Temporally restricted expression of transcription factor βFTZ-F1: significance for embryogenesis, molting and metamorphosis in Drosophila melanogaster

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SUMMARY

FTZ-F1, a member of the nuclear receptor superfamily, has been implicated in the activation of the segmentation gene fushi tarazu during early embryogenesis of Drosophila melanogaster. We found that an isoform of FTZ-F1, βFTZ-F1, is expressed in the nuclei of almost all tissues slightly before the first and second larval ecdysis and before pupation. Severely affected ftz-f1 mutants display an embryonic lethal phenotype, but can be rescued by ectopic expression of βFTZ-F1 during the period of endogenous βFTZ-F1 expression in the wild type. The resulting larvae are not able to molt, but this activity is rescued again by forced expression of βFTZ-F1, allowing progression to the next larval instar stage. On the other hand, premature expression of βFTZ-F1 in wild-type larvae at mid-first instar or mid-second instar stages causes defects in the molting process. Sensitive periods were found to be around the time of peak ecdysteroid levels and slightly before the start of endogenous βFTZ-F1 expression. A hypomorphic ftz-f1 mutant that arrests in the prepupal stage can also be rescued by ectopic, time-specific expression of βFTZ-F1. Failure of salivary gland histolysis, one of the phenotypes of the ftz-f1 mutant, is rescued by forced expression of the ftz-f1 downstream gene BR-C during the late prepupal period. These results suggest that βFTZ-F1 regulates genes associated with ecdysis and metamorphosis, and that the exact timing of its action in the ecdysone-induced gene cascade is important for proper development.

Key words: FTZ-F1, Nuclear hormone receptor, Drosophila melanogaster, Ecdysone, Molting, Metamorphosis, BR-C

INTRODUCTION

Larval molting and metamorphosis of the insect are induced by pulses of ecdysteroid hormones. In Drosophila melanogaster, the pulse of ecdysteroids that occurs during the first or second larval instar triggers molting of the cuticle, which includes renewal of the mouth hooks, spiracles and trachea. During the larva-to-adult metamorphosis, one or more successive ecdysone pulses induce a complex series of behavioral and morphological changes (Riddiford, 1993).

A role for ecdysteroids in gene expression has been inferred from the puffing pattern of the salivary gland polytene chromosomes (Becker, 1959). As the ecdysteroid titer peaks at the end of the third larval stage, internolt puffs prominent during the mid-third instar regress, and a small set of early puffs is induced rapidly in direct response to ecdysteroids. Subsequently, more than 100 late puffs are induced, while the early puffs regress. Several hours after the pulse of ecdysone, mid-prepupal puffs are induced, which regress upon the next ecdysone pulse, occurring during the late prepupal period. This ecdysteroid pulse induces the prepupal-pupal transition and the formation of late prepupal puffs. These puffing patterns have been reproduced in cultured salivary glands by addition or removal of ecdysteroid (Ashburner, 1972; Richards, 1976). Ashburner et al. (1974) proposed a model for the genetic control of polytene chromosome puffing during metamorphosis by ecdysone. Supporting Ashburner’s model, studies in molecular cloning have revealed that some of the ecdysone-inducible genes encode transcription factors containing DNA-binding motifs (Andres and Thummel, 1992; Thummel, 1995, 1996). Most of these genes encode two or more protein isoforms, and isoform-specific functions in different organs have been postulated (Emery et al., 1994; Fletcher and Thummel, 1995; Bayer et al., 1997; Bender et al., 1997; Schubiger et al., 1998). These regulatory genes are expressed in a time-specific manner and are likely to induce
or repress other ecdysone-responsive genes (Andres and Thummel, 1992, 1995, 1996). These observations illustrate the presence of a complex ecdysone-induced gene cascade. However, studies to date have mostly focused on early and late ecdysone-inducible genes, and little is known about the regulatory genes that are expressed immediately after the pulse of ecdysone, and the importance of the timing of expression of these genes.

FTZ-F1, a member of the nuclear hormone receptor superfamily (Lavorgna et al., 1991; Ueda et al., 1992), has been identified as a potential regulator of the transcription of fushi tarazu (ftz) in Drosophila (Ueda et al., 1990). Two isoforms, α- and β-FTZ-F1, are transcribed from the same gene: they share a common C-terminal region but contain different N-terminal regions (Lavorgna et al., 1991, 1993). αFTZ-F1 is expressed in early embryos, concomitant with ftz expression. βFTZ-F1 expression, on the other hand, is detected in late-stage embryos (predominantly after 16 hours of embryogenesis), larvae, prepupae and adults, when ftz expression is absent (Ueda et al., 1990; Lavorgna et al., 1993). The chromosome locus 75CD containing the FTZ-F1 gene forms a puff during the mid- preupal period, concomitant with FTZ-F1 expression and mRNA production (Lavorgna et al., 1993; Murata et al., 1996). The importance of ftz-1 expression during the early metamorphic period has been demonstrated by using a hypomorphic ftz-1 mutant that shows defects in the prepupa-to-pupa transition including failure in head eversion, histolysis of salivary glands and leg elongation (Broadus et al., 1999). This role is supported by findings that βFTZ-F1 is necessary for expression of late preupal genes, BR-C, E74A, E75A and E93, which are expressed after the ecdysteroid peak that induces the prepupa-to-pupa transition, providing evidence that βFTZ-F1 provides competence for these genes to respond to the ecdysteroid peak (Woodard et al., 1994; Broadus et al., 1999). βFTZ-F1 has also been found to positively regulate the EDG84A gene during the mid- to late preupal period before the ecdysteroid peak (Murata et al., 1996). BmFTZ-F1, a silkworm (Bombyx mori) homolog of FTZ-F1, is expressed during the late period of each molting stage after the ecdysteroid pulse (Ueda and Hirose, 1990). Exposure to and subsequent withdrawal of ecdysteroids results in induction of BmFTZ-F1 expression (Sun et al., 1994). The precise timing of βFTZ-F1 expression and its function remained to be analyzed in Drosophila.

