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

Masa-aki Yamada[1], Takehide Murata[1,*], Susumu Hirose[1], Giovanni Lavorgna[2,‡], Emiko Suzuki[3,4] and Hitoshi Ueda[1,§]

[1]Department of Genetics, The Graduate University for Advanced Studies, and Department of Developmental Genetics, National Institute of Genetics, Mishima, Shizuoka-ken 411-8540, Japan
[2]Laboratory of Molecular Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA
[3]Department of Fine Morphology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[4]CREST, Japan Science and Technology Corporation
*Present address: Biogenic Resources Center, RIKEN Tsukuba Institute, Koyadai 3-1-1, Tsukuba, Ibaraki 305-0074, Japan
‡Present address: Instituto Scientifico H San Raffaele, DIBIT, Via Olgettina, 58, 20132 Milano, Italy
§Author for correspondence (e-mail: hueda@lab.nig.ac.jp)

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, intermolt 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- prepupal period, concomitant with βFTZ-F1 expression and mRNA production (Lavorgna et al., 1993; Murata et al., 1996). The importance of *ftz-f1* function during the early metamorphic period has been demonstrated by using a hypomorphic *ftz-f1* 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 prepupal 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 prepupal 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 βFTZ-F1 and analyzed the function of *ftz-f1* 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 *y¹ Df(1)w⁶⁷ᶜ¹*. All stocks not explicitly described in the text are described elsewhere (Lindsley and Zimm, 1992). *hsL332* (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-f1ᵉˣ⁵* and *ftz-f1ᵉˣ⁷*, P-element excision lines from *ftz-f1²⁰⁹*, were obtained from Dr Antoine Guichent. *l(3)03649* 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-f1ᵉˣ⁷* 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 glycine 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′-CAATGATATCCAGTGCAG-3′) and a mutated 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 *Xho*I and inserted between the two *Xho*I 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-f1* mutant
Embryos produced from *y w; ftz-f1ᵉˣ⁷/TM3, y⁺* or *y w; ftz-f1ᵉˣ⁷ 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 embyogenesis. 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,

1989). For prepupal period analysis, white prepupae of the host strain *y w*, *y w*; *ftz-f1^{ex7}/l(3)03649* or *y w*; *ftz-f1^{ex7} hs-βFTZ-F1/l(3)03649* or *y w*; *hs-βFTZ-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 Bownes, 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}/l(3)03649* or *y w*; *hs-BR-C ftz-f1^{ex7}/l(3)03649* 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-F1mC/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

---

Fig. 1. Temporal expression patterns of βFTZ-F1 detected by western blot during the larval, prepupal and early pupal stages. (A) Larval stage in the *hs-βFTZ-F1/+* line. (B) Larval stage around second ecdysis in the *y w* line. (C) Prepupal and early pupal stages in the *hs-βFTZ-F1/+* line. Time (hours AEL) and larval instars are shown at the top of A and B. Samples of larvae in food (F) and wandering larvae (W) at 102-117 hours AEL were loaded in different lanes. Time (hours) relative to puparium formation is shown at the top of C. Positions of arrowheads represent the 95-kDa βFTZ-F1 protein (Lavorgna et al., 1993; Ueda et al., 1990). Growth speed is slightly different between *y w* and *hs-βFTZ-F1/+* lines. The latter line was used in A and C for comparison with the data shown in Fig. 6. Similar results were obtained with the *hs-βFTZ-F1/+* line and the host strain *y w*. Two bands at 38 kDa and 110 kDa are thought to be unrelated proteins that crossreact with the antibody, because they were not detected in the nuclear extract (data not shown).

