In vivo analysis of the helix–turn–helix motif of the *fushi tarazu* homeo domain of *Drosophila melanogaster*

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We report a systematic mutational analysis of the helix–turn–helix motif (HTH) of the *fushi tarazu* (*ftz*) homeo domain (HD) of *Drosophila*. We started out by testing the function of chimeric *ftz* proteins containing either a part of the *Sex combs reduced* (*Scr*) or the *muscle segment homeobox* (*msh*) HDs. By complementation tests in transgenic flies, cotransfection assays in cultured *Drosophila* cells and in vitro DNA-binding assays, we have found that the *ftz* activity is retained in the *ftz*–*Scr* chimera but is lost in the *ftz*–*msh* chimera, which is defective in binding to an *Antennapedia* (*Antp*)-class target site. Further studies with a series of back-mutants of the *ftz*–*msh* chimera have revealed that a set of class-specific DNA backbone-contacting residues in the HTH, particularly Arg-28 and Arg-43, are required for efficient target site recognition and, hence, full *ftz* activity both in vitro and in vivo.

[Key Words: Homeo domain; helix–turn–helix motif; DNA–protein interaction; gene complementation]

Received February 13, 1992; revised version accepted April 9, 1992.

Many of the regulatory genes involved in the control of development share a common sequence element of 180 bp, the homeo box, which encodes a 60-amino-acid motif, the homeo domain (HD; for review, see Scott et al. 1989; Gehring et al. 1990). The amino acid sequences of known HDs are conserved in evolution from yeast to higher vertebrates and plants (Shepherd et al. 1984; Scott et al. 1989; Ruberti et al. 1991). In vitro DNA-binding studies (Desplan et al. 1988; Hoey and Levine 1988; Müller et al. 1988) and structural studies (Qian et al. 1989; Kissinger et al. 1990; Otting et al. 1990; Wolberger et al. 1991) have revealed that HDs are sequence-specific DNA-binding domains that consist of three or four α -helices separated by a loop and a turn. Two of the α -helices, located in the carboxy-terminal portion of the HD, correspond to the helix–turn–helix (HTH) motif, which was initially identified in prokaryotic gene regulatory proteins as a sequence-specific recognition module (for reviews on HTH motifs, see Harrison and Aggarwal 1990; Steitz 1990).

On the basis of amino acid sequence similarities, HDs can be assigned to different classes (Scott et al. 1989). Although many of the HD proteins belonging to different classes share significant sequence similarity and are able to recognize similar or identical DNA sites in vitro

(Desplan et al. 1988; Hoey and Levine 1988), some of the distantly related HDs have been shown to have distinct DNA-binding specificities. Systematic analyses of the amino acid sequences in the recognition helices have revealed that a single change at position 9 in the recognition helix is sufficient to change the target gene specificity of *Drosophila* homeo proteins in yeast (Hanes and Brent 1989), in vitro (Treisman et al. 1989; Percival-Smith et al. 1990), and in flies (Schier and Gehring 1992). Structural studies on the *Antennapedia* (*Antp*) and *engrailed* (*en*) HD–DNA complexes have confirmed that the ninth residue of the recognition helix makes critical contacts with specific bases in the major groove (Kissinger et al. 1990; Otting et al. 1990). However, many HD proteins that have identical recognition helices have differential functions in development (Kuziora and McGinnis 1989; Gibson et al. 1990; Mann and Hogness 1990; for review, see Hayashi and Scott 1990), indicating that residues outside of the recognition helix may as well contribute to DNA binding and/or functional specificities.

For the characterization of the biologically functional amino acids of the *Antp*-class HDs, the pair-rule gene *fushi tarazu* (*ftz*) provides an excellent model system. The *ftz* HD amino acid sequence is identical to that of *Antp* HD at 50 of 60 positions and seems to interact with *Antp*-class target sites in an almost identical manner to that of *Antp* HD, as demonstrated by chemical interference experiments (Percival-Smith et al. 1990). Owing to the compact structure of its genetic unit, *ftz* provides a

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simple system that allows functional assessment of various HD mutations in vivo. A relatively short DNA fragment of ~10 kb that contains 6.1 kb of 5' enhancer and promoter elements, as well as the coding and the 3' trailer sequences, is able to rescue *ftz*[−] embryos to adulthood when introduced into flies by P-element-mediated transformation (Hiromi et al. 1985).

We have thus utilized this gene rescue system as a complementation assay to monitor the activity of mutations in and around the HTH of the *ftz* protein in vivo. To investigate the molecular basis of the phenotypic defects observed in transgenic flies, the mutated proteins were also tested for their *trans*-activation activity in *Drosophila* tissue culture cells and for their DNA-binding activity to an *Antp* class target site in vitro. We show that (1) the HTH of the *ftz* HD can be heavily mutated without abolishing *ftz* function in vivo; (2) a particular set of amino acids outside of the recognition helix of the *ftz* HTH motif controls efficient target site recognition both in vitro and in vivo; and (3) the activities of the different mutants are similar when monitored by the three different assay systems used in this study. The information derived from a given mutation is, however, increased by the qualitative and quantitative differences of the different assay systems.

Results

Gene rescue by chimeric proteins

The *ftz*–*Scr* (*ftz4*) and the *ftz*–*msh* (*ftz5*) chimeras were constructed using the *Xmn*I and *Bgl*II sites, which are conserved in *ftz* (Laughon and Scott 1984), *Sex combs reduced* (*Scr*) (LeMotte et al. 1989) and *muscle segment homeobox* (*msh*) (Robert et al. 1989; B. Jacq, U. Walldorf, and W.J. Gehring, in prep.) homeo box sequences. These fusions introduce 5 and 19 amino acid changes, respectively, into the *ftz* HD. However, the ninth position of the recognition helix (Gln 50; helix 3) is conserved between the three genes (Fig. 1B). An in-frame deletion in the *ftz* HD has also been constructed (*ftz*ΔHD).

Transgenic lines were established for the *ftz*⁺, *ftz4*, *ftz5*, and *ftz*ΔHD constructs, and their abilities to rescue *ftz* mutant embryos was assayed in vivo as described in Materials and methods. About half of the *ftz*⁺ transformant lines obtained with the P-element pCrfl (Fig. 1A) rescued a *ftz*^{9H34}/*ftz*^{9O93} mutant background to adulthood (see Table 1 and comments below). The five amino acid changes in *ftz4* did not affect the functionality of the chimeric protein, as most of the *ftz4* transformant lines also gave rescue to adulthood in the *ftz* rescue assay (see Table 1).