To study the role of FTZ-F1 in postembryonic development, we determined the precise expression periods of FTZ-F1 and analyzed the function of ftz-1 using more severely affected mutants than those previously analyzed. We also examined the importance of the timing of expression of this factor. Our results establish that temporally restricted expression of βFTZ-F1 is important for late embryogenesis, the normal molting process and early metamorphosis.

MATERIALS AND METHODS

Stocks
Abbreviation y w is used for y w Df(1)w67c1. All stocks not explicitly described in the text are described elsewhere (Lindsley and Zimm, 1992). hsL32 (Murata et al., 1996) was used as a line carrying the heat-shock promoter-FTZ-F1 cDNA fusion gene and referred as hs-βFTZ-F1. Flies were raised as described in Murata et al. (1996). ftz-1ex7 and ftz-1ex7, P-element excision lines from ftz-1209, were obtained from Dr Antoine Guinchet. f[3]035649 was provided by the Bloomington Drosophila Stock Center. Flies carrying Z1, Z2, Z3 or Z4 transgenes, which can express isoforms of the BR-C gene product under the control of the heat-shock promoter, were obtained from Dr Cynthia A. Bayer. Other flies were obtained from the Genetic Strain Research Center in the National Institute of Genetics. We recombined the hs-βFTZ-F1, Z1 or Z4 transgene with the ftz-1ex7 allele.

Western blotting
Staged larvae were prepared by collecting eggs for 3 hours and harvesting the developing larvae at 3-hour intervals. Larvae of unusually large or small size were not used. Larvae in food and wandering larvae 102-107 hours after egg laying (AEL) were harvested separately. Samples of prepupa and pupa were prepared by picking newly formed white prepupae at 1- or 2-hour intervals and harvesting every 2 hours. Samples taken 3 and 12 hours before puparium formation were prepared by harvesting wandering larvae according to Karim and Thummel (1992). About 2 μg of staged larvae and prepupae were homogenized in an Eppendorf tube containing 5 μl of 2× Laemmli sample buffer (Sambrook et al., 1989). Samples were heated at 95°C for 5 minutes, after which 5 μl of water was added. Half of the sample was boiled for 5 minutes, subjected to 8% SDS-PAGE and blotted onto nitrocellulose filter for 10 hours at 60 V in 25 mM Tris, 192 mM glycin solution containing 20% methanol (Sambrook et al., 1989). The filter was incubated with a polyclonal antibody against FTZ-F1 (Lavorgna et al., 1993) (1:10,000 dilution) in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20 at room temperature for 1 hour. Protein was detected using an alkaline phosphatase-conjugated goat anti-rabbit IgG detection system (Promega) or a horseradish peroxidase-linked goat anti-rabbit IgG ECL detection system (Amersham).

Immunohistochemistry
For antibody staining of larvae and prepupae, animals were dissected in phosphate-buffered saline and fixed with 2% paraformaldehyde in 100 mM Pipes (pH 7.0), 2 mM EGTA (pH 8), 1 mM MgSO4 for 20 minutes. The subsequent steps were performed as described previously (Hayashi et al., 1993). Primary antibody against FTZ-F1 (Murata et al., 1996) was diluted 1:20,000.

Plasmid construction and germ line transformations
A DNA fragment carrying a mutation in the DNA binding domain of FTZ-F1 was generated by PCR using a pair of primers within the white gene (5′-CAATGATATCCAGTGCA-3′) and a mutant primer in the DNA binding domain of FTZ-F1 (Ueda et al., 1992). phs-FTZ-F1, a plasmid containing a βFTZ-F1 cDNA insert in pCaSpeR-hs vector (Murata et al., 1996), was used as a template for PCR mutagenesis and amplification. The amplified fragment was digested with XhoI and inserted between the two XhoI sites of the phs-FTZ-F1. The resulting plasmid was designated as phs-βFTZ-F1mC. P element-mediated germ line transformations and transgenic fly lines, designated hs-βFTZ-F1mC, were established according to Murata et al. (1996).

