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Regulation of the *EDG84A* Gene by FTZ-F1 during Metamorphosis in *Drosophila melanogaster*

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The transcription factor FTZ-F1 is a member of the nuclear hormone receptor superfamily and is transiently expressed during the mid- and late prepupal periods in *Drosophila melanogaster*. A putative pupal cuticle gene, *EDG84A*, is expressed slightly following FTZ-F1 expression during the prepupal period and carries a strong FTZ-F1 binding site between bases 100 and 92 upstream of its transcription start site. In this study, *EDG84A* mRNA was found to be prematurely expressed upon heat induction of FTZ-F1 in prepupae carrying the heat shock promoter–FTZ-F1 cDNA fusion gene construct. Transgenic fly lines having the 0.8-kb region of the *EDG84A* promoter fused to *lacZ* expressed the reporter gene in a tissue- and stage-specific manner. Base substitutions in the FTZ-F1 binding site within the 0.8-kb promoter abolished expression of *lacZ*. These results strongly suggest that the *EDG84A* gene is a direct target of FTZ-F1. Deletion studies of the *cis*-regulatory region of the *EDG84A* gene revealed that space-specific expression in imaginal disc-derived epidermis is controlled by the region between bp −408 and −104 from the transcription start site. The region between bp −408 and −194 is necessary to repress expression in a posterior part of the body, while the region between bp −193 and −104 carries a positive element for activation in an anterior part of the body. These results suggest that FTZ-F1 governs expression of the *EDG84A* gene in conjunction with putative tissue-specific regulators.

The steroid hormone ecdysteroid acts as a temporal signal in inducing tissue-specific morphological changes in insects. In the fly *Drosophila melanogaster*, several low ecdysteroid pulses have been observed during the mid- and late third larval periods, and a high-titer pulse at the end of the larval stage terminates the wandering and induces puparium formation and metamorphosis. This pulse starts 5 h before puparium formation and lasts until 3 h after puparium formation. A brief low-titer ecdysteroid peak around 10 h after puparium formation triggers head eversion, one obvious marker for the prepupa-to-pupa transition (30, 32).

The effects of ecdysteroid pulses on gene expression have been inferred from the puffing pattern of salivary gland polytene chromosomes between the mid-third instar and the early pupal period (9) and also from their reproduction in cultured salivary glands by addition of 20-hydroxyecdysone or its removal and drug inhibition studies (5, 7, 29, 31). These studies identified five sets of ecdysteroid-regulated puffs: intermolt, early, late, mid-prepupal, and late-prepupal puffs. Ashburner et al. (6) proposed a model for the ecdysteroid-mediated control of genes encoded in the intermolt, early, and late puffs during metamorphosis. According to this model, ecdysteroid bound to its receptor represses the intermolt puffs, induces the early puffs, and represses some late puffs. In addition, induced early-puff products induce late puffs and repress early puffs. Recent studies on molecular cloning of ecdysone-regulated genes provided significant support for the model of Ashburner et al. First, the nuclear receptor EcR has been shown to bind ecdysteroid (22) and regulate target genes through heterodimerization with another nuclear receptor, USP (16, 28, 37, 40, 51, 52). Second, some of the ecdysone-inducible genes have been found to encode transcription factors containing DNA binding motifs (2, 21, 38). These genes are expressed in a temporally specific manner (1, 19–21, 36, 38, 42) and are likely to induce or repress other ecdysone-responsive genes (1, 2, 10, 11, 18). However, most of the studies were concentrated on the early and late ecdysone-regulated genes, and little is known about the mechanism of regulation of gene expression after the pulse of ecdysteroids.

The chromosome locus *75CD*, containing the *FTZ-F1* gene, forms a puff at the mid-prepupal stage, about 6 to 9 h after puparium formation, concomitant with expression of FTZ-F1 mRNA (23). FTZ-F1 is a member of the nuclear hormone receptor superfamily (24, 46) in *D. melanogaster* and has two protein isoforms, early and late FTZ-F1 (also called as α- and βFTZ-F1, respectively), which are transcribed from the same gene; they share a common C-terminal region but contain different N-terminal regions (23, 24). Early FTZ-F1 is expressed in blastoderm embryos, while late FTZ-F1 is expressed in late-stage embryos (predominantly after 16 h of embryogenesis), the late stages of first- and second-instar larvae, and mid- to late prepupae (23, 26a, 46). A counterpart of FTZ-F1, designated BmFTZ-F1, has been found in the silkworm *Bombyx mori* (45). BmFTZ-F1 mRNA is expressed at the end of each molting stage after the pulse of ecdysteroids and is inducible in cultured silk glands with exposure to and subsequent withdrawal of 20-hydroxyecdysone (39).

