Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1450–1454, February 1993
Genetics

Functional specificity of the homeodomain protein fushi tarazu: The role of DNA-binding specificity *in vivo*

(*Drosophila*/transcriptional regulation/development)

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Contributed by Walter J. Gehring, October 21, 1992

ABSTRACT **The mechanisms determining the functional specificity of *Drosophila* homeodomain proteins are largely unknown. Here, the role of DNA-binding specificity for the *in vivo* function of the homeodomain protein fushi tarazu (ftz) is analyzed. We find that specific DNA binding is an important but not sufficient determinant of the functional specificity of ftz *in vivo*: The ftz DNA-binding specificity mutant ftzQ50K retains partial ftz wild-type activity in gene activation and phenotypic rescue assays. Furthermore, specificity mutations in a ftz-*in vivo* binding site only partially reduce enhancer activity as compared to null mutations of this site. Despite bicoid-like DNA-binding specificity ftzQ50K does not activate natural or artificial bcd target genes in the realms of ftz. These results are discussed in the light of recent observations on the mechanism of action of the yeast homeodomain protein α2.**

Homeodomain proteins are DNA-binding transcription factors involved in multiple gene regulatory and developmental decisions (1–3). The homeodomain constitutes the DNA-binding domain of these proteins (4–6). Structural studies have revealed that the very divergent homeodomains of Antennapedia, engrailed, and Matα2 have almost identical three-dimensional structures and DNA-binding modes (7–10). They are folded in three helices with helices 2 and 3 forming the helix–turn–helix motif also found in many prokaryotic DNA-binding proteins. Helix 3 lies in the DNA major groove as a recognition helix, and the N-terminal arm of homeodomains makes contacts in the DNA minor groove.

Despite the related sequences and structures of homeodomains, the more than 30 *Drosophila* homeodomain proteins have very distinct functions during development (11–14). The question emerges of how the functional specificity of homeodomain proteins is determined. The unique spatial and temporal expression profiles of homeodomain proteins contribute to their specificity of action (15, 16). However, ectopic expression assays have demonstrated that different homeodomain proteins can induce very distinct fates in a given group of cells (16–20). The analysis of wild-type and chimeric *Drosophila* homeotic proteins in these assays has indicated that sequences in and immediately flanking the homeodomain are important determinants of target specificity *in vivo* (19–22). Different homeotic proteins exert only minor distinctions in their preference for different binding sites *in vitro* (23–26). Thus, it is not clear whether functional specificity differences are due to differences in DNA-binding specificity and/or in specific protein–protein interactions.

The best understood determinant of the DNA-binding specificity of homeodomain proteins is homeodomain position 50, which is located in recognition helix 3 (27–32). Structural studies on homeodomain–DNA complexes have shown that this amino acid is in close proximity to the 2 bp preceding the ATTA core common to many homeodomain binding sites (8, 9). In the case of the fushi tarazu (ftz) homeodomain, which contains a glutamine at position 50 (as do all the homeotic selector proteins), *in vitro* studies have shown high-affinity binding to CCATTA or CAATTA motifs (4, 23, 33). Changing this site to GGATTA, a motif found in binding sites for the homeodomain protein bicoid (bcd) (34), reduces *in vitro* binding affinity by more than an order of magnitude (30, 31). Substituting Gln-50 with lysine, as found at position 50 in the bcd homeodomain, restores high-affinity binding of the mutant ftz homeodomain (ftzQ50K) to the mutant site GGATTA. Concurrently, the DNA-binding specificity mutant ftzQ50K has a reduced affinity for CAATTA or CCATTA motifs, as compared with ftz (30, 31).

Recent studies on the regulation of the *ftz* gene have confirmed the importance of position 50 in the DNA-binding specificity of homeodomain proteins *in vivo* (32). The striped expression of *ftz* during early embryogenesis is, in part, controlled by an autoregulatory feedback mechanism involving ftz protein and two *ftz* autoregulatory enhancer elements (33, 35). Changing ftz-*in vitro* binding sites to GGATTA in one of these autoregulatory elements (AEs) reduces enhancer activity *in vivo*. This down-regulatory effect is specifically suppressed by introducing the compensatory DNA-binding specificity mutant ftzQ50K (32). These experiments demonstrate a direct autoregulatory feedback mechanism in the regulation of *ftz* and underline the important role of position 50 in the DNA-binding specificity of homeodomain proteins *in vivo*.

