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# A phosphorylation site in the Ftz homeodomain is required for activity

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**The *Drosophila* homeodomain-containing protein Fushi tarazu (Ftz) is expressed sequentially in the embryo, first in alternate segments, then in specific neuroblasts and neurons in the central nervous system, and finally in parts of the gut. During these different developmental stages, the protein is heavily phosphorylated on different subsets of Ser and Thr residues. This stage-specific phosphorylation suggests possible roles for signal transduction pathways in directing tissue-specific Ftz activities. Here we show that one of the Ftz phosphorylation sites, T263 in the N-terminus of the Ftz homeodomain, is phosphorylated *in vitro* by *Drosophila* embryo extracts and protein kinase A. In the embryo, mutagenesis of this site to the non-phosphorylatable residue Ala resulted in loss of *ftz*-dependent segments. Conversely, substitution of T263 with Asp, which is also non-phosphorylatable, but which successfully mimics phosphorylated residues in a number of proteins, rescued the mutant phenotype. This suggests that T263 is in the phosphorylated state when functioning normally *in vivo*. We also demonstrate that the T263 substitutions of Ala and Asp do not affect Ftz DNA-binding activity *in vitro*, nor do they affect stability or transcriptional activity in transfected S2 cells. This suggests that T263 phosphorylation is most likely required for a homeodomain-mediated interaction with an embryonically expressed protein.**

*Keywords*: cAMP-dependent protein kinase/*Drosophila*/*fushi tarazu*/homeodomain/phosphorylation

## Introduction

Homeodomain proteins constitute a large family of eukaryotic transcription factors that share a common 60 amino acid DNA-binding domain referred to as the homeodomain (reviewed in Gehring *et al.*, 1994a; McGinnis, 1994; Sharkey *et al.*, 1997). In all higher eukaryotes, homeodomain proteins play a major role in patterning the embryonic body plan. Loss-of-function or gain-of-function mutations cause transformations in regional identity that can be quite spectacular. In *Drosophila*, for example, antennae can be transformed into legs, eyes or mouth parts (Schneuwly *et al.*, 1987; Mann and Hogness, 1990; Lin and McGinnis, 1992; Zeng *et al.*, 1993; Halder *et al.*, 1995; Aplin and Kaufman, 1997). These same antennal transformations can be induced by mis-expressing the vertebrate homologs of the same genes (Malicki *et al.*, 1990; McGinnis *et al.*, 1990; Zhao *et al.*, 1993; Halder *et al.*, 1995), emphasizing the functional as well as structural conservation of these proteins.

Homeodomain proteins are thought to control patterning processes by coordinating the expression of specific sets of target genes. Indeed, the ability of homeodomain proteins to bind specific DNA sequences *in vitro*, and to regulate gene expression in cultured cells, has been well documented (Jaynes and O'Farrell, 1988; Han *et al.*, 1989; Krasnow *et al.*, 1989; Winslow *et al.*, 1989; Gehring *et al.*, 1994b). The specificity of action that each homeodomain protein exhibits *in vivo*, however, has not yet been duplicated *in vitro*. In fact, homeodomain proteins tend to bind short A/T-rich consensus sites that are often indistinguishable from one another (reviewed in Hayashi and Scott, 1990; Kalionis and O'Farrell, 1993; Gehring *et al.*, 1994b). A concerted effort has been made in the past few years to determine how specificity of action is achieved *in vivo*.

One potential source of specificity is through interactions with other DNA-binding transcription factors. Indeed a number of DNA-binding partners and cofactors have been identified in the past few years (reviewed in Mann and Chan, 1996). Some of these partners change DNA-binding specificity whereas others alter the ability to activate or repress adjoining promoters. Another potential source of specificity is the covalent addition of phosphate groups. Phosphorylation has been shown to affect a variety of transcription factor properties including structure, subcellular localization, DNA-binding affinity and specificity, and the ability to activate transcription (reviewed in Hunter and Karin, 1992; Karin, 1994; Hill and Treisman, 1995). All homeodomain proteins examined thus far are phosphorylated (Gay *et al.*, 1988; Krause *et al.*, 1988; Krause and Gehring, 1989; Odenwald *et al.*, 1989; Tanaka and Herr, 1990; Gavis and Hogness, 1991; Kapiloff *et al.*, 1991; Lopez and Hogness, 1991; Segil *et al.*, 1991; Wall *et al.*, 1992; Ronchi *et al.*, 1993; Bourbon *et al.*, 1995; Caelles *et al.*, 1995; Coqueret *et al.*, 1996; Zannin *et al.*, 1996; Jaffe *et al.*, 1997; Zwilling *et al.*, 1997), indicating a likelihood for similar types of phosphorylation-induced changes in activity.

Here, we use the *Drosophila* homeodomain protein Fushi tarazu (Ftz) as a model to examine the importance of phosphorylation on homeodomain protein function and specificity. Ftz is expressed during three stages of embryogenesis: first in alternating segmental primordia, then in differentiating neurons in the central nervous system (CNS), and finally in portions of the gut and posterior epidermis (Hafen *et al.*, 1984; Carroll and Scott, 1985; Krause *et al.*, 1988). *ftz* mutant embryos lack alternate segmental regions (Wakimoto *et al.*, 1984) and exhibit transformations in neuronal identities (Doe *et al.*, 1988).

Ftz was one of the first homeodomain proteins shown to be phosphorylated (Krause *et al.*, 1988; Krause and Gehring, 1989). Phosphorylation occurs on equivalent numbers of serine and threonine residues, with as many as 16 phosphates per molecule. Interestingly, phosphorylation also occurs in a tissue- and stage-specific fashion (Krause and Gehring, 1989).

In this study, we report that Ftz residue threonine 263 (T263), which is in the N-terminal arm of the homeodomain, is phosphorylated *in vitro* in a cAMP-dependent fashion by *Drosophila* embryo extracts and by purified cAMP-dependent protein kinase (PKA). To test whether this phosphorylation event is required for Ftz activity, we mutated T263 to Ala and Asp to mimic the unphosphorylated and constitutively phosphorylated states, and then subcloned the mutant DNAs into a 10 kb fragment of *ftz* genomic DNA, capable of rescuing *ftz*$^{-}$ embryos to adulthood (Hiromi *et al.*, 1985). The reconstituted genes were transformed into flies by P-element-mediated germline transformation and tested for their ability to rescue *ftz* mutant embryos. Ftz T263A mutant constructs failed to rescue *ftz*$^{-}$ flies to adulthood whereas wild-type and T263D Ftz constructs did. Phenotypic defects caused by the T263A mutation are described. The results indicate that T263 is phosphorylated when Ftz is in its active form, and that phosphorylation of T263 probably affects a protein–protein interaction.

## Results

### *Ftz phosphorylation by embryo extracts*

The Ftz protein, expressed in embryos, is heavily phosphorylated (Krause and Gehring, 1989). In order to label and map sites, and to identify the kinases responsible, we added bacterially expressed Ftz to staged *Drosophila* extracts in the presence of [$\gamma$-$^{32}$P]ATP. Figure 1A shows labeling of Ftz by extracts under a variety of conditions. Moderate labeling was achieved in extracts adjusted to pH 7.2 and 25 mM $MgCl_2$ (lane 2 versus lane 1). This low level labeling was largely blocked by the addition of inhibitors of protein kinase C (PKC) and calmodulin-dependent protein kinase (Cam PK) (lane 3). However, enhanced labeling could once again be achieved by the addition of cAMP (lane 6), indicating the presence of a PKA type kinase with specificity for Ftz. Using a series of deleted Ftz polypeptides, the sites of cAMP-dependent phosphorylation were mapped to the Ftz homeodomain (Figure 1B). Deletion of the Ftz homeodomain removed all detectable labeling (lane 3), whereas the homeodomain by itself (lane 4) incorporated label as efficiently as the intact Ftz protein.