In the prepupal stage, the pupal cuticle is formed by sequential production of epicuticle, exocuticle, and endocuticle layers from outside to inside. Immediately after the large pulse of ecdysteroids, the epicuticle is formed about 3 to 8 h after puparium formation, and then the exocuticle is formed 8 to 12 h after puparium formation (11, 49). After the small pulse of ecdysteroids, the endocuticle layer is formed. FTZ-F1 is expressed 6 to 12 h after puparium formation (23). This period starts slightly before, and overlaps, the time when the exocuticle is formed. Some of the genes encoding cuticle proteins have been cloned (4, 15). Among them, the *EDG84A* and *EDG78E* genes are induced by pulse exposure to 20-hydroxy-

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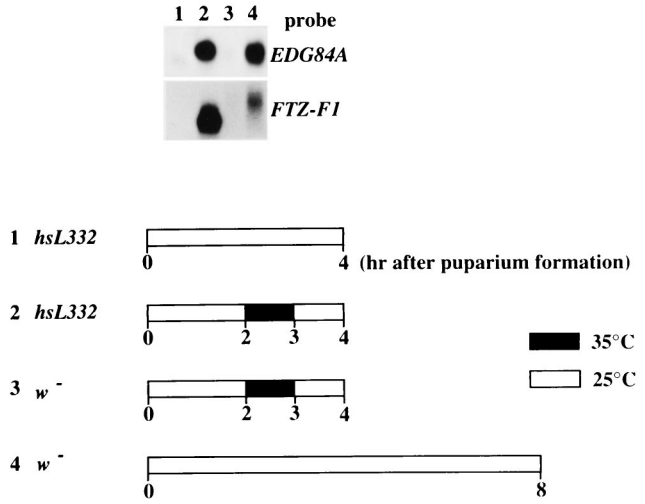

FIG. 1. Induction of *EDG84A* mRNA by forced expression of late FTZ-F1. Ten micrograms of total RNA from heat-shocked *hsL332* (lane 2) or w^- (lane 3) or untreated *hsL332* (lane 1) flies 4 h after puparium formation untreated or w^- flies at 8 h after puparium formation (lane 4) was electrophoresed and subjected to Northern blot hybridization with a ^{32}P-labeled genomic clone of either *EDG84A* or *FTZ-F1* as a probe. Top, autoradiogram; bottom, protocol of heat treatment.

ecdysone, and their products are assumed to be components of the exocuticle (4, 12). Furthermore, a relatively strong FTZ-F1 binding sequence (44, 47) is present at 100 and 500 bp upstream from the transcription start sites of the *EDG84A* and *EDG78E* genes, respectively. These observations suggest that the *EDG84A* and *EDG78E* genes are target candidates for FTZ-F1.

In the present study, we found that *EDG84A* mRNA is induced by premature expression of FTZ-F1 and that the FTZ-F1 binding site is essential for the expression. These results show that the *EDG84A* gene is a direct target of FTZ-F1. Furthermore, we found another regulatory elements for disc-derived epidermis-specific expression of this gene, suggesting cooperative regulation of the gene by stage- and tissue-specific factors.

MATERIALS AND METHODS

Stocks. The abbreviation w^- is used for *Df(1)w*^{67c1}*y*^{1}. All stocks not specifically described in this paper are described by Lindsley and Zimm (26). All of the flies used in this study had a w^- background. Flies were raised at 25°C on 8% cornmeal–4% ebios–10% glucose–0.7% agar medium containing propionic acid and *p*-butyl-*p*-hydroxybenzoate as an antifungal agent.

Staging of animals. Samples for prepupae and pupae were prepared by picking newly formed white prepupae at 1- or 0.5-h intervals and harvesting at the desired stage.

Construction of plasmids for germ line transformation. To construct *hsLFTZ-F1*, a pair of oligonucleotide primers was synthesized, one containing the sense-strand amino acids 1 to 5 (23), the translational start consensus sequence in *D. melanogaster* (boldface), and an *Eco*RI site (underlined) (5′-TCGAGGAATTC**AACATG**TTATTAGAAATG-3′), and the other containing the antisense sequence encoding amino acids 24 to 27 and an *Sph*I site (underlined) (5′-CAG CTGCATGCTGAACGGCGAT-3′), for use in PCR with a genomic clone of FTZ-F1, p32e20 (43a). The amplified fragments were digested with *Eco*RI and *Sph*I and then ligated into an *Eco*RI site of the vector pCaSpeR-hs (43) with a 1-kb *Sph*I-*Xho*I fragment of p32e20 and a 1.5-kb *Xho*I-*Eco*RI fragment of pC2, one of the FTZ-F1 cDNA clones (24).

To construct the *EDG84A* gene promoter-*lacZ* fusion gene, a 0.8-kb *Eco*RI-*Sau*3AI fragment containing 740 bp of the 5′-upstream region and 60 bp of the 5′ end of the transcribable region of *EDG84A* was inserted between the *Eco*RI and *Bam*HI sites of the pCaSpeRAUG-βgal vector (41). Mutations in the FTZ-F1 binding site and deletions were made by using PCR as described by Ueda et al. (47).