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
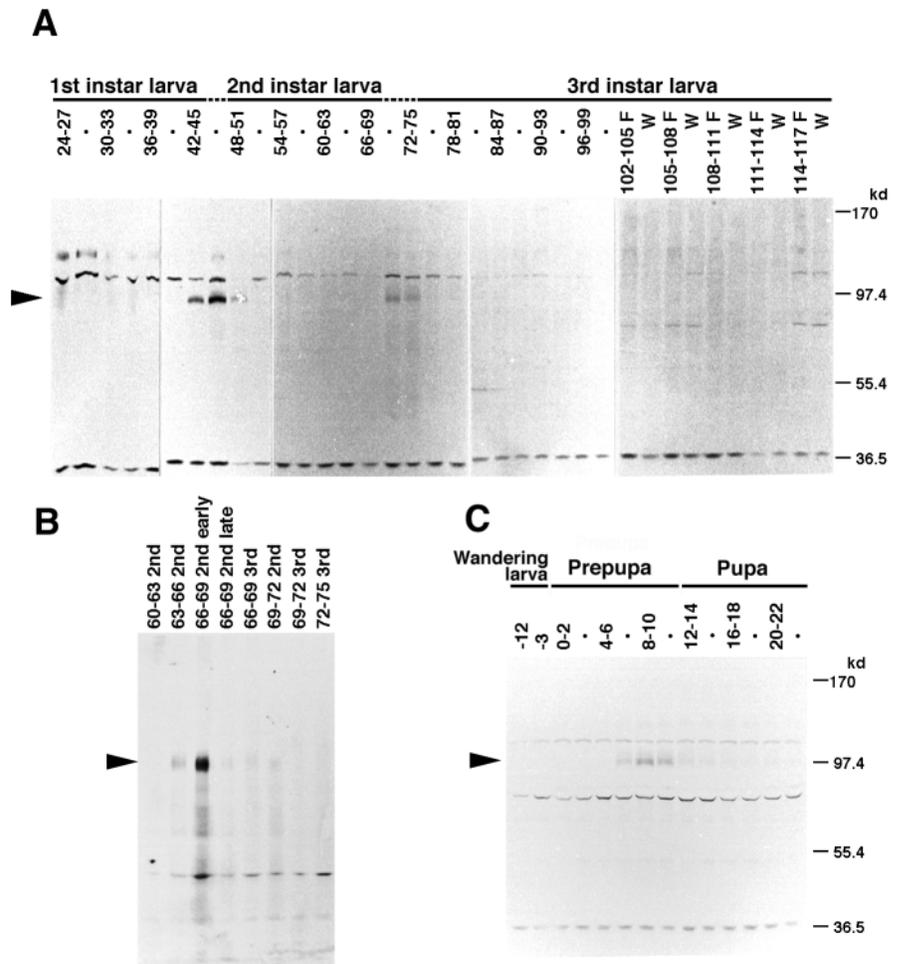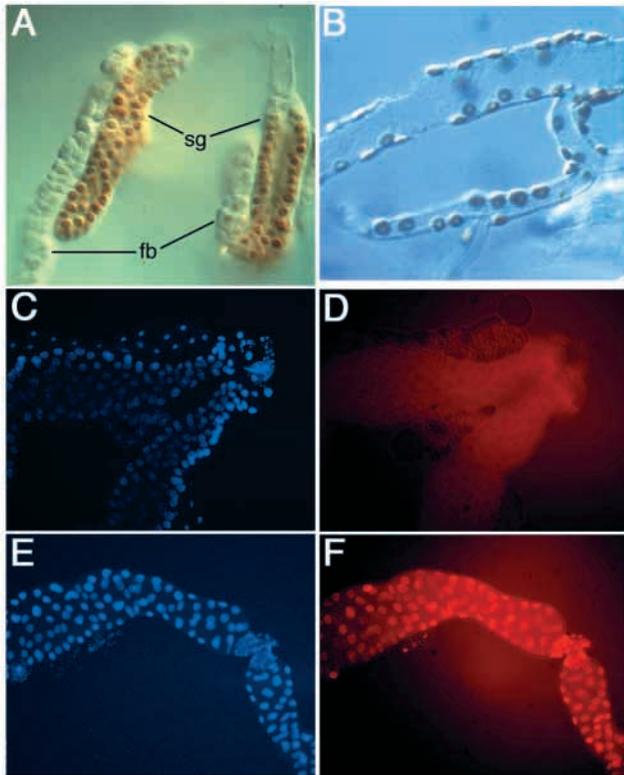
Fig. 2. Localization of FTZ-F1 protein to the nuclei of various tissues. (A) Salivary glands (sg) and fat bodies (fb) at the end of the first instar. (B) Trachea at the end of the first instar. (C,D) Salivary glands at 4 hours APF. (E,F) Salivary glands at 9 hours APF. (A,B) DAB staining with FTZ-F1 antibody, (C,E) DAPI staining, (D,F) Cy3 staining with FTZ-F1 antibody.

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 (Ueda 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*
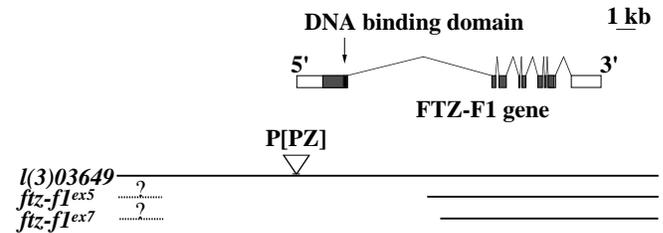

Fig. 3. Molecular map of the *ftz-f1* locus. Exons of βFTZ-F1 (boxes) and the open reading frame (tinted boxes) are indicated at the top. The zinc-finger DNA-binding region is indicated by a black box. The position of the P element insertion in *l(3)03649* allele is shown by a triangle. The position of the P element insertion in *ftz-f1^{209}* (Guichet et al., 1997) was not mapped in this region. The remaining regions after imprecise excision from *ftz-f1^{209}* 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 PCR. The deleted regions in excision lines were estimated by Southern blot hybridization using genomic DNA.