Heat-shock treatment of the ftz-1 mutant
Embryos produced from y w; ftz-1ex7/TM3, y+ or y w; ftz-1ex7 hs-βFTZ-F1/TM3, y+ parents were collected every 2 hours and subjected to heat-shock at 33°C for 60 minutes at different stages of embryogenesis. The number of hatched larvae were counted. Homozygous animals were identified by the y– marker. To assess rescue of the phenotype during the larval stage, surviving homozygous larvae were collected, transferred to new vials and subjected to heat treatment at 33°C for an hour within 42-47 hours AEL and then within 66-71 hours AEL. Larval stages were distinguished by the morphology of anterior spiracle (Ashburner,
Heterozygous males, y w; ftz-f1/+ lines were collected and heat-shocked at 33.5°C for 1 hour and then reared at 25°C. Viability was calculated by counting the number of animals that had developed to stage P4 or P12 (Bainbridge and Bowles, 1981). For induction of the BR-C transgene in the ftz-f1 mutant, progeny of y w; hs-BR-C ftz-f1 ex7/TM3 y+ female and y w; l(3)03649/TM3 y+ male or y w; hs-BR-C; ftz-f1 ex7/TM3 y+ female and y w; l(3)03649/TM3 y+ male were used. White prepupae of y w; hs-BR-C/+; ftz-f1 ex7/TM3 y+ or y w; hs-BR-C ftz-f1 ex7/TM3 y+ were collected and heat-shocked at 37°C for 30 minutes at 10 hours APF and allowed to recover at 25°C.

**Viability assays to see effect of forced expression of βFTZ-F1**

Heterozygous males, y w; hs-βFTZ-F1/TM3 or y w; hs-βFTZ-F1 m/C/TM3 were crossed with y w females. Synchronized larvae were obtained by allowing the flies to lay eggs for 3 hours and maintained at 25°C. The synchronously developing larvae were heated at 33.5°C for 60 minutes at various larval stages from 21 to 108 hours AEL, and then maintained at 25°C. The dominant bristle shape marker Stubble (Sb) and wing shape marker Serrate (Ser) carried on TM3 were used for discrimination between balancer/+ and hs-βFTZ-F1/+ adults. Two other independent lines carrying the hs-βFTZ-F1 gene were also used, in consideration of the position effect on the expression of the transgene.

**Electron microscopy**

Electron microscopy was performed as described by Kaznowski et al. (1985). Samples were observed at around 80 hours AEL.

**RESULTS**

**Developmental expression profile of βFTZ-F1**

The function of FTZ-F1 during postembryonic development was assessed first by investigating the temporal expression profile of FTZ-F1 in whole animals during the larval, prepupal and early pupal stages by western blotting. As shown in Fig. 1A, expression of the 95-kDa βFTZ-F1 protein was detected from 42 to 51 hours after egg laying (AEL) and from 69 to 75 hours AEL. These periods are around the first and second larval ecdyses. However, it was not clear from this first experiment whether FTZ-F1 is expressed before or after ecdysis because growth of individual larvae in a population is not completely synchronized even under optimal rearing conditions. To determine more precisely the timing of expression of βFTZ-F1 around the second larval ecdysis, western blotting analysis was performed using larvae separated into stages not only by time after egg laying but also by the morphology of the anterior spiracle. As shown in Fig. 1B, βFTZ-F1 was detected strongly in earlier larvae of the second instar population (66-69 hours AEL) but not in later larvae of the second instar population (66-69 hours AEL) or in the third instar larvae (66-69 hours AEL). Furthermore, at 69-72 hours AEL, βFTZ-F1 was not detectable in either second or third instar larvae. These results suggest that βFTZ-F1 expression occurs for a short time during the late stage of each instar and ceases before ecdysis. The difference in the timing of expression of βFTZ-F1 between Fig. 1A,B is presumably due to a slight difference in the growth rate between the y w; hs-βFTZ-F1/+ line and the host strain y w. Indeed, y w larvae reached ecdysis earlier than y w; hs-βFTZ-F1. During the prepupal and early pupal periods, βFTZ-F1 protein was mainly detected 8-12 hours after puparium formation (APF), though a
low level of expression was detected 6-8 hours and 12-16 hours APF (Fig. 1C). This stage is around the transition from the prepupa to pupa. Expression of βFTZ-F1 protein has been observed previously in late-stage embryos (Üeda et al., 1990). From these results, we conclude that expression timing of βFTZ-F1 is strictly restricted.

Spatial expression pattern of βFTZ-F1

The tissue distribution of βFTZ-F1 protein was examined by immunostaining. An antibody against βFTZ-F1 stained the nuclei of most larval tissues at 44-46 hours AEL: for example, the salivary gland (Fig. 2A), fat body (Fig. 2A), trachea (Fig. 2B), ring gland, epidermis, guts and Malpighian tubules (data not shown). Staining of gonads was not detectable (data not shown). Similar nuclear staining patterns were observed in stage-16 embryos, larvae at approximately 72 hours AEL (data not shown) and prepupae at 9 hours APF (e.g. staining of salivary glands shown in Fig. 2E,F). No staining was observed in either prepupal tissues at 4 hours APF (Fig. 2C,D) or larval tissues at 60-63 hours AEL, (data not shown), consistent with the temporal expression profile described above. These results clearly show that βFTZ-F1 is expressed in most tissues during particular stages and that the protein is localized to the nucleus.