Germ line transformations. P-element-mediated germ line transformations were performed as described by Rubin and Spradling (34). The host *Drosophila* strain, the w^- strain, was injected with a solution of DNA (200 μg of constructed CaSpeR-AUG-βgal vector per ml plus 100 μg of pπ25.7wc per ml as a helper plasmid). The resultant G_0 flies were crossed individually with w^-; *CyO*/*Gla* flies. G_1 progeny showing the w^+ activity were mated with w^-; *CyO*/*Gla* flies, and w^+ males were also crossed with w^-; *TM3*/*Pr e* flies. G_2 progeny showing the w^+ activity were mated with w^-; *CyO*/*Gla* or w^-; *TM3*/*Pr e* flies. Homozygous lines were obtained through a backcross of these obtained lines. These crosses yielded linkage group assignments for the most of the transformant lines. At least five independent lines were established for each construct and subjected to analyses.

Western blotting (immunoblotting). A prepupa was homogenized in an Eppendorf tube containing 50 μl of 1× Laemmli sample buffer. Samples were heated at 95°C for 5 min and then electrophoresed through an 8% polyacrylamide–sodium dodecyl sulfate Laemmli gel. Proteins were transferred to a nitrocellulose filter by electrophoresis for 10 h at 60 V in 25 mM Tris–192 mM glycine solution containing 20% methanol. The filter was incubated with a polyclonal antibody against FTZ-F1 (1:50,000 dilution) and a polyclonal antibody against β-galactosidase (Cappel) in 20 mM Tris-HCl (pH7.5)–150 mM NaCl–0.5% Tween 20 containing 5% skim milk at room temperature for 1 h, and then signals were detected by using horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (Cappel) and the ECL detection system (Amersham).

Detection of expression pattern of the *lacZ* gene. To detect β-galactosidase activity in tissues of larvae and prepupae, animals were dissected in phosphate-buffered saline (10 mM sodium phosphate buffer [pH 7.5] and 150 mM NaCl) and fixed in phosphate-buffered saline containing 0.1% Triton X-100, 4% paraformaldehyde, and 1% glutalaldehyde for 12 min. Samples were rinsed with phosphate-buffered saline three times and stained with 0.2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in staining buffer [1× phosphate-buffered saline, 1 mM $MgCl_2$, 0.1% Triton X-100, 3.1 mM $K_4Fe(CN)_6$, and 3.1 mM $K_3Fe(CN)_6$] at 25°C for 12 h.

Gel mobility shift assay. Nuclear extract was prepared as described by Ueda et al. (46) except that frozen prepupae were crushed into a fine powder in a mortar

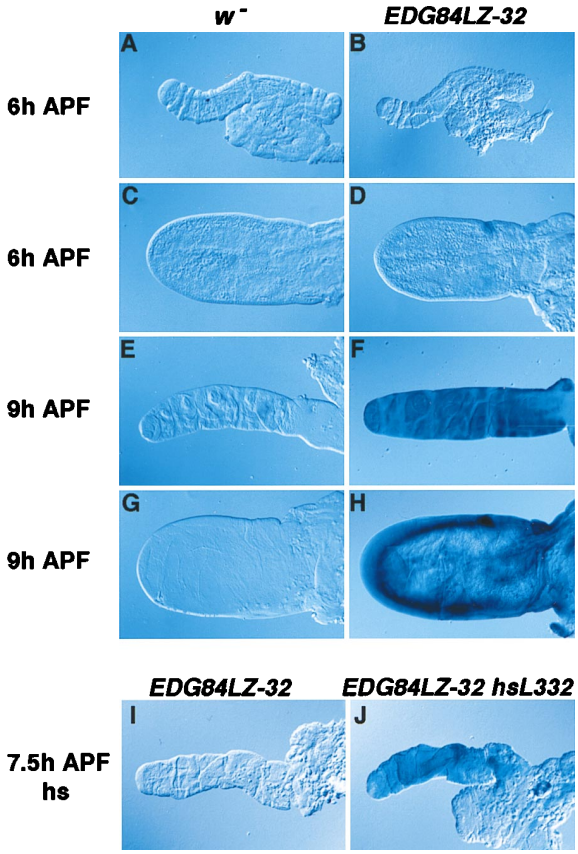

FIG. 2. The 0.8-kb promoter region is sufficient for stage- and tissue-specific expression of the *EDG84A* gene. Patterns of X-Gal staining of the leg (A, B, E, F, I, and J) and wing (C, D, G, and H) imaginal discs of the w^- host strain (A, C, E, and G), the *EDG84LZ-32* line (B, D, F, H, and I), or the *EDG84LZ-32 hLC332* line (J) at 6 h (A, B, C, and D), 9 h (E, F, G, and H), or 7.5 h (I and J) after puparium formation (APF) are shown. Prepupae were heat treated (hs) at 32°C for 1 h at 2 h after puparium formation (I and J).