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-f1^{ex5}* and *ftz-f1^{ex7}* are embryonic lethal alleles and have been established by a P-element excision from a maternal effect *ftz-f1* mutant *ftz-f1^{209}* (Guichet et al., 1997). Endpoints of the 3′ side in these deletions were mapped to the first intron, but identification of the 5′ endpoint was not possible due to the lack of a genomic clone covering this region (Fig. 3). Therefore, it is possible that other genes could be also deleted in 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-f1^{ex7}* suggested that βFTZ-F1 is necessary for late embryogenesis. To confirm this, we tried to rescue *ftz-f1^{ex7}* by expressing βFTZ-F1 under heat-shock control. As shown in Fig. 4, *ftz-f1^{ex7} 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-f1^{ex7} hs-βFTZ-F1* homozygous embryos hatched. Lethality was not rescued by heat-shock performed at other times or by heat treatment of *ftz-f1^{ex7}* 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.
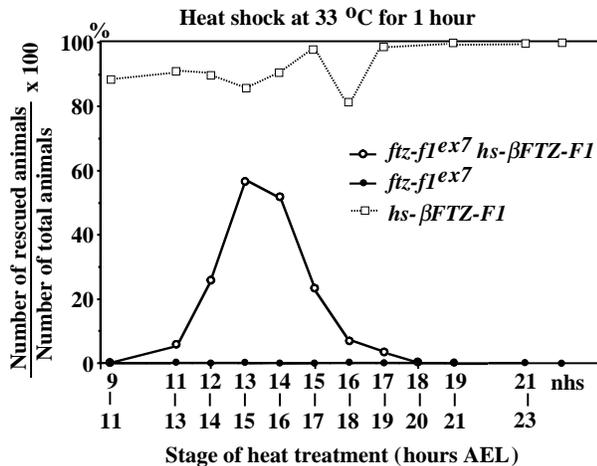
Fig. 4. Stage-specific rescue of *ftz-f1* mutants during embryogenesis. Embryos collected every 2 hours from *y w*; *ftz-f1^ex7^/TM3, y+, y w*; *ftz-f1^ex7^ hs-βFTZ-F1/TM3, y+*, or *y w; hs-βFTZ-F1* parents were heat shocked at 33°C for an hour at various developmental times and then incubated at 25°C. Mutant homozygous animals were identified using the *y* marker.

FTZ-F1 is necessary for larval molting

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, cepharopharyngeal 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
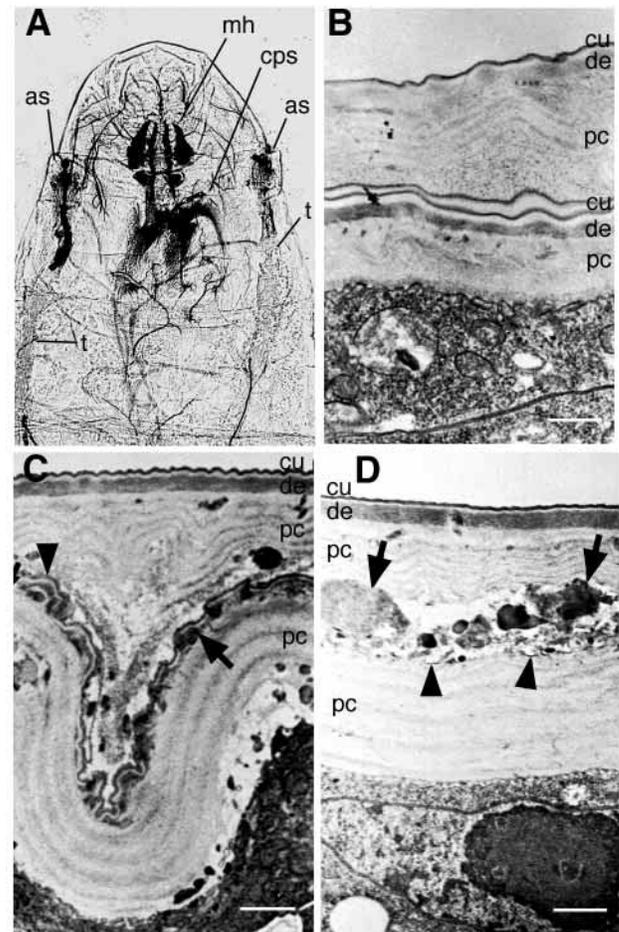

