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Binding Site–Dependent Direct Activation and Repression of In Vitro Transcription by Drosophila Homeodomain Proteins

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Summary

***Fushi tarazu* and *engrailed* are two of the genes required for proper segmentation of the Drosophila embryo. Their protein products Fushi tarazu and Engrailed (Ftz and En) each contain a homeodomain and have been shown to act as transcriptional regulators in transient expression experiments in a Drosophila cell culture system. We used an in vitro transcription system to test whether the effects of Ftz and En on transcription were direct or indirect. Purified Ftz directly activates in vitro transcription by binding to homeodomain binding sites inserted upstream of the TATA box of the Drosophila *hsp70* promoter. Equimolar amounts of purified En repress this activation by competition with Ftz for binding to these sites. These results indicate that Ftz and En act directly as transcription factors and suggest that such homeodomain proteins regulate development by combinatorial transcriptional control.**

Introduction

Segmentation of the early Drosophila embryo is controlled by the sequential action of the segmentation genes (gap genes, pair-rule genes, and segment polarity genes), which interact in a cross-regulatory network in response to maternal signals deposited into the egg (Nüsslein-Volhard et al., 1987; Ingham, 1988). The role of the gap genes is to read and interpret coarse positional information encoded by maternal effect genes such as *bicoid* (Driever and Nüsslein-Volhard, 1989; Driever et al., 1989). They interact with each other to control expression of the three pair-rule genes *hairy*, *runt*, and *even-skipped* (Gergen and Butler, 1988; Howard et al., 1988; Goto et al., 1989; Harding et al., 1989), which in turn set up the expression of another class of pair-rule genes including *fushi tarazu* (Carroll and Scott, 1985). Segmentation is achieved when the segment polarity genes *engrailed* and *wingless* are expressed in 14 stripes of expression corresponding to the 14 segments (Kornberg et al., 1985; DiNardo et al., 1985; Baker, 1987). Like many of the genes involved in early Drosophila development, both *fushi tarazu* (Kuroiwa et al., 1984; Laughon and Scott, 1984) and *engrailed* (Poole et al., 1985; Fjose et al., 1985) contain a homeobox (McGinnis et al., 1984; Scott and Weiner, 1984).

The homeobox is an evolutionarily conserved DNA sequence that encodes a 60 amino acid protein domain, the homeodomain (reviewed in Gehring and Hiromi, 1986; Scott et al., 1989). The homeodomain was proposed to be a DNA binding domain because of its similarity to the helix-turn-helix motif present in bacterial (Laughon and Scott, 1984; Pabo and Sauer, 1984) and yeast DNA binding proteins (Laughon and Scott, 1984; Shepherd et al., 1984). Indeed, all homeodomain proteins are localized to the nucleus, and in vitro DNA binding studies have demonstrated that the homeodomain is a sequence-specific DNA binding domain (Desplan et al., 1985, 1988; Beachy et al., 1988; Hoey and Levine, 1988; Laughon et al., 1988; Mihara and Kaiser, 1988; Müller et al., 1988a; Driever and Nüsslein-Volhard, 1989).

The *fushi tarazu* gene product, Fushi tarazu (Ftz), and the *engrailed* gene product, Engrailed (En), expressed in Escherichia coli, bind to the consensus sequence TCAATTAAAT, named NP. This motif is found in clusters in the *engrailed* regulatory region (Desplan et al., 1988; Hoey and Levine, 1988). This sequence will be called the homeodomain binding site because many homeodomain proteins (e.g., Even-skipped and Zerknüllt) also recognize it (Hoey and Levine, 1988; Treisman et al., 1989). Recent transient expression experiments in Drosophila cell lines have shown that several homeodomain proteins can regulate transcription of genes containing binding sites specific for each homeodomain protein in their promoters (Jaynes and O'Farrell, 1988; Driever and Nüsslein-Volhard, 1989; Han et al., 1989; Krasnow et al., 1989; Winslow et al., 1989). These in vitro results suggest that Ftz activates transcription, while En represses it. However, they do not show whether these homeodomain proteins act directly or indirectly (e.g., via other intermediates) to regulate transcription.

To understand whether the homeodomain proteins Ftz and En act directly as transcription factors, we used an in vitro transcription system from human cells. We show here that the purified Ftz protein can activate transcription directly upon binding to the NP consensus sequence, while equimolar amounts of purified En prevent this activation by competing with Ftz for binding to the NP sites. We reported elsewhere that En can also repress transcription by competition with the TATA box binding protein (TFIID) for binding to the TATA box (Ohkuma et al., 1990). This effect requires 4-fold more En protein than the competition with Ftz. We propose that both mechanisms could be used for the negative regulation of transcription by En.

Results

Purification of Ftz and En Proteins

To test whether Ftz and En are binding site–dependent transcriptional regulators, we purified these proteins from overexpressing E. coli cells by using a specific oligonucleotide column containing three repeats of the homeodomain protein binding sequence NP (TCAATTAAAT) (Ohkuma et al., 1990). Since we had previously shown that Ftz and En could both bind to this NP sequence (Desplan