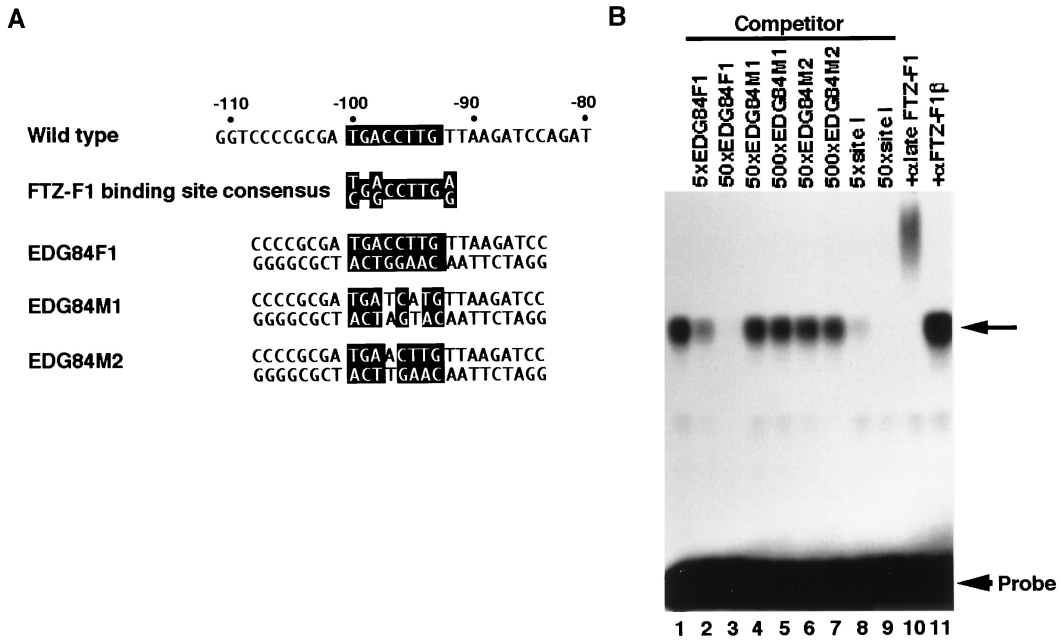

FIG. 3. (A) Nucleotide sequence around the FTZ-F1 binding site located 100 bp upstream from the transcription start site of the *EDG84A* gene (4) and its mutants M1 and M2. Bases which fit the best binding sequence of FTZ-F1 are in white letters in black boxes. Numbers above the sequence represent the positions of nucleotides relative to the transcription start site. The nucleotide sequences of oligonucleotides EDG84F1, EDG84M1, and EDG84M2 used for a gel mobility shift assay are shown. (B) Binding of late FTZ-F1 to EDG84F1 oligonucleotides and disruption of binding by mutation. Gel mobility shift assay was performed with nuclear extracts from prepupae 8 h after puparium formation and ^{32}P-labeled EDG84F1 oligonucleotide. The amounts of unlabeled competitors are indicated above lanes 2 to 9. Either anti-late FTZ-F1 serum or anti-FTZ-F1β serum was added in lanes 10 and 11, respectively. The positions of the late FTZ-F1-EDG84F1 probe are marked by an arrow.

under liquid nitrogen before homogenization. The gel mobility shift assay was performed as described by Ueda and Hirose (45). One microliter of anti-FTZ-F1 serum or anti-FTZ-F1β serum (27) was added to the reaction mixture for supershifting by a specific antibody.

RNA extraction and Northern (RNA) blotting. Total cellular RNA of prepupae was prepared by the guanidium thiocyanate method followed by centrifugation in cesium chloride solution essentially as described by Sambrook et al. (35). Northern blotting was performed as described by Sun et al. (39).

Organ culture. Imaginal discs of prepupae at 2 h after puparium formation were rinsed with 5/6× Grace's insect culture medium (Gibco) and cultured in this medium for 12 h at 25°C.

RESULTS

Induction of the *EDG84A* gene by forced expression of FTZ-F1. We examined whether *EDG84A* mRNA is induced by ectopic expression of FTZ-F1 in a transgenic fly line, the *hsL332* line, which carries late FTZ-F1 cDNA fused to a heat shock promoter. When the *hsL332* line was heat treated for 1 h at 2 h after puparium formation, FTZ-F1 mRNA was transcribed from the transgene (Fig. 1, lane 2); this mRNA is smaller than endogenous late FTZ-F1 mRNA (lane 4). Concomitant with the forced expression of FTZ-F1, *EDG84A* mRNA also accumulated (Fig. 1, lane 2) to a level similar to that normally observed 8 h after puparium formation (lane 4). These mRNAs were not detected either in heat-shocked animals of the host strain (Fig. 1, lane 3) or in the non-heat-shocked transformant animals 4 h after puparium formation (lane 1). These results suggested that the *EDG84A* gene is a target of FTZ-F1.