Molecular mapping of ftz-f1 mutations

Next, we characterized the genomic organization of the ftz-f1 locus and mapped the ftz-f1 mutation sites (Fig. 3). l(3)03649 has been identified as a P-element induced ftz-f1 mutant. Eggs produced from germline clones of heterozygous female mothers displayed embryonic lethal phenotype (Yu et al., 1997), with a zygotic effect of larval and prepupal lethality. Molecular analysis revealed that the P-element is located near the transcription start site of the βFTZ-F1 gene (Fig. 3). Western blotting analysis showed that the timing of βFTZ-F1 expression was not changed in the mutant but its level is reduced compared with the wild type (data not shown). ftz-f1ex5 and ftz-f1ex7 are embryonic lethal alleles and have been established by a P-element excision from a maternal effect ftz-f1 mutant ftz-f1209 (Guichet et al., 1997) was not mapped in this region. The remaining regions after imprecise excision from ftz-f1209 are indicated by lines. A dashed line represents an uncertain boundary. The position of the P element insertion in the l(3)03649 allele was determined by lines. The deleted regions in excision lines were estimated by Southern blot hybridization using genomic DNA.

The zinc-finger DNA-binding region is indicated by a black box. The open reading frame (tinted boxes) are indicated at the top. Exons of βFTZ-F1 (boxes) are completely deleted and trans-heterozygotes with these alleles. These are thought to be null mutants, because the DNA binding domain and the promoter region of the βFTZ-F1 gene are completely deleted and trans-heterozygotes with l(3)03649 displayed a phenotype indistinguishable from heterozygotes of l(3)03649 over Df(3L)Cat DH104, a deficiency including this region (Guichet et al., 1997).

Rescue of ftz-f1 mutants by temporally restricted expression of hs-βFTZ-F1

In early embryos, maternally supplied αFTZ-F1 is necessary for embryogenesis (Guichet et al., 1997; Yu et al., 1997). On the other hand, expression of βFTZ-F1 in late embryogenesis and the embryonic lethality of ftz-f1ex7 suggested that βFTZ-F1 is necessary for late embryogenesis. To confirm this, we tried to rescue ftz-f1ex7 by expressing βFTZ-F1 under heat-shock control. As shown in Fig. 4, ftz-f1ex7 hs-βFTZ-F1 embryos did not hatch without prior heat treatment. However, when heat-shock was performed 13-16 hours AEL, a time that corresponds to the period of endogenous FTZ-F1 expression, about 40% of the ftz-f1ex7 hs-βFTZ-F1 homozygous embryos hatched. Lethality was not rescued by heat-shock performed at other times or by heat treatment of ftz-f1ex7 without the hs-βFTZ-F1 gene. These results strongly suggest that temporally restricted expression of βFTZ-F1 during late embryogenesis is important for normal embryonic development.
Function of βFTZ-F1 in Drosophila

The rescued ftz-f1 ex7 hs-βFTZ-F1 homozygous first instar larvae survived for a few days, but could not progress to second instar larvae even 24 hours after hatching. Since endogenous FTZ-F1 is expressed just before first instar ecdysis in wild-type animals, mutant larvae were heat-shocked again at 33°C for 60 minutes during the late period of the first instar stage (within 42-47 hours AEL). About 30% of the mutant larvae succeeded in ecdysis and progressed to second instar larvae (Table 1). Again, they survived for a few days, but could not progress to third instar larvae. These larvae had two pairs of mouth hooks, cephalopharyngeal skeletons, anterior and posterior spiracles, double tubular tracheae (Fig. 5A) and cuticle for the third instar in addition to that for the second instar (Fig. 5B), indicating that these larvae prepared the structures appropriate to third instar larvae but failed in the second ecdysis. The rescued second instar larvae progressed to third instar larvae upon heat shock at 33°C for 60 minutes during the late period of the second instar stage (within 66-71 hours AEL), although efficiency of the rescue was very low (2.5% progressed to prepupae). From these results, we conclude that expression of βFTZ-F1 is necessary for larval molting.

**FTZ-F1 is necessary for larval molting**

Table 1. Rescue of the first instar larvae by hs-βFTZ-F1

<table>
<thead>
<tr>
<th>Number of total first instar larvae used in each experiment</th>
<th>Number of living larva after heat treatment</th>
<th>% rescued</th>
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<tr>
<td></td>
<td>Total</td>
<td>1st instar</td>
</tr>
<tr>
<td>ftz-f1 ex7 hs-βFTZ-F1</td>
<td></td>
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</tr>
<tr>
<td>hs</td>
<td>exp. 1</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>exp. 2</td>
<td>188</td>
</tr>
<tr>
<td>nhs</td>
<td>exp. 1</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>exp. 2</td>
<td>71</td>
</tr>
<tr>
<td>yw, hs</td>
<td>exp. 1</td>
<td>60</td>
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</tbody>
</table>

First instar larvae rescued in embryonic stage by heat treatment within 14-16 hours AEL were picked up and heat treated within 42-47 hours AEL. The number of the first or second instar living larvae were scored within 54-59 hours AEL.

hs, heat shocked; nhs, non-heat shocked; exp., experiment.