None of the *ftz5* and *ftz*ΔHD lines were able to complement a *ftz*[−] mutant. However, the inspection of the cuticle of *ftz5*/+;*ftz*^{9H34}/*ftz*^{9O93} embryos indicated that the 19 amino acid changes present in *ftz5* did not completely abolish *ftz* activity, because such individuals frequently showed a partial rescue of the *ftz* denticle belt pattern (cf. Fig. 2,B with C–E). Furthermore, embryos

carrying two copies of the *ftz5* gene showed an improved cuticular rescue phenotype (Fig. 2F; see also Fig. 3), although up to three copies of the *ftz5* gene still failed to rescue a *ftz*[−] embryo to adulthood. In contrast, all *ftz*ΔHD lines produced an apparent *ftz* pair-rule phenotype, as did all five transformant lines that harbor a destructive frameshift mutation in the *ftz5* context at position 45 of helix 3 (data not shown). Retention of partial activity of the *ftz5* gene is also supported by the fact that transgenic flies expressing the *ftz5* gene under the control of the heat-inducible *hsp70* promoter produced a so-called anti-*ftz* phenotype (Struhl 1985; Ish-Horowicz et al. 1989), when 2.5- to 3.5-hr-old embryos were heat-shocked for 20 min, although the frequency was considerably lower than that observed in control heat shock *ftz*⁺ and *ftz4* lines (see Fig. 2I,J). These results indicate that *ftz5* is a hypomorphic *ftz* allele, whose phenotype is dependent on its gene dosage.

The 19 amino acid changes introduced into the *ftz* protein by the construction of the *ftz5* allele raised the possibility that the cause for the reduced activity could be protein instability, possibly owing to the change in the composition of the hydrophobic core of the *ftz* protein (see Fig. 1B; for review, see Pakula and Sauer 1989). However, the *ftz5* protein could be detected immunologically in the nuclei of developing *ftz5*; *ftz*^{9H34}/*ftz*^{w20} embryos, and the *ftz* stripe pattern was maintained approximately as long as the wild-type pattern (cf. Fig. 4,K with L). Furthermore, *ftz5* expression was detected in the embryonic central nervous system with the same intensity and for the same period of time as the wild-type protein (data not shown), indicating that *ftz5* is properly expressed and localized in *Drosophila* embryos. Although these experiments cannot rule out minor differences in protein stability between *ftz*⁺ and *ftz5*, they support the view that differential stability is not responsible for the defect of the *ftz5* allele.

Comments on the in vivo *ftz* rescue assay

Several points are worth noting regarding the general features of the *ftz* complementation assay. First, Hiromi et al. (1985) had shown that one copy of the transformation vector prf20 corresponding to the P-element P[ry⁺,*ftz*G] could rescue the lethality of the *ftz* mutation in eight of eight independent transformant lines tested. In this construct, the *rosy* (*ry*) gene, which serves as a transformation marker, and the *ftz* gene are transcribed in tandem. Transgenic flies harboring prf19, a transformation vector in which the *ry* gene and the *ftz* gene are transcribed in opposite orientations (Y. Hiromi, unpubl.), gave rescue only in three of six established lines (see Table 1). One may explain this difference compared with prf20 by the fact that the *ftz* control elements could be more exposed to nearby silencer elements. The different orientation of transcription of the *ry* and the *ftz* genes in the two constructs could also influence *ftz* activity. The transgenic lines obtained with the newly constructed transformation vector pCrfl (see Fig. 1A) showed a rescue frequency

Table 1. Survival indices of the rescuing lines in the *ftz* complementation test

P element	Line	Linkage ^a	SI (<i>ftz</i> ^{9H34}) ^b	SI (<i>ftz</i> ^{w20}) ^c
P[ry ⁺ , <i>ftz</i> ⁺] ^d	1.5.1	X	0.61	0.72
	1.8.1	II	0.32	0
	1.13.3	II	0.68	0.69
P[ry ⁺ , <i>ftz</i> ⁺] ^e	3.43.2	II	0.30	0.11
	j3.7	II	0.30	0.11
	j3.8	X	0.31	0
	j3.17	X	0.56	ND
	j3.18	CyO	0.87	0.48
P[ry ⁺ , <i>ftz</i> 4]	4.26.1	IV	0.78	0.81
	4.26.1j1	CyO	0.46	0.08
	j4.1.1	X	0.24	0
	j4.14.1	Sp	0.21	0
	j4.18.2	Sp	0.33	0.04
P[ry ⁺ , <i>ftz</i> 57]	19.39.5	X	+	0
	19.68.2	II	0.05	0
P[ry ⁺ , <i>ftz</i> 58]	20.16.3	II	+	0
	20.17.4	II*	0.27	+
P[ry ⁺ , <i>ftz</i> 59]	21.48.4	II	+	0
	21.76.3	II*	0.40	0.19
	21.84.6	II*	0.30	0.13
P[ry ⁺ , <i>ftz</i> 11]	11.44.1	X	0.25	0
	11.44.2	II	0.35	0
P[ry ⁺ , <i>ftz</i> 12]	j15.7	CyO	0.49	0.32
	j15.15	CyO	0.66	0.66
	j15.16	CyO	0.26	0
	15.15.3	II	0.15	0
	15.23.4	II	0.41	0

Survival index (SI) was calculated as described in Materials and methods. (+) Occasional survivors in independent experiments. For P[ry⁺, *ftz*4], only 5 of the 14 rescuing lines are shown. (ND) Not done.

^a(X) X chromosome; (II) second chromosome; (CyO) CyO balancer chromosome; (Sp) *sternopleural* (homozygous lethal second chromosome marker); (*) line carrying two inserts on a single chromosome, as shown by genomic Southern blotting.

^bSurvival indices in *ftz*^{9H34}/*ftz*^{9O93} background.

^cSurvival indices in *ftz*^{9H34}/*ftz*^{w20} background.

^d*ftz*⁺ on prf19 (Y. Hiromi, unpubl.). The orientation of the *ftz*⁺ gene in this P element is opposite to the one shown in Fig. 1 (see text).

^e*ftz*⁺ on pCrfl (see Fig. 1). This vector was used for the mutational analysis presented in this paper.

similar to prf19 (see Table 1), even though the plasmid structure differs from prf20 only in a slightly different polylinker region. We have no reasonable explanation for this observation.

Second, for the *ftz* rescue assay we have used two different transheterozygous mutant backgrounds (see Table 1). It is evident that the calculated survival indices (SIs) are generally higher in the *ftz*^{9H34}/*ftz*^{9O93} than in the *ftz*^{9H34}/*ftz*^{w20} background. The same tendency is also observed regarding the embryonic rescue phenotype in the two mutant backgrounds (data not shown). This phenomenon can be interpreted in light of the available im-

munolocalization experiments. *ftz*^{9H34} and *ftz*^{w20} are *ftz* null-alleles (see also Materials and methods). However, as shown in Figure 4M, *ftz*^{9O93} (Jürgens et al. 1984) homozygous embryos establish a seven-striped, yet abnormal, *ftz* staining pattern. The *ftz*-dependent even-numbered *en* (DiNardo et al. 1988; Lawrence and Johnston 1989) stripes are not formed (data not shown). In a *ftz*^{9H34}/*ftz*^{9O93} background, the *ftz*^{9O93} protein therefore could contribute to the activation of the *ftz* transgene, although it might not affect the potential of the transgene to activate transcription of the segment polarity gene *en*.

Finally, recent studies have revealed that the seven-stripe pattern emerging from the transcription of P[ry⁺, *ftz*G] differs temporally and spatially from that observed for the endogenous *ftz* gene (L. Pick, in prep.). This may indicate that the 10-kb fragment characterized by Hiromi et al. (1985) does not contain all necessary regulatory elements to direct the full *ftz* expression pattern. On the other hand, it is possible that the *ftz* gene can only be properly expressed when located in the context of the entire ANT complex.

