**Ftz-F1 is a cofactor in Ftz activation of the *Drosophila* *engrailed* gene**

Brian Florence²,*, Antoine Guichet³, Anne Ephrussi³ and Allen Laughon¹,†

¹Departments of Genetics and Medical Genetics and ²Program in Cellular and Molecular Biology, University of Wisconsin, 445 Henry Mall, Madison, Wisconsin 53706, USA  
²Developmental Biology Programme, European Laboratory of Molecular Biology, Meyerhofstrasse 1, D-69117, Heidelberg, Germany  
*Current address: Department of Biology, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0357, USA  
†Author for correspondence (e-mail: alaughon@facstaff.wisc.edu)

**SUMMARY**

The *fushi tarazu* pair-rule gene is required for the formation of alternating parasegmental boundaries in the *Drosophila* embryo. *fushi tarazu* encodes a homeodomain protein necessary for transcription of the *engrailed* gene in even-numbered parasegments. Here we report that, within an *engrailed* enhancer, adjacent and conserved binding sites for the Fushi tarazu protein and a cofactor are each necessary, and together sufficient, for transcriptional activation. Footprinting shows that the cofactor site can be bound specifically by Ftz-F1, a member of the nuclear receptor superfamily. Ftz-F1 and the Fushi tarazu homeodomain bind the sites with 4- to 8-fold cooperativity, suggesting that direct contact between the two proteins may contribute to target recognition. Even parasegmental reporter expression is dependent on Fushi tarazu and maternal Ftz-F1, suggesting that these two proteins are indeed the factors that act upon the two sites in embryos. The two adjacent binding sites are also required for continued activity of the *engrailed* enhancer after Fushi tarazu protein is no longer detectable, including the period when *engrailed*, and the enhancer, become dependent upon *wingless*. We also report the existence of a separate negative regulatory element that apparently responds to *odd-skipped*.

Key words: segmentation, *engrailed*/*enhancer, fushi tarazu, Ftz-F1, Ftz, *Drosophila*

**INTRODUCTION**

The *Drosophila* segment polarity gene *engrailed* (*en*) plays a critical role in the generation and maintenance of parasegments, the fundamental units of the insect body plan. Expression of *en* and *wingless* (*wg*) in adjacent stripes establishes and maintains the parasegmental boundary (Martinez Arias et al., 1988; DiNardo et al., 1988; Heemskerk et al., 1991; Vincent and O’Farrell, 1992) and organizes patterning across each segment (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Heemskerk and DiNardo, 1994). Initiation, refinement and maintenance of the *en* expression pattern is controlled separately. Initiation is in response to patterns of pair-rule gene expression (Howard and Ingham, 1986; DiNardo and O’Farrell, 1987; Lawrence et al., 1987; Ingham et al., 1988; Manoukian and Krause, 1993; Fujioka et al., 1995). Later still, maintenance of the pattern becomes dependent upon repression by *Polycomb* group genes (Moazed and O’Farrell, 1992).

Models for pair-rule regulation of *en* involve overlapping patterns of putative activators and repressors (DiNardo and O’Farrell, 1987; Ingham et al., 1988; Manoukian and Krause, 1993; DiNardo et al., 1994; Mullen and DiNardo, 1995; Fujioka et al., 1995). In odd parasegments, graded expression of *even-skipped* (*eve*) establishes the *en* stripes by setting the boundaries of the activator *paired* (*prd*) and the repressors *runt* and *sluggy paired* (Fujioka et al., 1996). Expression of *en* in even parasegments results from activation by *fushi tarazu* (*ftz*). Only the anterior cells of each *ftz* stripe express *en* and this restriction is dependent upon *odd-skipped* (*odd*) and *naked* (*nkd*) (DiNardo and O’Farrell, 1987; Benedyk et al., 1994; Mullen and DiNardo, 1995). Repression of *en* by *odd* may be partially indirect since *odd* reduces the level of *ftz* expression in the posterior two thirds of each *ftz* stripe (Mullen and DiNardo, 1995).

Surprisingly, the Ftz homeodomain is not required for activation of *en*, for autoactivation of *ftz*, or for repression of *wg* (Fitzpatrick et al., 1992; Copeland et al., 1996; Hyduk and Percival-Smith, 1996). The Prd protein has been implicated in the homeodomain-independent repression of *wg* by Ftz: with or without its homeodomain, Ftz is unable to repress residual *wg* expression in *prd* mutant embryos and the N-terminal half of the Ftz protein (i.e., lacking the homeodomain) binds Prd in vitro (Copeland et al., 1996). However, Prd is not required for *ftz*-dependent activation of *en* in even-parasegmental stripes since these stripes are still present in *prd* mutants (DiNardo and O’Farrell, 1987).