**Fig. 4.** Stage-specific rescue of ftz-f1 mutants during embryogenesis. Embryos collected every 2 hours from y w; ftz-f1 ex7/TM3, y+, y w; ftz-f1 ex7 hs-βFTZ-F1/TM3, y+, or y w; hs-βFTZ-F1 parents were heat shocked at 33°C for an hour at various developmental times and then incubated at 25°C. Mutant homozygous animals were identified using the y marker.

**Fig. 5.** Morphology of an ftz-f1 mutant during the larval stage. (A) Morphology of the anterior part of the body of the y w; ftz-f1 ex7 hs-βFTZ-F1 animal, which was rescued at embryonic and first instar stages by hs-βFTZ-F1 transgene expression and arrested at the second instar stage. (B-D) Electron micrographs of a cross section of larval cuticle in the ftz-f1 ex7 hs-βFTZ-F1 animal arrested at the second instar stage (B) and in the hs-βFTZ-F1 animal arrested at the second instar stage by premature expression of βFTZ-F1 (C,D). Arrowheads and arrows indicate cuticulin-like layers and dense epicuticle-like layers, respectively. mh, mouse hooks; as, anterior spiracle; cps, cephalopharyngeal skeleton; t, trachea; ep, epicuticle; cu, cuticulin layer; de, dense epicuticle; en, endocuticle. Bar, 1 μm.
βFTZ-F1 in each instar is necessary for the normal molting process. The precise developmental stage of each individual rescued larva varied, which precluded the determination of the critical period of FTZ-F1 expression necessary for larval development.

To assess the importance of temporally restricted expression of βFTZ-F1 during the larval period, we analyzed the effect of forced hs-βFTZ-F1 expression on development of the wild-type animal. Progeny of hs-βFTZ-F1/TM3 males and y w females were heat shocked at various larval stages and the number of eclosed adults carrying the hs-βFTZ-F1 chromosome was compared to those carrying the balancer chromosome. As a control, hs-βFTZ-F1mC, which carries a point mutation disrupting DNA-binding activity (Ueda et al., 1992), was used instead of hs-βFTZ-F1. As shown in Fig. 6, animals carrying the hs-βFTZ-F1mC chromosome became adults as efficiently as those carrying the balancer chromosome (TM3/+) after heat shock, regardless of the specific developmental stage. Basically the same result was obtained when +/TM3 males were used instead of hs-βFTZ-F1mC/TM3 males (data not shown). In sharp contrast to this, we observed a drastic decrease in the number of adults bearing the hs-βFTZ-F1 chromosome when the larvae received a heat shock at around 36 or 60 hours AEL. The critical periods occurred slightly before the time of endogenous βFTZ-F1 expression at 45-48 hours and 69-72 hours AEL, and no endogenous βFTZ-F1 was detectable during these heat-sensitive periods (Fig. 1A).

Animals that were heat shocked during the critical period can survive for at least a few days, but they never progress to the next instar larva stage. These larvae have a similar morphological phenotype to the ftz-f1 mutant larvae, possessing two pairs of mouth hooks, cephalopharyngeal skeletons, anterior and posterior spiracles, double tubular tracheae (data not shown), and cuticle of the third instar in addition to that of the second instar 24 hours after heat shock (81-84 hours AEL). Fig. 5C,D shows typical examples of cuticle in the hs-βFTZ-F1 larvae, which were heat shocked at 57-60 hours AEL and reared at 25°C for 20 hours (77-80 hours AEL). The cuticle consists of two sets of epicuticle layers and lamellar endocuticle layers, indicating that these larvae failed in ecdysis in a similar manner to the ftz-f1 mutant (Fig. 5B).

Furthermore, the epicuticle layer of the third instar larvae was found to be abnormal. The layer was not even, (Fig. 5C) and in some cases was discontinuous (Fig. 5D). The temporally specific effects of forced expression were observed using other independently established hs-βFTZ-F1 lines, indicating that these findings are not the result of position effects of transgene expression. These results suggest that premature expression of βFTZ-F1 causes defects in cuticle formation, and that this, in turn, results in the failure of ecdysis. Normal development of the hs-βFTZ-F1mC line indicates that the effect of premature expression of βFTZ-F1 in the molting stage is achieved through the DNA-binding activity of the factor.

The observed molting defects in the ftz-f1 mutant and in the wild type upon premature expression of βFTZ-F1 demonstrate the importance of the stage-specific expression of the factor during the larval molting process.