Figure 2 shows that homeodomain-specific labeling could also be achieved with purified PKA. As with the cAMP-dependent extract labeling, the homeodomain was found to be both required and necessary for efficient labeling. In order to map the phosphorylated residue(s), extract and PKA-labeled homeodomains were subjected to tryptic digestion, thin-layer chromatography and solid-phase Edman degradation of isolated peptides (Figure 3). Ftz homeodomain labeled using embryo extract yielded two spots in the tryptic fingerprint (Figure 3A). The same two spots were labeled by PKA (Figure 3B and C).

Edman degradation of the two eluted peptides yielded $^{32}$P-labeled amino acid peaks at cycles 2 (Figure 3E) and 3 (not shown) for spots one and two respectively. A tryptic peptide near the N-terminus of the Ftz homeodomain is the only peptide that has a Ser or Thr residue at the second position (T263). Indeed, phosphorylation of T263 was confirmed by Edman degradation of undigested homeodomain and release of label at cycle 11 as expected (Figure 3F). Further confirmation of phosphorylation at T263 was obtained by changing the site to an alanine residue. Figure 1B (lane 5) shows that labeling of the mutant T263A homeodomain by embryo extract was markedly reduced, and Figure 3D shows that spot #1 in the tryptic fingerprint of the T263A homeodomain disappeared as expected. The same results were obtained when labeling was performed using purified PKA (Figure 2, lane 5, and data not shown).

Edman degradation analysis suggests that spot #2 corresponds to the homeodomain residue T269. Both sites, T263 and T269, are within PKA consensus sites. Notably, T263 is also in the analogous position to mapped PKA kinase sites in the POU class homeodomains of Pit-1 and Oct-1 (Kapiloff *et al.*, 1991; Segil *et al.*, 1991). Other protein kinases, including PKC, casein kinase I (CKI), casein kinase II (CKII), mitogen-activated protein kinase (MAPK), S6 protein kinase (S6K) and glycogen synthase kinase-3 (GSK-3), failed to phosphorylate this site. Phosphorylation by PKA, on the other hand, was equal in efficiency to phosphorylation of a PKA consensus site (heart muscle kinase peptide; data not shown).

### *In vivo analysis of T263 mutants*

In order to test whether T263 is phosphorylated *in vivo*, and to determine the role of phosphorylation, Ftz T263A and T263D mutant expression constructs were injected into flies, and the transgenic constructs tested for their ability to functionally replace the endogenous *ftz* gene. The *ftz* open reading frame, along with 6.1 kb of 5′ and 2 kb of 3′ DNA, is sufficient to direct normal *ftz* expression and to fully rescue *ftz* mutant embryos (Hiromi *et al.*, 1985). Rescue constructs were introduced into the genome by P-element-mediated germline transformation (Spradling and Rubin, 1982). For each mutation, four transformant lines, all on the second chromosome, were used for subsequent analyses. These, along with lines carrying wild-type *ftz* transgenes, were crossed to flies carrying the *ftz* alleles $ftz^{9H34}$ and $ftz^{w20}$, both of which are protein null. The resultant lines, P[*ftz*]; $ftz^{9H34}/ftz^{w20}$, were then tested for viability.

Table I shows the survival index (SI = No. of rescued flies obtained/No. of rescued flies expected) values for flies carrying wild-type, T263A and T263D transgenic *ftz* genes in the $ftz^{9H34}/ftz^{w20}$ mutant background. The wild-type control construct rescued flies to adulthood with SI values ranging from 21 to 71%. These values are in the same range as those found in previous studies (Hiromi *et al.*, 1985; Furukubo-Tokunaga *et al.*, 1992). These observed variations in rescuing capacity are likely to be due to reduced or ectopic *ftz* expression brought about by adjoining sequences at the various sites of P-element insertion.

In contrast to the wild-type *ftz* construct, the T263A mutant construct showed no ability to rescue: all four transformant lines gave SI values of zero. On the other

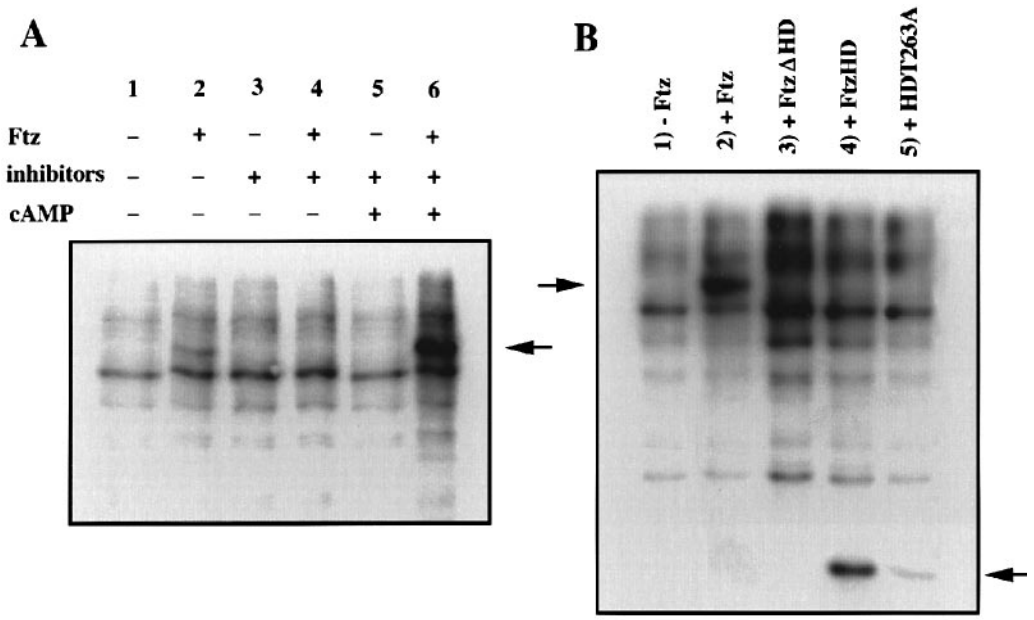


**Fig. 1.** Labeling of Ftz by embryo extracts. Extracts of 0–6 h embryos were used to phosphorylate Ftz polypeptides *in vitro* in the presence of [$\gamma$-$^{32}$P]ATP. Following labeling, proteins were electrophoresed on PAGE gels and the gels autoradiographed. (**A**) Stimulation of Ftz phosphorylation by cAMP. Lane 1, extract alone; lane 2, extract + Ftz; lane 3, extract + Ftz + PKC/Cam PK inhibitors; lane 4, extract + PKC/Cam PK inhibitors + Ftz; lane 5, extract + PKC/Cam PK inhibitors + cAMP; lane 6: extract + PKC/Cam PK inhibitors + Ftz + cAMP. (**B**) Mapping of Ftz cAMP-dependent phosphorylation sites. Full-length, deleted and T263A Ftz polypeptides were labeled in the presence of embryo extract, PKC and Cam PK inhibitors and cAMP. Lane 1, – Ftz; lane 2, + Ftz; lane 3, + FtzΔHD (homeodomain deleted). The protein migrates just below the full-length protein and above the major labeled band just below (determined by Western, not shown). Lane 4, Ftz homeodomain (amino acids 254–314); lane 5, Ftz homeodomain with T263A substitution. Arrows indicate the positions of full-length and homeodomain Ftz polypeptides.