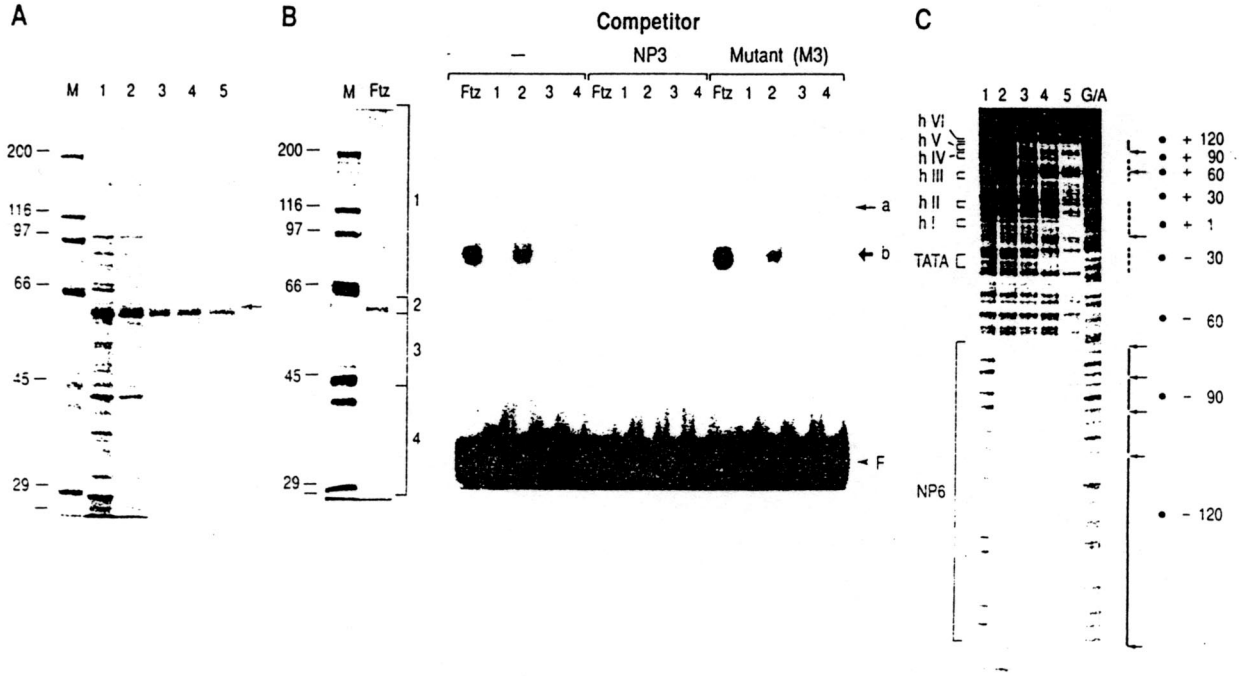

Figure 1. Purification of Ftz

(A) Coomassie blue staining of an SDS–polyacrylamide gel. Proteins (about 200 DNA binding units, see Table 1) from each column fraction were run on a 10% SDS–polyacrylamide gel at 200 V for 4 hr, and proteins were stained with Coomassie blue (Laemmli, 1970). Lane 1, crude cell extract; lane 2, dialysis pellet; lane 3, Sephacryl S300 fraction; lane 4, FPLC Superose 12 fraction; lane 5, specific oligonucleotide (NP3)–Sepharose fraction. Lane M, molecular size markers as indicated in kd. The 60 kd Ftz band is shown by an arrow.

(B) Renaturation of affinity-purified Ftz after SDS–polyacrylamide gel electrophoresis. Left: silver-stained gel of a purified fraction of Ftz. The regions 1 to 4 correspond to sections (slices 1–4) cut out of a non-silver-stained lane where 1 μg of Ftz was run in parallel (see text). Right: the material recovered from each slice was used in a gel shift assay using 2 fmol of end-labeled NP3 as a probe, without competitor or with 400 fmol of either specific (NP3) or mutant (M3) oligonucleotide. Ftz lanes show the shift obtained with the purified Ftz fraction prior to gel electrophoresis. Lanes 1 to 4 correspond to the four slices. The bands specifically shifted by Ftz are indicated by arrows (a and b). The arrowhead (F) indicates free probe.

(C) DNAase I footprinting analysis of En binding to the NP6-containing Drosophila *hsp70* promoter. Footprinting reactions were performed on a HindIII–XmnI fragment of NP6-HZ50pL (–175 to +209), which was labeled at the 3′ end of the transcribed strand. A G+A sequencing reaction was run in the adjacent lane. Amounts of En used are as follows: lane 1, no protein; lane 2, 40 ng; lane 3, 120 ng; lane 4, 225 ng; lane 5, 400 ng. The strongly protected regions are indicated by thick lines, and the weakly protected regions are indicated by broken lines. The hypersensitive sites are indicated by arrows. The location of the TATA box, NP sites, and other Ftz binding sites are indicated. The Ftz binding sites other than NP6 and the TATA box are designated hI–hIV.

et al., 1988), we used the same affinity column for their purification. Although Ftz remained insoluble after removal of guanidine–HCl used for extraction from E. coli, we were able to solubilize Ftz by running the extract through a Superose 12 column (FPLC, Pharmacia). The soluble preparation was purified on the specific oligonucleotide (NP3) affinity column. A unique 60 kd band was detected in the final preparation by SDS–gel electrophoresis (Figure 1A). This band comigrated with the ^{35}S-labeled Ftz band (data not shown). For an unambiguous identification of this 60 kd polypeptide, we performed a functional renaturation of the band subsequent to SDS–gel electrophoresis (Hager and Burgess, 1980) and examined its NP3 binding activity. Proteins were extracted from SDS–gel slices 1–4 (Figure 1B, left) and analyzed by a gel shift assay. The recovered NP3 binding activity was present in the 57–63 kd region (slice 2). The Ftz protein shifted the labeled probe as one major band (b) and one faint band (a) (Figure 1B, right). These two bands were specifically competed only by the NP3 oligonucleotide. The results of each step of purification are summarized in Table 1. The overall purification was about 20-fold, while the recovery of NP3 binding activity was 17%. A slightly different procedure was applied to the purification of En, which is not insoluble after the first renaturation (see Experimental Procedures). Figure 2 presents the various steps of purification as well as the renaturation experiment, which demonstrates that En is a 69 kd band binding to DNA. Table 2 summarizes the recovery and purification of En.

We used DNAase I footprinting to examine the binding of purified Ftz and En to the *hsp70* promoter containing six repeats of the NP sequence (NP6-HZ50pL; Figure 3C). Forty nanograms of Ftz was sufficient to show a strong footprint on the homeodomain binding sites (Figure 1C, lane 2). Larger amounts of Ftz (400 ng) also bound downstream of these sites (Figure 1C, lane 5). The affinity of En for these binding sites was 3- to 4-fold stronger than that of Ftz (data not shown).