Although autoactivation of *ftz* can occur independently of
...the homodomain (Fitzpatrick et al., 1992), analysis of the autoregulatory ftz enhancer element has shown that Ftz can also activate by binding directly to the autoregulatory enhancer by means of its homodomain (Schier and Gehring, 1992). This suggests that homeodomain-dependent and -independent mechanisms of transcriptional activation are at least partially redundant. Consistent with this model, a Ftz-dependent enhancer contained within the first en intron contains multiple homeodomain binding sites (Kassis et al., 1989; Kassis, 1990). However, homeodomain-DNA contact is clearly insufficient for normal target recognition since tandem arrays of consensus binding sites fail to mediate a Ftz-dependent pattern of reporter expression in embryos (Vincent et al., 1990; Nelson and Laughon, 1993; Zeng et al., 1994). It is therefore likely that Ftz requires a cofactor for target recognition, analogous to the way in which Extradenticle (Exd)/Pbx proteins act as cofactors for Hox proteins (Pieffer and Weischaus, 1990; Rassolob and Weischaus, 1994; van Dijk and Murre, 1994; Chan et al., 1994; Neuteboom et al., 1995; Chang et al., 1995, 1996).

Ftz utilizes its homodomain to bind en regulatory sequences, homeodomain binding sites should be required for activation. Sequences required in addition to homeodomain binding sites would be candidates for cofactor-binding sites and could be tested for their ability to facilitate target recognition by Ftz. If Ftz functions primarily through protein-protein interactions, the DNA sequences required for Ftz activation would be expected to contain binding sites for the transcription factor(s) with which Ftz interacts, and need not contain Ftz-binding sites.

The approximately 1 kb Ftz-responsive en enhancer provided a reasonable starting point for identifying sequences that are necessary for target recognition and activation by Ftz. We found that binding sites for Ftz and for Ftz-F1, contained within a 37 bp block of conserved sequence, were essential for activity of the en enhancer. When multimerized, this element directed Ftz-dependent reporter transcription in embryos. The native enhancer and the multimerized conserved block are dependent upon Ftz and maternal Ftz-F1 for the ability to activate transcription of pair-rule stripes. Our results support the homeodomain-DNA contact model and identify Ftz-F1 as a putative Ftz cofactor. The results also provide evidence that negative regulation of en in even parasegments is at least partially dependent upon direct interaction of a repressor with sequences located within the intron.

MATERIALS AND METHODS

Deletion analysis

Fragments of the en intron were generated by cleavage at unique restriction sites or by exonuclease III digestion (Erase-a-base, Promega Corp.). These were ligated directionally into the EcoRI and BamHI sites of CaSpeR such that the intron sequences were located 140 bp from the mini-white start of transcription. The mini-white gene was used as a reporter to detect en enhancer activity, as previously described (Kassis, 1990). This avoids the problem of targeted transposon insertion associated with en promoter (Kassis et al., 1992). In each case, the patterns shown or described are representative of three or more independent transformant lines. White transcripts were detected in embryos by in situ hybridization using a digoxigenin-labeled antisense white riboprobe (Tautz and Pfeifle, 1989). Hybridizations and washes were done at 57°C. The 4 bp substitutions introduced into hd1 and nhr1 (shown in Fig. 1) were generated by PCR using primers containing the basepair substitutions and confirmed by DNA sequencing. The base pair substitutions created HindIII and EcoRV sites within hd1 and nhr1, respectively, which were used to reconstitute the intact B-I fragment. 

RESULTS

Elements required for Ftz activation of the en intron enhancer

Enhancers that mediate Ftz-dependent even-parasegmental expression of en have been mapped upstream of the promoter (DiNardo et al., 1988) and in the first intron (Kassis, 1990). The intron sequence is about 30% conserved between D. melanogaster and D. viridis (Kassis et al., 1986), most of which falls into 16 discrete blocks (Fig. 1). By footprinting, we iden-
Cofactor-facilitated Ftz activation of *engrailed*

---

**Fig. 1.** Ftz- and Ftz-F1-binding sites within the *en* intron. A map at the top shows open boxes lettered A-P indicating the location of conserved sequence blocks from the sequence of Kassis et al. (1986), and arrows indicating the location of Ftz homeodomain binding sites identified by DNasel footprinting (see Fig. 5 for methods). Solid arrows indicate binding sites that most closely match the Antp class consensus (Laughon, 1991; Ekker et al., 1994), open arrows indicate sites that match the Cad-binding site consensus (Dearolf et al., 1989). Ovals mark sequences similar to the Ftz-F1-binding site consensus (Ueda and Hirose, 1991). Sequences of these sites are listed below the map grouped as hd, cad or nhr (nuclear hormone receptor) sites in the order that they appear from left to right on the map. Binding site consensus sequences for the Ftz homeodomain, for Cad and for Ftz-F1 are also shown and mismatches with these are shaded.