Establishment of *ftz* and *en* expression patterns in *ftz*5 embryos

ftz (Wakimoto and Kaufman 1981; Laughon and Scott 1984) and *en* (Kornberg 1981; Fjose et al. 1985) are components of the regulatory hierarchy establishing the metameric pattern of the *Drosophila* embryo (for review, see Howard 1990). The product of the pair-rule gene *ftz* regulates the expression of the segment polarity gene *en* in the even-numbered parasegments either directly or indirectly. In the absence of *ftz* activity, no even-numbered *en* stripes are formed (DiNardo et al. 1988). The striped *ftz* expression is regulated by two *cis*-regulatory elements located 5' of the *ftz* gene: the zebra and the *ftz*-dependent, enhancer-like upstream element (see Fig. 1A; Hiromi et al. 1985; Hiromi and Gehring 1987; Pick et al. 1990; Schier and Gehring 1992).

In an attempt to further characterize the molecular defects of the *ftz*5 allele, we examined the expression patterns of *ftz* (Carroll and Scott 1985) and *en* (DiNardo et al. 1985) by antibody staining in a *ftz*^{9H34}/*ftz*^{w20} mutant background and compared it with wild-type and *ftz*ΔHD/+; *ftz*^{9H34}/*ftz*^{w20} individuals. The hypomorphic character of *ftz*5 is evident at cellular blastoderm (Fig. 4A–D). *ftz* expression levels are drastically reduced in *ftz*ΔHD, one-copy and two-copy *ftz*5 embryos as compared with wild type. The uneven and weak *ftz* stripe pattern is almost entirely preserved in one-copy *ftz*5 embryos during early germ-band extension (Fig. 4F). In contrast, at that stage, the intensity of staining in two-copy *ftz*5 embryos is almost wild-type-like (Fig. 4G), emphasizing the dosage-dependent character of the *ftz*5 gene. This expression is maintained nearly as long as in wild-type embryos and contrasts to the rapid fading in *ftz*ΔHD and single-copy *ftz*5 embryos (cf. *ftz* expression patterns in Fig. 4I–L).

Figure 2. Embryonic rescue phenotypes of *ftz5* and its derivatives. Ventral cuticular patterns of first instar larva are presented. (A) Wild-type denticle belt pattern. (T1–T3) Thoracic denticle belts; (A1–A8) abdominal denticle belts. (B) *ftz^{9H34}/ftz^{9O93}* larva. The size of the *ftz* larva is reduced owing to the deletion of alternate segments. (C–E) Typical examples of the IAE group (*ftz5*, *ftz51*, *ftz53*, *ftz60*) larvae carrying one copy of the transgene. Their transgenes harbor the tripeptide Ile Ala Glu (IAE) substitutions at position 28–30. Arrowheads indicate partially or completely restored denticle belts. The larvae shown in C–E are preparations from *ftz60* (line 22.31.1). (F) Example of a larva carrying two copies of the *ftz5* gene (line 5.85.5). Note the improvement of the denticle belt pattern. (G,H) Typical examples of the RRR group (*ftz56*, *ftz57*, *ftz58*, *ftz59*) larvae. Their transgenes gain the Arg stretch at positions 28–30. (G) An example from *ftz56* (line 18.37.1); (H) an example from *ftz57* (line 19.10.3). (I,J) Anti-*ftz* phenotypes obtained with *ftz* (line 7.4.1) and *ftz5* (line 9.23.1) heat shock lines. Note the reciprocal denticle belt pattern compared with B.

At the germ-band elongation stage, *en* is expressed in 14 evenly spaced stripes along the anterior–posterior axis (Fig. 4P; DiNardo et al. 1985). While even-numbered *en* stripes were completely absent in the *ftz* Δ H Δ D embryos (Fig. 4I), the *en* patterns established in one- and two-copy *ftz5* embryos correlate well with the dosage dependence found for the *ftz* expression pattern (cf. Fig. 4N with O). In the former case, only patches of *en*-expressing cells can be detected at some of the expected positions (triangles in Fig. 4N). In the latter, 13 *en* stripes were formed (Fig. 4O). The second *en* stripe was almost completely missing, most probably the result of the insufficient expression levels of the first *ftz* stripe in the preceding stages of development (open triangles in Fig. 4N,O). The expression of the other even-numbered *en* stripes is mostly restricted to the ventral side of the embryo. Occasionally, patches of *en*-expressing cells can be observed more laterally. This may be a direct cause of the initially low *ftz* protein levels and might imply that higher *ftz* protein levels are required to transcriptionally activate *en* expression laterally. On the other hand, the eighth *en*

stripe looks more normal (data not shown). This correlates well with the enhanced staining in the fourth *ftz* stripe at the cellular blastoderm stage (arrowhead in Fig. 4C) and with the preferential rescue of the denticle belt in the third abdominal segment later in the embryogenesis of *ftz5* individuals (see Fig. 3).

An intriguing aspect of the *en* expression pattern in *ftz5* embryos is the uneven spacing between neighboring *en* stripes established after the onset of germ-band extension (cf. the distance between arrows and triangles in Fig. 4N–P). The width of even-numbered parasegments was narrower in *ftz5* embryos than normal because the distance between *en* stripes 2 and 3, 4 and 5, and 6 and 7 became smaller than in wild type. The irregular spacing was less apparent in the posterior parts of the embryo. A similar, although complementary, pattern of *en* expression has been described for *even-skipped* (*eve*) hypomorphic (DiNardo and O'Farrell 1987; Frasch et al. 1988) and *ftz* hypermorphic alleles (Kellerman et al. 1990), which express *en* stripes 1 and 2, 3 and 4, 5 and 6, and so on, closer together. It thus appears that the correct position-

Figure 3 Statistical analysis of embryonic rescue phenotypes. The denticle belt patterns of first-instar larvae of *ftz5* and *ftz5* revertants were analyzed for the potential of the mutant proteins to rescue the *ftz* embryonic phenotype. Denticle belts in the abdominal segments A1, A3, A5, and A7 were scored individually in a *ftz^{9H34}/ftz^{2O93}* background as described in Materials and methods. The rescue data for each transgenic line are plotted horizontally for each denticle belt individually. Standard deviations are indicated by thin lines at the end of each bar. The vertical axis lists the *ftz* alleles and the denticle belts A1, A3, A5, and A7. All six *ftz*ΔHD lines analyzed did not show any rescue and are not included. (Solid bars) Ile-28 Ala-29 Glu-30 (IEA) group mutants (*ftz5*, *ftz51*, *ftz53*, and *ftz60*); (open bars) Arg-28 Arg-29 Arg-30 (RRR) group mutants (*ftz56*, *ftz57*, *ftz58*, and *ftz59*). The denticle belt scores of the two-copy *ftz5* larvae are also shown by open bars (data for line 5.85.6 are shown). Numbers in parentheses (top right) indicate the number of embryos that were scored showing a partial rescue phenotype and the number of lines analyzed per mutant. Only transgenic lines with SI values <0 were included in this analysis.

ing of *en* stripes and therefore of the parasegmental boundaries is dependent on a proper dosage balance between the pair-rule genes *eve* and *ftz*.