Importance of FTZ-F1 expression during the mid-prepupal period

The function of FTZ-F1 during the prepupal period was analyzed using ftz-f1<sup>ex7</sup>/l(3)03649 trans-heterozygotest. Most ftz-f1<sup>ex7</sup>/l(3)03649 trans-heterozygotes died before reaching the prepupal stage, but 3% of them did progress to prepupae under our rearing conditions. The development of the survivors arrested before prepupal stage P4 (Bainbridge and Bownes, 1981), when the air bubble moves from the middle to the anterior position, the pupae withdraw to the posterior end in pupal sacks, mouth hooks are expelled, anterior spiracles separate from dorsal trunks, and the head everts (compare Fig. 7A,B). This arrest was rescued by forced expression of FTZ-F1. Thus, some of the heat-shocked mutant prepupae carrying the hs-βFTZ-F1 gene were able to become pupae (Fig. 7C) and a fraction of them developed until at least stage P12 (Fig. 7F). Efficient rescue was observed when the mutant animals carrying the hs-βFTZ-F1 gene were heat shocked at 6-10 hours APF (Fig. 8). This corresponds to the period of endogenous FTZ-F1 expression. Rescue of pupation after 12 hours APF might be due to a developmental delay in the mutant. These results indicate the importance of FTZ-F1 expression during the mid- to late prepupal period for the prepupa-to-pupa transition.

We also examined the effects of βFTZ-F1 expression in the wild type during various developmental stages around the prepupal period (Fig. 8B). Heat treatment during the mid- to late prepupal period and early pupal period had no effect on development of the hs-βFTZ-F1 line. However, heat treatment before 4 hours APF arrested development before stage P4,
although the host strain was not affected by heat treatment in these early stages. Premature expression of bFTZ-F1mC also did not affect development (data not shown). These results indicate that premature expression of bFTZ-F1 disrupts pupation through its binding to DNA. From the results of the rescue experiments and the effect of premature expression, we conclude that temporally restricted expression of FTZ-F1 is essential for the prepupa-to-pupa transition.

**Role of FTZ-F1 on metamorphic changes in various organs**

Larval salivary glands are generally histolyzed about 15 hours APF in *Drosophila melanogaster* (Jiang et al., 1997). In the *ftz-f1* mutant during metamorphosis, (A) *ftz-f1*^ex7 hs-βFTZ-F1/FT1TM3, y+ at 24 hours APF. (B) *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygote at 24 hours APF. (C) *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygote at 24 hours APF rescued by heat treatment at 8 hours APF. (D) *ftz-f1*^ex7 hs-βFTZ-F1/FT1TM3, y+ at 4 days APF. (E) *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygote at 4 days APF rescued by heat treatment at 6 hours APF. (G) Salivary glands of the *ftz-f1*^ex7/l(3)03649 trans-heterozygote at 24 hours APF. (H) CNS of the *ftz-f1*^ex7/l(3)03649 trans-heterozygote at 24 hours APF. (A) Rescue of the developmentally arrested *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygotes in prepupal stage by heat treatment. (B) Developmental arrest in the *ftz-f1*^ex7/l(3)03649 trans-heterozygotes, hs-βFTZ-F1+/+ and y w by heat treatment. Staged animals at 25°C were heat shocked at 33.5°C for an hour and then reared at 25°C. Efficiency of the developmental arrest or rescue was observed one day or five days APF.

**Fig. 7.** Morphology of the *ftz-f1* mutant during metamorphosis. (A) *ftz-f1*^ex7 hs-βFTZ-F1/FT1TM3, y+ at 24 hours APF. (B) *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygote at 24 hours APF. (C) *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygote at 24 hours APF rescued by heat treatment at 8 hours APF. (D) *ftz-f1*^ex7 hs-βFTZ-F1/FT1TM3, y+ at 4 days APF. (E) *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygote at 4 days APF rescued by heat treatment at 6 hours APF. (G) Salivary glands of the *ftz-f1*^ex7/l(3)03649 trans-heterozygote at 24 hours APF. (H) CNS of the *ftz-f1*^ex7/l(3)03649 trans-heterozygote at 24 hours APF.

**Fig. 8.** Importance of timing of expression for rescue of the *ftz-f1* mutant in early metamorphosis. (A) Rescue of the developmentally arrested *ftz-f1*^ex7 hs-βFTZ-F1/FT1l(3)03649 trans-heterozygotes in prepupal stage by heat treatment. (B) Developmental arrest in the *ftz-f1*^ex7/l(3)03649 trans-heterozygotes, hs-βFTZ-F1+/+ and y w by heat treatment. Staged animals at 25°C were heat shocked at 33.5°C for an hour and then reared at 25°C. Efficiency of the developmental arrest or rescue was observed one day or five days APF.