To determine whether the combination of Ftz- and Ftz-F1-binding sites is sufficient for Ftz-dependent activation, a construct containing three copies of block C (3×C) was built and tested. During stages 5-7, the 3×C construct showed a pattern of seven Ftz-like, 3- to 4-cell-wide transverse stripes (Fig. 3, bottom panel). Double-labeling to simultaneously detect endogenous *en* transcripts showed indirectly that reporter expression coincided with that of *ftz*. During late stage 7 and stage 8, the first stripe (parasegment 2) faded. The subsequent 6-stripe pattern was similar to the pattern reported for the Ftz-dependent pbx enhancer (Muller and Bienz, 1992). Crossing of 3×C into a *ftz* mutant background showed that expression was fully dependent upon Ftz. Crosses of 3×C males to females homozygous for a maternal effect *Ftz*-F1 mutation that eliminates expression of the α-Ftz-F1 isoform (Giuchet et al., 1997) showed that reporter expression was also fully dependent upon maternally derived α-Ftz-F1 (Fig. 4). These results suggest that Ftz and the α-Ftz-F1 act through their respective binding sites in block C to activate *en* transcription in even parasegments.

Removal of sequences from the 3′ end of the intron up to block G (construct B-F) altered the pattern by widening the stripes to 2-3 cells rather than the normal one-cell width (Fig. 3) and reduced the level of reporter expression, perhaps due to the loss of three Ftz-binding sites in block I. Removal of block F by a 17 bp truncation of B-F (construct B-E) completely eliminated reporter expression prior to stage 11 (Fig. 3). Block F lacks detectable Ftz-binding sites but contains a second Ftz-F1-binding site, nhr2 (Fig. 2). Thus there are two Ftz-F1-binding sites essential for activation by Ftz, one adjacent to the essential Ftz-binding site, hd1, and one located approximately 200 bp distant.

A small element is sufficient for activation by Ftz and Ftz-F1

---

### Table: Ftz- and Ftz-F1-binding sites within the enhancer

<table>
<thead>
<tr>
<th>Block</th>
<th>Consensus/Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CGAAGGTCACC</td>
</tr>
<tr>
<td>B</td>
<td>CGAAGGACACT</td>
</tr>
<tr>
<td>C</td>
<td>GCCAATTAGG</td>
</tr>
<tr>
<td>D</td>
<td>GCCAATTAAC</td>
</tr>
<tr>
<td>E</td>
<td>GCTAATTAGA</td>
</tr>
<tr>
<td>F</td>
<td>GCCAATTAGC</td>
</tr>
<tr>
<td>G</td>
<td>GCCAATTAGC</td>
</tr>
<tr>
<td>H</td>
<td>GTCAATTAAA</td>
</tr>
<tr>
<td>I</td>
<td>AGCAQTAAAC</td>
</tr>
<tr>
<td>J</td>
<td>AGCAATTAAT</td>
</tr>
<tr>
<td>K</td>
<td>GTCAATTAAC</td>
</tr>
<tr>
<td>L</td>
<td>GCCAATTAAC</td>
</tr>
<tr>
<td>M</td>
<td>GTCAATTAAC</td>
</tr>
<tr>
<td>N</td>
<td>GCCAATTAGC</td>
</tr>
<tr>
<td>O</td>
<td>GCCAATTAGC</td>
</tr>
<tr>
<td>P</td>
<td>GCCAATTAGC</td>
</tr>
</tbody>
</table>

---

To identify which, if any, of these Ftz-binding sites were required for Ftz-dependent activation, portions of the intron were tested for the ability to drive expression of a reporter gene mRNA in embryos, as detected by in situ hybridization (Figs 2, 3). Enhancer activity was not detectably altered by a deletion (construct A-1) that removed four Ftz-binding sites from the 3′ end of the intron. Deletions removing blocks A, B and the 5′-14 bp of block C had no effect on the spatial pattern of reporter expression. For these constructs, and those described below, double labeling to detect endogenous *en* transcripts was used to determine the registration of reporter stripes. Unless noted otherwise, reporter patterns were coincident with endogenous *en*.