***cis*-regulatory sequence for temporal and spatial expression of the *EDG84A* gene.** The *EDG84A* gene is specifically expressed in imaginal discs at the mid-prepupal stage (12). To delimit the *cis*-regulatory region that governs the temporal and spatial expression of the *EDG84A* gene, transgenic fly lines carrying the 0.8-kb *EDG84A* promoter region fused to *lacZ* (*EDG84LZ*) were established, and the pattern of expression of the reporter gene was examined at the mid-prepupal period. Strong β-galactosidase activity was detected in both the leg and wing discs at 9 h after puparium formation (Fig. 2F and H). In contrast, X-Gal staining was not observed in either the leg or wing disc 6 h after puparium formation (Fig. 2B and D), except for small patched staining in the wing disc (Fig. 2D). Similar stage-specific expression of the reporter gene was observed in the eye-antenna discs and the anterior part of the epidermis derived from the imaginal discs 9 h after puparium formation (data not shown). On the other hand, transgene-dependent X-Gal staining was not observed in the fat body, proventriculus, mid gut, or salivary gland either 6 or 9 h after puparium formation (data not shown). Essentially the same pattern of X-Gal staining was observed in prepupae in 13 independent transformant lines (data not shown). The observed expression timing and disc epidermis-specific expression of the transgene were basically identical to expression of the endogenous *EDG84A* gene as reported by Fechtel et al. (12), suggesting that the 0.8-kb promoter region has enough information not only for temporal control but also for tissue-specific control of the gene.

Induction of the *EDG84A* transgene through premature expression of FTZ-F1. To examine whether the *EDG84LZ* transgene is also induced by premature expression of FTZ-F1, prepupae of the *EDG84LZ*, *hsL332*, or *EDG84LZ hsL332* line were heat shocked at 32°C for 1 h at 3 h after puparium formation, and expression of the reporter gene was detected by X-Gal staining 7.5 h after puparium formation. Staining in imaginal discs was detected upon heat shock in the *EDG84LZ hsL332* line (Fig. 2I) but not in the *hsL332* (data not shown) or *EDG84LZ* line (Fig. 2J). These results clearly show that the *EDG84LZ* transgene can be induced by premature expression of FTZ-F1.

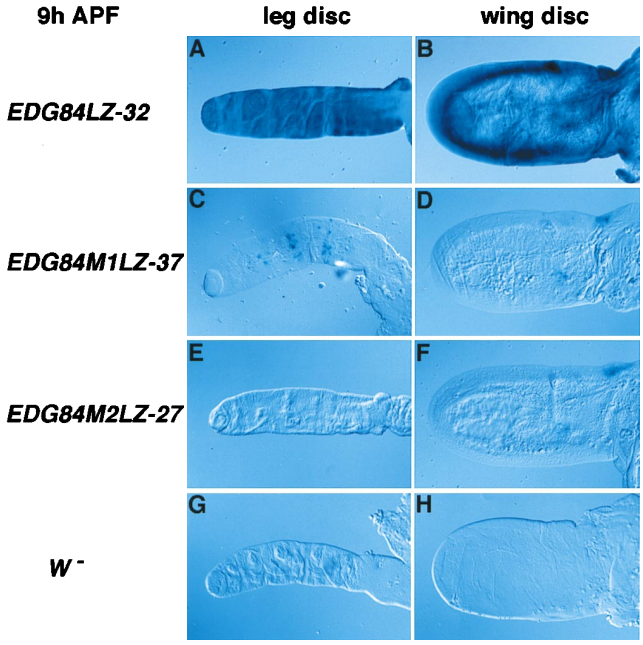

FIG. 4. The FTZ-F1 binding site is required for expression of the *EDG84A* promoter-*lacZ* fusion gene. Shown are patterns of X-Gal staining of the leg (A, C, E, and G) and wing (B, D, F, and H) imaginal discs in transgenic animals carrying the wild-type promoter *EDG84LZ-32* (A and B) or FTZ-F1 binding site mutant promoters *EDG84M1LZ-37* (C and D) and *EDG84M2LZ-27* (E and F). Patterns of X-Gal staining in the w^- host strain (G and H) 9 h after puparium formation (APF) are also shown as controls.

The FTZ-F1 binding site is essential for the expression of the *EDG84A* gene. Figure 3A shows the sequence around 100 bp upstream from the transcription start site of the *EDG84A* gene. Eight base pairs of the boxed 9-bp sequence matched the best FTZ-F1 binding sequence (44, 47). Gel mobility shift assay using a 25-bp oligonucleotide of this region, designated the EDG84F1 oligonucleotide (Fig. 3A), gave a strong band of protein-DNA complexes (Fig. 3B, lane 1). Supershift of the band by anti-late FTZ-F1 antibody (Fig. 3B, lane 10) but not by anti-FTZ-F1β antibody (lane 11) suggests that this shift was caused by late FTZ-F1. Although the T residue at position −92 does not fit the best binding sequence, this may cause only a two- to threefold reduction in the binding of FTZ-F1, judging from our previous results (44). This prediction was confirmed by a gel mobility shift competition assay (Fig. 3B, compare lanes 2 and 3 with lanes 8 and 9). We made two different base substitution mutants with mutations in the FTZ-F1 binding site. M1 has two base substitutions while M2 has a single base substitution (Fig. 3A). No competition in the gel mobility shift by late FTZ-F1 was observed in the presence of 500 times more mutant competitor, suggesting a more than 100 times reduction of affinity in either M1 or M2 (Fig. 3B, lanes 2 to 7). The 0.8-kb promoter fragment carrying M1 or M2 was fused to *lacZ*, and transgenic fly lines bearing these fusion genes were established and designated *EDG84M1LZ* or *EDG84M2LZ* lines, respectively.