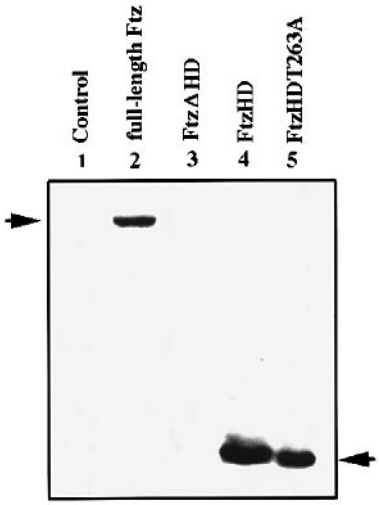


**Fig. 2.** Phosphorylation of Ftz by PKA. Full-length and deleted Ftz polypeptides were phosphorylated *in vitro* in the presence of purified PKA. Labeled polypeptides were separated on PAGE gels and autoradiographed. Lane 1, no Ftz added; lane 2, full-length Ftz; lane 3, FtzΔHD; lane 4, Ftz homeodomain; lane 5, Ftz homeodomain with T263A substitution. Arrows indicate the positions of the full-length and homeodomain Ftz polypeptides.

hand, the T263D construct was able to rescue mutant flies to adulthood, although less effectively than the wild-type construct (SI = 2–25% versus 21–71% for wild-type). Thus, the T263D protein probably exhibits minor irregularities in function or expression.

### *Cuticle patterns*

The rescue of flies to adulthood is a comprehensive measure of all *ftz* gene activities. Because *ftz* has several stage-specific developmental roles, reductions in SI values could result from either general or stage-specific defects. In order to test for segmentation-specific defects, we looked at the cuticles of larvae obtained from rescued embryos. We also examined the expression patterns of putative target genes required for *ftz*-dependent segmentation.

*ftz* mutant cuticles exhibit pairwise deletions of even-numbered parasegments (Furukubo-Tokunaga *et al.*, 1992, Schier and Gehring, 1993; and Figure 4B). Cuticle preparations of T263A embryos also exhibited pair-wise deletions of even-numbered parasegments (Figure 4C). Interestingly, however, the penetrance of the phenotype was quite variable, with some cuticles exhibiting complete deletion of *ftz*-dependent parasegments, while others showed few or no detectable defects. Although variable from cuticle to cuticle, defects in each *ftz*-dependent parasegment occurred with a characteristic frequency. These ranged from the most to least frequently affected as follows: parasegment (PS) 2 (97% affected)>PS6 (82%)>PS10, 4 (70%)>PS12, 14 (58%)>PS8 (36%) ($n$ = 482).

In contrast to T263A cuticles, cuticles from T263D flies were indistinguishable from wild-type (Figure 4D). A few cuticles (<1%) had minor abnormalities, but these were variable and also seen when wild-type rescue constructs were used. The ability of the T263D protein, and not the T263A protein, to rescue *ftz*-dependent segmentation suggests that T263 is normally in the phosphorylated state when the protein is active.

### *ftz autoregulation*

To trace the causes of cuticular defects observed with T263A mutant embryos, we examined the expression of

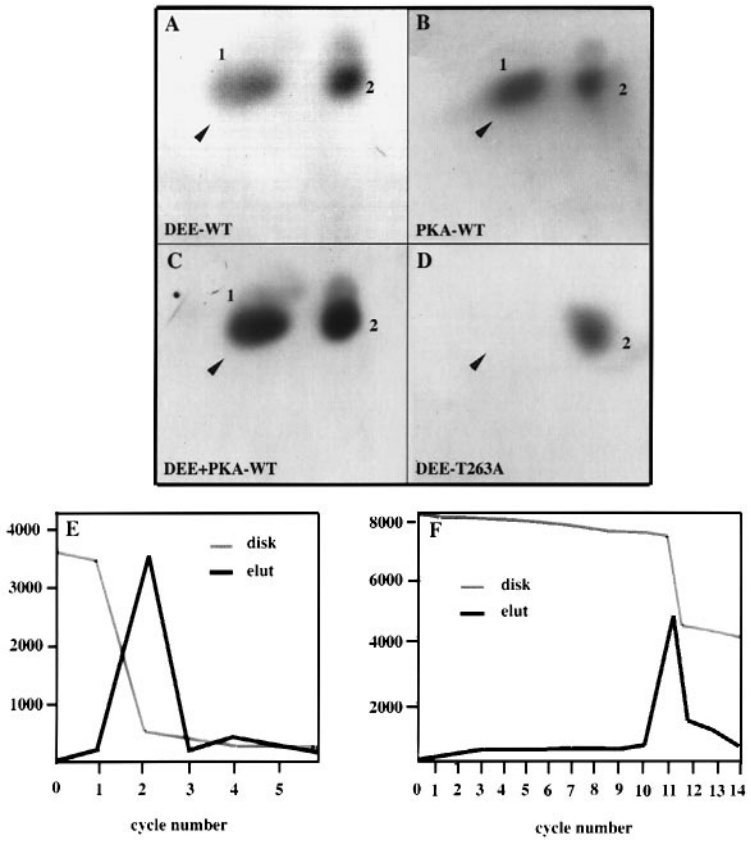


**Fig. 3**. Mapping cAMP- and PKA-dependent phosphorylation sites. Ftz homeodomain (HD), labeled *in vitro* by embryo extracts or purified PKA, was subjected to trypsin digestion, separated by thin-layer chromatography and autoradiographed. (**A**) Ftz HD labeled by embryo extract; (**B**) Ftz HD labeled by PKA and (**C**) an equal mix of extract- and PKA-labeled proteins. Spot #1 from (A) co-migrates with spot #1 from (B), as do the two #2 spots. (**D**) T263A HD labeled by embryo extract. Spot #1 is absent. (**E**) Manual sequencing of tryptic peptide #1. Label eluted at cycle 2. T263 is the only phosphorylatable residue within a tryptic peptide of the Ftz homeodomain that should be released at cycle 2. (**F**) Manual sequencing of undigested Ftz homeodomain. Label eluted at cycle 11. T263 is the seventh residue of the homeodomain, and the homeodomain construct used has four additional amino acids at its N-terminal end (confirmed by DNA sequencing; data not shown).