An intriguing observation in these studies was the finding that enhancer elements containing GGATTA-binding sites are more active than enhancer elements in which these sites are deleted or mutated to GGCCCC (32). These results suggested that ftz protein might still, although weakly, recognize binding sites with a changed specificity. In this report we describe experiments designed to learn more about the role of DNA-binding specificity in the *in vivo* function of the homeodomain protein ftz.

MATERIALS AND METHODS

DNA Methods. *ftz* transgenes *ftzQ50K* and *ftzΔHD* have been described (32, 36). They contain the entire 10-kb genomic region sufficient for rescue of ftz mutant animals (37). FtzQ50K contains a single amino acid change at position 50 in the ftz homeodomain from glutamine to lysine. FtzΔHD contains a deletion in the homeodomain. Fusion genes AE–BS2, AE–BS2BCD, and AE–BS2CCC were generated as follows. The sequences corresponding to upstream element

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Abbreviations: ftz, fushi tarazu; bcd, bicoid; AP, alkaline phosphatase; AE, ftz autoregulatory element; en, engrailed; A2 and A3, abdominal segment 2 and 3, respectively.

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position 2145–2574 were PCR-amplified by using oligonucleotide primers ASPE23 (32) and ASPE35 (5′-GGA TCC TCT AGA CGG GGC CCT TTT CTC TCA GTT TCT AAA A-3′) for AE–BS2, ASPE34 (5′-GGA TCC TCT AGA CTA ATC CCT TTT CTC TCA GTT TCT AAA A-3′) for AE–BS2BCD, and ASPE41 (5′-GGA TCC TCT AGA CGG GCC CCT TTT CTC TCA GTT TCT AAA A-3′) for AE–BS2CCC. Templates were AEΔC (32) for AE–BS2BCD and AE–BS2CCC, and AE–BS2BCD for AE–BS2. Amplified fragments were gel-isolated, digested with *Xba* I, and subcloned into the unique *Xba* I site of the lacZ reporter gene vector HZ50PL (35). Orientation and sequences were verified by dideoxynucleotide sequencing.

Fly Strains. *P* element-mediated transformation and establishment of balanced and homozygous transformant stocks were as described (35). For each AE–BS2 derivative, at least four independent lines were established and analyzed. To unambiguously identify embryos with mutations in the endogenous *ftz* gene (Figs. 2 and 3), fly lines were constructed containing a given transgene on the second chromosome in combination with the *ftz* amorphic mutation ftz^{9H34} on the third chromosome balanced over $TM3^{hb8}$ (W. Driever, personal communication). This balancer chromosome harbors the *hb* promoter–*lacZ* fusion gene pThb8 (38), which expresses β-galactosidase in the anterior portion of the embryo during blastoderm and germ-band extension stages. Embryos homozygous for ftz^{9H34} do not contain $TM3^{hb8}$ and can thus be identified by the absence of β-galactosidase expression in the anterior region. The ability of *ftzQ50K* to rescue the lethality caused by the mutant ftz combinations ftz^{9H34}/ftz^{w20} and ftz^{9093}/ftz^{9H34} was assayed as described (36, 37). The effect of *ftzQ50K* on the formation of cuticular structures was tested in the ftz null mutant combination ftz^{9H34}/ftz^{w20}. Cuticles were prepared as follows. Unhatched embryos were dechorionated, transferred to an Eppendorf tube containing 0.5 ml of heptane and 0.5 ml of methanol and "devitellinized" by vigorous shaking for several minutes. After being washed in methanol three times, embryos were pipetted onto a glass slide and, as soon as the methanol had evaporated, mounted in Hoyer's mountant/lactic acid, 1:1 (12). Embryos were cleared for 24 hr at 60°C.

