeland et al., 1996; Hyduk and Percival-Smith, 1996). Thus, interactions with other transcription factors are both required and sufficient for the recognition and regulation of specific target gene promoters.

To date, two cofactors have been identified that are required for proper Ftz function in vivo, the nuclear receptor protein Ftz-Factor 1 (Ftz-F1) (Guichet et al., 1997; Yu et al., 1997) and another homeodomain-containing protein Paired (Prd) (Copeland et al., 1996). Loss of Ftz-F1 expression during early embryogenesis causes a segmental phenotype that is indistinguishable from that of Ftz (Guichet et al., 1997; Yu et al., 1997). This phenotype is due to an inability of Ftz to regulate both positively and negatively regulated target genes (Guichet et al., 1997). A direct interaction between Ftz and Ftz-F1 is required for these processes. Interestingly, Ftz-F1 is not essential for the ability of Ftz to autoregulate its own expression (Guichet et al., 1997), indicating the existence of additional cofactors.

Two models have recently been put forward to explain how cofactors contribute to functional differences amongst Q50 homeodomain proteins. The first model suggests that cofactors target each protein to different sets of genes by contributing to their sequence-specific DNA binding properties (Mann and Chan, 1996; Mann and Affolter, 1998). Some classic examples in support of this model include interactions on specific response elements between members of the Extradenticle (Exd)/Pbx protein family and homeotic selector proteins such as Ultrabithorax (Ubx) (Chan et al., 1994; van Dijk and Murre, 1994; Chang et al., 1995). Similarly, it has been shown that Ftz and Ftz-F1 bind to adjacent sites on a functional reporter with as much as a 100-fold increase in affinity (Yu et al., 1997), suggesting that Ftz-F1 plays an important role in recruiting Ftz to these specific sites. Indeed, one of these reporters has also been tested with a homeodomain-deleted version of Ftz (Hyduk and Percival-Smith, 1996) and responds appropriately, indicating that cofactor interactions are sufficient for recruitment.

The second model proposes that, instead of modulating DNA binding properties, cofactors alter the ability of homeodomain proteins to regulate promoters to which they are already bound. These binding sites are thought to include all or most of the sites bound in vitro, meaning that these proteins would be extensively bound to most accessible promoters. Specificity of action is achieved by cofactor interactions that modify the regulatory potential of particular molecules in a site-specific fashion. Much of the support for this 'activity regulation' model comes from in vivo cross-linking studies to a number of promoters (Walter et al., 1994; Carr and Biggin, 1999). The two Q50 homeodomain proteins, Ftz and Even-skipped (Eve), were detected on most of the restriction fragments tested with similar relative levels of occupancy (Walter et al., 1994). It was also found that most embryonically expressed genes show some degree of pair-rule periodicity in their patterns of expression (Liang and Biggin, 1998), consistent with the possibility of direct regulation by Eve or Ftz. Additional support for this model comes from the observation that the homeodomain protein Deformed (Dfd) can bind to monomer DNA sites in vivo independently of cofactors such as Exd, but that this binding is apparently not sufficient for gene activation (Li et al., 1999a).

A major objective of this study was to search for additional evidence in support of either the selective binding or activity regulation models of Ftz cofactor function. A method was sought that would allow the monitoring of native target genes in an in vivo setting. This was achieved by expressing a chimeric Ftz transcription factor that contains the potent acidic activation domain of the herpes simplex virus protein VP16 in early embryos. The VP16 activation domain is fully modular in function and is capable of activating virtually any eukaryotic promoter in organisms ranging from yeast to man (Triezenberg et al., 1988). It can even convert transcriptional repressors into activators (Jimenez et al., 1996; Jimenez and Ish-Horowicz, 1997; Kramer et al., 1999). The strength and autonomy of the VP16 activation domain is explained in part by its ability to activate transcription via multiple mechanisms and at multiple levels. These include an ability to recruit histone acetyltransferases (Utley et al., 1998; Ikeda et al., 1999), to recruit ATP-dependent chromatin remodeling complexes (Neely et al., 1999) and to interact directly with components of the RNA polymerase holoenzyme (reviewed by Triezenberg, 1995, Stargell and Struhl, 1996). Taken together, these properties suggest that, when bound to Ftz, the VP16 activation domain should be able to activate promoters that are within regulatory distance of Ftz binding sites independently of Ftz cofactors. Thus, if Ftz function is strictly regulated at the level of DNA binding, FtzVP16 should regulate the same set of genes as Ftz. These genes might be activated to higher levels, but this may have little consequence on development since raising the levels of Ftz by as much as 2 fold has no apparent effect on segmental patterning (Lawrence and Pick, 1998; A. N. and H. M. K., unpublished observations). Conversely, if Ftz is widely distributed on most accessible promoters, as suggested by the activity regulation model, expression of FtzVP16 should result in the activation of genes that are not normally regulated by Ftz.

Our results suggest that Ftz specificity is primarily regulated at the level of DNA binding. Activity regulation then fine-tunes the times and regions that bound promoters can be regulated, as well as the magnitude of responses and whether they are positive or negative.

MATERIALS AND METHODS

Construction of Ftz-VP16 fusion and control constructs

FtzVP16 was made by removing a *Hae*III-*Eco*RI fragment from the C-terminal end of *ftz*, and replacing it with a *Sal*I-*Eco*RI restriction fragment that encodes the C-terminal activation domain of VP16

(Campbell et al., 1984). An analogous exchange was made using a homeodomain-deleted version of Ftz (FtzΔHD) (Fitzpatrick et al., 1992) to make FtzΔHDVP16. FtzΔC was made by removing the C-terminal *Hae*III-*Eco*RI fragment of *ftz*. FtzΔNVP16 was made by removing sequences from the FtzVP16 construct N-terminal to the *Xho*I site that is located in the N-terminal portion of the *ftz* homeobox. For the transfection studies, each of the constructs was inserted into the expression vector pPac (Krasnow et al., 1989). The same fragments were also inserted into the P element vector pHT4 (Schneuwly et al., 1987) for embryonic expression under control of the *hsp70* promoter.

As a consequence of the cloning strategies, non-Ftz amino acids were introduced at the termini of the Ftz polypeptides. In the Ftz, FtzΔHD, FtzVP16, FtzΔHDVP16 and FtzΔC constructs, the N-terminal sequence is MDPEFIKEEKLTMRDP-(T)3. In FtzΔNVP16, the N-terminal sequence is MDPEFELGTRGSSRV-(E)273. Non-Ftz amino acids at the C terminus of FtzΔC are Ftz(E)337-GGILV. The sequence at the Ftz-VP16 interphase also includes the additional amino acids: Ftz(E)337-GGIR-(T)480VP16.

Cell culture, transfections and CAT activity assays

Drosophila Schneider line 2 (S2) cells (Schneider, 1972) were grown as described previously (Di Nocera and Dawid, 1983). Transfections were performed using the calcium phosphate technique described by Krasnow et al. (1989). DNA mixtures included 2 μg of Ftz-expressing plasmids and 0.4 μg of the reporter plasmid pNP6CAT (Jaynes and O'Farrell, 1988). Cells recovered from each of the duplicate plates were split, with one half used for western blotting and the other half lysed for CAT activity measurements. CAT assays were performed as described by Seed and Sheen (1988).