Purified Ftz Directly Activates In Vitro Transcription by Binding to the Homeodomain Binding Sites

Recent transient expression experiments using Drosoph-

Table 1. Purification of Ftz

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Fold Purification	Yield (%)
Extract	50	300,000	6,000	1	100
Dialysis pellet	22	360,000	16,000	2.7	120
S300	4.5	120,000	27,000	4.5	40
Superose 12	1.5	99,000	66,000	11	33
Specific oligo (NP3)–Sepharose	0.52	50,000	97,000	16	17

One unit of binding activity is the amount of Ftz that, under standard gel shift conditions in the presence of 16 fmol of probe, retards 1 fmol of end-labeled probe. Oligo: oligonucleotide.

ila Schneider L2 cells have shown that Ftz activates transcription of promoters containing the NP sites (Jaynes and O'Farrell, 1988; Han et al., 1989). To test if Ftz could act directly on these sites, we added the purified Ftz protein to a cell-free transcription system from human (HeLa) cells. Since Mg^{2+} is critical for DNA binding by both Ftz and human general transcription factor IID (TFIID), we determined the optimum Mg^{2+} concentration for transcriptional activation by Ftz (Figure 3A). Ftz did not activate transcription at 8 mM Mg^{2+}, the concentration normally used in the cell-free system to achieve optimum TFIID activity (Figure 3A, lanes 9 and 10). This correlated with the observation that Ftz did not bind to the homeodomain binding sites of the NP6-HZ50pL fragment at 8 mM Mg^{2+} (data not shown). At Mg^{2+} concentrations between 0.5 and 4 mM, Ftz activated transcription (Figure 3A, lanes 1–8) with an optimum stimulation at 1 mM (Figure 3A, lanes 3 and 4). This Mg^{2+} concentration is suboptimum for TFIID and results in a lower basal level of promoter activity than that observed at higher Mg^{2+} concentrations. These results indicate that Ftz acts directly on transcription by binding to the NP sites. When increasing amounts of Ftz were added to the reaction, transcription of NP6-HZ50pL was significantly stimulated by 100 ng (Figure 3B, lanes 1–4), while only minimal effect was seen in the absence of the NP6 sites, even at 200 ng of Ftz (Figure 3B, lanes 5–8; see also Figure 4). This effect could be mediated by the weak binding sites downstream of the start site (Figure 1).

En Represses Ftz Activation by Competition for Binding to the Homeodomain Binding Sites

In transient expression experiments, En repressed Ftz-activated transcription of the *hsp70* promoter in the pres-

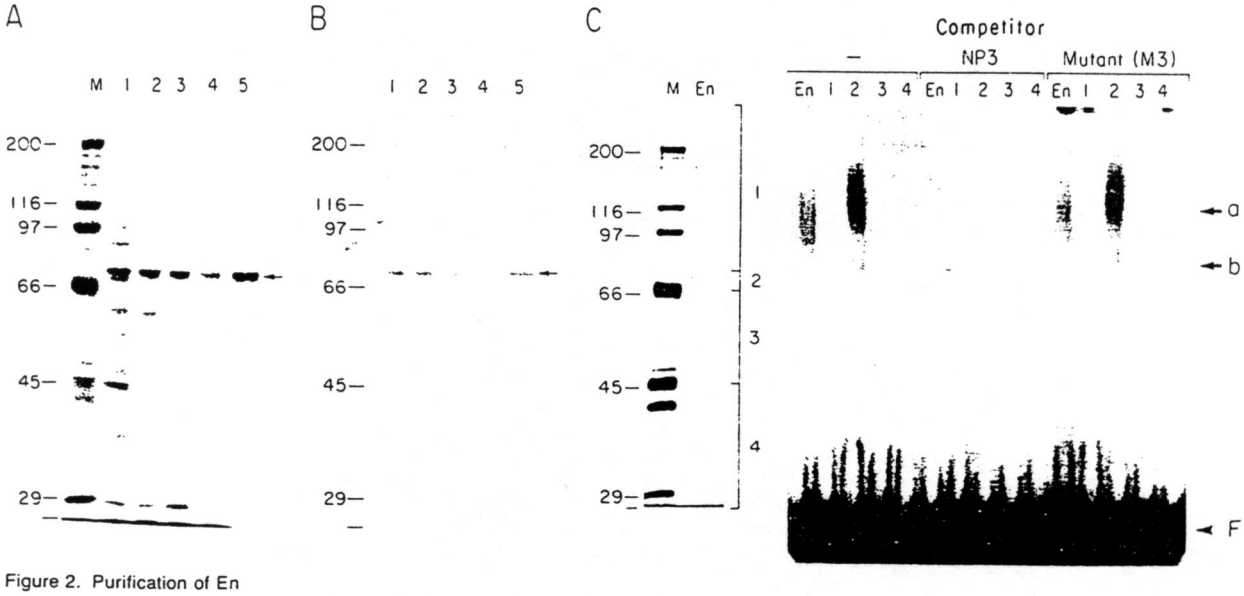

Figure 2. Purification of En

(A) Coomassie blue staining of an SDS–polyacrylamide gel. Proteins (about 200 DNA binding units, see Table 1) from each fraction were stained with Coomassie blue after SDS–polyacrylamide gel (10%) electrophoresis at 200 V for 4 hr (Laemmli, 1970). Lane 1, cell extract; lane 2, heparin–agarose fraction; lane 3, DE52 fraction; lane 4, mutant oligonucleotide (M3)–Sepharose fraction; lane 5, specific oligonucleotide (NP3)–Sepharose fraction. Lane M, molecular size markers as indicated in kd. The En band is shown by an arrow.

(B) Autoradiogram of an SDS–polyacrylamide gel. ^{35}S-labeled En in each fraction was detected by autoradiography. The lanes are the same as in (A). The En band is also shown by an arrow.

(C) Renaturation of affinity-purified En after SDS–polyacrylamide gel electrophoresis. Left: silver-stained gel of a purified fraction of En. The regions 1 to 4 correspond to sections (slices 1–4) cut out of a non-silver-stained lane where 1 μg of En was run in parallel (see text). Right: the material recovered from each slice was used in a gel shift assay containing 2 fmol of end-labeled NP3 without competitor or with 400 fmol of either specific oligonucleotide (NP3) or mutant oligonucleotide (M3). En lanes show the shift obtained by the En-purified fraction prior to gel electrophoresis. Lanes 1 to 4 correspond to the four cut slices. The bands specifically shifted by En are indicated by arrows. Arrowhead (F) indicates free probe.