Analysis of Expression Patterns. Detection of β-galactosidase activity in transgenic embryos was as described (32, 35). Antibody staining was done as described (39). The following antibodies were used: rabbit anti-hunchback (ref. 40; dilution 1:50), rat anti-empty spiracles (ref. 41; 1:400); rabbit anti-ftz (ref. 42; 1:300); mouse anti-engrailed (ref. 43; 1:300); mouse anti-β-galactosidase (Promega; 1:1000); alkaline phosphate (AP) coupled with rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark; 1:200); AP coupled to swine anti-rabbit IgG (Dakopatts; 1:200); AP coupled to goat anti-rat IgG (Cappel; 1:200). Detection of AP activity was as described (44).

RESULTS

Direct *in vivo* Interaction of ftz with a ftz-*in Vitro* Binding Site from the Engrailed Gene. To extend our previous observations on the direct interaction of ftz protein with ftz-binding sites in AE (32), we wished to test whether ftz can also directly interact with other ftz-*in vitro* binding sites. We chose to analyze homeodomain binding site BS2, a sequence located ≈2 kb upstream of the transcription start site of the engrailed (en) gene (4). The *in vitro* interaction of homeodomains and BS2 has been extensively analyzed at both the biochemical and structural levels (4, 8, 23, 30, 31). A single homeodomain peptide binds to this site *in vitro* with a K_d of $\approx 10^{-9}$ M (23). As the DNA region of *en* that contains binding site BS2 is only poorly characterized with respect to its regulatory function, the properties of BS2 were studied in the context of AE (32). It has been shown (32) that ftz-binding sites within this element are interchangeable. ftz-binding site D in AE was exchanged with BS2 (Fig. 1), and lacZ reporter gene expression was monitored in transgenic embryos. Fig. 1*c* shows that fusion gene AE–BS2 is strongly expressed. *In vitro* DNA-binding studies have shown that mutating the CCATTA motif in binding site BS2 to GGATTA, a motif found in bcd/ftzQ50K-binding sites, results in a 40-fold reduction of the binding affinity of ftz homeodomain (30, 31). Accordingly, mutating BS2 to GGATTA in AE–BS2 reduces enhancer activity *in vivo* (construct AE–BS2BCD in Fig. 1*e*). As expected for the direct *in vivo* interaction of ftz with BS2, this down-regulatory effect is specifically suppressed by the introduction of the DNA-binding specificity mutation *ftzQ50K* (Fig. 1*f*). We conclude that in the context of AE, ftz binds *in vivo* to a heterologous *in vitro* binding site from the *en* gene. Interestingly, we find that mutation of BS2 to GGCCCC (construct AE–BS2CCC in Fig. 1*g*) reduces enhancer activity much more drastically than mutating it to GGATTA (AE–BS2BCD in Fig. 1*e*). This result suggests that despite a 40-fold reduced *in vitro* affinity, ftz is able, although weakly, to recognize a specificity mutant of a ftz target site *in vivo*. These results extend our previous *in vivo* studies on the interaction of ftz with DNA target sites (32) and demonstrate that BS2, a nonpalindromic site that is recognized by a single DNA-binding moiety *in vitro* (23), can function as an *in vivo* target for homeodomain proteins. The role of BS2 in the regulation of *en* is unknown. Genetic studies suggest that *en* is a regulatory target gene for ftz (45). Our results show that ftz can, in the context of AE, directly interact with a sequence from the *en* cis-regulatory region.