**Table I.** Survival indices of Ftz T263 mutant offspring

| | Line | Linkage | No. rescued | SI |
|---|---|---|---|---|
| WT | 1 | II | 132; $n = 559$ | 0.708 |
| | 2 | II | 36; $n = 521$ | 0.207 |
| | 3 | II | 65; $n = 531$ | 0.367 |
| | 4 | II | 75; $n = 470$ | 0.479 |
| T263A | 1 | II | 0; $n = 278$ | 0 |
| | 2 | II | 0; $n = 278$ | 0 |
| | 3 | II | 0; $n = 495$ | 0 |
| | 4 | II | 0; $n = 443$ | 0 |
| T263D | 1 | II | 3; $n = 401$ | 0.022 |
| | 2 | II | 16; $n = 379$ | 0.126 |
| | 3 | II | 63; $n = 744$ | 0.254 |
| | 4 | II | 19; $n = 477$ | 0.119 |

SI values (No. of flies with appropriate phenotype/No. expected) are given for flies homozygous mutant for the endogenous *ftz* gene and carrying two copies of either a wild-type, T263A or T263D transgenic *ftz* gene. For each transgene, four independent lines were examined, all with inserts on the second chromosome (linkage group II). $n$ = No. of flies scored.

*ftz* target genes. The *ftz* gene itself is a well characterized target of Ftz activity. Ftz facilitates its own expression via binding and activation of an upstream enhancer element (Hiromi *et al.*, 1985; Hiromi and Gehring, 1987; Pick *et al.*, 1990; Schier and Gehring, 1992). Figure 5A and B shows typical patterns of *ftz* transcripts at two different stages of embryogenesis. In the T263A mutants, stripes of expression were weaker and narrower than normal (Figure 5E and F), except perhaps at the earliest stages of initiation (not shown) which are thought to be *ftz*-independent. The frequency and severity of defects were similar at the two later stages shown. Patterns of protein expression (not shown) were similar to those of the transcripts, with the same variations seen at the same stages. Protein was localized properly to the nucleus in all transgenic lines. These observations suggest, but do not prove, that it is the ability of Ftz to autoregulate its own promoter, and not its synthesis, stability or localization, that is affected by the mutation.

As with the cuticle preparations, the severity of defects in *ftz* expression patterns was highly variable. Some embryos were devoid of detectable expression while others were essentially normal. Certain stripes were more prone to defects than others. Although stripe defects varied from embryo to embryo, the average frequency of defects occurred in the following order: stripe 1>3>5, 2>6, 7>4. This correlates well with the prevalence of defective *ftz*-dependent parasegments in the T263A cuticle preparations (i.e. PS2>PS6>PS10, 4>PS12, 14>PS8). The T263D embryos, in contrast, produced normal patterns of *ftz* mRNA (Figure 5G and H) and protein (not shown).

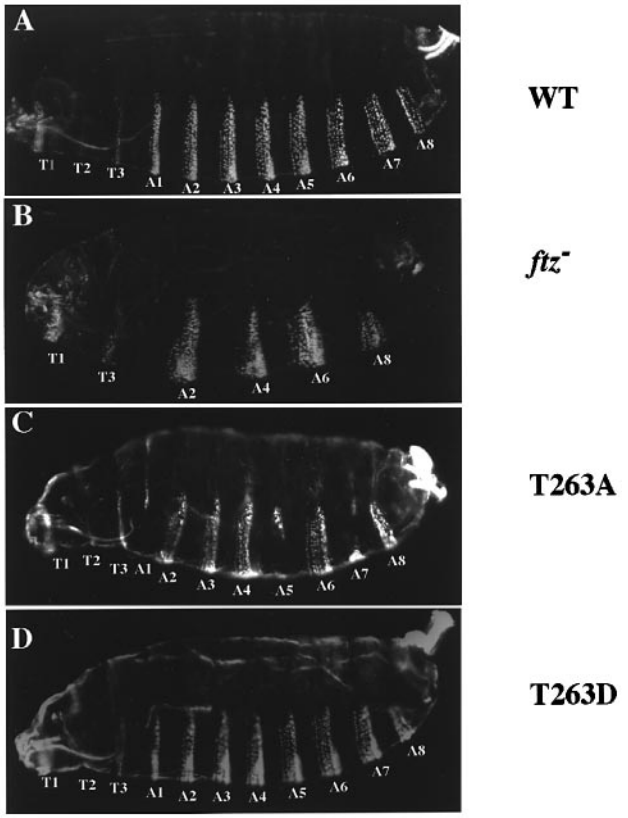


**Fig. 4.** Cuticle preparations of T263 mutant larvae. Cuticles were prepared from P[*ftz T263*] rescued larvae and visualized by dark field microscopy. (**A**) Wild-type larval cuticle; (**B**) homozygous *ftz* mutant cuticle; (**C**) typical Ftz T263A rescued cuticle; (**D**) typical Ftz T263D rescued cuticle.

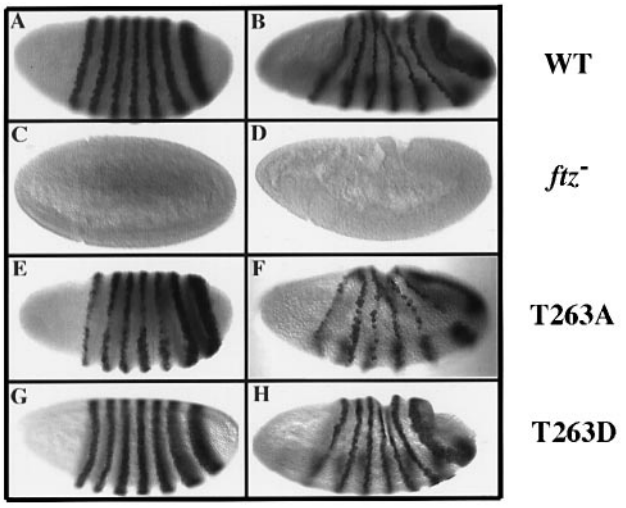


**Fig. 5.** *ftz* gene autoregulation in Ftz T263 mutants. *ftz* gene expression was examined by *in situ* hybridization at two different stages to test for defects in autoregulation. (**A** and **B**) Wild-type *ftz* expression; (**C** and **D**) homozygous *ftz* mutant embryo; (**E** and **F**) typical *ftz T263A* expression; (**G** and **H**) typical *ftz T263D* expression. In (E–H), homozygous *ftz*⁻ embryos were identified based on lack of β-galactosidase expression (see Materials and methods).

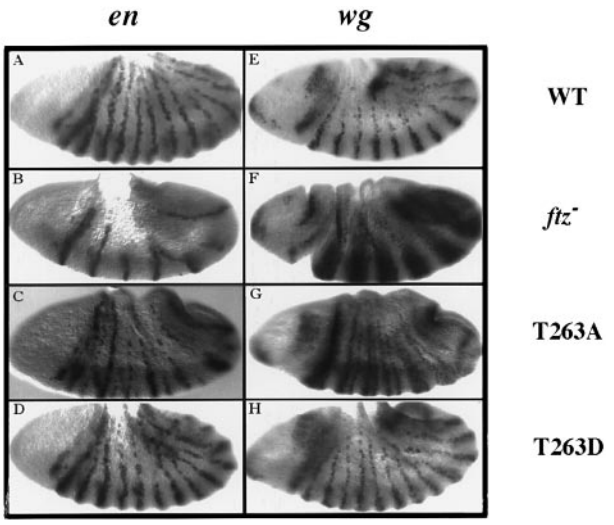


**Fig. 6.** *en* and *wg* expression in Ftz T263 mutants. Patterns of *en* mRNA expression are shown on the left (A–D) and patterns of *wg* mRNA expression on the right (E–H). From top to bottom, embryos shown are wild-type (**A** and **E**), *ftz*⁻ (**B** and **F**), Ftz T263A (**C** and **G**) and Ftz T263D (**D** and **H**). *ftz*-dependent *en* stripes are partially missing in the T263A mutant embryo and *wg* stripes are partially expanded. In contrast, *en* and *wg* stripes are normal in the T263D embryo.