Generation of transgenic flies

Transgenic flies were obtained by injecting the P element constructs described above into ry^{506} embryos as described by Rubin and Spradling (1982). Transgenic lines obtained for pHT4Ftz and pHT4FtzΔHD have been described previously (Fitzpatrick et al., 1992). Two independent lines with single copy insertions, one on the first chromosome and the other on the third, were obtained for pHT4FtzVP16. Both lines are non-viable when homozygous and maintained as heterozygous stocks over either FM6 or TM3. Two independent lines with single copy insertions were obtained for pHT4FtzΔHDVP16; one inserted on the second chromosome and the other on the third. These lines are also homozygous inviable, and are kept over CyO and TM3, respectively. One homozygous line was obtained for pHT4FtzΔC; a single copy insertion on the third chromosome. Finally, two independent homozygous lines were produced for pHT4FtzΔNVP16, one with a single copy insertion on the first chromosome, and the other with a single copy insertion on the second.

For western blots, Ftz polypeptides were detected using either anti-Ftz or anti-VP16 polyclonal antibodies and then alkaline phosphatase-coupled secondary antibodies.

Cuticle preparation and analysis

Embryos for cuticle preparations were collected on apple juice/agar plates for 30 minutes. Eight collections each of wild-type, HSFtzVP16 and HSFtz embryos were made and aged for different periods such that a series of 30 minute intervals was obtained. Each interval overlapped the preceding and the following one by 10 minutes (i.e. 1:40-2:10, 2:00-2:30, 2:20-2:50, etc.), spanning from 1:40 to 4:30 AEL. After aging at 25°C for the times indicated, embryos were heat-treated by immersion in a 36.5°C water bath, usually for 8 minutes, then rinsed with 25°C water and transferred to apple juice plates for further aging. Cuticles were prepared as described in Saulier-Le Drean et al. (1998).

In situ hybridization and immunocytochemistry

Embryos were collected on apple juice/agar plates for 1 hour, aged until either 2:00-3:00 or 2:30-3:30 hours old, and then heat-treated for either 8 or 4 minutes at 36.5°C. After heat-shock, embryos were allowed to recover for 25 minutes, fixed in 4% formaldehyde and stored in methanol. The effect of the heat treatment was determined by following target gene expression in heat-treated Oregon R embryos, treated in parallel.

In situ hybridization to whole-mount embryos using digoxigenin-labeled DNA probes was performed as previously described (Manoukian and Krause, 1992). Plasmids and primers for probe preparation have also been described elsewhere (Nasiadka and Krause, 1999). Following hybridization, probes were visualized using alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim). Cellularizing embryos were precisely staged by monitoring the position of inward-migrating plasma membranes.

Antibody staining was performed as described previously (Manoukian and Krause, 1992). Expression of Ftz deletion and fusion constructs in whole-mount embryos was detected with anti-Ftz polyclonal antibodies (Krause et al., 1988).

RESULTS

Enhancement of Ftz activity by the VP16 activation domain

The C-terminal 76 amino acids of Ftz encode a modular, glutamine-rich activation domain capable of efficiently activating transcription in cultured *Drosophila* S2 cells (Fitzpatrick et al., 1992). In the embryo, deletion of this region causes only a moderate loss of activity that is largely compensated for by higher levels of protein expression (Kellerman et al., 1990; Fitzpatrick et al., 1992). Hence, replacement of this region with the acidic activation domain of VP16 should result in a fusion protein that retains Ftz specificity while gaining the ability to activate target genes with greater autonomy and effectiveness. If so, then target genes might be activated at times and in places where the non-fused protein is incapable of acting. In addition, target genes that are normally repressed by Ftz might now be activated. Constructs generated to test this hypothesis are shown in Fig. 1A. In addition to the FtzVP16 fusion protein, several control constructs were made, including full-length Ftz, Ftz with the C-terminal 76 amino acids deleted (FtzΔC), the VP16 activation domain fused to the C-terminal 2/3 of the Ftz homeodomain (FtzΔNVP16) and homeodomain-deleted versions of Ftz (FtzΔHD) and FtzVP16 (FtzΔHDVP16).

Transient expression in *Drosophila* S2 cells was used as a first approximation of whether FtzVP16 retained the same DNA binding specificity as that of Ftz. Ftz constructs were subcloned under control of the *actin 5C* promoter (Krasnow et al., 1989). The reporter gene NP6CAT, which contains concatamerized Ftz binding sites upstream of a basal promoter (Desplan et al., 1988; Jaynes and O'Farrell, 1988), was used to monitor transcriptional activity. All proteins were expressed at similar levels when assayed by western blot analysis (data not shown). Results, shown in Fig. 1B, show that removal of the Ftz C-terminal activation domain results in an approximate six-fold drop in Ftz activity (15-fold activation versus 94-fold for full-length Ftz). Replacement of this region with the VP16 activation domain results in a two-fold increase in activity (192-fold activation versus 94-fold for full-length Ftz). This increase likely reflects the relative activities of the two activation domains; the Ftz C-terminal activation domain is a relatively strong one (Fitzpatrick et al., 1992), while the VP16

activation domain is arguably the most potent domain tested (Triezenberg et al., 1988). As has been previously shown, removal of the Ftz homeodomain drops transcriptional activity in this assay to near basal levels (Fitzpatrick et al., 1992). This was also true for the homeodomain deleted control constructs FtzΔHDVP16 and FtzΔNVP16. Similar results were obtained using another Ftz reporter gene (UbxCAT), and no responses were observed when a reporter gene with no Ftz binding sites (TATACAT) was used (data not shown). Taken together, these data show that FtzVP16 retains the DNA binding specificity of Ftz, and that in cultured cells, it is a better activator of transcription.

All constructs shown in Fig. 1A were next subcloned under control of the *hsp70* gene heat-inducible promoter (Schneuwly et al., 1987) and introduced into flies by P element-mediated germ-line transformation (Rubin and Spradling, 1982). At least two transgenic lines were obtained for each construct (see Materials and methods). To ensure that each construct was correct, and to compare levels of protein expressed, larvae were collected from transgenic lines, heat pulsed for 1 hour, allowed to recover for 30 minutes, lysed in SDS PAGE loading buffer and analyzed by western blotting. Fig. 1C shows that equivalent levels of Ftz and FtzVP16 are expressed, and that no major degradation products are observed. Each of the other proteins is also expressed at similar levels (Fitzpatrick et al., 1992; Copeland et al., 1996 and data not shown).

Protein levels were also assessed in embryos by immunocytochemistry. With an 8 minute heat pulse at 36°C, the standard duration of heat shock for our HSFtz lines (Nasiadka and Krause, 1999), cellular levels of ectopic protein expression were similar to one another, and at least two-fold lower than the cellular levels of endogenous Ftz protein (data not shown). The same induction conditions were used in all of the experiments that follow, except when monitoring genes whose expression is particularly sensitive to heat shock (*even-skipped* (*eve*), *runt* (*run*) and *gooseberry* (*gsb*)). For the latter, heat pulses of 4 minutes were used.

Effects of FtzVP16 on segmental patterning

FtzVP16 activity in vivo was first assessed by inducing ectopic expression at various developmental time points and then examining patterning defects in the cuticles of mature embryos. It has been shown previously that ectopic expression of Ftz between 2.5 and 3 hours after egg laying (AEL) gives rise to a pair-rule cuticular phenotype in which regions normally derived from odd-numbered (*ftz*-independent) parasegments are missing (Struhl, 1985; Ish-Horowicz and Gyurkovics, 1988; Ish-Horowicz et al., 1989). To compare the patterning activities of Ftz and FtzVP16, transgenic embryos were heat pulsed before, during and after this time interval, and then cuticles were prepared and examined.