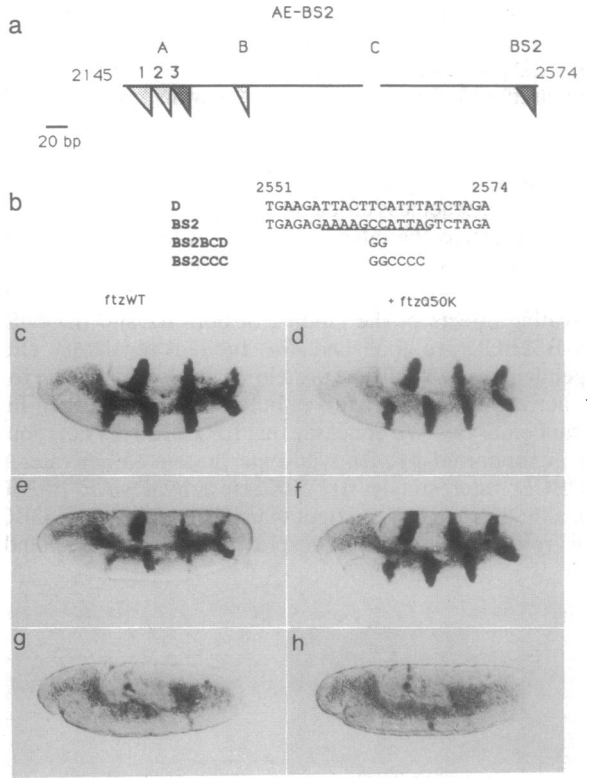

FIG. 1. Effect of *ftzQ50K* on fusion gene expression in wild-type embryos. (*a*) Structure of AE–BS2. ftz-*in vitro* binding site D in AE–ΔC was replaced with BS2. (*b*) BS2 (underlined) in AE–BS2 was mutated to BS2BCD and BS2CCC, respectively. Embryos were collected and stained from crosses of flies carrying the different AE–BS2 derivatives with flies harboring the *ftzQ50K* transgene. Expression directed by AE–BS2 (*c*, *d*), AE–BS2BCD (*e*, *f*), and AE–BS2CCC (*g*, *h*) in wild-type embryos (*c*, *e*, and *g*) or in wild-type embryos containing, in addition, one copy of the *ftzQ50K* transgene (*d*, *f*, and *h*).

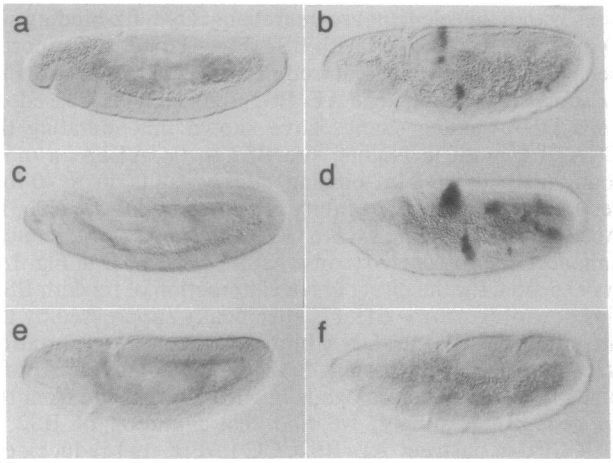

FIG. 2. Effect of *ftzQ50K* on fusion gene expression in ftz mutant embryos. The expression of AE–BS2 (*a*, *b*), AE–BS2BCD (*c*, *d*), and AE–BS2CCC (*e*, *f*) was analyzed in embryos that are homozygous for ftz^{9H34} (*a*, *c*, and *e*) or in embryos homozygous for ftz^{9H34} and carrying a copy of the *ftzQ50K* transgene (*b*, *d*, and *f*). Homozygous mutant embryos were identified as described.

In the foregoing experiments ftzQ50K-dependent suppression of cis-regulatory mutations was assayed in a context where endogenous ftz protein is present. We wanted to determine whether ftzQ50K could also activate AE–BS2BCD in a background devoid of ftz wild-type protein. Fusion genes AE–BS2, AE–BS2BCD, and AE–BS2CCC are inactive in ftz mutant embryos (Fig. 2). Strikingly, introduction of *ftzQ50K* into this genetic background leads to the activation of AE–BS2BCD (Fig. 2*d*). AE–BS2BCD is less active in *ftz* mutant as compared with *ftz* wild-type embryos transgenic for *ftzQ50K* (Fig. 2*d* vs. 1*f*). Three effects might be responsible for this result. (*i*) The *ftzQ50K* transgene is expressed at reduced levels in ftz mutant animals (see below, Fig. 3*c*) and might, therefore, interact less efficiently with AE–BS2BCD. (*ii*) In ftz mutant embryos the interaction of ftz wild-type protein with ftz-binding sites in AE–BS2BCD cannot contribute to enhancer activity. Consequently, the additive (or cooperative) activating effects of the binding of both ftz and ftzQ50K to AE–BS2BCD are abolished. (*iii*) Indirect regulatory effects dependent on ftz wild-type protein might also contribute to the full activity of AE derivatives but would be abolished in ftz mutant embryos. We conclude that ftzQ50K does not require, but is supported by, ftz wild-type protein to activate AE–BS2BCD. Interestingly, ftzQ50K also activates AE–BS2 (Fig. 2*b*), although to a lesser extent than it activates AE–BS2BCD. This result suggests that despite an altered DNA-binding specificity, ftzQ50K has a low affinity for regulatory elements recognized by wild-type ftz protein (see below).