Figure 4 shows X-Gal staining patterns in leg and wing imaginal discs of prepupae at 9 h after puparium formation under normal rearing conditions. No strong X-Gal staining was detected in leg and wing discs of the *EDG84M1LZ-37* and *EDG84M2LZ-27* lines, although strong X-Gal staining was observed in these discs of the *EDG84LZ-32* line, which harbors the wild-type construct. Essentially the same staining patterns were observed in all independent transgenic lines examined.

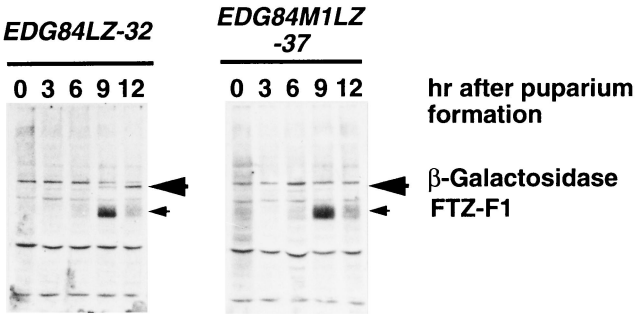

FIG. 5. Expression of the reporter gene as revealed by Western blot analyses. Total cellular proteins were subjected to Western blotting, and FTZ-F1 and β-galactosidase were visualized by using specific antibodies. The time after puparium formation is indicated above each lane. The large and small arrowheads represent the positions of β-galactosidase and late FTZ-F1, respectively.

These results were confirmed by Western blot analyses (Fig. 5). In the *EDG84LZ-32* line, carrying the wild-type promoter, β-galactosidase was not detected until 6 h after puparium formation, became detectable after 9 h, and accumulated until 12 h. However, β-galactosidase was not detected in the mutant *EDG84M1LZ-37* line even 12 h after puparium formation. Expression of the *lacZ* gene also was not found in prepupae of another mutant line, the *EDG84M2LZ* line (data not shown). From these observations, we concluded that the FTZ-F1 binding site located 100 bp upstream from the transcription initiation site is essential for expression of the *EDG84A* gene during the prepupal period.

No additional hormone is necessary for transcriptional activation by FTZ-F1. Since FTZ-F1 is a member of the nuclear hormone receptor superfamily, a secreted hormone might be necessary for transcriptional activation of the target gene by FTZ-F1. To examine this possibility, imaginal discs at 2 h after puparium formation were cultured in vitro. Induction of *lacZ* was clearly detected in the discs of the *EDG84LZ-32* line cultured for 12 h (Fig. 6A) but not in discs of the mutant *EDG84M1LZ-37* line (Fig. 6B). These results show that transcriptional activation by FTZ-F1 occurs without simultaneous addition of a hormone, although we cannot exclude the possibility that some substance present in imaginal discs at 2 h after puparium formation serves as a ligand for this orphan receptor.

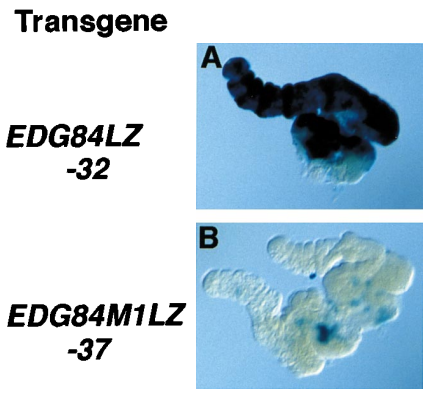

FIG. 6. No additional hormone is required for FTZ-F1-dependent activation of the *EDG84A* promoter in cultured discs. The leg discs in the *EDG84LZ-32* (A) or *EDG84M1LZ-37* (B) line 2 h after puparium formation were cultured for 12 h in the absence of 20-hydroxyecdysone and stained with X-Gal.

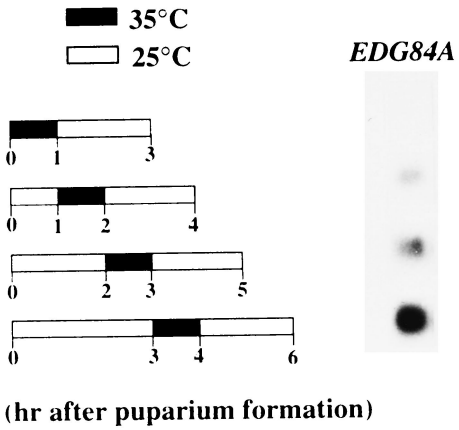

FIG. 7. Temporal regulation of the *EDG84A* gene requires not only FTZ-F1 but also another factor(s). *EDG84A* mRNA was detected by Northern blot analysis (right) with total RNA from prepupae of the *hsL332* line heat treated by the protocols shown (left).