Removal of block A (construct B-1) weakened and delayed the appearance of the early stripe pattern by 15-20 minutes (i.e., stripes appeared at the end of stage 7). Additional truncation of the *en* intron, removing block B (construct C-1) or the 5′-14 bp of block C, had no additional effect on the level or timing of early reporter expression. Removal of the remainder of block C (construct D-I) resulted in the loss of all Ftz-dependent activity. The deleted region contains one conserved binding site for Ftz (hd1) and a conserved sequence (nhr1) resembling the binding site consensus for Ftz-F1, a member of the nuclear receptor superfamily (Ueda et al., 1990; Lavorgna et al., 1991, 1993; Ueda and Hirose, 1991; Horner et al., 1995). Indeed, DNaasel footprinting determined that Ftz-F1 bound this site (as well second site nhr2, see below). Clustered mutations were introduced into each site in construct B-I (hd1* and nhr1*) and tested for effects on reporter expression. In each case, reporter expression was reduced to background level. A cross of B-I into a *ftz* mutant background showed that expression was fully dependent upon Ftz (data not shown). Crosses of A-I males to females homozygous for a maternal effect *Ftz*-F1 mutation that eliminates expression of the α-Ftz-F1 isoform (Giuchet et al., 1997) showed that reporter expression was also fully dependent upon maternally derived α-Ftz-F1 (Fig. 4). These results suggest that Ftz and the α-Ftz-F1 act through their respective binding sites in block C to activate *en* transcription in even parasegments.

---

To identify which, if any, of these Ftz-binding sites were required for Ftz-dependent activation, portions of the intron were tested for the ability to drive expression of a reporter gene mRNA in embryos, as detected by in situ hybridization (Figs 2, 3). Enhancer activity was not detectably altered by a deletion (construct A-1) that removed four Ftz-binding sites from the 3′ end of the intron. Deletions removing blocks A, B and the 5′-14 bp of block C had no effect on the spatial pattern of reporter expression. For these constructs, and those described below, double labeling to detect endogenous *en* transcripts was used to determine the registration of reporter stripes. Unless noted otherwise, reporter patterns were coincident with endogenous *en*.

Removal of block A (construct B-1) weakened and delayed the appearance of the early stripe pattern by 15-20 minutes (i.e., stripes appeared at the end of stage 7). Additional truncation of the *en* intron, removing block B (construct C-1) or the 5′-14 bp of block C, had no additional effect on the level or timing of early reporter expression. Removal of the remainder of block C (construct D-I) resulted in the loss of all Ftz-dependent activity. The deleted region contains one conserved binding site for Ftz (hd1) and a conserved sequence (nhr1) resembling the binding site consensus for Ftz-F1, a member of the nuclear receptor superfamily (Ueda et al., 1990; Lavorgna et al., 1991, 1993; Ueda and Hirose, 1991; Horner et al., 1995). Indeed, DNaasel footprinting determined that Ftz-F1 bound this site (as well second site nhr2, see below). Clustered mutations were introduced into each site in construct B-I (hd1* and nhr1*) and tested for effects on reporter expression. In each case, reporter expression was reduced to background level. A cross of B-I into a *ftz* mutant background showed that expression was fully dependent upon Ftz (data not shown). Crosses of A-I males to females homozygous for a maternal effect *Ftz*-F1 mutation that eliminates expression of the α-Ftz-F1 isoform (Giuchet et al., 1997) showed that reporter expression was also fully dependent upon maternally derived α-Ftz-F1 (Fig. 4). These results suggest that Ftz and the α-Ftz-F1 act through their respective binding sites in block C to activate *en* transcription in even parasegments.

Removal of sequences from the 3′ end of the intron up to block G (construct B-F) altered the pattern by widening the stripes to 2-3 cells rather than the normal one-cell width (Fig. 3) and reduced the level of reporter expression, perhaps due to the loss of three Ftz-binding sites in block I. Removal of block F by a 17 bp truncation of B-F (construct B-E) completely eliminated reporter expression prior to stage 11 (Fig. 3). Block F lacks detectable Ftz-binding sites but contains a second Ftz-F1-binding site, nhr2 (Fig. 2). Thus there are two Ftz-F1-binding sites essential for activation by Ftz, one adjacent to the essential Ftz-binding site, hd1, and one located approximately 200 bp distant.