Amino acid changes at the amino terminus of the HTH improve *ftz* activity in transgenic flies

To specify the important residues in *ftz5* responsible for the reduced *ftz* activity in vivo, a series of revertants were constructed and tested in transgenic flies. Furthermore, the second position of the recognition helix was mutated in the *ftz* wild-type context (*ftz11*, *ftz12*), as it represents the only exposed residue on the surface of helix 3 that is different in *ftz* and *ftz5* (see Fig. 1B). Reversions of *ftz5* to wild type at exposed residues at the amino terminus of the recognition helix, or buried residues contributing to the hydrophobic core, did not improve the rescue activity (Fig. 1B, *ftz51*, *ftz53*). Even more extensive reversions did not change the situation (*ftz60*). The cuticular preparations obtained with these rescue negative lines showed a similar embryonic cuticular phenotype as *ftz5* (see Fig. 3). In contrast, transformant lines harboring constructs that gained a consecutive stretch of 3 arginines at the amino terminus of the HTH motif (*ftz56*, *ftz57*, *ftz58*, *ftz59*) showed moderate to drastic improvements in the embryonic cuticular patterns (see Figs. 2G,H and 3). Moreover, one (albeit with very low SI values) or two copies of these constructs were able to rescue *ftz* embryos to adulthood (Fig. 1B, Table 1). However, the arginine stretch was not sufficient for a drastic increase in the rescue efficiency (*ftz56*). Additional reversions either in the loop between helix 1 and helix 2 (*ftz57*) or at the second position of helix 3 (*ftz58*) were required. Further reversions toward the wild-type situation did not significantly improve the activity of the *ftz* protein in vivo (*ftz59*). Mutants with single amino acid changes at the second position of the recognition helix (*ftz11* and *ftz12*) were still able to rescue *ftz* embryos to adulthood.

Cell culture experiments confirm the requirements of the arginine stretch for *ftz* activity

ftz has been shown to be a transcriptional activator in *Drosophila* tissue culture cells (Jaynes and O'Farrell 1988; Han et al. 1989; Winslow et al. 1989). To further dissect the HTH motif of the *ftz* HD, we examined the transcriptional stimulatory activities of the mutant *ftz* proteins in Schneider cells (S1W220; see Materials and methods). *ftz* protein producer plasmids were cotransfected with a reporter plasmid that has 11 copies of the *Antp*-class binding sites BS2 (Müller et al. 1988) upstream of a *hsp70*-chloramphenicol acetyl transferase (CAT) fusion gene. Consistent with the observations in transgenic flies, *ftz5* was defective in *trans*-activation in Schneider cells while *ftz4* retained wild-type activity (see relative *trans*-activation activities in Fig. 1B). The complete activity retention by *ftz4* protein agreed well with the observation that full-length *Scr* protein itself stimulated the same reporter gene expression in Schneider cells (data not shown). We were, however, unable to detect the expression in Schneider cells of any of our *ftz* constructs, either by direct immunostaining or Western blotting, presumably because of the rapid turnover rates

of *ftz* RNA and protein in *Drosophila* cells (Edgar et al. 1987; Kellerman et al. 1990).

The tissue culture assay data for the *ftz5* back-mutations confirmed the requirement for the arginine stretch at the amino terminus of the HTH. The effect of the reversion of the arginine stretch was drastic as demonstrated by the mutant pairs *ftz5* and *ftz56*, *ftz51* and *ftz58*, *ftz55* and *ftz59*, and *ftz60* and *ftz61* (see Fig. 1B). A limited substitution of the 3 arginines in the wild-type context completely abolished *ftz trans*-activational activity (*ftz85*), indicating that the requirement for the arginine stretch is intrinsic for *ftz*⁺ protein function.

Arg-28 restores ftz trans-activation activity in combination with other key residues

A computer analysis of the *Antp* HD structure (Qian et al. 1989; Billeter et al. 1990) suggested that Arg-30 in the Arg stretch in helix 2 forms an ion-pair bridge with Glu-19 in helix 1. Interestingly, the *ftz5* mutation substitutes Glu for Arg-30, breaking this helix 1–helix 2 bridge, whereas an ion pair is present in the original *msh* protein. To examine the role of this helix–helix bridge in stabilizing the alignment of the two helices, we introduced a single Glu mutation at position 30 (*ftz73*) in the *ftz59* context, which is fully active both in flies and in Schneider cells. We also introduced single mutations into wild-type *ftz* context that would disrupt the helix–helix bridge by like–like combinations of electrical charges (*ftz81* and *ftz82*), as well as a double mutation that reverses the ion-pair combination as found in *msh* (*ftz83*). However, none of these mutations significantly affected *ftz trans*-activation activity, indicating that a functional configuration of the *ftz* HD is not dependent on the charge stabilized interaction between the side chains of amino acids 19 and 30.

On the other hand, changing Arg-28 to Ile in the *ftz59* context led to significant suppression of the *trans*-activation activity (*ftz71*), and a single reversion of Ile-28 to Arg in *ftz55* completely restored wild-type activity (*ftz77*). The importance of Arg-28 to *ftz* function was further confirmed by a single substitution of Arg-28 to Ile in the wild-type context (*ftz84*), which resulted in ~50% reduction of *trans*-activation activity.

However, the activity of *ftz56* was still weak despite the preservation of Arg-28. It was not improved by a single back-substitution of Arg-39 to Ser (*ftz79*) in the turn between helix 2 and 3, where some of the λ phage positive control mutations have been mapped (Hochs-

child et al. 1983). On the other hand, *ftz* activity was stimulated either by the reversion at position 43 (cf. activities of *ftz56* and *ftz58*) or at residues 21–24 (*ftz57*). The combined contribution of Arg-28 and Arg-43 was further supported by the complete abolishment of *ftz trans*-activation activity by simultaneous mutations for these two residues (*ftz89*) while single substitutions for Arg-43 suppressed activity only weakly (*ftz11*, *ftz12*).

The result that both Arg-28 and Arg-43 are required for optimal *ftz trans*-activation activity agrees well with the structural information on the *Antp* HD–DNA complex, which is likely to be preserved for the *ftz* HD–DNA complex. The topological positions of Arg-28 and Arg-43 in the *Antp* HD–DNA complex (Otting et al. 1990) suggest that these two residues are directly involved in HD–DNA backbone interactions. Close to Arg-28, DNA backbone contacts by the highly conserved Tyr-25 and Arg-31 have been reported for the *en* and *Antp* HD–DNA complexes (Kissinger et al. 1990; Otting et al. 1990; see Table 2). Consistent with this, substitutions for Tyr-25 and Arg-31 significantly suppressed *ftz* transcriptional activity (*ftz86* and *ftz87*). Moreover, a double mutation for Tyr-25 and Arg-28 completely abolished *trans*-activation activity (*ftz88*), revealing the importance of the multiple DNA–backbone contacts at the amino terminus of the *ftz* HTH.