Rescue Activity. Homeodomain substitutions can change the regulatory specificities of homeotic proteins *in vivo* (19–22). It is not known whether these effects are caused by an altered DNA-binding specificity or other functional differences conferred by the heterologous homeodomains (22, 26). The fact that ftzQ50K has an altered DNA-binding specificity allowed us to assess in more detail the importance of sequence-specific DNA binding for the biological function of homeodomain proteins. The ability of the *ftzQ50K* gene to substitute for *ftz* gene activity was analyzed in a phenotypic rescue assay. In contrast to a transgene carrying the 10 kb of the wild-type *ftz* gene (36, 37), *ftzQ50K* cannot rescue ftz mutant animals to adulthood (data not shown). Cuticular structures of ftz mutant larvae show pattern deletions in a double segment periodicity (ref. 46; Fig. 3*h*). Surprisingly, ftz mutant embryos carrying one copy of the *ftzQ50K* transgene show a partial-to-complete rescue of the denticle belt of abdominal segment 3 (A3), one of the structures missing in ftz mutants (Fig. 3*i*). Furthermore, the posterior spiracles and filzkörper are well-differentiated in ftz mutant larvae transgenic for *ftzQ50K* (Table 1). Neither of these effects is observed in ftz mutant larvae harboring *ftzΔHD*, a *ftz* transgene containing a deletion in the homeodomain (ref. 36; Table 1). The two structures rescued by *ftzQ50K* correspond to parasegments 8 and 14 (47). Of the seven ftz stripes, stripes 4 and 7 are expressed in these domains during early embryogenesis (47–49).

To determine why parasegments 8 and 14 are most susceptible to rescue by *ftzQ50K*, we analyzed the expression of ftzQ50K protein in embryos devoid of endogenous ftz protein. As shown in Fig. 3*c*, ftzQ50K is detectable in seven stripes with stripes 4 and 7 most strongly expressed; this is most evident in the lateral and dorsal regions of the embryo. The expression pattern of *ftzQ50K* is partly reminiscent of zebra element–lacZ fusion genes or *ftz* transgenes impaired in autoregulation (refs. 35 and 36; A.F.S., unpublished results). Hence, *ftzQ50K* might be weakened in its autoregulatory capacities. The observation that ftzQ50K only partially activates fusion gene AE–BS2 supports this view (Fig. 2). As shown in Fig. 3*f* the high-level expression of *ftzQ50K* in parasegments 8 and 14 leads to the normal activation of the ftz target gene *en*. We conclude that the expression of a DNA-binding specificity mutant like ftzQ50K at sufficient levels allows the activation of ftz target genes (AE–BS2 and *en*) and confers partial rescue to a ftz mutant embryo.