A small element is sufficient for activation by Ftz and Ftz-F1

---

To determine whether the combination of Ftz- and Ftz-F1-binding sites is sufficient for Ftz-dependent activation, a construct containing three copies of block C (3×C) was built and tested. During stages 5-7, the 3×C construct showed a pattern of seven Ftz-like, 3- to 4-cell-wide transverse stripes (Fig. 3, bottom panel). Double-labeling to simultaneously detect endogenous *en* transcripts showed indirectly that reporter expression coincided with that of *ftz*. During late stage 7 and stage 8, the first stripe (parasegment 2) faded. The subsequent 6-stripe pattern was similar to the pattern reported for the Ftz-dependent pbx enhancer (Muller and Bienz, 1992). Crossing of 3×C into a *ftz* mutant background showed that expression was fully dependent upon Ftz. Crosses of 3×C males to females homozygous for a maternal effect *Ftz*-F1 mutation showed that expression was also fully dependent upon maternally derived Ftz-F1 (Fig. 4). Thus the Ftz and Ftz-F1 sites contained in block C are necessary and, when multimerized, sufficient for Ftz activation. Since multimerization of homeodomain binding sites alone failed to direct stripe formation in embryos (Vincent et al., 1990; Nelson and Laugthon, 1993; Zeng et al., 1994), it is clear that the Ftz-F1-binding sites are crucial for Ftz activity.
Cooperative binding of the Ftz homeodomain and Ftz-F1

We tested whether binding of Ftz-F1 to nhr1 could increase the affinity of the Ftz homeodomain for hd1 in vitro. Addition of Ftz-F1 protein reduced the concentration of Ftz homeodomain necessary to footprint hd1 by 4- to 8-fold (compare hd1 footprints in lanes 4 and 10 of Fig. 5), indicating that bound Ftz-F1 stabilizes binding of the Ftz homeodomain to the adjacent site. Similarly, the binding of Ftz-F1 to nhr1 was enhanced 4- to 8-fold by the addition of Ftz homeodomain. The proteins fail to bind specifically to the corresponding mutated sites (these are the same mutations, shown in Fig. 2, that eliminated reporter expression in embryos). Approximately the same level of cooperative binding was obtained in a footprinting experiment using Ftz-F1 and full-length Ftz (data not shown). These results suggest that Ftz-F1 may enhance the regulatory specificity of Ftz by directly contacting the Ftz homeodomain. A closely related Drosophila monomer nuclear receptor, DHR39 (also referred to as Ftz-F1B), Ohno and Petkovich, 1992; Ayer et al., 1993; Ohno et al., 1994; Horner et al., 1995), also binds to nhr1, but without effect on Ftz binding to hd1 (data not shown). In addition, block E, which contains a second Ftz-F1 site, is also required for enhancer activity. This second Ftz-F1 site (nhr2) is 50 bp from the nearest Ftz-binding site (cad2) and binding of Ftz-F1 to this site does not alter the affinity of the Ftz homeodomain for hd1 (or cad2).

Elements affecting expression during the wg-dependent phase

Reporter expression continues to be affected by blocks A, B, C, F and G as Ftz expression declines and disappears during stages 7-10 (Fig. 6). Both the hd1- and nhr1-binding sites affect this later expression. A-I is the smallest construct that produces a normal en pattern during these stages. A-I embryos continue to express even parasegmented stripes at high levels into stage 11. Odd parasegmented stripes appear later than the even stripes and the two become approximately equal in intensity by late stage 10 (~5 hours AEL). In wg mutant embryos, A-I expression begins to disappear near the end of stage 8, paralleling the requirement of wg for continued expression of en as Ftz and other pair-rule proteins disappear from the embryo (Heemskerk et al., 1990). Thus, as shown previously for the entire intron (Kassis, 1990), A-I contains elements that depend on wg signaling for transcriptional activation. Although blocks J-P appear dispensable for both regulation by Ftz and by Wg, these conserved sequences could perform redundant functions or contribute to regulation during later stages of development.

The expression of construct B-I was almost identical to that of A-I, but weaker in all reporter lines examined, suggesting that, as for early, Ftz-dependent expression, block A strengthens, but is not absolutely required for, later enhancer activity. By the beginning of stage 9, both construct C-I and 3'C-I showed little odd parasegmental staining in the ectoderm except at the very dorsal edge of the embryo, and even parasegmental expression had begun to fade (3'C-I in Fig. 6). Reporter RNA visible at this time was restricted mainly to the mesoderm. By the end of stage 9, a low level of ectodermal expression in the odd parasegments was evident in some embryos. Although even parasegmental expression had faded considerably, it was still stronger than the weak odd parasegmental expression. Taken together, these results suggest that the region containing block B responds to a factor that is required for, or strongly enhances, ectodermal expression of en during the Wg-dependent phase. Constructs D-I and B-I[ cad2+] showed no ectodermal expression during this interval. These results show that the hd1 and nhr1 sequences are required for continued enhancer activity in the ectoderm during the Wg-dependent phase. Ftz-dependent activation of en extends only until stage 9 indicating another factor may act on hd1 at later stages, or Ftz-
dependent early expression may be required in some other way for late expression, possibly to prevent the onset of repression.

