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

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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 sloppy paired (Fujioka et al., 1996). Expression of en in even parasegments results from activation by fushi tarazu (ftz). Only the most 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 Pereival-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 homedomain (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 homedomain (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; Ženg 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 (Peifer and Wieschaus, 1990; Rauskolb and Wieschaus, 1994; van Dijk and Murre, 1994; Chan et al., 1994; Neuteboom et al., 1995; Chang et al., 1995, 1996).

If Ftz utilizes its homedomain 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 hdl and nrhl (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 hdl and nrhl, respectively, which were used to reconstitute the intact B-I fragment. 3Ct was generated by ligation of a double-stranded synthetic oligonucleotide corresponding to the block C sequence shown at the top of panel A. The oligonucleotides had additional half-sites for BamHI and RflI at opposite ends, such that digestion with these enzymes after blunt end ligation yielded only tandemly arranged multimers.

Reporter constructs were examined in Df(1)w67C2 backgrounds that were otherwise wild type or contained the following mutations: ftz1, Df(3L)Carm; ftz-F1209, odd1, and wg17 (Lindsley and Zimm, 1992; Guichet et al., 1997).

#### DNase I footprinting

The Ftz homedomain (FtzHD2) was expressed in E. coli and purified as previously described (Florence et al., 1991). A partial α-Ftz-F1 cDNA (lacking the amino terminal 191 codons) was provided by C. Wu and used to over-express Ftz-F1 under T7 control in E. coli. Ftz-F1 containing extracts for footprinting experiments were prepared as follows (all steps ice cold): Cells from a liter culture were lysed in 3 ml of 6.4 M guanidine-HCl, 200 mM Tris/50 mM glucose (pH 9.5), 20 mM ZnSO4, 40 mM DTT. An equal volume of glycerol was added and the lysate was dialyzed for 8 hours against 100 volumes of 50 mM Tris/12.5 mM glucose (pH 9.5), 1 M guanidine HCl, 1 M NaCl, 10 mM ZnSO4, 1 mM DTT and 50% glycerol. A second 8-hour dialysis was done against 25 mM Tris/6 mM glucose (pH 9.5), 200 mM NaCl, 10 mM ZnSO4, 1 mM DTT, 0.5 mM PMSF and 50% glycerol. A third 8-hour dialysis was done against 25 mM Tris (pH 7.5), 100 mM NaCl, 10 mM ZnSO4, 0.5 mM EDTA and 50% glycerol, followed by centrifugation in a microfuge and precipitation of the supernatant with two volumes of 80% saturated ammonium sulfate. After centrifugation, the resulting precipitate was resuspended in the pH 7.5 dialysis buffer, minus glycerol and briefly centrifuged. The supernatant was dialyzed against the pH 7.5 buffer (with glycerol) and stored at −20°C. DHR39 was expressed in E. coli as a fusion to the carboxy-terminus of maltose-binding protein, as described by Ohno and Petkovich (1992). The resulting fusion protein contained amino acids 294-808 of DHR39 was prepared according to the same protocol described above for unfused Ftz-F1.

For footprinting, the Styl 5’ end was labeled with γ[32P] ATP using polynucleotide kinase, cut with EcoRI and gel purified. Labeled DNA (3.5x106 M) was incubated with the FtzHD2 protein and/or Ftz-F1 in 25 mM Tris (pH 7.5), 50 mM NaCl, 10 mM ZnSO4, 1 mM MgCl2, 0.1 mM DTT and 10% glycerol. After a 4 hour incubation on ice, one-tenth volume of 200 μg/ml sonicated herring sperm DNA was added immediately prior to addition of an equal volume of a 1:3 dilution of DNaseI (1U/ml, Promega Corp.). After 30 seconds, the reaction was stopped with one-tenth volume 10% SDS, 50 mM EDTA, 1 mg/ml sonicated herring sperm DNA and immediately extracted with an equal volume of phenol. Samples were then precipitated, washed with 70% ethanol, resuspended in 5 μl standard formamide/dye mix and fractionated on a denaturing 6% polyacrylamide/4 M urea gel.

### 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-
Ftz-F1 are also shown and mismatches with these are shaded. Site consensus sequences for the Ftz homeodomain, for Cad and for in the order that they appear from left to right on the map. Binding the map grouped as hd, cad or nhr (nuclear hormone receptor) sites (Ueda and Hirose, 1991). Sequences of these sites are listed below consensus (Laughon, 1991; Ekker et al., 1994), open arrows indicate binding sites that most closely match the Antp class identified by DNase I footprinting (see Fig. 5 for methods). Solid arrows indicating the location of Ftz homeodomain binding sites (i.e., stripes appeared at the end of stage 7). Additional tran-
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-F1β; 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 F, 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 parasegment stripes at high levels into stage 11. Odd parasegment 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 parasegment staining in the ectoderm except at the very dorsal edge of the embryo, and even parasegment 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-[hd1−] 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 is 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)Cut, 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
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-
deficient Ftz would still be able to contact DNA at a homeodomain binding site indirectly through a homeodomain-containing 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 speculating 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 disappeared. 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) (Orenic et al., 1990) encodes a second GLI-related protein, but ci is not required for the normal embryonic pattern of en (Hidalgo, 1991).

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 constructs 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, 1995; 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, 8 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; 3’C-I and B-I containing the 4 bp substitution in the nhr1 site express mesodermal stripes (about 10% of B-Ihd1- 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.
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