We first scored the number of wild-type cuticles obtained from each line as a function of the time of heat shock. As can be seen in Fig. 2B, induction of both Ftz and FtzVP16 causes a substantial decrease in the number of wild-type cuticles, with the maximal decrease centered at about 2.5 hours AEL. The two curves differ, however, in the number of non-wild-type cuticles obtained at earlier and later times. FtzVP16 is able to induce patterning defects with a modest increase in efficiency at both earlier and later stages of development.

Next, we closely examined the mutant cuticles to see if the FtzVP16-induced phenotypes were either similar to those induced by Ftz or novel. The most frequently observed phenotype for both constructs is the 'anti-*ftz*' pair-rule phenotype previously described by Struhl (1985; Fig. 2C). In both the HSFtz and HSFtzVP16 lines, the frequency of this phenotype (deletion of *ftz*-independent parasegments) peaks at about 50% when induction is initiated at about 2.5 hours AEL (Fig. 2D). The HSFtzVP16 line differs, however, in that this

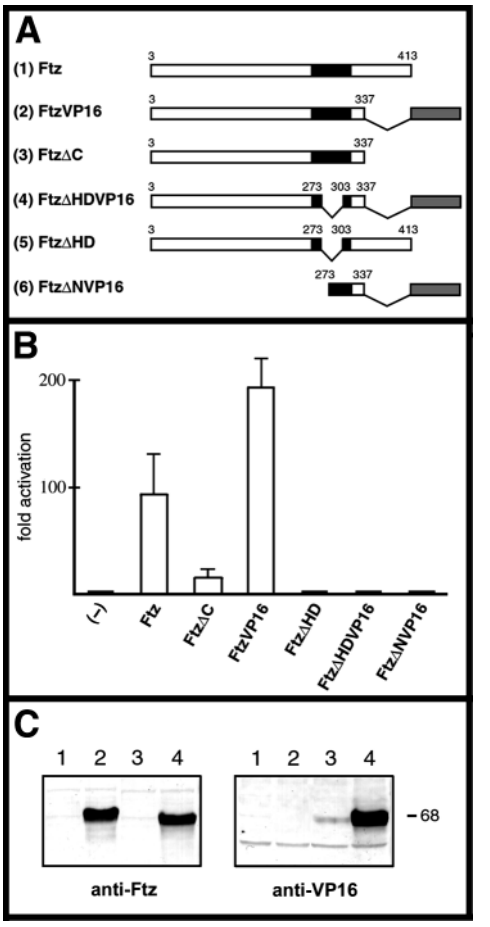

Fig. 1. Structure, activity and expression of Ftz derivatives. (A) Schematic representation of Ftz constructs: The Ftz polypeptide is presented as an open box with the homeodomain denoted in black. The VP16 activation domain is shown as a gray box. Internal deletions span the Ftz amino acid residues indicated by the numbers above. (B) Relative transactivation activity of Ftz and Ftz derivatives in *Drosophila* Schneider line 2 (S2) cells. Cells were transfected with the CAT (chloramphenicol acetyltransferase) reporter plasmid pNP6CAT alone (–) or together with the Ftz-expressing construct indicated. Transactivation activity was calculated as fold activation over basal reporter activity. The activities shown are the average of three transfections. Corresponding standard deviations are indicated. Addition of the VP16 activation domain significantly increases the activity of Ftz, while removal of the homeodomain reduces activity to near background levels. (C) Western blot analysis showing Ftz (lanes 1, 2) and FtzVP16 (lanes 3, 4) expression levels in transgenic larvae. Proteins were extracted from non-heat shocked (lanes 1, 3) and heat shocked (lanes 2, 4) larvae and detected using anti-Ftz (left panel) or anti-VP16 (right panel) antisera. Ftz and FtzVP16 migrate with relative molecular masses (M_r) of about 70 and 68, respectively. Both polypeptides are expressed at similar levels and are predominantly full-length.

phenotype can be induced a little earlier and with a somewhat higher efficiency.

Further examination of the FtzVP16-induced cuticles revealed several novel phenotypes not found amongst the Ftz-induced cuticles. These less abundant phenotypes are shown in Fig. 2E,G,I,K. The first phenotype (Fig. 2E) is characterized by fusions between alternate denticle belts. Most of these fusions (approx. 80%) occur in posterior regions. Disruptions of terminal structures are also observed in each of these cuticles. This phenotype peaks when heat pulses are administered between 1:40 and 2:20 AEL (Fig. 2F). Although the non-fused Ftz protein appears to generate a weaker form of this phenotype, its penetrance and severity are substantially lower than that induced by FtzVP16.

The second FtzVP16-specific phenotype can best be described as an extreme pair-wise fusion of denticle belts with the remaining denticles arranged in mirror-image symmetry (Fig. 2G). This phenotype peaks with a frequency of about 15% at 2:40-3:10 AEL (Fig. 2H). The third phenotype (Fig. 2I) exhibits deletions of both naked and denticle belt-containing portions of each segment. As with the previous phenotype, the remaining portions of each denticle belt appear to be duplicated with mirror image symmetry. This pattern is prevalent when heat pulses are administered at 3:00-3:30 AEL with a maximum frequency of about 17% (Fig. 2J). The final pattern (Fig. 2K) occurs when heat pulses are provided relatively late (3:30-4:00; Fig. 2L) in the responsive window. All denticle belts are deleted in this phenotype.

The segmental phenotypes described were not observed in heat shocked control embryos, nor in FtzΔNVP16 embryos. FtzΔC embryos yielded only wild-type and anti-*ftz* phenotypes, with the latter at a lower penetrance than those induced by full-length Ftz. Taken together, these controls show that the novel phenotypes generated by FtzVP16 are specific and most likely due to the enhanced abilities of the chimeric protein.

Effects of FtzVP16 on *ftz* autoregulation

The changes underlying the cuticle phenotypes described above can best be understood by monitoring the earliest changes in Ftz target gene expression patterns. These were monitored by in situ hybridization in cellularizing and gastrulating embryos. Embryos were fixed 25 minutes after a brief 8 minute heat shock to ensure that the effects observed are direct (Nasiadka and Krause, 1999). The best characterized target of Ftz is the *ftz* gene enhancer (Hiromi et al., 1985; Hiromi and Gehring, 1987; Pick et al., 1990; Schier and Gehring, 1992; Schier and Gehring, 1993). Multiple Ftz binding sites are present on the *ftz* enhancer, and reporter genes carrying portions of the *ftz* enhancer fail to respond when either the sites or Ftz is inactivated. Conversely, ectopic expression of Ftz results in expansion of endogenous *ftz* stripes (Ish-Horowicz et al., 1989) and stripes expressed by reporter