Temporal regulation of the *EDG84A* gene is also dependent on another factor(s). To learn whether temporal regulation of the *EDG84A* gene is controlled only by FTZ-F1, the inducibility of *EDG84A* mRNA by premature expression of FTZ-F1 was examined at different developing stages of prepupae. As shown in Fig. 7, the earlier the timing of heat shock, the lower the amounts of induced *EDG84A* mRNA. During these developmental stages, the levels of FTZ-F1 induced by heat shock were almost the same (data not shown). These results suggest that a factor(s) other than FTZ-F1 is involved in the temporal control of the *EDG84A* gene.

Several *cis* elements are involved in the spatial regulation of the *EDG84A* gene. To further investigate the regulation of the *EDG84A* gene, transgenic fly lines which carry a series of 5′ promoter deletions in *EDG84LZ* (Fig. 8, left) were established, and the expression pattern of the reporter gene was examined at 11 h after puparium formation (Fig. 8, right). The expression pattern of β-galactosidase in the prepupae of the *EDG84D04LZ* line, carrying −408 bp of the upstream region, was the same as that in the *EDG84LZ* lines, indicating that the sequence between bp −408 and +50 has enough information for stage- and tissue-specific expression. In the *EDG84D02LZ* line, having bp −193 to +50, additional staining was observed in the epidermis of a posterior part of the body, suggesting that a repressor in the posterior epidermis acts through sequence between bp −408 and −194. Deletion to bp −104 abolished expression in most of the epidermis in the anterior part of the body, although expression in the posterior part was retained. No expression in nonepidermal cells (salivary gland, gut, and fat body) was observed in the transgenic lines carrying any of the constructs shown in Fig. 8. These results suggest that expression in the anterior epidermis is activated through the region between bp −193 and −104 and that the sequence between bp −103 and +50 is responsible for ectopic expression in the posterior epidermis. No expression of β-galactosidase was detectable in prepupae 6 h after puparium formation in these deletion constructs, i.e., *EDG84D04LZ*, *EDG84D02LZ*, and *EDG84D01LZ* (data not shown). These results indicate that the *EDG84A* gene is temporally regulated through a small region around the FTZ-F1 binding site.

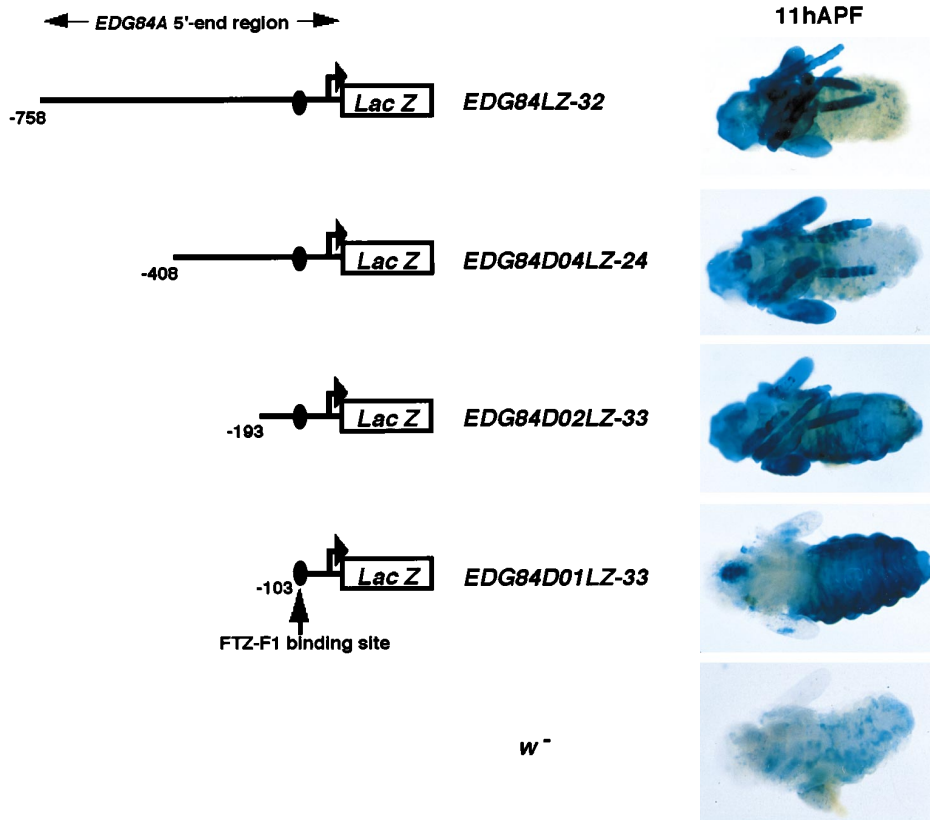

FIG. 8. Tissue-specific expression of the *EDG84A* 5′ end region-*lacZ* fusion gene in late prepupae detected by β-galactosidase activity. (Left) Transgene constructs; (right) patterns of X-Gal staining 11 h after puparium formation (APF).