Fig. 5. Morphology of an *ftz-f1* mutant during the larval stage. (A) Morphology of the anterior part of the body of the *y w*; *ftz-f1^ex7^ hs-βFTZ-F1* animal, which was rescued at embryonic and first instar stages by *hs-βFTZ-F1* transgene expression and arrested at the second instar stage. (B-D) Electron 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 cuticlin-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.

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

			Number of living larva after heat treatment			% rescued	
		Number of total first instar larvae used in each experiment	Total	1st instar	2nd instar	Living larvae	Total larvae
ftz-f1^ex7^ hs-βFTZ-F1							
hs	exp. 1	145	44	12	32	73	22
	exp. 2	188	93	30	63	68	34
nhs	exp. 1	55	32	32	0	0	0
	exp. 2	71	51	51	0	0	0
yw, hs	exp. 1	60	47	0	47	100	78

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.

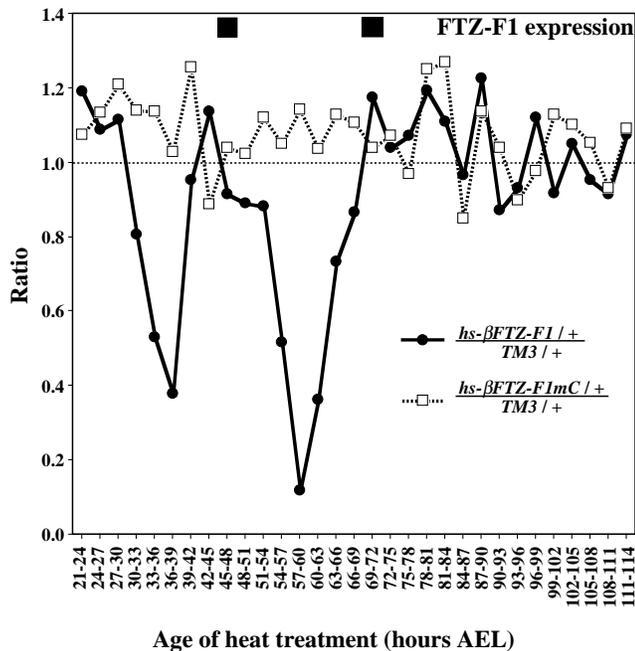hs, heat shocked; nhs, non-heat shocked; exp., experiment.

Fig. 6. Effects of forced expression of βFTZ-F1 during larval
development. Staged larvae at 25°C were heat shocked at 33.5°C for
an hour and then reared at 25°C. Shown is the ratio of the number of
hs-βFTZ-F1/+ flies emerged to that of balancer/+ flies in the progeny
from *hs-βFTZ-F1*/balancer males and +/+ females. As a control, the
hs-βFTZ-F1mC line, which carries a mutation in the DNA binding
domain, was used instead of *hs-βFTZ-F1*. Developmental stages
(hours AEL) at which larvae received the heat shock at 33.5°C are
shown at the bottom. Black boxes at the top represent the
developmental periods during which high levels of endogenous
βFTZ-F1 are detected.

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 occur 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, cepharopharyngeal
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-f1ex7*/*l(3)03649* trans-heterozygotes. Most
ftz-f1ex7/*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
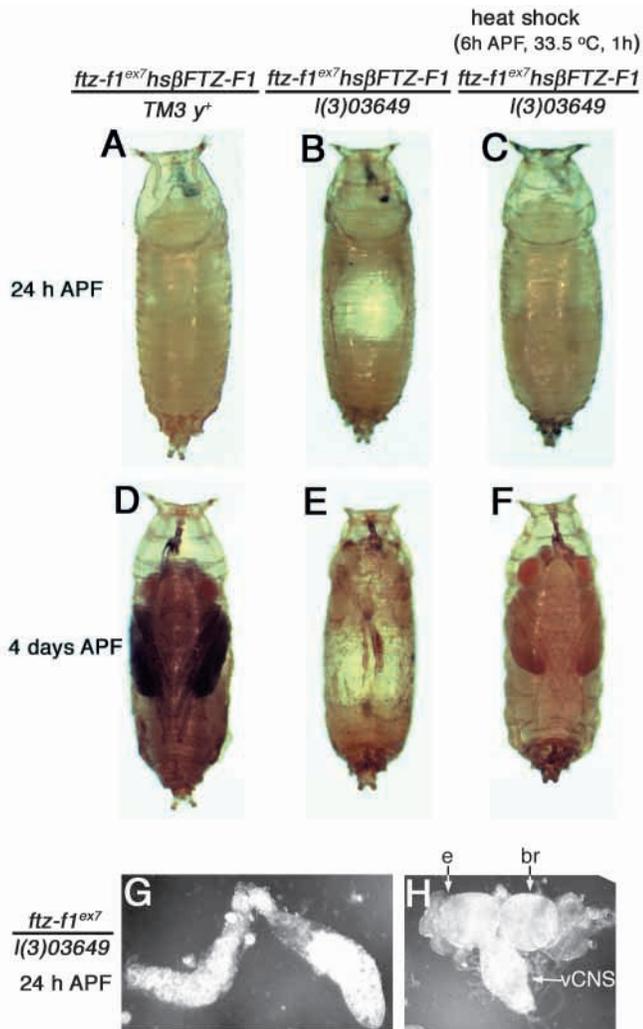before 4 hours APF arrested development before stage P4,