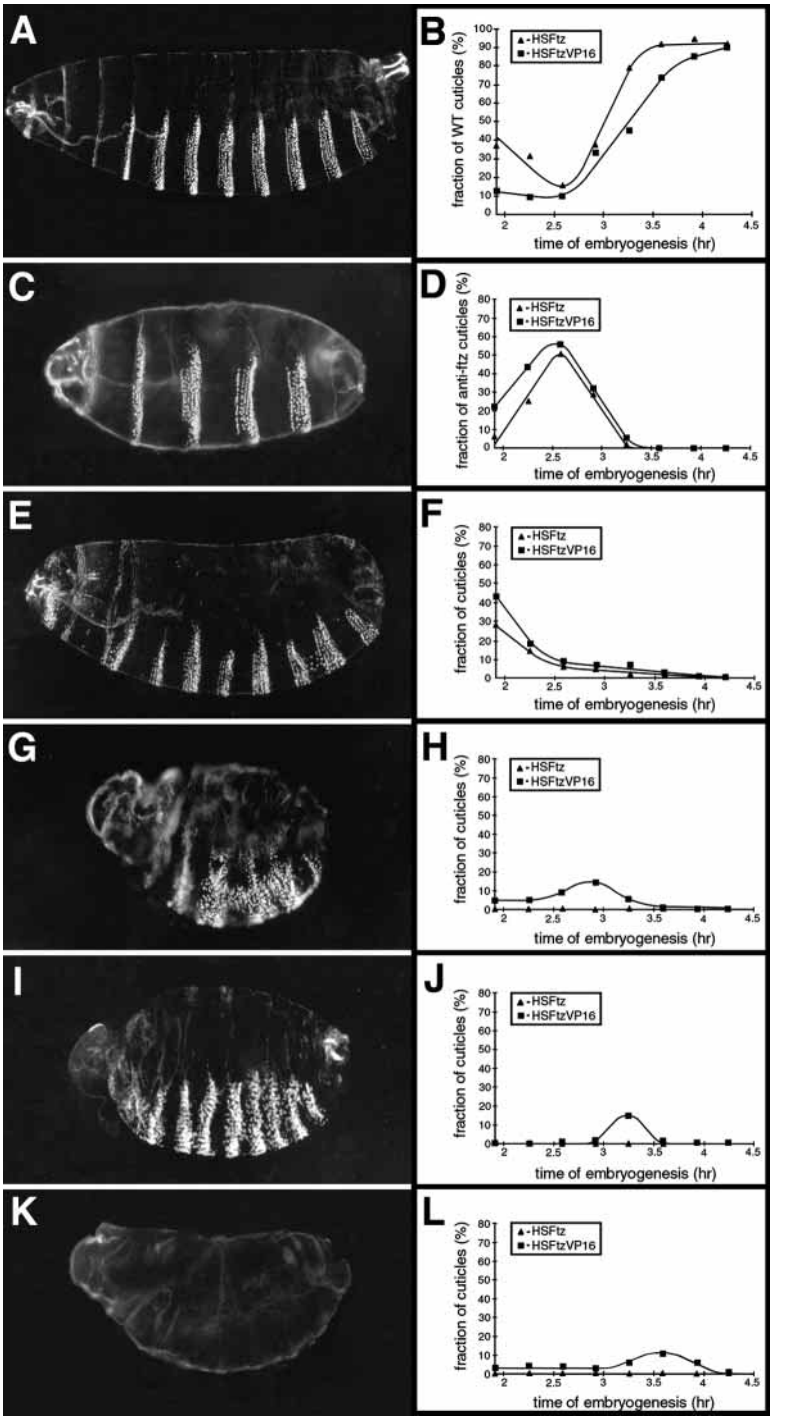

Fig. 2. Cuticular phenotypes caused by ectopic FtzVP16. Ftz and FtzVP16 were expressed ectopically in differentially staged embryos (aged between 1:30 and 4:30 AEL), and the embryos allowed to develop for 24 hours to secrete cuticle. Cuticular phenotypes observed are shown on the left (dark-field photomicrographs: A,C,E,G,I,K). Curves on the right (B,D,F,H,J,L) show the frequencies (Y axes) at which each of the phenotypes on the left was observed when induced at the times shown (X axes). A minimum of 200 cuticles were scored for each of the indicated times. Values obtained using HSFtz embryos are indicated by triangles, and values obtained using HSFtzVP16 embryos are indicated by squares. Although their effects are similar, FtzVP16 was able to induce novel phenotypes during times at which Ftz had no effect.

genes carrying these Ftz-responsive elements (Hyduk and Percival-Smith, 1996).

Fig. 3 shows that ectopically expressed Ftz begins to effectively activate the endogenous *ftz* gene during early stages of cellularization (early stage 5, 2:00-2:20 AEL). Endogenous *ftz* expression is expanded from an irregular set of initiating stripes (Fig. 3A) into a single broad band that fills the trunk portion of the embryo (Fig. 3B). FtzVP16 has a similar effect except that induction is more robust and uniform (Fig. 3C), and begins about 10-15 minutes earlier (not shown). Neither protein, however, is able to activate *ftz* in the terminal regions of the embryo.

As cellularization nears completion, *ftz* stripes are well-established and about 4 cells wide (Fig. 3D). At this time (late stage 5), ectopically expressed Ftz can no longer activate *ftz* throughout the trunk: the pattern remains clearly striped (Fig. 3E). In contrast, FtzVP16 retains the ability to effectively activate *ftz* throughout the trunk (Fig. 3F). By the time cellularization is complete (stage 6), endogenous *ftz* stripes are beginning to narrow (Fig. 3G). When Ftz is expressed ectopically, each *ftz* stripe widens anteriorly by approximately one cell in width (Fig. 3H). As observed at the earlier stage, FtzVP16 has a similar but more robust effect. Endogenous *ftz* stripes widen by about the same amount anteriorly, but are noticeably more intense (Fig. 3I). In addition, a novel set of weak stripes (marked by arrowheads) appears immediately behind each of the stronger stripes. These occupy the posterior regions of even-numbered parasegments. Similar responses are observed when Ftz or FtzVP16 is induced during later stages of normal *ftz* expression. Ectopic Ftz causes only a modest widening of endogenous *ftz* stripes (Fig. 3K), while FtzVP16 gains the ability to induce additional *ftz* stripes in the posterior regions of even-numbered parasegments (Fig. 3L). This capacity perdures somewhat beyond the time that *ftz* is normally expressed and beyond the time that ectopic Ftz has any effect (Fig. 3M-O).

To summarize, both Ftz and FtzVP16 are limited in terms of when and where they can activate the endogenous *ftz* gene. FtzVP16, however, activates *ftz* to somewhat higher levels, and exhibits broader temporal and spatial domains of activity. As seen with the cuticle preparations, the effects of FtzΔC are similar to those of Ftz, although weaker, and FtzΔNVP16 has no effects on *ftz* expression (data not shown). Similar results, although marginally weaker, were obtained with 4 minute heat shocks (data not shown).

Responses of the *engrailed* (*en*) gene

After *ftz*, the next best characterized Ftz target gene is the segment polarity gene *engrailed* (*en*). Transcription of *en* normally begins at the end of cellularization, with its 14, single-cell-wide stripes initiating in an anterior to posterior fashion (Fjose et al., 1985; Kornberg et al., 1985; Fig. 4D). The even-numbered stripes, which appear prior to adjacent odd-numbered stripes, are Ftz-dependent (Howard and Ingham, 1986; DiNardo and O'Farrell, 1987; Ingham et al., 1988). These broaden when Ftz is ectopically expressed (Ish-Horowicz et al., 1989; Fig. 4E) with the same response kinetics as *ftz* autoregulation (Nasiadka and Krause, 1999). Binding sites for Ftz and Ftz-F1 have been mapped within the *en* promoter, and mutation of these sites affects reporter gene responses (Florence et al., 1997).