Table 2. Purification of En

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Fold Purification	Yield (%)
Extract	50	170,000	3,400	1	100
Heparin–agarose	10	83,000	8,300	2.4	49
DE52	1.8	47,000	26,000	7.6	28
Mutant oligo (M3)–Sepharose	1.2	35,000	29,000	8.5	21
Specific oligo (NP3)–Sepharose	0.20	18,000	90,000	27	11

One unit of binding activity is the same as described in Table 1. Oligo: oligonucleotide.

ence of the NP sites (NP6-HZ50pL) (Jaynes and O'Farrell, 1988). This suggested that En works as a repressor by competing with Ftz for binding to the homeodomain binding sites. However, we have shown that 400 ng of En (a 10-fold excess of En over the number of binding sites) can repress in vitro transcription of the *hsp70* promoter, even in the absence of NP sites (Ohkuma et al., 1990). This repression is due to competition of En with TFIID for binding to the TATA box. The amounts of En that we used in this paper do not affect the basal transcription of a promoter and do not exhibit any footprint on the TATA box (Figures 1 and 4). When equimolar amounts of En and Ftz (75 ng) were added to the in vitro transcription system, the activation by Ftz seen in the absence of En was suppressed, and the promoter was expressed at its basal level (Figure 4A, lanes 4 and 5). The promoter that did not contain the NP6 sites was not activated by Ftz and was unaffected by 100 ng of En. These results indicate that, as suggested by the transient expression experiment, repression by En can occur by direct competition with Ftz for binding to its target sites.

We used a gel shift assay to examine whether En could indeed compete with Ftz for binding to the NP sites under conditions in which En can suppress the in vitro activation provided by Ftz. Figure 5 shows that En and Ftz exhibit different band shift patterns with a probe containing three copies of the NP consensus sequence (NP3). Ftz alone generated a major band, Ftz(b), that migrates faster than the two complexes, En(a) and En(b), formed by En. Addition of En to Ftz revealed that En competed very efficiently with Ftz for binding to NP3 (Figure 5, lanes 3–7), with equimolar amounts of the two proteins resulting in the almost exclusive binding of En (Figure 5, lanes 6 and 11). Similarly, no new band could be detected when Ftz and En were mixed, indicating that the two proteins cannot bind to the same fragment; rather, one excludes the other. It should also be noted that only very weak gel shift could be observed with a single NP site (data not shown). Furthermore, incubation of decreasing amounts of Ftz with a fragment containing several NP sites did not give rise to bands smaller than the Ftz(b) band. Therefore, it is likely that Ftz and En each bind cooperatively and that there is cooperativity only between proteins of the same species. The presence of the two different complexes with each protein can be explained by the presence of three sites in the DNA fragment and will be discussed later. It appears that Ftz has a weaker affinity for the homeodomain binding sites than En and that the replacement of Ftz by En on the sites of activation can explain the repression by En of transcriptional activation due to Ftz.

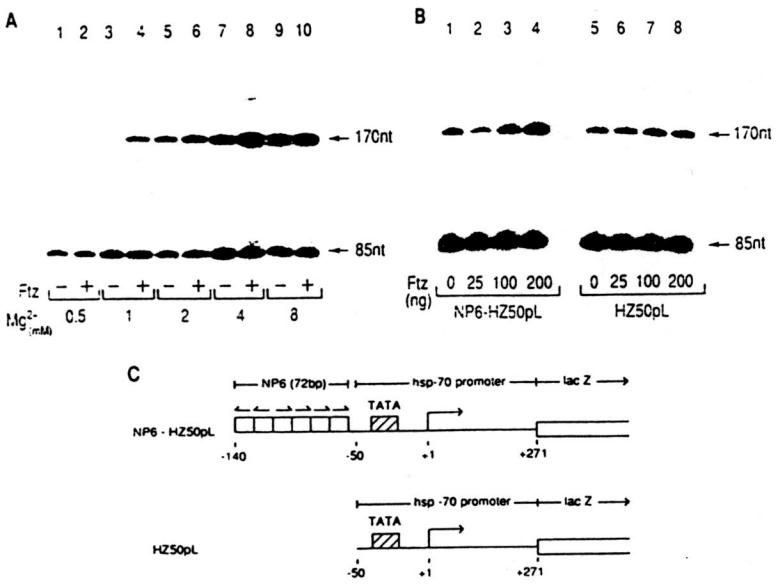

Figure 3. Transcriptional Activation by Ftz In Vitro

Primer extension analyses were carried out to examine whether Ftz activates transcription. (C) represents the two templates that contain the Drosophila *hsp70* promoter. NP6-HZ50pL contains six repeats of the homeodomain protein binding sequence (NP).

(A) Effect of Mg^{2+} on transcriptional activation by Ftz. NP6-HZ50pL (44 fmol) was used as a template. Transcription was carried out at various concentrations of Mg^{2+} with (+) and without (−) Ftz. The transcript from the *hsp70* promoter is detected as a 170 nucleotide band by primer extension. A 85 nucleotide ^{32}P-labeled fragment was added to the reaction as an internal control to estimate extraction efficiency.

(B) Effect of the homeodomain binding sites on the transcriptional activation by Ftz. The Mg^{2+} concentration was 1 mM. The same amount (44 fmol) of both templates was used. Amounts of Ftz added are as indicated.