heat shock
(6h APF, 33.5 °C, 1h)

$\dfrac{ftz\text{-}f1^{ex7}hs\beta FTZ\text{-}F1}{TM3\ y^+}$ $\dfrac{ftz\text{-}f1^{ex7}hs\beta FTZ\text{-}F1}{l(3)03649}$ $\dfrac{ftz\text{-}f1^{ex7}hs\beta FTZ\text{-}F1}{l(3)03649}$

24 h APF

4 days APF

$\dfrac{ftz\text{-}f1^{ex7}}{l(3)03649}$
24 h APF

e   br

vCNS

Fig. 7. Morphology of the *ftz-f1* mutant during metamorphosis. (A) *ftz-f1^{ex7} hs-βFTZ-F1/TM3, y+* at 24 hours APF. (B) *ftz-f1^{ex7} hs-βFTZ-F1/l(3)03649* trans-heterozygote at 24 hours APF. (C) *ftz-f1^{ex7} hs-βFTZ-F1/l(3)03649* trans-heterozygote at 24 hours APF rescued by heat treatment at 8 hours APF. (D) *ftz-f1^{ex7} hs-βFTZ-F1/TM3, y+* at 4 days APF. (E) *ftz-f1^{ex7} hs-βFTZ-F1/l(3)03649* trans-heterozygote at 4 days APF. (F) *ftz-f1^{ex7} hs-βFTZ-F1/l(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. br, brain; e, eye disc; vCNS, ventral CNS.

although the host strain was not affected by heat treatment in these early stages. Premature expression of βFTZ-F1mC also did not affect development (data not shown). These results indicate that premature expression of βFTZ-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-*
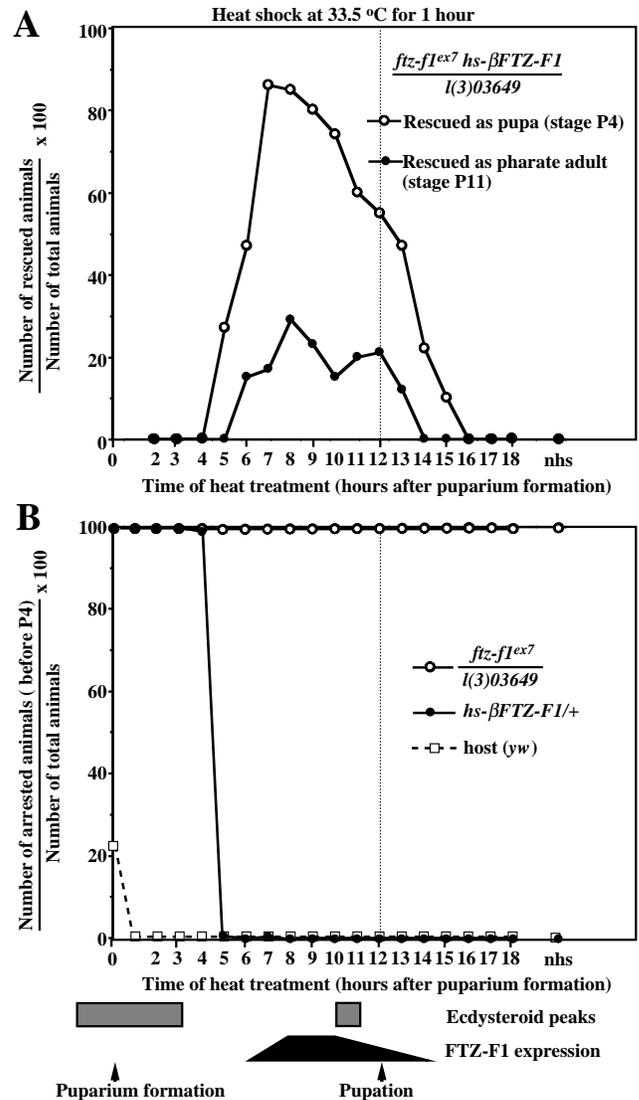