### *en and wg expression*

Ftz is thought to be a direct transcriptional activator of alternate stripes of the segment polarity gene *engrailed* (*en*) (DiNardo and O'Farrell, 1987; Kassis, 1990; Florence *et al.*, 1997). Conversely, transcription of alternate *wingless* (*wg*) stripes appears to be repressed by Ftz (Ingham *et al.*, 1988; Copeland *et al.*, 1996). Altered expression patterns of *en* and *wg* in T263A mutant embryos (Figure 6) correlated well with the defects observed in *ftz* expression patterns (Figure 5). *ftz*-dependent *en* stripes were generally weak or missing. Conversely, the *wg* stripes that normally are repressed by Ftz were expanded. As with *ftz* expression, the penetrance of these defects varied greatly from embryo to embryo and showed similar frequencies of segment-specific defects. No defects in *en* and *wg* stripes were observed in T263D embryos.

### *DNA-binding activity*

The results thus far indicate that the T263A mutation reduces Ftz activity, and that the effects of the T263D substitution are extremely minor. Since T263 resides in the DNA-binding homeodomain, we tested whether DNA-binding activity is affected by the mutations. Electrophoretic mobility shift assays (EMSAs) were performed using consensus Ftz-binding sites (Percival-Smith *et al.*, 1990; Pick *et al.*, 1990; Florence *et al.*, 1991). Wild-type, T263A and T263D homeodomains were expressed in reticulocyte lysates. Figure 7 shows that inherent DNA-binding activity of the homeodomain was not affected by either of the two mutations. The same result was achieved using a different Ftz-binding site and different concentrations of protein (data not shown). Thus, the effect of the T263A mutation on Ftz activity does not appear to be exerted at the level of protein–DNA recognition.

### *Transcriptional activity in cultured S2 cells*

A tissue culture co-transfection assay was used to characterize further the functional properties of Ftz T263 mutants.

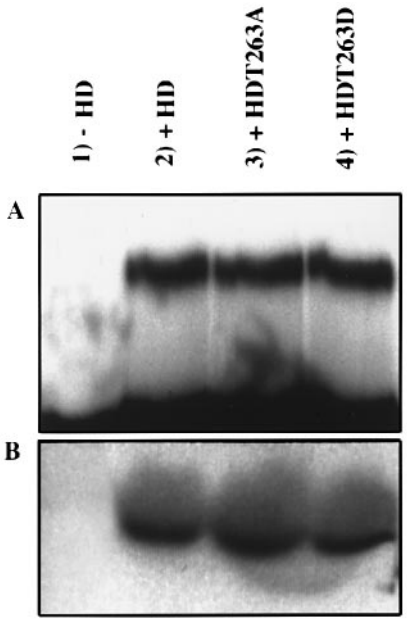


**Fig. 7.** Binding of Ftz T263 mutant homeodomains to DNA. (**A**) Wild-type and T263 mutant Ftz homeodomains were expressed in a reticulocyte lysate system and bound to a $^{32}$P-labeled Ftz consensus DNA-binding site GGGAAGCAATTAAGGAT (Percival-Smith *et al.*, 1990; Pick *et al.*, 1990). Complexes were resolved on non-denaturing polyacrylamide gels and visualized by autoradiography. Lane 1, no protein added; lane 2, wild-type homeodomain; lane 3, T263A homeodomain; lane 4, T263D homeodomain. All three polypeptides showed equivalent DNA-binding activity. (**B**) Western blot of *in vitro* expressed homeodomain polypeptides used in (A). Proteins were separated on 15% PAGE gels, transferred to nitrocellulose and detected using a polyclonal Ftz antiserum. Lane 1, no protein added; lane 2, wild-type homeodomain; lane 3, T263A homeodomain; lane 4, T263D homeodomain. All three polypetides were expressed at similar levels.

Wild-type, T263A and T263D Ftz expression constructs were co-transfected individually into *Drosophila* S2 cells together with the Ftz-responsive reporter plasmid 3′K TATA-CAT, which contains concatamerized Ftz-binding sites derived from a portion of the *en* promoter (Han *et al.*, 1989). All three Ftz expression constructs were able to activate the reporter gene with similar efficiency: ~15- to 16-fold over basal levels (Figure 8). Furthermore, co-transfection of the catalytic subunit of PKA (hatched columns in Figure 8), or treatment of cells with PKA stimulators or inhibitors (not shown), had no effect on the activities of the three Ftz proteins. Other Ftz-responsive reporter plasmids, such as Ubx-CAT (Krasnow *et al.*, 1989) and NP6-CAT (Jaynes and O'Farrell, 1988) also gave equivalent responses to the wild-type and mutant Ftz polypeptides (not shown). These results indicate that T263 phosphorylation does not affect the general ability of Ftz to bind and activate simple reporter genes in S2 cells. Rather, the effects of T263 phosphorylation are likely to depend on factors specifically expressed in the *Drosophila* embryo.

## Discussion

### *Evidence for T263 phosphorylation in vitro and in vivo*

We have shown that T263, in the N-terminal arm of the Ftz homeodomain, is phosphorylated specifically and efficiently *in vitro* by embryo extracts and PKA. Mutation of this site to alanine, which is generally a conservative substitution (Cunningham and Wells, 1989), resulted in complete loss of Ftz-rescuing activity *in vivo*. This lethality was attributed mainly to defects in *ftz*-dependent segmentation. In contrast, mutation of T263 to aspartate, a far less conservative substitution than alanine, but one which has been shown in functional and structural studies effectively to mimic threonine phosphate (see, for example, Cowley *et al.*, 1994; Kowlessur *et al.*, 1995; Napper *et al.*, 1996; Peverali *et al.*, 1996; Boekhoff *et al.*, 1997; Jaffe *et al.*, 1997), returned Ftz activities to near wild-type levels. This strongly suggests that T263 is phosphorylated *in vivo*, and that phosphorylation of this site is required for normal protein activity.

### *Stage specificity of T263 mutant defects*

The majority of T263A mutants died as embryos. Segmental defects, most prevalent in the head, are probably responsible for this lethality, as defects were observed in close to a quarter of the cuticles (97% of homozygous larvae). However, it is also possible that pattern abnormalities in the developing CNS or gut, where *ftz* is expressed later (Carrol and Scott, 1985; Krause *et al.*, 1988), also contributed to lethality. Defects incurred at these later stages could also account for the reduced rescuing capacity of the T263D mutant construct relative to a wild-type construct, since cuticle patterns of these animals appeared to be normal, yet SI values were significantly lower than for wild-type *ftz* constructs. Both gain-of-function and loss-of-function defects are possible, as cofactors and DNA targets may vary from tissue to tissue in their preference for phosphorylated or unphosphorylated Ftz. The phosphorylation state of T263 may also vary from tissue to tissue.