Expression of bcd Target Genes in *ftzQ50K* Transgenic Embryos. Previous studies on the function of the yeast transcriptional activator GAL4 in *Drosophila* have shown that reporter genes containing multiple GAL4-*in vitro* binding sites are activated in apparently every cell in which GAL4 is

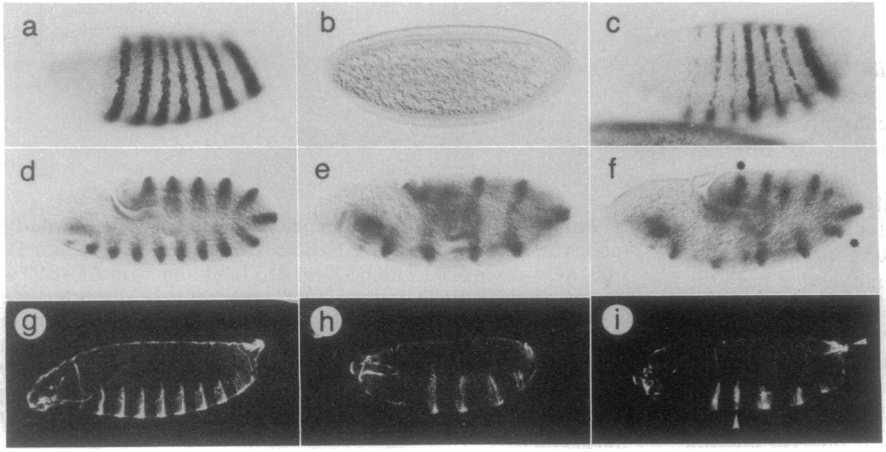

FIG. 3. Effect of *ftzQ50K* on expression of engrailed and formation of cuticular structures. Expression of ftz protein (*a*, *b*), ftzQ50K protein (*c*), and engrailed protein (*d*, *e*, and *f*) and formation of ventral cuticular structures (*g*, *h*, and *i*) in wild-type embryos (*a*, *d*, and *g*), embryos that are homozygous for ftz^{9H34} (*b*, *e*, and *h*), and embryos homozygous for ftz^{9H34} harboring a *ftzQ50K* transgene (*c*, *f*, and *i*). Engrailed stripes and cuticular structures corresponding to parasegments 8 and 14 are indicated. Note that engrailed stripes corresponding to parasegments 4, 10, and 12 are also partially activated by *ftzQ50K*.

Table 1. Effect of *ftzQ50K* on formation of cuticular structures in ftz mutant embryos

	Denticle belt A3, %			Posterior spiracles	
	None	Partial	Complete	and filzkörper, %	Scored, no.
No transgene	100	0	0	0	43
ftzΔHD 13.1	100	0	0	0	58
ftzΔHD 1.2	100	0	0	0	38
ftzQ50K 1	100	0	0	12	17
ftzQ50K 3	64	31	5	46	65
ftzQ50K 4	52	24	24	48	59

Ftz mutant embryos collected from the cross *ftz* transgene/*Cy*;*ftz*^{9H34}/*TM3* × +/+;*ftz*^{w20}/*TM3* were analyzed for rescue of the ftz embryonic phenotype. In this cross 50% of ftz mutant embryos should carry one copy of the *ftz* transgene. Ftz mutant embryos were identified according to characteristic pair rule-like defects. Only abdominal denticle belts and posterior spiracles/filzkörper were scored for rescue. With a few exceptions [one and three embryo(s) of the ftzQ50K4 line showed rescue of parasegment 10 and 12, respectively], only parasegments 8 and 14 showed rescue. Rescue was scored according to the extent of A3 denticle belt formation. Partial rescue corresponds to the development of a few denticle hairs or a denticle belt still fused to denticle belt A2. Full rescue corresponds to the formation of an A3 denticle belt no longer fused to A2. Rescue of parasegment 14 was scored according to the presence of clearly differentiated posterior spiracles and filzkörper.

expressed (50). In this regulatory system the target specificity of GAL4 seems to be determined only by the presence of GAL4-binding sites upstream of a minimal promoter. A similar mechanism might also be put forward to explain the functional specificity of homeodomain proteins. The presence of particular binding sites might be sufficient for target recognition and activation by a given homeodomain protein. Indeed, the oligomerization of bcd-*in vitro* binding sites creates bcd-responsive cis-regulatory elements (38).