Interestingly, Ftz-dependent activation of *en* begins well after the beginning of *ftz* gene autoregulation. Consistent with this delayed responsiveness, ectopic expression of Ftz fails to bring about premature activation of *en* (Fig. 4B). However, as even-numbered stripes begin to initiate, ectopic Ftz expression causes an anterior expansion of about one cell in width (Fig. 4E). This effect can be achieved until about mid-germ band

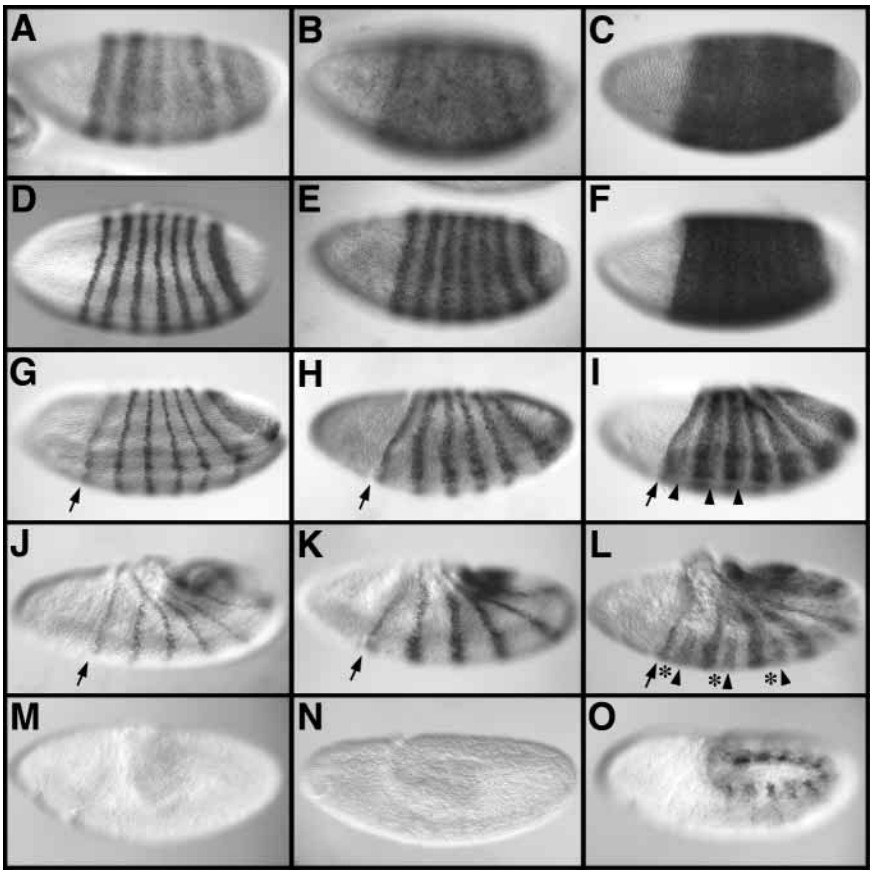

Fig. 3. Effects of ectopic Ftz and FtzVP16 on the endogenous *ftz* gene. *ftz* expression patterns are shown at five different stages: the onset of cellularization (A-C), the end of cellularization (D-F), late gastrulation (G-I), early germband extension (J-L) and advanced germ band extension (M-O). Control embryos are shown on the left (A,D,G,J,M), HSFtz embryos in the middle (B,E,H,K,N) and HSFtzVP16 embryos on the right (C,F,I,L,O). *ftz* transcripts were detected using a probe that hybridizes specifically to endogenous *ftz* gene mRNA. Arrows mark the position of the cephalic furrow in gastrulating and early germband extending embryos. Arrowheads in I and L indicate posterior regions of the first three even-numbered parasegments. Asterisks in L mark the middle of the first three even numbered parasegments (L). Note that FtzVP16 is able to induce ectopic *ftz* expression in regions and at times that Ftz cannot. However, its activity is still limited.

extension (approx. 3:30 AEL), after which time, ectopically expressed Ftz has no effect.

The effects of FtzVP16 on *en* are again different from those of Ftz and also differ somewhat from the types of effects that were observed on the *ftz* gene. As with *ftz*, *en* responses begin earlier than those induced by the non-fused Ftz protein. Weak induction is observed in the trunk of the embryo during the earliest stages of cellularization (approx. 2:10 AEL; Fig. 4C). Curiously, this early ability of FtzVP16 to activate *en* in the trunk is quickly lost, such that by mid-cellularization, FtzVP16 is only able to activate *en* in posterior regions of the head, and more weakly at the posterior tip of the embryo (Fig. 4F). By the end of cellularization, a reciprocal pattern of induction is once again detected; *en* is no longer activated in the terminal regions, and induction of seven pair-rule-like stripes occurs in the trunk (Fig. 4I). These stripes are formed by the anterior expansion of even-numbered *en* stripes such that they fuse with the odd-numbered stripes in front. Expression is, however, still excluded from the posterior regions of even-numbered parasegments. Interestingly, FtzVP16 was able to activate *ftz* within the latter regions, but not in the middle portions of odd-numbered parasegments (compare Fig. 4I to Fig. 3I). Thus, the factors that limit FtzVP16 activity spatially are promoter-specific, and for these two genes, nearly complementary.

Effects on *en* expression by the control constructs FtzΔC and FtzΔNVP16 were essentially the same as observed on *ftz*; FtzΔC had the same effects as Ftz (although weaker), and FtzΔNVP16 had no effects at all (data not shown). Similar responses were also observed when 4 minute heat shocks were used to induce transgene expression (data not shown). These trends were found with all other target genes tested, and will not be described further.

Effects of FtzVP16 on negatively regulated target genes

Previous tests conducted on the transcriptional activities of Ftz in vitro and in cultured cells only revealed its ability to function as a transcriptional activator (Jaynes and O'Farrell, 1988; Han et al., 1989; Winslow et al., 1989; Ohkuma et al., 1990). In the developing embryo, however, Ftz also appears to function as a transcriptional repressor (Ingham et al., 1988; Copeland et al., 1996; Nasiadka and Krause, 1999). The best-characterized negative target of Ftz is the segment polarity gene *wingless* (*wg*). In *ftz* mutant embryos, *wg* stripes expand to fill regions where Ftz is normally expressed (Ingham et al., 1988), and when Ftz is expressed ectopically, odd-numbered *wg* stripes are repressed (Ish-Horowicz et al., 1989) with the same rapid kinetics as observed for *ftz* gene autoregulation (Nasiadka and Krause, 1999).