### *Variability in the T263A phenotype*

An interesting aspect of the T263A segmental phenotype was its variability. Cuticle patterns and Ftz target gene patterns ranged from wild-type in appearance to patterns that resembled *ftz* nulls. Also, while certain *ftz*-dependent segments were more likely to be affected than others, the segments affected within any given embryo were highly variable. Similar observations were made by Furukubo-Tokunaga *et al.* (1992) in a study where multiple mutations were introduced into the Ftz homeodomain. This variability suggests that there must be considerable variation within individual embryos in terms of *ftz* gene regulators and/or Ftz target gene co-regulators. This possibility is consistent with variations that we have observed in the normal initiating patterns of *ftz* and Ftz target genes: the intensities and widths of initiating stripes tend to vary from embryo to embryo, and yet normally end up equal in intensity and width later on (unpublished data). This variability may be unimportant in wild-type embryos, but may be critical when Ftz activity drops below threshold levels.

Interestingly, the T263A substitution caused a more severe phenotype than those caused by many of the previously generated homeodomain mutations, even though all of the latter included multiple substitutions. Often, those substitutions included residues in helix 3 which makes key contacts with DNA. The relative severity

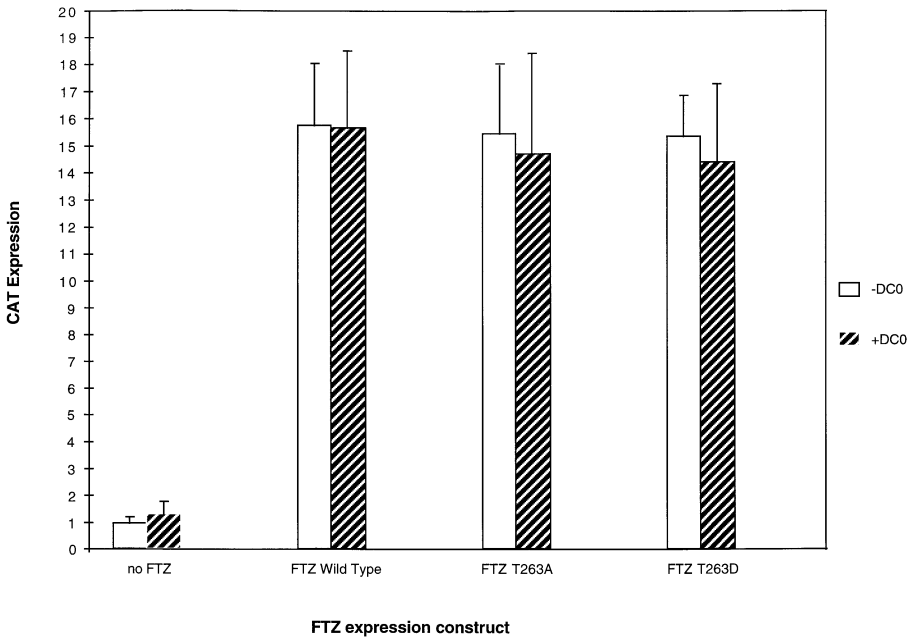


**Fig. 8.** Transcriptional activity of Ftz T263 mutants in cultured cells. Wild-type and T263 mutant *ftz* cDNAs were placed under control of the actin 5C promoter and transfected into cultured *Drosophila* S2 cells together with the Ftz-dependent reporter gene 3K' TATA-CAT (open columns). Duplicate transformations contained in addition a construct that expresses the catalytic subunit of PKA (cross-hatched columns). Levels of CAT expression were determined immunologically and then corrected for minor variations in levels of Ftz expression. Levels of CAT expression are given as levels relative to the level of basal promoter activity, which was assigned a value of one. All three Ftz proteins had equivalent levels of activity, and activity was not altered by co-expression of PKA.

of our T263 substitution suggests that this residue plays a particularly important role in Ftz homeodomain function.

### *Activities affected by T263*

The autoregulatory nature of *ftz* gene expression during its segmental phase of expression (Hiromi and Gehring, 1985; Pick *et al.*, 1990; Schier and Gehring, 1992) makes it difficult to determine which aspects of Ftz activity are compromised. When Ftz activity is lowered, expression levels are lowered. This, in turn, would lead to reduced effects on other target genes such as *en* and *wg*. Hence, the T263A effects could be due either to a general reduction in overall activity, or to a specific defect in autoregulation.

For the same reason, a possibility that we cannot rule out is that the T263A defect is simply one of reduced stability. However, three points argue against this likelihood. First, no differences in stability were observed when mutant and wild-type Ftz proteins were expressed in cultured cells under the control of a heterologous promoter. Second, patterns of mRNA and protein in the embryo were similar. Third, protein expression levels approaching wild-type occasionally were achieved, and subsequent stability was normal.

Another possibility is that the T263A mutation affects DNA-binding properties. Homeodomain proteins make most of their base-specific contacts within the DNA major groove using helix 3 of the homeodomain. However, the N-terminal arm of the homeodomain makes additional DNA contacts, most often non-specifically, with phosphate residues on the minor groove side (Gehring *et al.*, 1994b). In Ftz, these non-specific contacts are made by Arg residues at positions 3 and 5 of the N-terminal arm (Percival-Smith *et al.*, 1990; Qian *et al.*, 1994). T263 is at position 7, and does not appear to contact the DNA molecule. However, it is conceivable that the addition of a large, negatively charged phosphate group could affect this interaction. This appeared not to be the case, however, as the presence of either Thr, Ala or Asp at this position made no difference in binding of Ftz to consensus binding sites *in vitro* or in cultured cells. Nevertheless, the possibility still exists that interactions with sites other than those tested might be affected, as it has been shown that phosphorylation at this position does alter the DNA-binding properties of Pit-1 and Oct-1 (Kapiloff *et al.*, 1991; Segil *et al.*, 1991; Caelles *et al.*, 1995). Alternatively, binding site specificity could be affected indirectly by altering the ability of Ftz to interact with a specificity-conferring cofactor.

### *Proteins that interact with Ftz*

The fact that T263A and T263D mutations had no observable effect on Ftz DNA binding or general transactivation activities suggests that phosphorylation of this site may modulate a protein–protein interaction. Indeed, the N-terminal arms of several homeodomain proteins are thought to serve as specificity-conferring contact points with other proteins (reviewed in Mann, 1996). We have shown previously that, when expressed at sufficient levels from a heterologous promoter, a Ftz polypeptide with its DNA-binding activity destroyed is still capable of regulating target genes *in vivo* (Fitzpatrick *et al.*, 1992; Copeland *et al.*, 1996). This DNA-binding-independent activity is somewhat lower when the Ftz homeodomain is completely versus partially (middle of helix 1 to middle of helix 3) deleted (H.M.Krause and A.Percival-Smith, unpublished observations). These results suggest that protein–protein interactions play a particularly important role

```
FTZ     DSKRTRQTYTR
ANT     ERKRGRQTYTR
UBX     LRRRGRQTYTR
PRD     KQRRCRTTFSA
SCR     ETKRQRTSYTR
I-POU   AGEKKRTSIAA
Pit-1   RKRKRRTTISI
Oct-1   RRRKKRTSIET
EN      DEKRPRTAFSS
EVE     SVRRYRTAFTR
```

**Fig. 9.** Comparison of homeodomain N-terminal sequences. Amino acid sequences at the beginning of several homeodomains are shown for comparison. The PKA consensus ($RX_{1-2}T/S$), including T263, is found in several other homeodomains. This includes the POU class where this residue has been shown to be a substrate for PKA. In a more divergent homeodomain subclass, the corresponding residue is an alanine, further demonstrating the conservative nature of this substitution.

in recruiting Ftz to specific sites on DNA, and that portions of the homeodomain may serve as contact points. If so, then phosphorylation of T263 could affect these interactions.