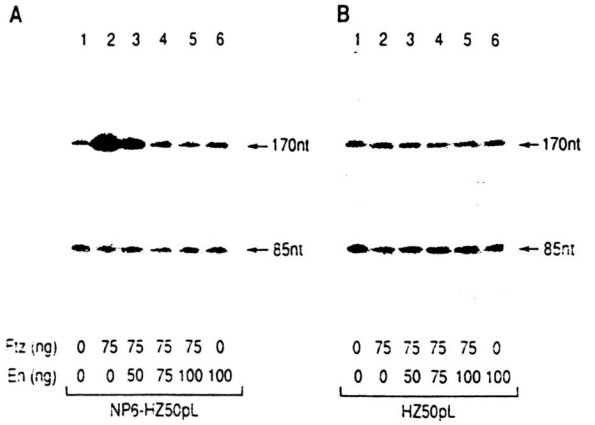

Figure 4. Repression of Ftz-Activated Transcription by En In Vitro

Primer extension analysis was carried out to examine the effect of En on Ftz-activated transcription. The Mg^{2+} concentration was 1 mM, and 44 fmol of NP6-HZ50pL (A) or HZ50pL (B) was used as template. Ftz and En were added at the same time. Amounts of Ftz and En used are as indicated. Arrows indicate the bands described in Figure 2.

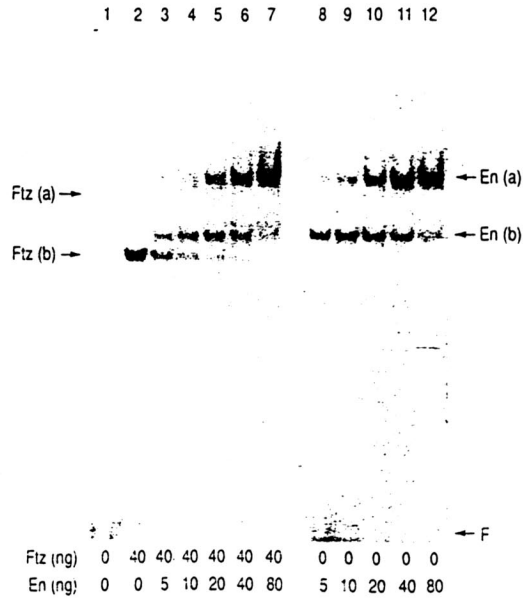

Figure 5. Competition between En and Ftz for Binding to the Homeodomain Binding Sequences

The gel shift assay was carried out as in Experimental Procedures under transcriptional conditions, except that nucleotides were omitted and the DNA concentration was 10-fold lower. Probe (2 fmol of ^{32}P-NP3) was incubated with En and/or Ftz as indicated. The free probe (F) is shifted by Ftz as a major band, Ftz(b), and by En as two bands, En(a) and En(b).

En Represses Ftz Activation Even When Ftz Has Been Prebound to the Homeodomain Binding Sites

We examined the ability of En to compete with Ftz that had been prebound to the homeodomain binding sites. En could repress Ftz-induced transcriptional activation of NP6-HZ50pL whether or not the template had been preincubated with Ftz (Figure 6A). Gel shift assays showed that preincubation with Ftz did not affect the extent of competition by En (Figure 6B). Ftz appeared to be easily replaced by En even after preincubation of the promoter with Ftz. Thus Ftz does not form a stable protein–DNA complex that would resist competition by En.

This mechanism of repression is different from the mechanism that we proposed for the repression of basal transcription by higher concentrations of En. In this case, binding of En to the TATA box effectively competed with TFIID and prevented expression of the gene. Preincubation of TFIID with the promoter prevented this repression, presumably because TFIID forms a stable committed complex that cannot be disrupted by En (Ohkuma et al., 1990). The two mechanisms do not exclude each other,

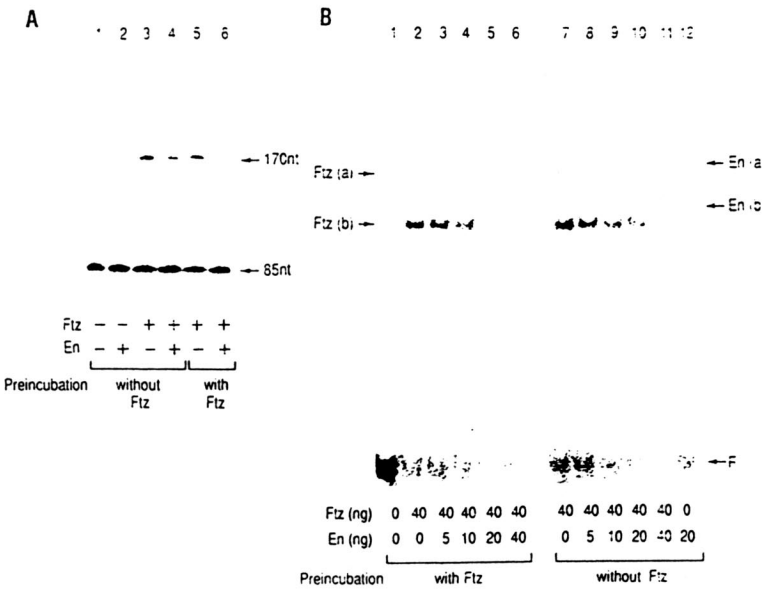

Figure 6. Effect of Preincubation with Ftz on Transcription and Binding to the Homeodomain Binding Sites

(A) Effect of preincubation of NP6-HZ50pL with Ftz on the transcriptional repression by En. Primer extension analysis was carried out as described in Figure 2. Lanes 1 to 4, preincubation without Ftz; lanes 5 and 6, preincubation with 100 ng of Ftz. The En (100 ng) and Ftz (100 ng) proteins were added at the time of the transcription reaction as indicated. The 170 nucleotide product and 85 nucleotide internal control are indicated.

(B) Effect of preincubation with Ftz on the competition by En. The gel shift assay was carried out as in Figure 4. Lanes 1 to 6, preincubation with Ftz; lanes 7 to 12, preincubation without Ftz. The amounts of Ftz or En proteins are indicated. Bands shifted by Ftz or En are also indicated.