**ftz-f1**^ex7/l(3)03649 trans-heterozygotes, however, larval salivary glands were still present even 24 hours APF (Fig. 7G). The morphology of the central nervous system undergoes drastic changes around the time of pupation. In the mutant, the optic lobes did not expand appropriately, and the subesophageal neuromeres and the thoracic neuromeres did not separate (Fig. 7H). Arrested development was also manifested as leg elongation, edge constriction of the wing disc, and morphological changes in the midgut and Malpighian tubules (data not shown). These results suggest that βFTZ-F1 is necessary for development of many organs during the early stages of metamorphosis. Many organs were affected, as expected from the ubiquitous expression pattern of βFTZ-F1. However, we cannot exclude the possibility that these effects...
Table 2. Rescue of salivary gland histolysis by expression of BR-C under control of the heat shock promoter

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Heat shock</th>
<th>Total number of animals examined</th>
<th>Persistent (%)</th>
<th>Not persistent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No transgene</td>
<td>nhs</td>
<td>6</td>
<td>100 (6)</td>
<td>0 (0)</td>
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<tr>
<td></td>
<td>hs</td>
<td>11</td>
<td>100 (11)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Z1‡</td>
<td>nhs</td>
<td>8</td>
<td>100 (8)</td>
<td>0 (0)</td>
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<tr>
<td></td>
<td>hs</td>
<td>15</td>
<td>27 (4)</td>
<td>73 (11)</td>
</tr>
<tr>
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<td>nhs</td>
<td>13</td>
<td>92 (12)</td>
<td>8 (1)</td>
</tr>
<tr>
<td></td>
<td>hs</td>
<td>16</td>
<td>50 (8)</td>
<td>50 (8)</td>
</tr>
<tr>
<td>Z3</td>
<td>nhs</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>0 (0)</td>
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<tr>
<td></td>
<td>hs</td>
<td>13</td>
<td>92 (12)</td>
<td>8 (1)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent the number of animals that showed the indicated phenotype at 24 hours APF.
‡Transgene encoding Tn-Q1-Q2-Z1 isoform was used in this experiment.
A similar result was obtained by expressing Q1-Q2-Z1 isoform (data not shown).
hs, heat shocked; nhs, non-heat shocked.

DISCUSSION

Temporally restricted expression of \(\beta\)FTZ-F1

Prior to the present study, precise temporal expression profiles of \(\beta\)FTZ-F1 were poorly analyzed; the factor had been identified as present during late embryogenesis by gel mobility-shift analysis (Ueda et al., 1990) and its mRNA was found to be expressed 6-10 hours into prepupal development (Andres et al., 1993; Lavorgna et al., 1993). This paper provides a comprehensive survey of the expression of \(\beta\)FTZ-F1 protein during postembryonic development. The results obtained show that the factor is transiently expressed slightly before each larval ecdysis, and before pupation. Ecdysteroid peaks are present at the middle of embryogenesis and each larval instar, and a strong ecdysteroid pulse, which triggers puparium formation and metamorphosis, peaks slightly before puparium formation (Riddiford, 1993). The timing of expression of \(\beta\)FTZ-F1 was always found to follow the ecdysteroid peak (Fig. 9). In the silkworm \(B.\) mori, BmFTZ-F1 mRNA was detected just before both larval-larval and larval-pupal ec dyses and eclosion. These BmFTZ-F1-positive periods also follow the ecdysteroid pulse, and BmFTZ-F1 mRNA can be induced in cultured silk glands by a 6-hour exposure to 20-hydroxyecdysone followed by 6 hours in hormone-free medium (Sun et al., 1994). Furthermore, \(\beta\)FTZ-F1 mRNA is induced in cultured organs of prepupae at 0 or 6 hours APF in a low-hormone medium (Woodard et al., 1994). All these observations indicate that \(\beta\)FTZ-F1 and BmFTZ-F1 belong to a class of unique transcription factors whose expression is regulated in a temporally specific manner governed by ecdysteroid titer.

Importance of temporally restricted expression of \(\beta\)FTZ-F1

We show here that temporally restricted expression of \(\beta\)FTZ-F1 during mid- to late embryogenesis and early metamorphosis is necessary to rescue the ftz-f1 mutant. Furthermore, we show of specific isoforms of \(\beta\)C can work downstream of ftz-f1 in the pathway responsible for salivary gland histolysis.
that premature expression of βFTZ-F1 at specific periods during postembryonic development is lethal, even though forced expression has no obvious effect when performed during any other stage of development (Fig. 9). The critical periods occur close to the ecdysteroid peak and before the time of endogenous βFTZ-F1 expression in all three developmental stages: late first instar, late second instar and early prepupa. These observations suggest a relationship between the ecdysone-induced cascade and the lethal phase. Several mechanisms can be envisaged to explain the temporally specific effect of forced βFTZ-F1 expression. First, co-expression of the target genes for βFTZ-F1 and some ecdysone-inducible genes that are normally expressed earlier than βFTZ-F1 may disturb development. Second, βFTZ-F1 may repress some ecdysone-inducible genes that are normally expressed earlier than βFTZ-F1. Premature repression of these genes may result in the developmental defects. Third, untimely induction of the target genes for βFTZ-F1 may be always detrimental, but cofactor(s) or conditions required for the action of βFTZ-F1 may be present before or during the expression of endogenous βFTZ-F1 and then disappear. In this case, ecdysone may induce the cofactor(s) or competence for βFTZ-F1 function. Finally, premature expression may affect expression of the endogenous FTZ-F1 gene. Woodard et al. (1994) suggested that βFTZ-F1 expression represses its own gene. However, this possibility appears to be unlikely because most FTZ-F1 expressed from the transgene was found to disappear within 3 hours and expression of the endogenous FTZ-F1 gene was observed in heat-treated animals that underwent developmental arrest during molting or the prepupal period (H. U., unpublished observation).