Two Ftz-interacting proteins previously have been shown to interact specifically with Ftz; Paired (Prd) (Copeland *et al.*, 1996) and αFtz-F1 (Guichet *et al.*, 1997; Yu *et al.*, 1997). It has also been observed that αFtz-F1 and a Ftz polypeptide containing the homeodomain, along with short stretches of flanking amino acids, bind cooperatively to adjacent sites on DNA (Florence *et al.*, 1997). However, preliminary experiments with our Ftz T263 mutants suggest that the phosphorylation state of T263 does not affect either the Ftz–Prd or the Ftz–Ftz-F1 interaction. Hence, a yet to be identified protein expressed in embryos is likely to be the relevant target.

### *Conservation of Thr263*

Figure 9 shows the N-terminal sequences of several homeodomain proteins. Homeodomains can be assigned to different subclasses based on amino acid sequence similarities. In approximately half of all HOX proteins (groups 5–9), residue 7 of the homeodomain (analogous to Ftz T263) is either a Thr or Ser residue, while position 5 is an Arg residue (Gehring *et al.*, 1994a; Sharkey *et al.*, 1997). This conserves the PKA recognition site, and suggests that each of these proteins probably shares the ability to be phosphorylated at this position. This includes the more divergent POU class homeodomain proteins Pit-1 and Oct-1, which like Ftz are phosphorylated efficiently by PKA, or a PKA-like kinase *in vitro* (Kapiloff *et al.*, 1991; Segil *et al.*, 1991; Caelles *et al.*, 1995). It is also worth noting that some homeodomain proteins, such as En and Even-skipped (Eve), normally possess alanines at position 7. Hence, our substitution of Thr263 with Ala is a conservative one that is unlikely to exert its effect at the level of general homeodomain structure. This is consistent with our findings that this substitution has no apparent effect on DNA-binding activity, transactivating activity (in S2 cells), protein stability or subcellular localization.

### *Identity of the T263 kinase*

For Pit-1, the serine in the analogous position of Ftz T263 could be phosphorylated *in vitro*, either by PKA or an M-phase-specific kinase (Kapiloff *et al.*, 1991, Caelles *et al.*, 1995). However, PKA-dependent phosphorylation could not be verified in cultured cells (Fischberg *et al.*, 1994; Okimura *et al.*, 1994). As with Pit-1, phosphorylation of Ftz T263 may or may not be mediated by PKA *in vivo*. Biochemical and genetic approaches to test this relationship (data not shown) have provided ambiguous results: cultured cells failed to provide enough metabolically labeled Ftz for analysis, and results obtained with mutations in the PKA catalytic subunit gene *DCO* (Kalderon and Rubin, 1988; Lane and Kalderon, 1993) have been difficult to interpret, due in part to the pleiotropic nature of the *DCO* mutant phenotype.

### *Homeodomain proteins and phosphorylation*

All homeodomain proteins tested thus far are phosphorylated (Gay *et al.*, 1988; Krause *et al.*, 1988; Krause and Gehring, 1989; Odenwald *et al.*, 1989; Tanaka and Herr, 1990; Gavis and Hogness, 1991; Kapiloff *et al.*, 1991; Lopez and Hogness, 1991; Segil *et al.*, 1991; Wall *et al.*, 1992; Ronchi *et al.*, 1993; Bourbon *et al.*, 1995; Caelles *et al.*, 1995; Coqueret *et al.*, 1996; Zannin *et al.*, 1996; Jaffe *et al.*, 1997; Zwilling *et al.*, 1997). However, studies testing the relevance of these phosphorylation events are relatively few. For the vertebrate homeodomain proteins (Kapiloff *et al.*, 1991; Segil *et al.*, 1991; Fischberg *et al.*, 1994; Okimura *et al.*, 1994; Caelles *et al.*, 1995; Van Renterghem *et al.*, 1995; Coqueret *et al.*, 1996; Zannin *et al.*, 1996; Poleev *et al.*, 1997; Yan and Whitsett, 1997), tests have only been conducted *in vitro* and in cultured cells, often with conflicting results (Kapiloff *et al.*, 1991; Fischberg *et al.*, 1994; Okimura *et al.*, 1994; Caelles *et al.*, 1995). In the case of *Drosophila* homeodomain proteins, four have been tested for the effects of phosphorylation. Ultrabithorax (Ubx) was shown to exist as multiple-phosphate isoforms *in vivo*, but functional significance could not be ascertained (Gavis and Hogness, 1991; Lopez and Hogness, 1991). Bicoid phosphorylation levels were shown to correlate with activity of the *torso/D-raf* signaling cascade (Ronchi *et al.*, 1993). Phosphorylation of sites mapped on the En protein enhanced DNA-binding affinity by ~2-fold *in vitro* (Bourbon *et al.*, 1995). More recently, several CKII sites on the Antp protein were found to affect activity both *in vitro* and *in vivo* (Jaffe *et al.*, 1997). These sites were distributed outside of the homeodomain.

This is the first study where the functional relevance of a phosphorylation site within the highly conserved homeodomain has been investigated *in vivo*. We have shown that T263 is crucial for Ftz function in developing embryos and, unlike other homeodomain protein sites, is likely to be phosphorylated when the protein is active. The conservation of this site in other homeodomain proteins suggests that many of these sites will also be phosphorylated, and that phosphorylation at this position may be a general mechanism for modulating homeodomain protein activities.

## Materials and methods

### *Site-directed mutagenesis and construction of Ftz expression vectors*

Site-directed mutagenesis was performed using *Pfu* DNA polymerase (Stratagene) following the instructions provided by the manufacturer, and using the template plasmid pGemF1 (Krause *et al.*, 1988). The T263A

and T263D mutations were introduced by two-step PCR mutagenesis. The T263A mutation was generated using the primer HK9 (CCCGTCA-GGCGTACACC) together with a T3 primer (Pharmacia Biotech Inc.), and the T263D mutation using primers HK16 (CGGGTGTAATCCTG-ACGGG) and HK17 (GCCTCCGAGGATGTCGACTACTTGG). In the second step, T3 or HK17 primers were used as appropriate, together with the first round PCR products, to generate products containing the amino two-thirds of the protein. Amplified fragments were gel-isolated, digested with *Sal*I and subcloned into pBlueftz (Furukubo-Tokunaga *et al.*, 1992). Orientation and sequences were verified by dideoxynucleotide sequencing. *ftz*-containing *Xmn*I fragments were then removed from the recombinant pBlueftz plasmids and subcloned into pBKMftzGΔX (Furukubo-Tokunaga *et al.*, 1992) to reconstitute the full-length *ftz* open reading frame together with necessary 5′ and 3′ promoter regions.

*ftz* full-length and ΔHD bacterial expression constructs have been described previously (Krause *et al.*, 1988; Hyduk and Percival-Smith, 1996). To construct T263 mutant homeodomain constructs, PCR was used to amplify the homeodomain portions of the wild-type and mutant pBKMftzG plasmids described above. The 5′ primer used was GATGG-ATCCCGACTCGAAACGCACCCG and the 3′ primer was GCCGGAT-CCTACGTGCGATCCTTCTTCG. The products contain *Bam*HI sites at each end and encode four non-homeodomain residues, Gly–Leu–Asp–Pro, at their N-terminal ends. The PCR products were treated with *Bam*HI and then placed under T7 promoter control in the vector pET-3b. Positive clones were verified by DNA sequencing.