Construct B-F displayed a distinctive pattern of stage 9-10 expression. During stage 9, as Ftz-dependent activation ended, expression in the ventral half of the embryo disappeared (Fig. 6). Odd-parasegment stripes appeared at this time but similarly only in the dorsal half of the embryo. In addition, reporter expression began to appear between the odd and even stripes. B-F expression was strongest in PS8-15, weaker in PS6-7, weakest in PS4-5 and absent in PS1-3. The loss of expression ventrally is very similar to loss of en expression in extradenticle (exd) mutant embryos (Peifer and Wieschaus, 1990), suggesting the Exd homeodomain protein may interact with sequences contained within the G-I interval. Further truncation, removing nhr2 (construct B-E), resulted in loss of detectable enhancer activity, consistent with involvement of Ftz-F1 in continued expression during the Wg-dependent phase. Interestingly, the 3xC construct shows continued expression until late stage 10, well after the time when Ftz disappears (Carroll and Scott, 1985; Krause et al., 1988). During stage 9, 3xC stripes become restricted to the dorsal half of the embryo.

**DISCUSSION**

Cofactors have been shown to play an important role in target recognition by several homeodomain proteins. Based on deletion analysis and footprinting of the ftz-UAS enhancer, it has been suggested that Ftz-F1 might function as a cofactor for Ftz (Schier and Gehring, 1993; Han et al., 1993). Matches to the Ftz-F1 consensus also have been noted in other Ftz-dependent enhancers (Schier and Gehring, 1993). Recently, a P element insertion that disrupts α-Ftz-F1 function has been identified and shown to confer a recessive maternal effect pair-rule phenotype that resembles the ftz phenotype (Guichet et al., 1997). The resulting embryos fail to express en in even parasegments, while the blastoderm pattern of ftz mRNA and protein is normal.

Our results show that a Ftz-F1-binding site is necessary and sufficient in combination with a Ftz-binding site for Ftz-dependent enhancer activity. We also found that Ftz and maternally derived α-Ftz-F1 are necessary for transcriptional activation by the enhancer and by a multimerized segment containing the Ftz- and Ftz-F1-binding sites. Cooperative binding of the Ftz homeodomain and Ftz-F1 to conserved binding sites in vitro indicate that Ftz-F1 may increase the target specificity of Ftz by means of a direct interaction between the two proteins. Taken together, these results suggest that α-Ftz-F1 functions as a Ftz cofactor. Interestingly, early embryonic expression of en is normal in embryos homozygous for Df (3L)Cat, which removes Ftz-F1 (A. L., unpublished results). This confirms that only the maternally expressed α-Ftz-F1 isoform, and not the zygotically expressed β-Ftz-F1, is required for en activation (Guichet et al., 1997). While Ftz-F1 has been implicated in both positive and negative regulation of the ftz gene via dual regulatory elements (Dearolf et al., 1989; Topol et al., 1991; Tsai and Gergen, 1995), our results point only to a positive role in the transcription of en. Although our results indicate that Ftz and Ftz-F1 act directly upon en, we cannot strictly rule out the possibility that other regulatory proteins might act as intermediates.

Cooperative binding of Ftz-F1 and the Ftz homeodomain to block C in vitro suggests that cofactor function is a result of direct contact between Ftz-F1 and the Ftz homeodomain, and that homeodomain-DNA interactions contribute to regulation. Guichet et al. (1997) report that α-Ftz-F1 binds to immobilized Ftz regardless of whether the homeodomain is present. Our failure to detect a difference in the interaction of Ftz-F1 with the Ftz homeodomain versus full-length Ftz may have been due

![Fig. 3. w + reporter expression during the Ftz-dependent phase (stage 5-8/9). Shown are the stage 7-8 patterns of w + transcripts in embryos containing the indicated reporter construct, except for the panel labeled 'en', which shows the corresponding pattern of endogenous en transcripts. A-I resembles the normal en pattern in even parasegments; B-I expression is weaker and delayed in comparison with A-I; D-I and B-I constructs containing 4 bp substitutions in the hd1 or nhr1 sites fail to express the reporter, even as late as stage 9 (stage embryos 9 shown for hd1 and nhr1-); B-F shows a widened three-cell-wide pattern of stripes (stage 7/8 in upper panel, stage 9 in lower panel) which is similar to even parasegmental en expression in an odd mutant (Benedyk et al., 1994); B-E fails to express the reporter; 3xC shows six 3- to 4-cell-wide stripes (corresponding to Ftz stripes 2-7) and weak parasegment 2 expression (Ftz stripe 1). Even parasegmental expression of A-I, B-I and 3xC is abolished in a ftz mutant background (data not shown).](image-url)
to the use in our experiments of a truncated α-Ftz-F1 protein that lacked 191 N-terminal amino acids [i.e., approximately half of the α-Ftz-F1 isoform-specific region that is generated by alternative splicing (Lavorgna et al., 1993)]. While cooperative binding of N-terminally truncated Ftz-F1 and the Ftz homeodomain may be limited to adjacent sites, the two full-length proteins may be capable of interacting while bound to distant sites, providing an explanation for the essential Ftz-F1-binding site (nhr2) that did not mediate cooperative binding in our experiments.