If Ftz is indeed a direct repressor of *wg*, then it is quite possible that addition of the strong VP16 activation domain to Ftz will convert it from a repressor of *wg* into an activator. Fig. 5B shows the strong repression of alternate *wg* stripes that is observed 25 minutes after ectopic expression of Ftz. The effect

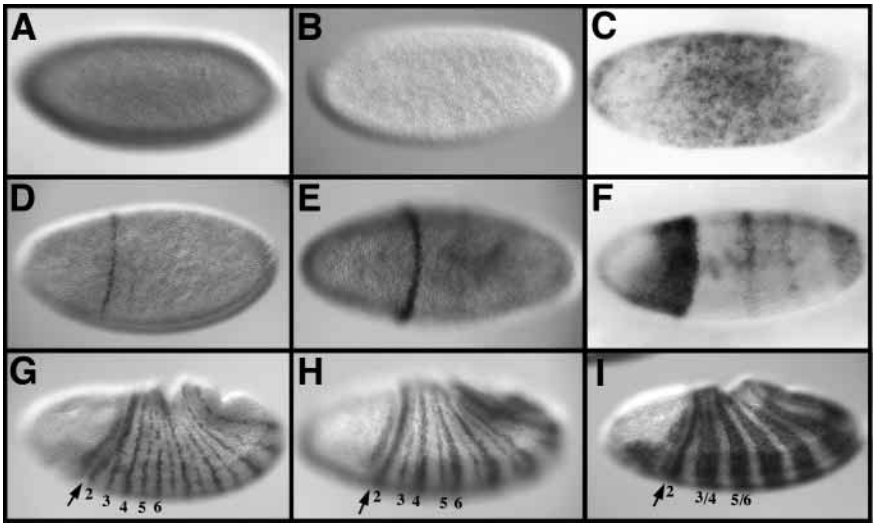

Fig. 4. Effects of FtzVP16 on *engrailed* (*en*). Expression of *en* transcripts is shown at three different stages: during early cellularization (A-C), mid-cellularization (D-F) and early germband extension (G-I). Control embryos are shown on the right (A,D,G), HSFtz embryos in the middle (B,E,H) and HSFtzVP16 embryos on the right (C,F,I). Arrows mark the position of the cephalic furrow, and stripes are numbered up to stripe 6. Stripe 1 in G-I is located within the cephalic furrow and is therefore undetected. Note that FtzVP16 overcomes some, but not all, of the spatial and temporal limitations imposed on Ftz.

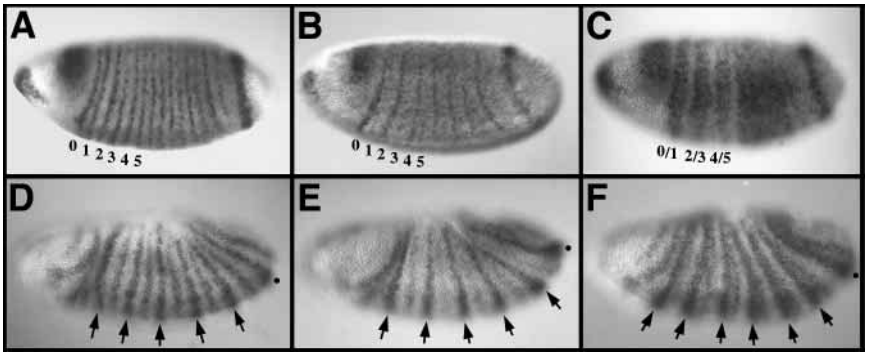

Fig. 5. Effects of FtzVP16 on genes that are repressed by Ftz. The effects of ectopic Ftz and FtzVP16 expression are shown on the expression patterns of *wg* (A-C) and *slp* (D-F) in control (A, D), HSFtz (B,E) and HSFtzVP16 (C,F) embryos. The first six stripes of *wg* are numbered. Arrows (and dots) mark the primary stripes of *slp*, which are expressed at the posterior edges of even-numbered parasegments. Ectopic Ftz represses alternate stripes of *wg* (B) and *slp* (E), whereas ectopic FtzVP16 activates both genes throughout odd-numbered parasegments (C,F). FtzVP16 also induces *wg* expression in the 8th and 10th even-numbered parasegments (C).

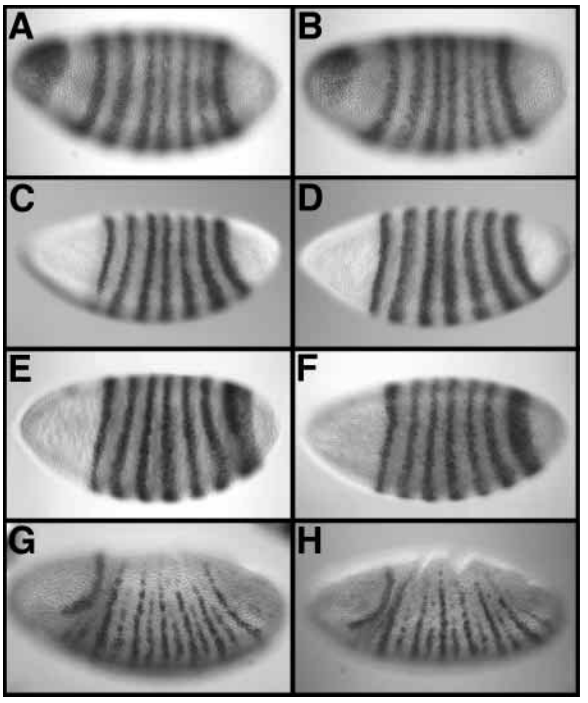

Fig. 6. Effects of FtzVP16 on non-Ftz target genes. mRNA expression patterns are shown for control embryos on the left (A,C,E,G) and HSFtzVP16 embryos on the right (B,D,F,H). Embryos stained for *h* (A,B), *eve* (C,D) and *run* (E,F) are shown at the end of cellularization (end of stage 5), while those stained for *gsb* expression (G,H) are shown at gastrulation (stage 6). No effect is observed on any of these genes by ectopic expression of FtzVP16.

of FtzVP16 within the same short recovery time was quite the opposite (Fig. 5C). Instead of repressing *wg*, FtzVP16 caused a rapid and substantial broadening of *wg* stripes. Expression expanded into odd-numbered parasegments, and in abdominal regions, into parasegments 8 and 10 as well.

The pair-rule gene *sloppy-paired* (*slp*) is another gene that appears to be directly repressed by Ftz (Nasiadka and Krause, 1999). Like *wg*, *slp* stripes expand into Ftz expression domains in *ftz* mutant embryos, and are repressed when Ftz is expressed ectopically (Nasiadka and Krause, 1999; Fig. 5E). As with *wg*, the repression caused by ectopic Ftz is converted into activation when the VP16 activation domain is fused to Ftz. FtzVP16 causes a rapid expansion of *slp* stripes into odd-numbered parasegments (Fig. 5F), much as seen for *wg*. As observed with the responses of *ftz* and *en*, the regions in which activation of *wg* and *slp* occurs are limited both spatially and temporally. Taken together, these results provide further evidence that Ftz is capable of acting as a transcriptional repressor, and that *wg* and *slp* are direct targets of Ftz repression.

Effects of FtzVP16 on suspected non-target genes

In a previous study, four of nine genes tested appeared not to be direct targets of Ftz. These genes, *even-skipped* (*eve*), *hairy* (*h*), *runt* (*run*) and *gooseberry* (*gsb*), showed either no response or a significantly delayed response to pulses of ectopically expressed Ftz (Nasiadka and Krause, 1999). In vivo UV-cross-linking studies, however, indicate that Ftz is evenly distributed along the promoter of at least one of these genes, *eve* (Walter et al., 1994). It is possible that ectopically expressed Ftz fails to affect these genes due to the absence of available cofactors or the presence of overriding repressors. Hence, we expressed FtzVP16 to see if such limitations existed and whether they could be overcome.