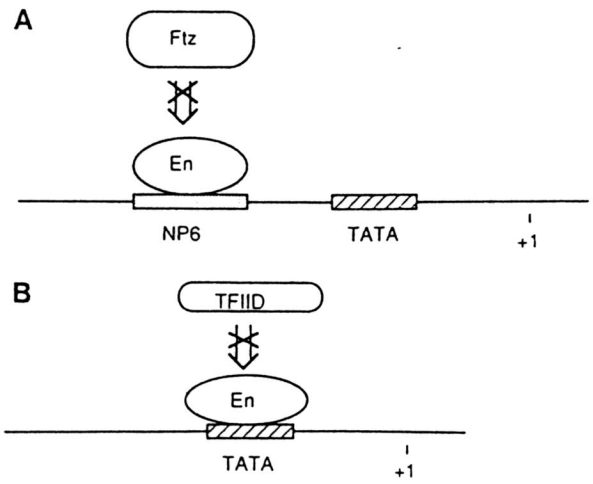

Figure 7. Model for the Transcriptional Repression by En

(A) Competition at the upstream sequence. At a low concentration, En binds to the homeodomain binding (NP6) sites, but not to the TATA box, and competes with Ftz for binding to the NP6 sites. Activation by Ftz is eliminated and transcription lowered to the basal level.

(B) Competition at the TATA box. At a high concentration (10-fold molar excess of protein over the number of En binding sites), En binds to the TATA box and competes with TFIID. As a result, the basal level of transcription is reduced to near zero.

and we can indeed observe two stages of repression of the NP6-HZ50pL promoter stimulated by Ftz using different concentrations of En (data not shown). At 75 ng of En, the activation of the *hsp70* promoter by 75 ng of Ftz is totally suppressed, but the promoter is still expressed at its basal level. At 400 ng, the basal level is completely repressed, and transcription is annulled. We interpret this in terms of a competition with Ftz for the first repression and a competition with TFIID for further repression (Figure 7).

Discussion

In this paper, we present direct evidence that the Drosophila homeodomain protein Ftz activates in vitro transcription, while equimolar amounts of the homeodomain protein En were sufficient to suppress this activation. En appears to act by competing with Ftz for binding to the homeodomain binding sites. These results are in agreement with the results obtained in transient expression experiments in a Drosophila cell culture system (Jaynes and O'Farrell, 1988; Han et al., 1989).

Purification of Ftz and En

This study was made possible by high level expression of homeodomain proteins in bacteria. We purified Ftz and En on an oligonucleotide affinity column with NP sites. The major difference between Ftz and En is that Ftz is highly insoluble when guanidine–HCl is removed from the E. coli extract. Krause et al. (1988) also reported that purified Ftz was insoluble in the absence of urea. This problem of insolubility is frequently encountered for proteins overexpressed in E. coli and is a major obstacle to the purification of such proteins. The purification procedure employed here suggests that this insolubility is not an intrinsic property of Ftz, but that it is due to the presence of contaminants in the preparation. A similar conclusion was reached for the dnaA protein by Sekimizu et al. (1988). They suggested that the protein was insoluble because of its presence in inclusion bodies and that phospholipids remained bound to the protein upon extraction with denaturing agents, thus making it insoluble. In the present case, rapid renaturation on a sizing column (Pharmacia FPLC Superose) gave rise to a soluble Ftz preparation, presumably because both the contaminants and guanidine–HCl were removed. This method should be very useful in purifying other insoluble proteins from E. coli. En was easily purified by combination of conventional and affinity columns because it is mostly soluble, even in the absence of guanidine–HCl.

Ftz Activates Transcription by Binding to the Homeodomain Binding Sites

Recent transient expression experiments have suggested that Ftz is a transcriptional activator (Jaynes and O'Farrell, 1988; Fitzpatrick and Ingles, 1989; Han et al., 1989; Winslow et al., 1989). To understand the direct role of Ftz, we performed in vitro transcription experiments in a heterologous system containing human general transcription factors (TFIIB, TFIID, TFIIE, and RNA polymerase II) (reviewed in Nakajima et al., 1988). Since purified Ftz could not bind at the high concentration of Mg^{2+} (8 mM) that is normally used to optimize conditions for in vitro transcription (Ohkuma et al., unpublished data), we determined that 1 mM Mg^{2+} was the optimum concentration for Ftz activation of a Drosophila *hsp70* promoter containing NP sites. This activation was dependent on the presence of the binding sites, and the extent of activation was correlated with the amount of Ftz protein added to the transcription reaction. These results show that Ftz activates transcription directly by binding to the homeodomain binding sites.

Several mammalian transcription factors have been shown to contain highly divergent (about 30% amino acid identity) homeodomain sequences (Bodner et al., 1988; Clerc et al., 1988; Ingraham et al., 1988; Ko et al., 1988; Müller et al., 1988b; Scheidereit et al., 1988; Sturm et al., 1988). These proteins are also related to each other by a second conserved sequence called the POU-specific box (Herr et al., 1988). Many experiments have shown that these proteins act directly on transcription. However, many of their properties, including their DNA binding (Sturm and Herr, 1988; Treisman et al., 1989), are different from that of the Drosophila developmental homeodomain proteins. Ftz is the first non-POU homeodomain protein that has been shown to activate in vitro transcription directly.

En Suppresses Activation by Ftz by Competition for the Homeodomain Binding Sites

We examined the suppression by En of the activation by Ftz described above. The 5-fold activation mediated by exogenous Ftz could be suppressed by adding the same amount of En, which returned the activity of the NP6-

containing promoter to its basal level. Preincubation with Ftz prior to addition of En resulted in the same effect. Our interpretation of these observations is that En competes strongly with Ftz for binding to the NP sites, thus preventing its positive action on the promoter.

Using a gel shift assay, we indeed showed that Ftz was excluded from a fragment containing several binding sites (NP3) by equimolar amounts of En. Preincubation with Ftz did not change the nature of this competition. There are two possible explanations for this result. Since the affinity of Ftz for the NP sites is about 4-fold lower than that of En, En might be able to displace Ftz very efficiently when the two proteins are mixed. Alternatively, a strong cooperativity of the binding of En could exclude Ftz. If this cooperativity was occurring only between molecules of the same species, it would allow a more efficient suppression of Ftz activation by En. Although there is no direct demonstration that the binding of homeodomains is cooperative, we have failed to observe interactions of Ftz or En, even at high levels, with a single NP site in a gel shift assay (Ohkuma et al., unpublished data). The gel shift pattern given by En shows that one band, En(b), is shifted at low En concentration, while another band, En(a), appears progressively with increasing En concentration. The En(b) decreases while En(a) increases. Two En or Ftz molecules (or dimers) would bind cooperatively to two of the three NP sites. When more En or Ftz is added, another molecule would bind noncooperatively to the remaining site and shift the NP3 fragment to the En(a) or Ftz(a) positions. We reported (Desplan et al., 1988) that a β-galactosidase–En fusion protein bound to three NP sites in a pairwise cooperative way.