The ftzQ50K homeodomain has a similar *in vitro* DNA-binding specificity as bcd (30–32, 34). The "GAL4 model" for homeodomain protein target recognition would suggest that this property is sufficient to activate bcd target genes in the expression domains of ftzQ50K, as these genes contain bcd/ftzQ50K-*in vitro* binding sites. To test this hypothesis the expression of several bcd target genes was analyzed in wild-type embryos transgenic for *ftzQ50K* (Fig. 4). We tested hunchback (*hb*) (34), empty spiracles (41, 51), and bcd-responsive reporter genes containing oligomerized bcd-binding sites (pThb10, -11, -15, and -16 in ref. 38). As shown in Fig. 4, no deviation from wild-type expression was detectable (e.g., no expression in seven stripes was seen). This finding is consistent with the observation that flies harboring one or two copies of the *ftzQ50K* transgene show no obvious phenotypic abnormalities (data not shown). Experiments

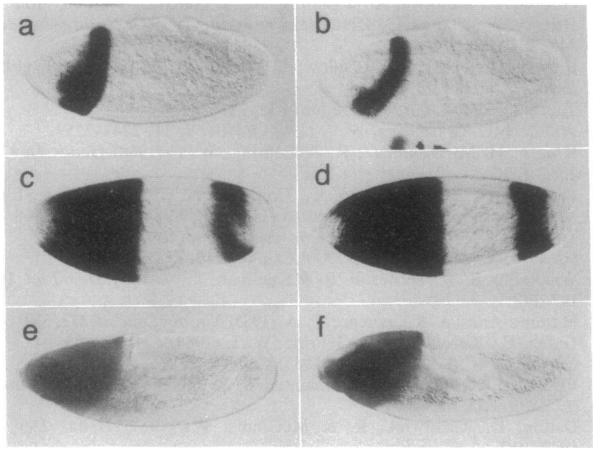

FIG. 4. Expression of bcd target genes in embryos transgenic for *ftzQ50K*. The expression of hunchback (*a*, *b*), empty spiracles (*c*, *d*), and fusion gene pThb11 (*e*, *f*) was analyzed in wild-type embryos (*a*, *c*, and *e*) and embryos harboring two copies of the *ftzQ50K* transgene (*b*, *d*, and *f*). Note that also at later embryonic stages no deviation from wild-type expression was detectable in ftzQ50K embryos.

involving the ectopic expression of bcd have shown that the *hb* gene is still competent to be activated by bcd at the cellular blastoderm stage (W. Driever, personal communication). As ftzQ50K is expressed at this time (see below), the inability of ftzQ50K to act like bcd might be due to the absence of bcd protein sequences in ftzQ50K other than Lys-50 in the homeodomain. Our results are consistent with the previous finding that oligomerized ftz-*in vitro* binding sites are not sufficient to constitute a ftz-responsive element (52). These observations contradict the simple view that homeodomain binding sites are sufficient to constitute a homeodomain protein-responsive element *in vivo*.

DISCUSSION

In this report we have described experiments designed to study the role of DNA-binding specificity for the *in vivo* function of the *Drosophila* homeodomain protein ftz. Several of our observations underline the important role of the sequence-specific interaction of ftz with its regulatory target sites for efficient *in vivo* function. (*i*) Enhancer activity is reduced by specificity mutations that convert a ftz-binding site (containing a CCATTA motif) into a bcd-binding site (GGATTA). (*ii*) The DNA-binding specificity mutant ftzQ50K cannot fully replace ftz in gene activation and phenotypic rescue assays. (*iii*) ftz binds preferentially to CA/CCATTA motifs, whereas ftzQ50K prefers GGATTA sites.

However, we find that DNA-binding specificity *per se* is not sufficient to explain the functional specificity of homeodomain proteins like ftz and ftzQ50K. (*i*) ftzQ50K-*in vitro* binding sites in the context of bcd target elements are not sufficient for ftzQ50K-dependent gene activation. Concurrently, embryos transgenic for *ftzQ50K* do not show any obvious phenotypic abnormalities. Hence, in the case of ftz, an altered DNA-binding specificity does not seem to result in a new regulatory specificity. (*ii*) An enhancer element in which the specificity of a single ftz-binding site is mutated from CCATTA to GGATTA is more active than an enhancer element in which this site is deleted or mutated to GGCCCC. ftz seems capable of weakly recognizing target sites with a changed specificity. Additionally, ftzQ50K can weakly activate a ftz target element (AE–BS2). These findings indicate that homeodomain proteins with a given DNA-binding specificity *in vitro* can recognize quite divergent sites *in vivo*. Our results underline the importance of low- and medium-affinity binding sites in the recognition of enhancers by homeodomain proteins (32, 38). (*iii*) In gene activation and phenotypic rescue assays ftzQ50K retains ftz activity and specificity when expressed at high levels. This observation suggests that homeodomain proteins with very different *in vitro*