Fig. 6B,D,F,H shows that all four genes fail to show any response to FtzVP16 within the 25 minute response time that all the other direct responses were observed. This was true for all stages tested, suggesting that Ftz is either not bound to these promoters, or that it is bound to sites that are somehow non-functional.

The majority of FtzVP16 activity is homeodomain-independent

In previous studies, we have shown that Ftz can regulate the majority of known target genes when the DNA binding activity of its homeodomain is compromised (Fitzpatrick et al., 1992; Copeland et al., 1996; Hyduk and Percival-Smith, 1996). These results suggest that Ftz can be recruited to response elements on target gene promoters via specific interactions with cofactors bound at those sites. In order to explore the temporal, spatial and promoter-specific properties of these cofactors, we compared the regulatory abilities of FtzVP16 and FtzΔHDVP16 on three Ftz target genes.

Fig. 7 shows the responses of *ftz* (A,B), *odd* (C,D) and *en* (E,F) in embryos just beginning to cellularize (early stage 5, approx. 2:10 AEL). For the first two genes, *ftz* and *odd*, equivalent levels of activation are achieved by both FtzVP16 and FtzΔHDVP16 within the trunk region of the embryo. In contrast, the early response of *en*, which can be induced by FtzVP16 but not Ftz, fails to occur when the homeodomain of FtzVP16 is removed (compare 7E and F). This homeodomain dependence is not observed later during the normal temporal window of *en* expression (compare 7G and H).

Several conclusions can be drawn from these results. First, Ftz is able to regulate the *ftz* and *odd* genes as early as stage 5. This activity is boosted by fusion of Ftz to the VP16 activation domain. Second, binding to the *ftz* and *odd* promoters can occur independently of the homeodomain, indicating that cofactors necessary for promoter recruitment are present and active at this early stage. Third, in contrast to the other two promoters, binding of Ftz to the *en* promoter is homeodomain-dependent in stage 5 embryos. However, even with the homeodomain present, transcriptional activation at this stage fails to occur unless the bound protein is fused to the VP16 activation domain.

DISCUSSION

Selective binding versus activity regulation

As described earlier, there are two prevalent models that seek to explain how homeodomain proteins achieve their unique developmental roles in vivo (Biggin and McGinnis, 1997). The selective binding model argues that homeodomain proteins regulate specific target genes with the aid of cofactors that direct them to a specific subset of their many potential binding sites (Mann, 1995; Mann and Chan, 1996). The activity regulation model, on the other hand, argues that homeodomain proteins are distributed rather evenly along the promoters of most genes (Walter et al., 1994; Carr and Biggin, 1999), and that interacting cofactors act primarily by modulating their

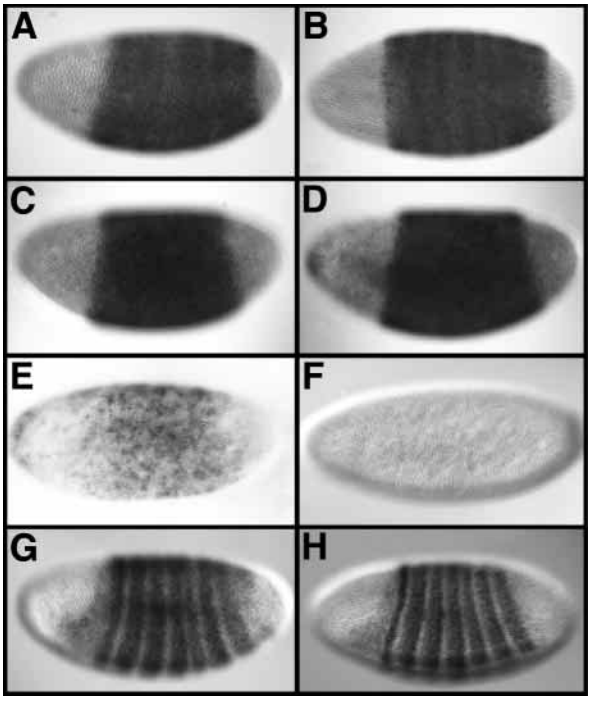

Fig. 7. The Ftz homeodomain is dispensable for most FtzVP16 activities. Expression of the *ftz* (A,B), *odd* (C,D) and *en* (E,F,G,H) genes is shown in HSFtzVP16 (A,C,E,G) and HSFtzΔHDVP16 (B,D,F,H) embryos during early cellularization (A-F) or late cellularization (G,H). With the exception of early activation of *en*, the Ftz homeodomain is not required for any of the observed FtzVP16 activities

transcriptional activity (Biggin and McGinnis, 1997; Li and McGinnis, 1999; Li et al., 1999a). Importantly, the two models also differ in the number of predicted target genes. The selective binding model predicts a relatively limited number of targets while the activity regulation model predicts a very large number of targets. The data obtained with our FtzVP16 fusion protein and actual target genes suggest that Ftz binds and regulates a limited number of target genes and that target gene selection occurs primarily by cofactor effects on the affinity for specific sites.

One of the clearest results in favor of selective target gene binding was the limited number of observed FtzVP16-responsive genes. Four of the nine genes monitored in this study (*eve*, *hairy*, *runt* and *gooseberry*) were previously suggested to be non-target genes (Nasiadka and Krause, 1999). Consistent with this finding, all four failed to respond to FtzVP16 at any of the developmental stages tested, suggesting that Ftz does not bind to the promoters of these genes. This result is inconsistent, however, with those of previous in vivo cross-linking experiments. Immunoprecipitation of Ftz molecules, cross-linked to DNA by UV irradiation showed that restriction fragments within a 7 kb region of the *eve* promoter, and within several other promoters including *ftz*, could all be detected with similar efficiencies (Walter et al., 1994). A possible explanation for this apparent discrepancy is that our assay measures promoter activity while the cross-linking experiments measured promoter occupancy. In other words, FtzVP16 may be able to bind to the *eve*, *hairy*, *runt* and *gooseberry* promoters, but the VP16 activation domain may for some reason be incapable of activating them. This explanation, however, is not consistent with the strong activation of *eve* that is induced by the VP16 activation domain when fused to the *eve* repressor protein Runt (Jimenez et al., 1996). Similarly, the VP16 activation domain is able to activate *runt* when fused to the *runt* repressor protein Hairy (Jimenez et al., 1996). Alternative explanations for the inability of FtzVP16 to activate these promoters are that there may be promoter-specific repressors of VP16 that specifically target the Ftz portion of the fusion protein, or that FtzVP16 binds to regions of these promoters that are somehow non-functional. A third explanation is that Ftz only binds these promoters at high levels of expression. The cellular concentrations of FtzVP16 that we induced are approximately 50% or less of endogenous levels of Ftz. We have found in past studies that these levels are more than sufficient to rescue *ftz*-dependent segmentation (Fitzpatrick et al., 1992; Copeland et al., 1996). Also, heterozygous animals with only one wild-type copy of the gene develop normally (Wakimoto and Kaufman, 1981). Hence, sites detected by cross-linking in wild-type animals may represent a significant number of non-essential sites that are only bound when excess protein is present.