The results of Guichet et al. (1997) are consistent with experiments demonstrating that the endogenous en and wg genes respond to Ftz protein that lacks a homeodomain ( Fitzpatrick et al., 1992; Copeland et al., 1996; Hyduk and Percival-Smith, 1996). In light of these facts, our results present a paradox: the Ftz homeodomain is dispensible for Ftz activation of endogenous genes, but we have shown that a Ftz homeodomain binding site is required for activity of the en intron enhancer. A possible explanation lies in the suggestion (Hyduk and Percival-Smith, 1996) that homeodomain-binding.

**Fig. 4.** w+ reporter expression of A-I and 3xC is abolished in Ftz-F1 mutant embryos. A-I expression in wild-type (A,B) and in Ftz-F1 mutant embryos (C,D). A and C, stage 6; B and D, stage 9. In the embryos C and D, the reporter is not expressed. 3xC expression in wild-type (E,F) and in Ftz-F1 mutant embryos (G,H). E and G, stage 6; F and H, stage 9. In the embryos G and H, the reporter is not expressed. A,B,D-H, lateral view; C, dorsal view.

**Fig. 5.** DNase I protection of block C by the Ftz homeodomain and Ftz-F1. Ftz homeodomain and N-terminally truncated Ftz-F1 (Lavorgna et al., 1991) purified from *E. coli* were used to protect a StyI-EcoRI fragment, end-labeled at the StyI site located 41 bp 5′ of block C. Footprints are labeled on the right-hand side of the sequencing gel. Sequential two-fold increases in Ftz homeodomain or Ftz-F1 concentration are indicated by the ramps at the top of the gel. Lanes 1-5 and 10-14 contain 4×10^{-9} M to 6×10^{-8} M Ftz homeodomain. Lanes 6-9 contain 1:240- to 1:30-fold dilutions of Ftz-F1-containing extracts. No protein was added for lane 1 and G/A marks the G+A cleavage ladder. The hd1 footprint is apparent at a 4- to 8-fold lower homeodomain concentration in the presence of Ftz-F1 (compare lanes 5 and 11). Also, the nhr1 footprint intensifies as the Ftz homeodomain concentration increases. This result has been repeated twice each with two preparations of Ftz-F1 protein. hd8 and nhr3 are overlapping low affinity Ftz and Ftz-F1-binding sites that lie outside of conserved block C.
deficient Ftz would still be able to contact DNA at a homeo-
domain binding site indirectly through a homeodomain-con-
taining cofactor. A candidate is the Prd homeodomain protein,
which interacts directly with the N terminus of Ftz (Copeland et al., 1996). Prd is also required for Ftz-homeodomain-
mediated repression of wg (Copeland et al., 1996). We
propose an expansion of the Hyduk and Percival-Smith model
for ‘Ftz homeodomain-independent’ regulation by speculate-
ning that, without its homeodomain, Ftz is still able to bind Ftz-F1 and Prd. These interactions allow cooperative binding of Prd (via its homeodomain) and Ftz-F1 to sites normally occupied
by Ftz and Ftz-F1, thus maintaining target specificity. This
model does not exclude a role for the Ftz homeodomain; by
providing an additional means of contacting DNA, the Ftz
homeodomain may contribute to the stability of enhancer-
bound multiprotein complexes without being absolutely
required for activation of transcription. The ability of the Ftz
homeodomain to function in the regulation of target genes is
supported by experiments in which its substitution for the En
homeodomain generated a chimeric En-FtzHD-En protein
capable of repressing genes that are normally
activated by Ftz (John et al., 1995).

The observed elimination of later stage en intron reporter
expression by mutation of either a Ftz- or a Ftz-F1-binding site
might be due to a need for initiation of a transcriptionally active state by Ftz in
order for transcription to continue after Ftz has dis-
appeared. If this is the case, any cis regulatory element
that affects Ftz-dependent activation would alter later
expression as well, in agreement with our results.
Alternatively, or in addition, a second homeodomain
protein (or proteins) might be required for continued
enhancer activity during the Wg-dependent phase. In
this respect, it is interesting to note that the minimal
Ftz-responsive 3xC reporter construct continues to be
expressed in dorsal even-parasegmental stripes after
Ftz protein is no longer detectable in the ectoderm.