DNA-binding specificities (in our case ftz versus ftzQ50K but not ftzQ50K versus bcd) can retain some overlap in the recognition of binding sites. The hypomorphic, not neomorphic, nature of ftzQ50K leads us to conclude that specific DNA binding is an important, but not sufficient, determinant of the functional specificity of ftz *in vivo*. Future studies have to determine whether the results on the ftz protein also hold true for other homeodomain proteins.

The results reported here might be interpreted in the light of recent observations on the mechanism of action of the yeast homeodomain protein $\alpha 2$. Target specificity of this protein is determined by the interaction with both a particular DNA-binding site and the accessory protein MCM1 (53, 54). These combined clues seem to mark regulatory regions to be efficiently and specifically recognized by $\alpha 2$ protein. Target recognition in this and other cases (see ref. 55) seems to result from multiple interactions. Specificity is not determined by a single high-affinity interaction but by the overall efficiency of various individual interactions. Based on this paradigm, our results suggest that multiple interactions of ftz protein not only with DNA target sites but also with auxiliary protein factors determine the functional specificity of this homeodomain protein *in vivo*. Weakening the efficiency and specificity of a single regulatory interaction (in this study, DNA binding) would only partially impair regulatory function because the interaction with other components remains efficient and partially compensating. Furthermore, the formation of regulatory complexes depends not only on the mutual affinities of the interacting components but also on their concentrations. According to this model, a homeodomain protein that is impaired in some interactions would still be active when expressed at sufficient levels. The ftz-like activity of ftzQ50K in parasegments 8 and 14 supports this proposal. The model of multiple interactions could also explain why ftzQ50K does not have an apparent new regulatory specificity *in vivo*. As specificity would be the product of multiple, more or less efficient, interactions, an obvious change of functional specificity would not be gained by changing DNA-binding specificity alone; this change would occur only upon altering also the efficiency of other interactions.

Apart from our results, two recent reports support the model of multiple interactions. In a mutational analysis of the helix–turn–helix motif of ftz, Furukubo-Tokunaga *et al.* (36) found that mutations that weaken the efficient *in vitro* interaction of ftz with DNA target sites still have some wild-type activity *in vivo*. As in our studies, PS8 and -14 seem to be the structures most easily rescued (36). Secondly, Fitzpatrick *et al.* (56) have reported that the heat-shock-mediated ectopic expression of a ftz protein lacking part of the homeodomain can induce an anti-ftz phenotype. This protein is likely to exert its biological effect mainly via protein–protein interactions. The individual contributions of protein–DNA and protein–protein interactions to the function of ftz remain speculative. However, we point out that in our phenotypic rescue assay the different activities of ftz, ftzQ50K, and ftzΔHD demonstrate that specific DNA binding makes an important contribution to ftz *in vivo* activity.

In summary, the studies on the ftz protein suggest that high-specificity, high-affinity homeodomain–DNA interactions are neither sufficient nor absolutely required for the target regulation and the biological activity of homeodomain proteins. Apart from DNA binding, multiple interactions with auxiliary factors seem to contribute strongly to the functional specificity of homeodomain proteins. It will be the challenge of the future to identify the factors that interact with homeodomain proteins in target regulation and recognition.

We thank Markus Affolter and Martin Müller for helpful discussions; Ken Cadigan and Markus Affolter for critically reading the manuscript; H. Krause, D. Tautz, P. Lawrence, U. Walldorf, W. Driever, and M. Müller for providing antibodies and fly strains; and W. Driever for communicating results before publication. This work was supported by the Kantons of Basel.

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