In vitro studies reveal the requirement of the in vivo-defined key residues for DNA binding

To directly determine the residues required for efficient target site recognition and to quantify their contribution to it, in vitro DNA band-shift experiments were performed. Full-length protein was expressed in reticulocyte lysates (Fig. 5A) and its binding activity to the *Antp*-class target site BS2 (Müller et al. 1988) was examined (Fig. 5B). Despite the identity of the ninth residue in the recognition helices (see Fig. 1B), *ftz5* and *msh* proteins did not bind the target DNA (Fig. 5B, lanes e,g), whereas wild-type *ftz* (Fig. 5B, lane b), *ftz4* (lane d) and *Scr* (lane f) proteins did.

The DNA-binding defect of *ftz5* HD was further confirmed with a 73-amino-acid HD peptide synthesized in vitro. The binding activity of the *ftz5* HD was undetectable (Fig. 5C, lanes c,e) even after prolonged exposure of the film indicating that it is reduced at least 50- to 100-fold as compared with wild type (lanes b,d). Mutant HDs that gained the Arg stretch of helix 2 bound the DNA significantly better (Fig. 5C and DNA-binding activities

Figure 4. *ftz* and *en* protein expression patterns in *ftz* Δ HD (line 13.13.1), *ftz5* (line 5.85.6), and wild-type embryos. Embryos are shown anterior to the left, ventral side down. *ftz* protein was detected with a polyclonal anti-*ftz* serum (Krause et al. 1988) and an alkaline phosphatase-conjugated secondary antibody (dark blue); *en* was detected with a monoclonal anti-*en* antibody (Patel et al. 1989) and HRP-conjugated secondary antibody (brown). (A,E,I) *ftz* Δ HD/+; *ftz*^{9H34}/*ftz*^{w20}; (B,F,J,N) *ftz5*/+; *ftz*^{9H34}/*ftz*^{w20}; (C,G,K,O) *ftz5*/*ftz5*; *ftz*^{9H34}/*ftz*^{w20} (D,H,L,P) wild type; (M) homozygous *ftz*^{9O93}. Compare with the wild-type *ftz* staining in H. (A–D) Cellular blastoderm stage; (E–H,M) early germ-band extension stage; (I–L) mid-germ-band extension stage; (N–P) extended germ-band stage. Open triangle in C points to the weakly expressed first *ftz* stripe. Arrowheads in C indicate the slightly enhanced staining of the fourth and seventh *ftz* stripes. Arrows in N–P point to *en* stripes 1, 3, 5, and 7. The first *en* stripe is labeled with the number 1. Open triangles in N and O indicate the second *en* stripe barely detectable in *ftz5* embryos. Solid triangles in N and O point to *en* stripes 4 and 6. Note the uneven spacing of the *en* pattern in N and O compared with P.

Table 2. The DNA backbone contacting amino acids at the amino terminus of the HTH motifs of HDs

HD	Residue			
	25	28	31	43
<i>Antp</i> class	Tyr	Arg	Arg ^a	Arg
<i>Dfd</i> class	—	—	—	—
<i>AbdB</i> class	—	—/Lys ^b	—	—
<i>labial</i> class	—	—	—	Thr
<i>en</i> class	—	Glu	—	Ala/Ser
<i>eve</i> class	—	—	—	Ser/Thr
<i>paired</i> class	—	Ile/Val	—	Ala
<i>hox-1.5</i> class	—	—	—	—
<i>hox-2.4</i> class	—	—	—	—
<i>caudal</i>	—	Ile	Lys ^b	—
<i>cut</i>	—	Val	Ile	—
<i>bcd</i>	—	Ala	Leu	Ala
MAT α 2	—	Thr	Leu	Ile
Oct-1	Lys ^b	Ser	Ile	Glu

Amino acid sequence data and classification is from Scott et al. (1989). Residue identical to *Antp* HD is shown by a dash. Amino acid(s) are indicated where the residue is different from *Antp* HD.

^aWith the exception of *hox-11* and *r5*.

^bLysine may be able to make similar DNA backbone contact.

in Fig. 1B). This is shown clearly by the comparisons of *ftz5* (Fig. 5C, lane e) and *ftz56* (lane h), *ftz51* (lane f) and *ftz58* (lane j), *ftz55* (lane g) and *ftz59* (lane k), and *ftz60* (lane v) and *ftz61* (lane w). Binding activity was significantly suppressed by single substitutions for the key residues Arg-28 (*ftz84*, lane n), Arg-31 (*ftz86*, lane o), and Tyr-25 (*ftz87*, lane p).

Single back-mutations at position 43 improved DNA binding, although weakly. Compared with *ftz5* (lane e), *ftz51* (lane f) showed detectable binding activity. DNA-binding activity was also improved in *ftz58* (lane j) compared with *ftz56* (lane h), where only Arg-43 is substituted between the two mutants. Moreover, single Ala (*ftz11*, lane l) and Thr (*ftz12*, lane m) substitutions at position 43 in the wild-type context suppressed DNA-binding activity.

To determine the binding activities of some of the mutant HDs more precisely, the 73-amino-acid HD peptides were expressed in *Escherichia coli* and purified to near homogeneity. Many of the *ftz5* derivatives were insoluble in bacterial lysates and could not be purified by our standard protocol, but some of the key mutant proteins were purified, and their K_d values were determined (see K_d values listed in Fig. 1B). Those K_d values are in good

Figure 5. In vitro DNA-binding activities of mutant HD proteins. [A] In vitro synthesis of full-length HD proteins. Proteins synthesized in reticulocyte lysate were analyzed by SDS-10% PAGE. (a) Reticulocyte lysate incubated without RNA template; (b) *ftz* wild type (c) *ftz4*; (d) *ftz5*; (e) *Scr*; (f) *msh*. *ftz* proteins appear as a doublet that may be caused by proteolysis in the lysate. *Scr* protein migrates faster than standards, probably owing to its amino acid composition (Le-Motte et al. 1989). Positions of known molecular mass markers are indicated (in kD). [B] In vitro DNA-binding activities of full-length proteins. The in vitro-translated full-length proteins were incubated with an *Antp* site probe (F1-60) that has one BS2 site. Bound (B) and free (F) probes were separated through 8% acrylamide gel in 0.25× TBE. (a) Lysate incubated without RNA template; (b) *ftz* wild type; (c) *ftz* wild type with specific competitor (100-fold BS2-18); (d) *ftz4*; (e) *ftz5*; (f) *Scr*; (g) *msh*. [C] DNA-binding activity of in vitro-translated mutant *ftz* HDs. In vitro-synthesized 73-amino-acid *ftz* HD was incubated with the F1-60 probe. Probe bound by *ftz* HD (small arrowhead) was separated from free (F) probe through 8% acrylamide gels in 0.25×TBE. Protein amount was standardized based on [³⁵S]methionine incorporation. (a) Lysate incubated without template RNA; (b) *ftz* wild-type HD with 117-amino-acid *Antp* HD (the *Antp* HD RNA was cotranslated with *ftz* HD to serve as an internal control for in vitro translation and DNA binding); (c) *ftz5* HD with 117 amino-acid *Antp* HD; (d) *ftz* wild type; (e) *ftz5*; (f) *ftz51*; (g) *ftz55*; (h) *ftz56*; (i) *ftz57*; (j) *ftz58*; (k) *ftz59*; (l) *ftz11*; (m) *ftz12*; (n) *ftz84*; (o) *ftz86*; (p) *ftz87*; (q) 117-amino-acid *Antp* HD alone; (r) *ftz* wild type with specific competitor (100-fold BS2-18); (s) *ftz* wild type; (t) *ftz53*; (u) *ftz59*; (v) *ftz60*; (w) *ftz61*; (x) *ftz4*; (y) *ftz41*. Probe bound by the *Antp* HD is indicated by the large arrowhead. The amount of *ftz* HD used in b and c was one-quarter of that used in other lanes. Proteins used in a–r and s–y were synthesized with independent sets of RNA templates. *ftz* wild type and *ftz59* were included in both experiments for comparison.

agreement with the relative DNA-binding activities determined with the in vitro-synthesized HDs. In particular, single amino acid substitutions at positions 25, 28, and 31 at the amino terminus of the HTH increased the K_d values by 7- to 17-fold as compared with wild type.