A second argument in favor of the selective binding model is that the most frequent segmental phenotype generated by ectopically expressed FtzVP16 was the same as that generated by full-length Ftz. If Ftz were widely distributed on the majority of *Drosophila* promoters, then addition of the VP16 activation domain should have resulted in the spatial and temporal misexpression of many of these genes. This in turn would lead to dramatically different segmental phenotypes, perhaps even lethality prior to the secretion of cuticle. Similarly, inappropriate activation of Ftz target genes before and after the time that Ftz is normally expressed should also have lead to novel segmental phenotypes. However, FtzVP16 had no obvious effects on segmental patterning when expressed at those stages of development. Although FtzVP16 was able to induce several novel phenotypes within the normal temporal window of *ftz* expression, these were relatively low in frequency and could be explained by the observed effects on known target gene expression patterns.

The limited effects of FtzVP16 on known target genes and segmental phenotypes compares well with results obtained with two other Q50 homeodomain-VP16 fusion proteins. Ectopic expression of UbxVP16 (Li and McGinnis, 1999) using the ubiquitously expressing *armadillo* promoter generated relatively normal cuticles with mixed Ubx and Antp-like segmental transformations in the thorax and head (Li and McGinnis, 1999). DfdVP16 expressed in the same way generated stronger segmental defects, but the transformed segments were nevertheless Dfd-like in identity (Li et al., 1999a). Amongst the suspected target and non-target genes monitored in those studies, only one responded inappropriately and this may have been an indirect effect (Li et al., 1999a).

Another result in favor of the selective binding model was the ability of the homeodomain-deleted versions of Ftz and FtzVP16 to regulate Ftz target genes. These proteins have no capacity to bind DNA on their own (Fitzpatrick et al., 1992), and yet are capable of regulating all of the Ftz target genes investigated (Fitzpatrick et al., 1992; Copeland et al., 1996). This suggests that promoter binding can be mediated by cofactors that are either bound, or are able to bind, to specific

sites on Ftz target genes. Indeed, analysis of a *ftz* reporter gene containing a portion of the *ftz* autoregulatory element has shown that recruitment of Ftz can be achieved by both homeodomain-dependent and homeodomain-independent mechanisms (Schier and Gehring, 1993; Hyduk and Percival-Smith, 1996). Protein-protein interactions such as these have been shown to change equilibrium constants for DNA by two to three orders of magnitude (Ptashne, 1992). For example, Ftz-F1 can increase the affinity of Ftz for flanking binding sites by 100 fold (Yu et al., 1997). At low protein concentrations, these interactions would favour binding to the small subset of Ftz binding sites that have Ftz-F1 (or other cofactor) binding sites nearby.

Cofactors have also been shown to affect the binding site specificity of other Q50 homeodomain proteins. The best characterized of these is a divergent homeodomain protein called Extradenticle (Exd). Exd interacts with Hox proteins via conserved YPWM motifs found N-terminal to their homeodomains (Mann and Chan, 1996). Hox-Exd heterodimers bind bipartite response elements, and by changing the spacing and sequence of these elements, binding and responsiveness can be changed from one Hox protein to another (Chan et al., 1994, 1997; Ryoo and Mann, 1999).

Several other criteria support our conclusion that Ftz specificity is determined primarily at the level of promoter binding. First, the number of potential Ftz binding sites greatly outnumbers the amount of protein molecules available. In vitro DNA binding studies have shown that there are approximately 10-20 Ftz binding sites per kb of genomic DNA. The *Drosophila* genome contains about 3×10^5 kb of DNA per diploid genome (Miklos and Rubin, 1996). Hence, as many as 6×10^6 molecules of Ftz would be required to occupy all of these sites. This number is more than two orders of magnitude higher than the 15,000 molecules previously estimated to be expressed in blastoderm stage nuclei (Krause and Gehring, 1988). This is the stage when Ftz protein levels are highest and as stated earlier, dosage studies have shown that Ftz can regulate target genes effectively at levels that are 2-4 times lower (Wakimoto and Kaufman, 1981; Fitzpatrick et al., 1992; A. N. and H. M. K., unpublished observations). Indeed, immunolocalization shows that, when expressed at functional levels in salivary glands, bands of Ftz and other Q50 homeodomain proteins are detected at somewhere between 50 and several hundred discrete chromosomal locations (Serrano et al., 1995; Botas and Auwers, 1996; H. M. K., unpublished observations). Taken together, these data suggest that the number of target genes bound and regulated by Q50 homeodomain proteins range in the low hundreds, not thousands.

The role of activity regulation

Our results show that activity regulation also plays an important role in Ftz function, but that this role is mainly to refine the temporal and spatial windows of target gene regulation and to modulate levels of expression. This conclusion is supported by the following results. First, five of the genes tested (*ftz, odd, slp, en* and *wg)* could be activated ectopically by FtzVP16 in regions and at times that Ftz could not induce a response. This shows that Ftz has the ability to bind to these promoters, but that it must be bound in an inactive state. For Ftz to function in these cells, it probably requires the addition of requisite cofactors, the removal of repressors or both. For the five genes listed above, the VP16 activation domain was able to overcome some of these limitations.

The regulation by Ftz of *en* is a good example of this type of temporal and spatial refinement in activity. Our results with FtzVP16 showed that Ftz can bind to the *en* promoter during the time that *ftz* autoregulation and *odd* activation are well under way. However, the ability of Ftz to activate *en* is normally delayed until cellularization is completed (approx. 45 minutes). This delay may be necessary to allow other *en* regulators to resolve into the complex patterns of expression that are required for *en* to initiate in 14 narrow stripes.

Like most homeodomain proteins, Ftz has the ability to function as both a transcriptional activator and repressor. This dual capacity suggests a requirement for distinct activity-regulating cofactors. However, differential activity can also be achieved, at least in part, by binding to different sites on different genes. For example, the response elements required for repression of the *Distalless* gene by Ubx (Vachon et al., 1992) and activation by Dfd (O'Hara et al., 1993) are different. This also appears to be the case for activation of the *dpp* gene by Ubx and its repression by Abd-A (Capovilla et al., 1994; Sun et al., 1995; Capovilla and Botas, 1998). The different cofactors that help recruit the three proteins to these sites may also be partly responsible for their differences in transcriptional activity. For example, Exd is thought to generally work as a coactivator, acting in part to alter Hox protein conformation (Li et al., 1999a). Other factors bound in the vicinity of these sites are also likely to play a major role in activity regulation (see for example Li et al., 1999b).

A better sense of the relative contributions made to functional specificity by binding site selection and activity regulation, and the number of genes regulated by each protein will require more comprehensive methods of identifying and monitoring target gene responses. Methods capable of mapping binding sites in vivo with greater resolution and with higher levels of sensitivity will also be needed.

Homeodomain protein potential and the need for regulators

In addition to showing that positively acting cofactors are important for Ftz specificity, our data implicates the actions of powerful negative regulators that limit its temporal and spatial domains of activity. The strength and diversity of these negative regulators was emphasized by their ability to suppress the actions of the fused VP16 activation domain despite its previously reported reputation of strength and autonomy. It may be the low DNA binding specificity of the homeodomain that has necessitated this need for diverse mechanisms of repression, since low DNA specificity provides the potential to regulate a large number of inappropriate target genes. Indeed, a rapidly growing number of homeodomain proteins have been shown to be capable of functioning as oncogenes or proto-oncogenes (Rabbitts, 1994; Look, 1997), and oncogenicity can be conferred by fusions to other transcriptional activators (Hunger et al., 1991). Further studies will be required to identify many of the cofactors and inhibitors that modulate Ftz activity and to determine how they do so.

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