The mechanism of suppression of Ftz activation is clearly distinct from the mechanism that we proposed for the repression of a basal promoter by En, which is mediated by competition of En with TFIID for binding to the TATA box (Ohkuma et al., 1990). In this latter case, the TATA box–containing promoter was completely silenced by relatively high concentrations of En, which were similar to the amounts of Even-skipped protein used by Biggin and Tjian (1989) to repress transcription of the *Ultrabithorax* promoter. In our experiments, En did not repress transcription when the promoter had been preincubated with TFIID, presumably because TFIID forms a committed complex with the promoter that cannot be disrupted by En. In Figure 7, we propose two possible models for transcriptional repression by En. At a low concentration, similar to that at which Ftz stimulates transcription, En competes with Ftz for binding to the NP sites, the transcriptional activation by Ftz is suppressed, and the promoter returns to its basal level (Figure 7A). At a higher concentration (10-fold molar excess of protein over the number of En binding sites), En competes with TFIID for binding to the TATA box (Ohkuma et al., 1990). As a result, the basal level of transcription is repressed (Figure 7B).

In sum, the simplest model based on the present data is that En (and maybe other homeodomain proteins such as Even-skipped; Biggin and Tjian, 1989; Han et al., 1989) competes with other activating homeodomain proteins with similar specificities. This model is quite attractive in view of the large number of homeodomain proteins sharing strongly related specificities (Hoey and Levine, 1988; Treisman et al., 1989) that interact in a cross-regulatory network and are often expressed at the same developmental stage in the same cell. Alternatively, En could interact directly with the general transcription machinery, at the level of the TATA box. This competition with TFIID appears to be more specific to En than to Ftz, but could be shared by other homeodomain proteins such as Even-skipped, which has been shown to act as a general repressor (Biggin and Tjian, 1989; Han et al., 1989). In this case, both the presence of homeodomain binding sites and a specific sequence of the TATA box would be required for the repression. Combination of such transcriptional regulatory mechanisms could play a role in controlling the expression of the developmental genes during morphogenesis.

Experimental Procedures

Recombinant Plasmids

The expression plasmid pGEMF1, which expresses Ftz protein in E. coli under the control of the T7 promoter, was a gift of H. Krause (Krause et al., 1988). The pAR-*engrailed* expression plasmid was constructed by Hoey and Levine (1988). The plasmid HZ50pL, constructed by Hiromi and Gehring (1987), contains the Drosophila *hsp70* promoter from −50 to +271 from the transcription start site. The plasmid NP6-HZ50pL, constructed by Jaynes and O'Farrell (1988), contains six repeats of the NP consensus sequence (Desplan et al., 1988) inserted into the KpnI–XbaI site of HZ50pL.

Oligonucleotides and Affinity Column

A specific oligonucleotide (NP3) that contains three repeats of the homeodomain binding consensus sequence (Desplan et al., 1988) was prepared by annealing the synthetic oligonucleotides NP3T (5′-GGAATTCACTCGGATCCTCAATTAAATGATCAATTAAATGATCAATTAAATGAGTCGACG-3′) and NP3B (5′-CGTCGACTCATTTAATTGATCATTTAATTGATCATTTAATTGAGGATCCG-3′). A mutant oligonucleotide (M3) that contains 4 bp changes (bases underlined) in each En binding consensus sequence was prepared from synthesized oligonucleotides: M3T (5′-GGAATTCACTCGGATCCTCAGTCGGATGATCAGTCGGATGATCAGTCGGATGAGTCGACG-3′) and M3B (5′-CGTCGACTCATCCGACTGATCATCCGACTGATCATCCGACTGAGGATCCG-3′). An affinity column was made by coupling of CNBr-activated Sepharose CL-6B with the above mentioned double-stranded oligonucleotides (NP3 or M3) by the method of Kadonaga and Tjian (1986). The coupling efficiency was approximately 0.25 mg of oligonucleotides per ml of column resin.

Purification of Ftz

The Ftz-induced E. coli extract was prepared essentially by the method of Hoey et al. (1988), except that the cell extract was initially resuspended in buffer Z, 0.1 M KCl containing 4 M guanidine–HCl. Buffer Z contains 25 mM HEPES (pH 7.8), 0.2 mM EDTA, 20% glycerol, and 0.1% Triton X-100 and was adjusted to 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 1 mM dithiothreitol prior to use. Ftz expression was induced from the plasmid pGEMF1 in the E. coli strain BL21 (DE3) by IPTG (Studier and Moffatt, 1986; Rosenberg et al., 1987). ^{35}S-labeled Ftz was also prepared as a marker for the purification of Ftz (Studier and Moffatt, 1986). The labeled cell extract was prepared by the same method, and 2 μCi of the labeled cell extract was mixed with the unlabeled extract. This mixture (50 mg) was dialyzed against buffer Z, 0.1 M KCl containing 1 M guanidine–HCl for 2 hr and then against the same buffer without guanidine–HCl for 16 hr. The precipitate that developed was collected by centrifugation at 10,000 × g for 15 min. The pellet was resuspended by sonication in 5 ml of buffer Z, 0.1 M KCl containing 2 M guanidine–HCl. Soluble material was loaded directly onto a 300 ml Sephacryl S300 (Pharmacia) column, equilibrated with buffer Z, 0.1 M KCl containing 2 M guanidine–HCl.