Discussion

Mutational analysis of a protein of known function is most instructive when combined with information about its three-dimensional structure. Outstanding examples for the power of such approaches are the detailed studies on the prokaryotic gene regulatory proteins such as λ repressor (Sauer et al. 1990). Recent high-resolution nuclear magnetic resonance (NMR) and X-ray crystallographic studies of the *Antp* (Qian et al. 1989; Billeter et al. 1990; Otting et al. 1990), *en* (Kissinger et al. 1990), and *Mata2* (Wolberger et al. 1991) HDs provide a solid basis for the interpretation of the increasing number of HD mutations of the homeo protein super family. These structural studies have shown that HDs with highly divergent amino acid sequences can adopt almost congruent folds and that they contact their target DNA in similar ways. In vitro DNA-binding studies on the *ftz* and *Antp* HDs also support this view (Affolter et al. 1990; Percival-Smith et al. 1990). In this paper we have presented a systematic mutational analysis of the *ftz* HTH motif. We have shown that the two class-specific DNA backbone-contacting amino acids, Arg-28 and Arg-43, are important determinants for the *ftz* protein function both in vivo and in vitro. Our results support the concept that the DNA-binding affinity of the HD needs to be optimized for full function but can withstand a large amount of variation. We discuss the results in light of the available HD structures, assuming similar protein folds for all of the mutants.

Values of different assay systems for the analysis of gene function

Besides the implications of this study on HD function, our data allow an assessment of the values of different experimental systems for the analysis of gene function. We have tested many of the mutants in vivo for complete *ftz* rescue potential to adult viability and for embryonic patterning activity. We have also tested them in vitro for DNA-binding activity and in cultured *Drosophila* cells for *trans*-activation activity. In general, the data obtained by these different assay systems correlate well (Figs. 1B and 6); however, several points are worthy of discussion in more detail.

1. The in vitro DNA-binding and, in particular, the cell culture systems do not allow the monitoring of some of the in vivo activities, for example, the weak embryonic patterning activity of the *ftz5* protein. In the presence of all of the natural set of regulatory proteins, the modular structure of transcription factors (for review, see Frankel and Kim 1991) may still allow

Figure 6. Comparison of *ftz* HD mutant activities assayed by the three systems. The in vitro DNA-binding activity and the transcriptional stimulatory activity of each mutant HD are plotted (percent wild-type values listed in Fig. 1B). The *ftz* rescue activity of each mutant in transgenic flies is indicated, (●) Rescue competent; (○) rescue incompetent. (×) Rescue activity not tested. See rescue data listed in Fig. 1B and Table 1.

the *ftz5* protein to cooperate with other *trans*-acting factors and therefore to compensate to some extent for its greatly abolished activity in vitro.

2. The in vivo assay system in transgenic flies and, also to some extent, the cell culture assay system seem to be buffered against small activity changes between different mutants that can be monitored by the in vitro DNA-binding assay. For example, this assay reproducibly detected low DNA-binding activities of some of the *ftz5* revertants (*ftz51*, *ftz53*, *ftz55*) that could not be distinguished from *ftz5* in tissue culture cells and in transgenic flies.
3. A particular set of specific amino acids improved the *ftz* protein activity when reverted simultaneously. Comparisons of the results obtained for *ftz5*, *ftz51*, *ftz56*, and *ftz58* (stepwise reversions of the three arginines at positions 28–30 and Arg-43) show that this apparent cooperative action is manifested in vivo and in cell culture systems more clearly than in the in vitro DNA-binding assay system.
4. The requirement of positions 21–24 of the *ftz* HD is conditional depending on the amino acid context in which they are placed and on the assay system in which they are tested. The improvement in vivo and in tissue culture cells between *ftz56* and *ftz57* is intriguing because it is induced without significantly changing the in vitro DNA-binding activity. The four residues are solvent exposed (Billeter et al. 1990) and could therefore be involved in association with other transcription factors.
5. As little as sixfold reduction of the in vitro DNA-binding activity from the wild-type level may lead to a severe defect of *ftz* function in flies.

In summary, our results show that the data obtained with the three different assay systems are consistent with one another and support the validities of the in

vitro and in vivo assays for detailed mutational analyses. However, reflecting the qualitative and quantitative differences, the three assay systems are complementary to each other in monitoring the effects of various amino acid substitutions.

Stability and flexibility of the *ftz* HTH

Except for *ftz* Δ HD, all of our mutants retained at least some activity in vivo (see Table 1; Fig. 3). This fact is especially astonishing for *ftz5*, where almost all but the highly conserved residues (Tyr-25, Arg-31, Leu-38, and Leu-40) have been exchanged between positions 21 and 45 of the *ftz* HD. Several lines of evidence suggest that the *ftz5* protein is stably folded. Because most of the *ftz5* core-forming side chains are identical with those of the *msh* HD, the overall *ftz5* HD fold might be more similar to the *msh* HD leading to a slight change in the HD conformation (e.g., a slightly shifted alignment of the helices), which, in turn, could result in an attenuated protein–DNA interaction (Lim and Sauer 1991). However, all attempts to increase the activity of *ftz5* by reverting core amino acids (particularly positions 35 and 45, which are thought to be especially important to the proper alignment of helices 2 and 3) failed to improve the *ftz5* defectiveness in vivo and in tissue culture cells, although a small increase in DNA-binding activity was observed in vitro. Instead, our results have assigned the responsible amino acids for the reduced *ftz5* activity to a few residues that are located on the surface of the HD. In *ftz58*, for example, four reversions at positions 28–30 and 43 are sufficient to restore the activity close to wild-type level in vivo. Furthermore, the results of *ftz71–ftz73* clearly show that, among the three residues at the amino terminus of the HTH, only position 28 is of crucial importance. These results indicate that as a whole, the defect of *ftz5* may be traced back to mainly two (Arg-28 and Arg-43) of the 19 amino acid changes. The substitution of these two residues in wild-type protein (*ftz89*) is sufficient to abolish *ftz*-dependent *trans*-activation in Schneider cells.