Fig. 8. Importance of timing of expression for rescue of the *ftz-f1* mutant in early metamorphosis. (A) Rescue of the developmentally arrested *ftz-f1^{ex7} hs-βFTZ-F1/l(3)03649* trans-heterozygotes in prepupal stage by heat treatment. (B) Developmental arrest in the *ftz-f1^{ex7}/l(3)03649* trans-heterozygotes, *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.

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 Malpigian 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

Transgene	Heat shock	Total number of animals examined	Persistent (%)*	Not persistent (%)*
No transgene	nhs	6	100 (6)	0 (0)
	hs	11	100 (11)	0 (0)
Z1‡	nhs	8	100 (8)	0 (0)
	hs	15	27 (4)	73 (11)
Z2	nhs	13	92 (12)	8 (1)
	hs	16	50 (8)	50 (8)
Z3	nhs	-	-	-
	hs	7	100 (7)	0 (0)
Z4	nhs	9	100 (9)	0 (0)
	hs	13	92 (12)	8 (1)

*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.

are not due to cell-autonomous responses but are caused by indirect responses through the endocrine system.

Broad Complex acts downstream of *ftz-f1* in the histolysis of salivary glands

Some of the observed phenotypes in the *ftz-f1* mutant during the early pupal stage are similar to those in *BR-C* mutants. Since *BR-C* expression during the late prepupal period depends on FTZ-F1 expression (Woodard et al., 1994; Broadus et al., 1999), we tried to rescue the *ftz-f1* mutant phenotype by forced expression of *BR-C*. When the Z1 isoform of *BR-C* was expressed under heat-shock control at 10 hours APF, larval salivary glands disappeared in most of the animals by 24 hours APF (Table 2), although other *ftz-f1* mutant phenotypes were not found to be rescued. On the other hand, when the Z2 isoform was expressed under control of the heat-shock promoter in the *ftz-f1* mutant, salivary glands remained present in half of the animals. When Z3 or Z4 isoform was overexpressed, salivary glands generally persisted. These results suggest that expression

of specific isoforms of *BR-C* can work downstream of *ftz-f1* in the pathway responsible for salivary gland histolysis.

DISCUSSION

Temporally restricted expression of βFTZ-F1

Prior to the present study, precise temporal expression profiles of βFTZ-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 βFTZ-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 βFTZ-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 ecdyses 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, βFTZ-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 βFTZ-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 βFTZ-F1

We show here that temporally restricted expression of βFTZ-F1 during mid- to late embryogenesis and early metamorphosis is necessary to rescue the *ftz-f1* mutant. Furthermore, we show