### Expression and partial purification of Ftz polypetides

Ftz full-length, ΔHD and homeodomain polypeptides were expressed in BL21:DE3 pLysS cells (Rosenberg *et al.*, 1987). Cells were grown at 37°C to an $A_{600}$ of 0.6–0.8, induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for a further 2–3 h. Cells were pelleted, resuspended in phosphate-buffered saline (PBS) + 0.1% Triton and sonicated. Pellets were resuspended in guanidine buffer (6 M guanidine HCl, 50 mM Tris pH 8.0), sonicated and centrifuged once again. An equal volume of cold isopropanol was added dropwise to the supernatant and spun again to remove nucleic acids. Four volumes of cold methanol were then added to precipitate Ftz polypeptides. Pellets were dissolved in guanidine buffer and then run over Centricon size exclusion columns (Amicon, Inc.). Full-length protein was run over a Centricon 30 column equilibrated in 20 mM Tris, pH 8.0, 0.5 M NaCl, 1 mM EDTA and 10% glycerol. Concentrated protein was diluted in the same buffer (2 ml) and spun twice more prior to recovery and addition of glycerol to 20% final concentration. Homeodomain polypeptides were purified similarly, except that they were run over Centricon 10 columns first, and then the flow-throughs concentrated on Centricon 3 columns. Final protein concentrations were ~0.5 mg/ml, and proteins were 50–90% pure.

### In vitro kinase assays

*Drosophila* 0–6 h embryo whole cell extract (kindly provided by S.Mason) was prepared according to the method of Kamakaka *et al.* (1993). Kinase assays were performed following protocols provided by Upstate Biotechnology, Inc. Thirty μl reactions contained 5 mM MOPS, pH 7.2, 8 mM β-glycerol phosphate, 1.5 mM EGTA, 0.3 mM sodium orthovanadate, 0.3 mM dithiothreitol, 25 mM $MgCl_2$, 150 μM ATP, 10 μCi of [$\gamma$-$^{32}$P]ATP (4500 Ci/mmol, ICN radiochemicals) and 1 μl of embryo extract (5 mg/ml). Where indicated, 100 ng of full-length or deleted Ftz polypeptide, 10 μl of inhibitor cocktail [6 μM PKC inhibitor peptide, 6 μM Cam PK inhibitor (Upstate Biotechnology, Inc.)] and/or 10 μM cAMP were added. For PKA reactions, 0.5 μg of PKA catalytic subunit cocktail (Upstate Biotechnology, Inc.) was added in place of embryo extract. Reactions were incubated at 30°C for 10 min, stopped by the addition of SDS–PAGE loading buffer and boiled for 5 min. Proteins were resolved on a 12% polyacrylamide gel, transferred to nitrocellulose and detected by autoradiography.

### Phosphopeptide mapping and phosphoamino acid analysis

Wild-type and mutant $^{32}$P-labeled Ftz homeodomains were separated on 15% PAGE gels and blotted to nitrocellulose. Labeled bands were excised from the membrane and tryptic fingerprinting performed according to the method of van der Geer *et al.* (1993). Labeled phosphopeptide spots were scraped from the thin-layer chromatography plates and the peptide eluted with 100–200 μl of fresh 0.05M $NH_4HCO_3$. Peptides were then immobilized on a Sequelon disk and subjected to manual Edman degradation using a Millipore protein sequencing kit (Sequelon AA) as described by the manufacturer.

### Construction of P-element rescue vectors

*ftz*-containing *Kpn*I fragments from wild-type and T263 mutant pBKMftzG plasmids were subcloned into the P-element construct pC20K (Hiromi *et al.*, 1985; Furukubo-Tokunaga *et al.*, 1992) which contains the marker *rosy.* P-element-mediated germline transformation was as described (Spradling and Rubin, 1982). Four independent, homozygous viable, second chromosome insertion lines were isolated for each construct. These were crossed to $ftz^{9H34}$/TM3 and $ftz^{w20}$/TM3 flies to generate P[*ftz*];$ftz^{9H34}$/TM3 and P[*ftz*]; $ftz^{w20}$/TM3 stocks, which in turn were mated to give P[*ftz*]; $ftz^{9H34}/ftz^{w20}$ offspring. The TM3 balancer used was marked with a *hunchback–lacZ*-containing reporter gene to allow unambiguous identification of homozygous mutant offspring.

### Cuticle preparations, in situ hybridization and immunolocalization

Cuticle preparations were as described by Manoukian and Krause (1992). Double *in situ* hybridization (Lehmann and Tautz, 1994) antibody detection, to visualize *ftz*, *en* or *wg* transcripts together with *hunchback*-directed β-galactosidase expression, was performed as described in Manoukian and Krause (1992). Vectors and probes for *ftz*, *en* and *wg in situ* hybridization were as described (Copeland *et al.*, 1996; Guichet *et al.*, 1997). Ftz protein was detected as previously described (Krause *et al.*, 1988) using a Ftz polyclonal antiserum and the ABC kit from Vectastain.

### Transient transfection assays

For cell culture transfection assays, cDNAs encoding Ftz mutant proteins were subcloned into the expression vector pPac (Krasnow *et al.*, 1989). PKA expression was achieved using the plasmid pAct-DC0 described by Norris and Manley (1992). Schneider line-2 (S2) cells were grown at 25°C in Schneider medium (Gibco-BRL) containing 5% fetal bovine serum (heat-inactivated, Gibco-BRL). DNA transfections were performed using a CellPhect transfection kit (Pharmacia) and the reporter 3K′-TATA-CAT (Han *et al.*, 1989). DNA precipitates contained 0.5–2 μg of pPacFtz, as indicated, 1 μg of reporter plasmid, 1 μg of pAct-DC0 where indicated and enough pPac plasmid to make 3 μg of total DNA. Following transfection and cell growth, as described in the CellPhect kit directions, cell extracts were prepared by freeze-thawing three times and pelleting cell debris using a 1 min centrifugation at 12 000 r.p.m. Levels of CAT expression were determined by Western blotting with an anti-CAT monoclonal antibody (5 Prime-3 Prime, Inc), detection using an enhanced chemiluminescence kit (ECL, Boehringer Mannheim) and quantitation using a Bio-Rad PhosphorImager. Levels of Ftz in the same samples were determined similarly except that anti-Ftz antiserum (Krause *et al.*, 1988) was used instead of anti-CAT antibodies.

### Electrophoretic mobility shift assays

*In vitro* translations of wild-type and mutant Ftz homeodomains were performed using the TNT T7 Coupled Reticulocyte Lysate System (Promega) and unlabeled amino acids. After translation, proteins were separated on a 15% PAGE gel and quantitated by ECL Western blotting and PhosphorImager analysis. Equal amounts of protein were used for the EMSAs, performed as described by Percival-Smith *et al.* (1990) using the double-stranded DNA substrate GGGAAGCAATTAAGGATCCC (Percival-Smith *et al.*, 1990; Pick *et al.*, 1990).

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