Our results also imply that a number of residues in helix 2 and the following turn can be substituted without significant loss of activity. It is interesting to note that two (*ftz4*) or three (*ftz57*, *ftz58*, and *ftz59*) of the helix 2 residues (positions 32, 33, and 36) that have been shown to be important to the in vitro association of Oct-1 and VP16 (Stern et al. 1989) are changed in these mutants without abolishing *ftz*-like activity in vivo.

The importance of HD–DNA backbone contacts in target site recognition

In addition to specific base-pair interactions, DNA sugar-phosphate backbone contacts have been shown to play important roles in DNA recognition by prokaryotic HTH regulatory proteins (for review, see Harrison and Aggarwal 1990; Steitz 1990). First, they contribute to a significant part to the free energy required for the formation of a DNA–protein complex. Second, they provide crucial

reference points to correctly dock the recognition helix against the major groove of the DNA (Pabo et al. 1990). Third, it has been proposed for the *trp* repressor–operator complex that the protein–DNA backbone contacts might be adopted to read out the DNA backbone shape that is dependent on the DNA sequence (Luisi and Sigler 1990).

Structural studies on the *Antp*, *en*, and *Mat α 2* HD–DNA complexes have identified multiple DNA backbone contacts, most of which are made by the highly conserved Arg and Lys residues (Kissinger et al. 1990; Otting et al. 1990; Wolberger et al. 1991). We have shown that the highly conserved residues Tyr-25 and Arg-31 contacting the DNA backbone make important contributions to the formation of the *ftz* HD–DNA complex. In addition, we have shown that the class-specific residues Arg-28 and Arg-43 are crucial to the optimum DNA binding. This is consistent with the recent high-resolution NMR data on a ^{13}C -labeled *Antp* HD–DNA complex, which have located the side chains of Arg-28 and Arg-43 in close proximity of the DNA backbone (Y.-Q. Qian, G. Otting, M. Billeter, and K. Wüthrich, pers. comm.). The requirement of Arg-43 is also consistent with the methylation and ethylation interference data on the *ftz* HD–DNA complex (Percival-Smith et al. 1990). Moreover, a DNA backbone contact at position 42 is involved in the *Mat α 2* HD–DNA complex, demonstrating that the amino terminus of the HD recognition helix is in a position to contact DNA, despite its different positioning in the major groove as compared with the prokaryotic HTH motif.

Residues 28 and 43 are less conserved within the homeo protein family (see Table 2) and could therefore contribute to a higher diversity of DNA-binding specificity among homeo proteins. Slight structural differences in the overall protein folding might result in different requirements for DNA backbone contacts in different HD–DNA complexes. Alternatively, it is possible that the 2 arginines exert a more active contribution such as modification of the positioning of the recognition helix in the major groove or indirect readout of the target sequence. Although the positioning of the recognition helices is conserved between the *Mat α 2* and *en* HD–DNA complexes (Wolberger et al. 1991), positions 28 and 43 are substituted in the two HDs. A comparative analysis of the *Antp* and *en* HD–DNA complexes will shed more light on the importance of different sets of DNA backbone contacts established by different HDs.

Materials and methods

P-element transformation vectors

pCrfl (see Fig. 1A) was constructed by inserting the 10-kb *KpnI* fragment containing the *ftz*⁺ gene (*ftzG* fragment in Hiromi et al. 1985) into the *KpnI* site of pC20K. pC20K is a Carnegie-20 (Rubin and Spradling 1983) derivative in which the *PstI* site in the polylinker was replaced by a *KpnI* site. To mutagenize the *ftz* homeo box, a *SalI*-cassette (see Fig. 1A) was subcloned into different suitable vectors. The mutants *ftz4* and *ftz5* were obtained by replacing the *XmnI*–*BglII* fragment in the *ftz* homeo

box with the homologous fragment from *Scr* or *msh*, respectively. *ftz* Δ HD is an in-frame deletion between Glu-17 and Ile-47 of the *ftz* HD. Mutants *ftz*11, *ftz*12, *ftz*41, and *ftz*51–*ftz*61 (see Fig. 1B) were obtained by site-directed mutagenesis with Amersham's oligonucleotide-directed in vitro mutagenesis system 2. Correct clones were selected by sequencing and subsequently inserted into the transformation vector pCr3. pCr3 is pCr1 with the *Sall* cassette deleted, leaving *Sall* as a unique cloning site.

The three heat shock vectors pNH*ftz*, pNH*ftz*4, and pNH*ftz*5 were constructed by inserting the pGEMf1 (Krause et al. 1988)-derived *ftz*⁺, *ftz*4, and *ftz*5 cDNA fragments into the unique *Not*I site of pNHT4 (Gibson et al. 1990). These *ftz* heat shock vectors differ slightly from the one published in Krause et al. (1988) by a different leader sequence and by the replacement of the second amino acid (Ala) of the *ftz*-coding region by the tripeptide Arg Asp Pro.

Drosophila strains and P-element-mediated transformation

The *ftz* alleles used in this study were *ftz*^{9H34}, *ftz*^{9O93} [ethylmethanesulfonate (EMS) induced; Jürgens et al. 1984] and *ftz*^{w20} (Wakimoto et al. 1984). *ftz*^{w20} is associated with a 4.9-kb insertion in the *ftz* gene (Weiner et al. 1984); The molecular lesions of the other two alleles are not known. *ftz*^{9O93} homozygous embryos produce considerable amounts of the mutant protein (see Fig. 4M). *ftz* protein is barely detectable (Lawrence and Johnston 1989) in homozygous *ftz*^{w20} embryos. *ftz* protein was not detectable in *ftz*^{9H34} homozygous embryos (data not shown). Germ-line transformations were performed as described by Rubin and Spradling (1982). The recipient strain was *ry*⁵⁰⁶ in all cases. Some of the transformant lines (marked j in the line names in Table 1) were generated by the jump–start technique of Robertson et al. (1988). Jump–start insertions on the second chromosome were isolated on the *CyO* and *Sp* chromosomes originating from the line *CyO/Sp; ry*⁵⁰⁶/*Sb P[ry*⁺, Δ 2-3]/*TM6 Ubx*.

ftz rescue assay

The ability of the mutant *ftz* transgenes to rescue *ftz* lethality was assayed by a similar crossing scheme as described by Hiromi et al. (1985), except that different marker genes were used. Adult survivors heterozygous for two *ftz* alleles were identified by the *ebony* marker gene. The SI was calculated by setting the number of *ebony* flies in relation to the number of the phenotypically wild-type progeny of the same cross using the following equation: SI = [*ebony*]/[+ /2]. The SI values shown in Table 1 represent the average of at least two independent experiments.

Cuticular preparations of embryos were made either by the protocol of Van der Meer (1977) for nondevitellinized embryos or according to Wieschaus and Nüsslein-Volhard (1986) for devitellinized embryos.

The statistical analysis of the denticle belt patterns of mutant embryos presented in Figure 3 was based on nondevitellinized preparations. A semistatistical approach was taken to analyze the different *ftz* mutants in a *ftz*^{9H34}/*ftz*^{9O93} background. *ftz* embryos have only four abdominal denticle belts owing to segmental fusions and deletions (Wakimoto and Kaufman 1981). Most of the *ftz* mutants analyzed in this study retain at least some activity leading to an embryonic rescue phenotype, which manifests itself in partial or complete formation of denticle belts that are missing in *ftz* embryos. Reformation of denticle belts in the abdominal segments, A1, A3, A5, and A7, was scored individually in the following way: (1) no denticle hairs = 0; (2) partially formed denticle belt = 0.5; and (3) com-

pletely restored denticle belt = 1. The scores for each of the four segments were added up and divided by the number of embryos showing an embryonic rescue phenotype.