**Function of FTZ-F1 in cuticle formation**

The insect cuticle is composed of layers of film. Sequential formation of different layers, cuticle, epicuticle and endocuticle, is observed beginning approximately 12 hours before the next ec dysis (Fristrom and Fristrom, 1993; Kaznowski et al., 1985). βFTZ-F1 is expressed after a new epicuticle layer for the next instar appears. Premature expression of βFTZ-F1 induced disruption of the epicuticle. These observations highlight the importance of βFTZ-F1 in the formation of normal cuticle structure and suggest that some of the target genes of βFTZ-F1 are involved in the process of cuticle formation. In particular, the importance of the timing of expression of these genes is demonstrated. It has been shown that some pupal cuticle proteins are expressed in a stage-specific manner during prepupal periods (Doctor, 1985; Apple and Fristrom, 1991; Wolfgang et al., 1994). We have shown that βFTZ-F1 regulates the EDG78E and EDG84A genes, which encode putative pupal cuticle proteins (Murata et al., 1996). These observations suggest that βFTZ-F1 is responsible for the stage-specific expression of cuticle proteins during the prepupal stage.

**Phenotype of the ftz-f1 mutant**

The demonstration of developmental arrest in the ftz-f1 mutants and its rescue by forced expression of βFTZ-F1 indicate that the expression of βFTZ-F1 during mid- to late embryogenesis, larval molting and early metamorphosis is important for the progression of each developmental stage. Some of the observed phenotypes at the prepupa-to-pupa transition, i.e. failure of head eversion, histolysis of salivary glands and leg elongation, are consistent with phenotypes reported for another ftz-f1 mutant (Broadus et al., 1999). In addition to these phenotypes, the mutant we analyzed showed defects in morphogenesis of the CNS, midgut, wing and Malpighian tubules that have not been reported previously. This is probably due to differences between the mutants. The hypomorphic mutant used in this study is thought to be more severely affected than that reported previously, since it was found to completely arrest at stage P3, while most animals bearing the latter mutation developed to become pharate adults.

Mutants of various ecdysteroid-regulated genes present similar phenotypes to the ftz-f1 mutant. EcR mutants and a DHR3 mutant are embryonic lethal, and larval molting defects are observed in EcR-B isof orm-specific mutants, conditionally rescued EcR null mutants and a up5 mutant (Carney et al., 1997; Hall and Thummel, 1998; Li and Bender, 2000; Schubiger et al., 1998). We postulate that part of the phenotype can be attributed to misregulation of the FTZ-F1 gene. EcR, up5, BR-C, E74A and E74B mutants show defects during the late third instar and prepupal periods (Bender et al., 1997; Kiss et al., 1988; Fletcher and Thummel, 1995). Most of the phenotypes of these mutants appear before stage P3, which is earlier than the βFTZ-F1 expression during the prepupal period, as expected from the timing of expression of these factors (Andres et al., 1993). Some BR-C mutants show phenotypes quite similar to the ftz-f1 mutant. Histolysis of salivary glands is arrested in an rhp mutant of the BR-C locus and separation of the CNS between the subesophageal neuromeres and the thoracic neuromeres does not occur in the BR-C mutants (Restifo and White, 1991, 1992). The BR-C gene is expressed around pupariation and also during the late prepupal period. The latter expression is dependent on βFTZ-F1 in addition to the ecdysteroid pulse (Woodard et al., 1994), and BR-C mRNA is drastically reduced during the late prepupal period in the ftz-f1 mutant (Broadus et al., 1999). We showed that efficient degradation of larval salivary glands occurs upon ectopic expression of the Z1 isof orm of BR-C during the late prepupal period in the ftz-f1 mutant. This result is consistent with the abundant expression of the Z1 isof orm in the salivary glands (Emery et al., 1994) and with failure of salivary gland histolysis in an rhp mutant (Restifo and White, 1992), because rhp+ function is supported by the Z1 isof orm of BR-C (Bayer et al., 1997). This result also suggests a BR-C+ function downstream of ftz-f1 in the process of salivary gland cell death.

A large ecdysteroid pulse is present during the early to middle pupal stages, followed by a decline in ecdysteroid titer which results in eclosion. Preliminary western blot experiments indicated that βFTZ-F1 is also expressed during the late pupal stage, although the precise timing of expression has not been determined yet (H. U., unpublished observation). Expression of BmFTZ-F1 mRNA just before eclosion is also observed in the silkworm. Significant portions of the ftz-f1ex7 hs-βFTZ-F1/1/3J03649 trans-heterozygotes rescued at the prepupal stage by heat treatment developed at least until stage P12, but failed to eclose. These results suggest that βFTZ-F1 expression during the late pupal stage is necessary for late pupal development.

In summary, βFTZ-F1 is expressed in highly restricted periods of development and plays an important role in late embryogenesis, the molting process and early metamorphosis by regulating its target genes.
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