Regulation by Ftz has been the main focus of this
report, but several additional elements uncovered in
our analysis deserve brief discussion. Block A affects
expression level but not pattern, and we note that it
contains an 8-out-of-9 bp match to the consensus
binding site of GLI, a human protein that is closely
related to Opa (Benedyk et al., 1994). Opa has been
shown to up-regulate en expression through repression
of odd and the activation wg (Benedyk et al., 1994).
The presence of a putative Opa-binding site in block
A suggests that Opa may also exert a direct effect on
en. The segment polarity gene cubitus interruptus (ci)
(Orenci et al., 1990) encodes a second GLI-related
protein, but ci is not required for the normal embryonic

The en intron enhancer responds to Ftz in only the
anterior-most cell column of each Ftz stripe. Deletion
of the G-I interval widens the pattern of reporter
expression to all Ftz-expressing cells, paralleling the
patterns of en or of full-length en intron-reporter con-
structs in odd mutant embryos (DiNardo and
O’Farrell, 1987; Kassis, 1990; Benedyk et al., 1994;
data not shown). These results suggest that Odd
directly represses en by binding to one or more sites
within the 300 bp G-I interval. The separation of repressing
from activating elements is consistent with repression by
‘quenching’, which may be a more general mechanism than
repression by direct competition for binding to DNA (Johnson,
1985; Gray and Levine, 1996). Recently, nkd has been shown
to contribute to repression of en in even parasegments (Mullen
and DiNardo, 1995), leaving open the possibility that the G-I
interval could respond to nkd as well.

Because deletion of block C or block F eliminated detectable
reporter expression, our analysis does not reveal whether
blocks D and E contribute to enhancer activity. However,
we note that block D contains a striking match (11 out of 11 bp
for D. virilis, 9 out of 11 bp for D. melanogaster) to the binding
site consensus for the mammalian transcription factors LEF-1
and TCF-1, LEF-1 and XTcf-3, members of the LEF-1/TCF-1
subgroup of HMG domain proteins, interact with β catenin and
have been implicated as a nuclear effectors of Wnt signaling
(Behrens et al., 1996; Molenaar et al., 1996), suggesting that a
homologous Drosophila protein(s) regulates transcription in
response to wg.

![Fig. 6. w+ reporter expression during the Wg-dependent phase. Shown are
mid-to late-stage-9 patterns of w+ transcripts in embryos containing the
indicated reporter construct, except for the panel labeled ‘en’, which shows the
corresponding pattern of endogenous en transcripts. A-I now shows expression
in both even and odd parasegments; B-I expression is weaker than A-I, 3C-I
and B-I containing the 4 bp substitution in the nhr1 site express mesodermal
stripes (about 10% of B-I(hd1-/-) embryos show a faint B-I pattern at late stage
9); D-I, B-E and B-I containing the 4 bp substitution in the nhr1 site fail to
express the reporter; B-F expression fades ventrally at stage 9 and laterally
shows an irregular pattern of expression between the strong even
parasegmental stripes, and an increase in stripe intensity in the anterior-to-
posterior direction; 3xC is similar to B-F except that expression is limited to
even parasegments.](image)
It is striking that an enhancer as large and structurally complex as the B-I segment of the en intron [eight conserved blocks totaling about 200 bp (Kassis et al., 1986)] is critically dependent on single binding sites for two factors. In contrast, redundancy among Ftz-binding sites has been well documented for the ftz- UAS autoregulatory element (Schier et al., 1992; 1993; Han et al., 1993). Indeed, it is possible that the block C binding sites may be dispensable in the context of the complete intron, with its additional conserved blocks and Ftz-binding sites. Nonetheless, analysis of the B-I interval has demonstrated the important role played by a cofactor in facilitating Ftz-dependent transcriptional activation.

We thank Judy Kassis, Carl Wu and Martin Petkovich for generously providing plasmids and fly strains. For communicating unpublished data, we thank John Copeland, Henry Krause, Judy Kassis and Mary Whitely. We are also indebted to Sean Carroll, Henry Krause and Bill McGinnis for comments on the manuscript. This work was supported by the American Cancer Society and the Milwaukee Foundation (A. L.), the EMBL (A. E. and A. G.) and a fellowship from the Ministere de la Recherche et du Travail (A. G.). This is Laboratory of Genetics publication number 3408.

REFERENCES


(Accepted 2 December 1996)