Fig. 9. Timing of events correlated with βFTZ-F1 expression in the embryo (A), larva (B) and prepupa to early pupa (C). Timing of ecdysteroid pulses (Riddiford, 1993) (black boxes), timing of βFTZ-F1 expression (gray boxes), critical periods for rescue (shaded boxes) and critical periods for generating the premature expression phenotype (open boxes) are indicated. Expression of βFTZ-F1 occurs a few hours after the ecdysteroid pulse in embryos, each instar larva and prepupae. Rescue of *ftz-f1* mutants is possible when βFTZ-F1 is induced from a transgene during the periods of endogenous βFTZ-F1 expression in wild type. Forced expression of βFTZ-F1 is deleterious when it is expressed slightly before endogenous expression.
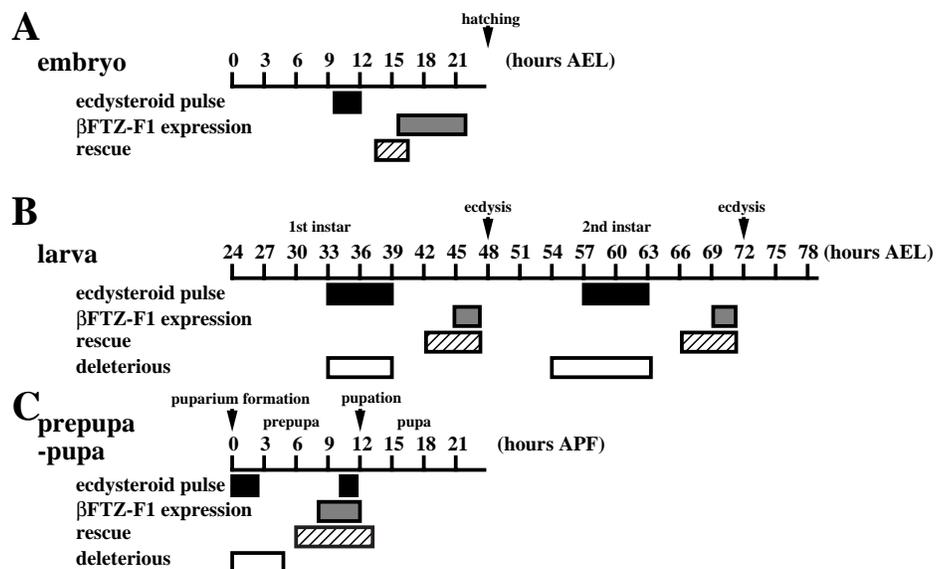
that premature expression of βFTZ-F1 at specific periods during postembryonic development is lethal, even though forced expression has no obvious effect when performed during any other stage of development (Fig. 9). The critical periods occur close to the ecdysteroid peak and before the time of endogenous 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, cuticlin, epicuticle and endocuticle, is observed beginning approximately 12 hours before the next ecdysis (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* isoform-specific mutants, conditionally rescued *EcR* null mutants and a *usp* 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*, *usp*, *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 *rbp* mutant of the *BR-C* locus and separation of the CNS between the subesophgeal 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 isoform of BR-C during the late prepupal period in the *ftz-f1* mutant. This result is consistent with the abundant expression of the Z1 isoform in the salivary glands (Emery et al., 1994) and with failure of salivary gland histolysis in an *rbp* mutant (Restifo and White, 1992), because *rbp+* function is supported by the Z1 isoform 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/l(3)03649* 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.

REFERENCES

Andres, A. J., Fletcher, J. C., Karim, F. D. and Thummel, C. S. (1993). Molecular analysis of the initiation of insect metamorphosis: A comparative study of *Drosophila* ecdysteroid-regulated transcription. *Dev. Biol.* **160**, 388-404.

Andres, A. J. and Thummel, C. S. (1992). Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. *Trends Genet.* **8**, 132-138.

Apple, R. T. and Fristrom, J. W. (1991). 20-hydroxyecdysone is required for, negatively regulates, transcription of *Drosophila* pupal cuticle protein genes. *Dev. Biol.* **146**, 569-582.

Ashburner, M. (1972). Patterns of puffing activity in the salivary gland chromosome of *Drosophila* VI. Induction by ecdysone in salivary glands of *D. melanogaster* cultured *in vitro. Chromosoma* **38**, 255-281.

Ashburner, M. (1989). Drosophila: *A Laboratory Handbook.* Cold Spring Harbor Laboratory Press, New York.

Ashburner, M., Chihara, C., Meltzer, P. and Richards, G. (1974). Temporal control of puffing activity in polytene chromosome. *Cold Spring Harbor Symp. Quant. Biol.* **38**, 655-662.

Bainbridge, S. P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. Exp. Morph.* **66**, 57-80.

Bayer, C. A., Kalm, L. v. and Fristrom, J. W. (1997). Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the *Broad-Complex* during *Drosophila* metamorphosis. *Dev. Biol.* **187**, 267-282.

Becker, H. J. (1959). Die puffs der speicheldrüsenchromosomen von *Drosophila* melanogaster. *Chromosoma* **10**, 654-678.

Bender, M., Imam, F. B., Taibot, W. S., Ganetzky, B. and Hogness, D. S. (1997). Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91**, 777-788.

Broadus, J., McCabe, J. R., Endrizzi, B., Thummel, C. S. and Woodard, C. T. (1999). The *Drosophila* βFTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell* **3**, 143-149.

Carney, G.E., Wade, A.A., Sapra, R., Goldstein, E.S. and Bender, M. (1997). DHR3, an ecdysone-inducible early-late gene encoding a *Drosophila* nuclear receptor, is required for embryogenesis. *Proc. Natl. Acad. Sci. USA* **94**, 12024-12029.

Doctor, J., Fristrom, D. and Fristrom, J. W. (1985). The pupal cuticle of *Drosophila*; Biphasic synthesis of pupal cuticle proteins in vivo and in vitro in response to 20-hydroxyecdysone. *J. Cell Biol.* **101**, 189-200.

Emery, I. F., Bedian, V. and Guild, G. M. (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* **120**, 3275-3287.