Each fraction (3 ml) was assayed for ^{35}S radioactivity, protein concentration, and NP3 binding activity. Protein concentration was determined by the method of Bradford (1976). Active fractions (15 ml) were pooled. Two hundred microliters was then loaded onto a Superose 12 column (Pharmacia FPLC HR 16/50) equilibrated with buffer Z, 0.1 M KCl without guanidine–HCl and eluted at a flow rate of 0.3 ml/min. Active fractions were recovered after repetition of this step and were loaded onto a 1.5 ml specific oligonucleotide (NP3) affinity column equilibrated with the same buffer containing 1.4 μg/ml poly(dI-dC)•(dI-dC). The column was washed and eluted by stepwise KCl concentration in buffer Z; it was washed with 6 ml of 0.1 M KCl followed by 2 ml of 0.2 M KCl in buffer Z. The protein was eluted with 2 ml of 0.4 M KCl. The eluted material was stored at −70°C.

Purification of En

The En-expressing cell extract was prepared by the same method as Ftz, except that the cells were transformed with pAR-*engrailed* recombinant plasmid (Hoey et al., 1988). The cell extract (50 mg of protein) containing 2 μCi of ^{35}S-labeled En was loaded directly onto a 6 ml heparin–agarose (Bio–Rad) column equilibrated with buffer Z, 0.1 M KCl. The column was washed with 30 ml of buffer Z, 0.1 M KCl and eluted with a 0.1 to 1.0 M KCl linear gradient in buffer Z (40 ml). Each fraction (2 μl) was assayed for ^{35}S radioactivity, protein concentration, and NP3 binding activity. Active fractions (250–400 mM KCl) were pooled. This sample was diluted to 0.1 M KCl and loaded onto a 6 ml DEAE–cellulose (DE52, Whatman) column equilibrated with buffer Z, 0.1 M KCl. The column was washed with 30 ml of the same buffer and eluted with a 0.1 to 0.6 M KCl linear gradient in buffer Z (30 ml). Each fraction (2 μl) was assayed, and active fractions (100–200 mM KCl) were pooled. The pooled DE52 fractions were diluted to 0.1 M KCl and loaded onto a 1.5 ml mutant oligonucleotide (M3) affinity column equilibrated with buffer Z, 0.1 M KCl and 1.4 μg/ml poly(dI-dC)•(dI-dC). The column was washed with 4.5 ml of the same buffer and eluted with a 0.1 to 0.6 M KCl linear gradient in buffer Z (10 ml). Active fractions (100–200 mM KCl) were pooled, diluted to 0.1 M KCl, and loaded onto a 1.5 ml specific oligonucleotide (NP3) affinity column equilibrated with the same buffer as the M3 column. The column was washed and eluted by stepwise KCl concentration in buffer Z, washed with 6 ml of 0.1 M KCl followed by 2 ml of 0.2 M KCl in buffer Z, then eluted with 2 ml of 0.4 M KCl.

Gel Shift Assay

The labeled NP sites (^{32}P-NP3) were prepared as a probe by elongation of a short nucleotide, LSalB (5′-CGTCGACTCA-3′), annealed to NP3T with [α-^{32}P]dATP, [α-^{32}P]dCTP (ICN), and the Klenow fragment of DNA polymerase I (New England BioLabs). Binding reactions were performed under the conditions used for the in vitro transcription experiments, except that the ribonucleotides were omitted and DNA concentration was 10-fold lower. Probe (2 fmol) was incubated with Ftz fractions at 30°C for 30 min, and protein–DNA complexes were resolved from free DNA by electrophoresis on a 4% acrylamide gel with running buffer containing 25 mM Tris–HCl (pH 8.3), 190 mM glycine, and 1 mM EDTA. Gel shift assays with En were performed by the same method, except that 90 mM Tris–HCl (pH 8.3) and 18 mM boric acid were added in the gel and running buffer instead of 25 mM Tris–HCl (pH 8.3) and 190 mM glycine.

Renaturation of Proteins after SDS–Polyacrylamide Gel Electrophoresis

Renaturation was performed as described by Hager and Burgess (1980), starting with 1 μg of purified Ftz or En. Gel shift assays were performed as described above with 8 μl out of 250 μl of renatured material and 2 fmol of the end-labeled probe (NP3). For the binding competition, 200-fold excess (400 fmol) of either specific oligonucleotide (NP3) or mutant oligonucleotide (M3) was used.

DNAase I Footprinting Assay with Purified Ftz

About 500 ng of a 384 bp HindIII–XmnI fragment of NP6-HZ50pL was end labeled by filling in the HindIII site with [α-^{32}P]dATP and [α-^{32}P]dCTP with the Klenow fragment of DNA polymerase I; 20 fmol (5 ng) of this fragment was incubated with various amounts of Ftz in 20 mM HEPES (pH 7.8), 60 mM KCl, 2 mM $MgCl_2$, 4 μg/ml poly(dI-dC)•(dI-dC), and 0.1 mg/ml bovine serum albumin for 30 min at 30°C. Two microliters of 20 μg/ml DNAase I was added and incubated for 30 s at 30°C. The reaction was stopped by the addition of 50 μl of stop buffer (20 mM EDTA, 0.6 M sodium acetate [pH 5.2], 0.2% SDS, 100 μg/ml yeast tRNA). The DNA was isolated by phenol–chloroform, and chloroform extractions were followed by ethanol precipitation. The DNA was then analyzed on an 8% sequencing gel. A "G+A" reaction was performed by the method of Maxam and Gilbert (1977).

In Vitro Transcription Assay

Transcription reactions were performed essentially as described in Sawadogo and Roeder (1985), except that 1 mM $MgCl_2$ (or as indicated) and 600 ng of supercoiled template DNA were used. The amounts of general transcription factors were the same as in Hai et al. (1988). Primer extension analysis of each transcript was done as described (Lillie et al., 1986). A 21-mer synthetic oligonucleotide (5′-GGTTGATTTCAGTAGTTGCAG-3′) was 5′ end labeled with [γ-^{32}P] ATP (Amersham) and T4 polynucleotide kinase (Boehringer Mannheim) and used as a primer. We chose not to use a basal promoter as an internal control for transcription because concurrent work has shown that high levels of En can affect basal transcription of a promoter (Ohkuma et al., 1990). An 85 nucleotide DNA fragment of the human immunoglobulin heavy-chain gene promoter was added to estimate the recovery of products after transcription reaction. The products of transcription (170 nucleotides) were analyzed on a 6% denaturing polyacrylamide gel.

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