Immunostaining of embryos

Antibody staining was performed essentially as described in LeMotte et al. (1989). The *ftz* antiserum was a rabbit polyclonal serum used at a dilution of 1 : 200 (Krause et al. 1988). The mouse monoclonal *en* antiserum (Patel et al. 1989) was used at a dilution of 1 : 100.

Heat shock experiments

Experiments were done as described (Struhl 1985), with minor modifications. Dechorionated embryos were placed on a wet blotting paper in a plastic petri dish. The dish was sealed and immersed into a water bath at 37°C for 20 min. The embryos were then allowed to develop at 25°C for 24 hr on fresh grape juice plates before they were mounted in Hoyer's medium as described above.

Plasmids for cell culture experiments

The *ftz* producer plasmid pAc*ftz* was constructed by inserting a 1.8-kb genomic fragment into the *Bam*HI site of pPac (Krasnow et al. 1989). In addition to the entire coding sequence of *ftz*, this insert has a 20-bp leader, 150-bp intron, and 420-bp trailer of *ftz* genomic DNA. The mutant DNAs constructed for the fly experiments were used for pAc*ftz*4, *ftz*41, and *ftz*51–*ftz*61. The *Xho*I–*Bgl*II fragment of pAc*ftz* was substituted by the mutant fragments. Mutants *ftz*71–*ftz*77 were constructed by inserting the corresponding mutant oligonucleotide directly between the *Xho*I and *Eco*RI sites (amino acids 16–34) of *ftz*59. The *Eco*RI site was incidentally created during mutant construction without changing the encoded amino acid. *ftz*79 was constructed in the same way except that a mutant oligonucleotide cassette between the *Eco*RI and *Bgl*II sites (amino acids 33–48) was used. Mutants *ftz*81–*ftz*88 were constructed by inserting the corresponding mutant oligonucleotide cassette between the *Xho*I and *Eco*RV (amino acids 16–34) sites of pAc*ftz* wild-type. Mutant clones were identified by directly sequencing the ampicillin-resistant clones. The list of oligonucleotides used for mutagenesis is available upon request. The reporter gene pF11CAT was constructed by subcloning oligomerized BS2-26 (AGCT-GAGAAAAAGCCATTAGAGAAGC; Müller et al. 1988) oligonucleotides and the *hsp*70 basic promoter (spanning –50 to +90) into the unique *Pst*I site of pC4CAT (Thummel et al. 1988).

Transfection assay with Drosophila tissue culture cells

Schneider line 1 (S1W220) cells were used for transfection experiments. S1W220 cells showed higher transfection efficiency than Schneider line 2 cells. Cells were grown at 25°C in Schneider medium containing 15% fetal bovine serum (heat inactivated, GIBCO). DNA transfection was done with calcium phosphate as described (Rio and Rubin 1985). Each transfection contained 3 μ g of producer, 1 μ g of reporter, and 6 μ g of pCOP β gal DNAs per plate. pCOP β gal expresses the β -galactosidase gene under *copia* long terminal repeat (LTR) and served as an internal control. The expression of pCOP β gal was not affected by the cotransfection of the *ftz* producer construct. After 42–48 hr, cells were harvested and extracts were prepared by freezing and thawing. All transfections were done in duplicate plates. CAT activities were determined by the fluor-diffusion method with

³H-labeled acetyl CoA as described by the distributor [New England Nuclear]. β -Galactosidase activities were determined with chlorophenol red- β -D-galactopyranoside (Boehringer Mannheim). CAT activity was normalized against the control β -gal activity. Transfection assays were repeated at least three times, and the values were averaged.

Determination of DNA binding activities with in vitro-synthesized proteins

DNA-binding activities of in vitro-synthesized proteins were determined as described by Hope and Struhl (1985). A 60-bp *Bam*HI fragment containing BS2-binding site (F1-60: GATC-CCCGCG GCCGCTT CTC TAATGGCTTT TTCTCAGCTG CCCGCGGGTA CCGGGGGATC; nucleotides corresponding to BS2-18 are underlined) was used as a probe. The synthesized protein was analyzed by separation through SDS-polyacrylamide gels, and the amounts of protein were estimated by TCA precipitation assay for [³⁵S]methionine according to the method of the supplier of the lysates (Promega). DNA-binding reactions were done at room temperature for 20 min in 20 mM HEPES (pH 7.9), 4% Ficoll 400, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.25 μ g/ml of BSA, and 50 μ g/ml of [poly(I-C)]. The protein-DNA complexes were resolved through 8% polyacrylamide gels in 0.25 $\times$ TBE buffer at room temperature. The bound and unbound fractions of the probe DNA were determined by laser computing densitometry with films of different exposure times.

K_d measurements with HD peptides synthesized in E. coli

ftz HD peptides (73 amino acids) were purified from *E. coli* as described for the *Antp* HD [Müller et al. 1988] except that the protein was induced at 18°C after growing up the cultures at 37°C to OD₆₀₀ = 1.0. The peptide expressed from the T7 expression vector [Studier and Moffatt 1986], pfop1, is identical to the one used in Percival-Smith et al. (1990). Mutant versions of pfop1 used in this study were pfop11, pfop12, pfop4, pfop41, pfop59, pfop84, pfop86, and pfop87. The numbers of these plasmids correspond to the mutant numbers in Figure 1B. Binding reactions for the saturation experiments were carried out as described by Affolter et al. (1990), with BS2-18 (GAGAAAA-AGCCATTAGAG) as a probe.

Acknowledgments

We thank Yan Qiu Qian, Gottfried Otting, Martin Billeter, and Kurt Wüthrich for sharing the structure information on the *Antp* HD-DNA complex before publication; Paul Baumgartner for Southern blot analyses of genomic DNAs; Hugo Bellen, Anthony Percival-Smith, and Alex Schier for discussions; and Ursula Weber for performing some of the initial P-element-mediated transformations. We thank Yash Hiromi for plasmids and advice to M.M. at early stages of this work, Beat Suter for help during the preparation of Figure 1, Henry Krause for *ftz* antibody, and Nipam Patel and Corey Goodman for *en* antibody. pPac and pC4CAT were kindly provided by Wolfgang Driever. We thank Alex Schier for critical reading of the manuscript, Maria Rizzieri for preparation of Schneider medium, and Erika Wenger-Marquardt for her help in typing. L.P. was supported by a Damon Runyon-Walter Cancer Fund Fellowship (DRG-912). K.F.-T. was supported partly by Mochida Foundation Japan for his travel expenses to Basel. This work was supported by grants from the Swiss National Science Foundation and the Kantons Basel-Stadt and Basel-Landschaft.

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Genes Dev. 1992, **6**:

Access the most recent version at doi:[10.1101/gad.6.6.1082](https://doi.org/10.1101/gad.6.6.1082)

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