Fletcher, J. C. and Thummel, C. S. (1995). The *Drosophila E74* gene is required for the proper stage-and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. *Development* **121**, 1411-1421.

Fristrom, D. and Fristrom, J. W. (1993). The Metamorphic Development of the Adult Epidermis. In *The Development of* Drosophila melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 843-898. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Guichet, A., Copeland, J. W. R., Erdély, M., Hlousek, D., Závorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H. M. and Ephrussi, A. (1997). The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature* **385**, 548-552.

Hall, B. L. and Thummel, C. S. (1998). The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. *Development* **125**, 4709-4717.

Hayashi, S., Hirose, S., Metcalfe, T. and Shirras, A. (1993). Control of imaginal cell development by the *escargot* gene of *Drosophila*. *Development* **118**, 105-115.

Jiang, C., Baehrecke, E. H. and Thummel, C. S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* **124**, 4673-4683.

Karim, F. D. and Thummel, C. S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J.* **11**, 4083-4093.

Kaznowski, C. E., Schneiderman, H. A. and Bryant, P. J. (1985). Cuticle secretion during larval growth in *Drosophila melanogaster*. *J. Insect. Physiol.* **31**, 801-813.

Kiss, I., Beaton, A. H., Tardiff J., Fristrom, D. and Fristrom, J. W. (1988). Interaction and developmental effects of mutations in the *Broad-Complex* of the *Drosophila melanogaster*. *Genetics* **118**, 247-259.

Lavorgna, G., Karim, F. D., Thummel, C. S. and Wu, C. (1993). Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl. Acad. Sci. USA* **90**, 3004-3008.

Lavorgna, G., Ueda, H., Clos, J. and Wu, C. (1991). FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. *Science* **252**, 848-851._

Li, T. and Bender, M. (2000). A conditional rescue system reveals essential functions for the *ecdysone receptor* (*EcR*) gene during molting and metamorphosis in *Drosophila. Development* **127**, 2897-2905._

Lindsley, D. L. and Zimm, G. (1992) *The genome of* Drosophila melanogaster. San Diego: Academic Press.

Murata, T., Kageyama, Y., Hirose, S. and Ueda, H. (1996). Regulation of the *EDG84A* gene by FTZ-F1 during metamorphosis in *Drosophila melanogaster*. *Mol. Cell. Biol.* **16**, 6509-6515.

Restifo, L. L. and White, K. (1991). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of the central nervous system in *Drosophila*. *Dev. Biol.* **148**, 174-194.

Restifo, L. L. and White, K. (1992). Mutation in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle, and gut. *Wilhelm Roux's Arch. Dev. Biol.* **201**, 221-234.

Richards, G. (1976). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster* IV. The mid prepupal period. *Dev. Biol.* **54**, 264-275.

Riddiford, L. M. (1993) Hormones and *Drosophila* Development. In *The Development of* Drosophila melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 899-940. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Second Edition. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schubiger, M., Wade, A. A., Carney, G. E., Truman, J. W. and Bender, M. (1998). *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* **125**, 2053-2062.

Sun, G.-C., Hirose, S. and Ueda, H. (1994). Intermittent Expression of BmFTZ-F1, a member of the nuclear hormone receptor superfamily during development of the silkworm *Bombyx mori*. *Dev. Biol.* **162**, 426-437.

Thummel, C. S. (1995). From embryogenesis to metamorphosis: The regulation and function of Drosophila nuclear receptor superfamily members. *Cell* **83**, 871-877.

Thummel, C. S. (1996). Flies on steroids-*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet*.**12**, 306-310.

Ueda, H. and Hirose, S. (1990). Identification and purification of a *Bombyx mori* homologue of FTZ-F1. *Nucl. Acids Res.* **18**, 7229-7234.

Ueda, H., Sonoda, S., Brown, J. L., Scott, M. P. and Wu, C. (1990). A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. *Genes Dev.* **4**, 624-635.

Ueda, H., Sun, G.-C., Murata, T. and Hirose, S. (1992). A novel-DNA binding motif abuts the zinc finger domain of insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding protein. *Mol. Cell. Biol.* **12**, 5667-5672.

Wolfgang, W. J., Fristrom, D. and Fristrom, J. W. (1986). The Pupal Cuticle of *Drosophila*. Differential ultrastructural immunolocalization of cuticle proteins. *J. Cell. Biol.* **102**, 306-311.

Woodard, C. T., Baehrecke, E. H. and Thummel, C. S. (1994). A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell* **79**, 607-615.

Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N. and Pick, L. (1997). The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz. *Nature* **385**, 552-555.