DISCUSSION

The present study showed that the *EDG84A* gene is a direct target of FTZ-F1. The *EDG78E* gene (4) carrying a FTZ-F1 binding site 500 bp upstream of the gene was induced by premature expression of FTZ-F1 (unpublished results), suggesting that this gene is also a target of FTZ-F1. The *EDG78E* and *EDG84A* genes encode putative cuticle proteins and are induced after the pulse of ecdysteroids during the prepupal period (4). It has been shown that several pupal cuticle proteins are expressed in a stage-specific manner during the prepupal and early pupal periods. Numerous different low-molecular-weight pupal proteins contained in the exocuticle are produced at the mid- to late prepupal periods, while high-molecular-weight proteins contained in the endocuticle are produced after pupation (11, 49). The products of the *EDG78E* and *EDG84A* genes are assumed to be components of the exocuticle because of their structural features, expression pattern, and ecdysteroid response (1, 4, 11–13, 49). These observations indicate that FTZ-F1 plays an important role in the formation of the pupal exocuticle.

We showed that FTZ-F1 activates the *EDG84A* gene through the FTZ-F1 binding site located 100 bp upstream from the transcription start site. Apple and Fristrom (4) indicated that this region shows a weak similarity to the ecdysone response element of the *hsp27* gene (33). The sequence in the M2 mutant has a more prominent similarity to the ecdysone response element (3) than the original sequence in the *EDG84A* gene. However, no expression of the reporter gene was observed in either the *EDG84M1* or *EDG84M2* mutant line, indicating that the presence of the FTZ-F1 binding site is important for the induction of the *EDG84A* gene. This ecdysone response element might be involved in the repression of this gene by ecdysteroids (4). The FTZ-F1 binding site is also recognized by another nuclear receptor, FTZ-F1β (also called DHR39) (8, 17, 27). However, FTZ-F1β may not be responsible for the activation of *EDG84A*, because *FTZ-F1*β is an early ecdysone response gene, and its mRNA is expressed from the mid-third larval instar to early prepupal periods (17), in which *EDG84A* is repressed. These results were confirmed by both gel mobility supershift assay with the FTZ-F1 binding site in the *EDG84A* promoter as a probe and Western blotting analysis with anti-FTZ-F1β (data not shown). Furthermore, the amounts of FTZ-F1β in prepupae 8 h after puparium formation were estimated to be less than 1/100 of those of late FTZ-F1 (Fig. 3). As FTZ-F1β has been shown to be a repressor of the target gene in cultured cells (8, 27), it might serve as a repressor of the *EDG84A* gene by competing with FTZ-F1 for the FTZ-F1 binding site.

Recently, Woodard et al. (50) showed that FTZ-F1 is necessary for the induction of three late prepupal puff genes, *E74*, *E75*, and *E93*. For the induction of these genes, another ecdysteroid peak around 10 h after puparium formation is necessary, in addition to expression of FTZ-F1. *EDG84A* mRNA was detected from 8 h after puparium formation (1). This expression was slightly before the small ecdysteroid peak around 10 h after puparium formation. Furthermore, we demonstrated that the *EDG84A* promoter-*lacZ* fusion gene was induced without addition of any hormone in cultured imaginal discs. These observations indicated that another ecdysteroid peak was not necessary for induction of the *EDG84A* gene by FTZ-F1.

Expression of FTZ-F1 was detected not only in imaginal discs but also in almost all other tissues. However, the *EDG84A* gene was expressed only in imaginal discs and the anterior part of epidermis, which is derived from imaginal discs (12). These results suggest that tissue-specific expression of the gene is determined by a factor(s) other than FTZ-F1. According to the results of deletion studies with the *cis*-regulatory element, space-specific expression in the imaginal disc-derived epidermis is controlled by the region between bp −408 and −104. These results suggest that both FTZ-F1 and putative tissue-specific regulators which act through the region between bp −408 and −104 work cooperatively for proper expression of the *EDG84A* gene.

In addition to the mid-prepupal period, FTZ-F1 is expressed in late embryogenesis (46) and late first instar and late second instar (unpublished observation), but *EDG84A* mRNA was not detected in these early periods of development (13). No induction of *EDG84A* mRNA or β-galactosidase activity was detected upon forced expression of FTZ-F1 in the mid-third instar larvae of the *EDG84LZ hsL332* line (unpublished observation). Even during the prepupal period, *EDG84A* mRNA was induced at a high level only in limited developmental stages. These observations suggest that stage-specific expression of *EDG84A* requires not only FTZ-F1 but also another factor(s). This factor may not be a hormone which binds to the ligand binding domain of FTZ-F1, because induction of *EDG84A* occurred in imaginal discs in a hormone-free medium. Recently, it has been shown that the timing of expression of the *EDG84A* gene shifts to an earlier stage in loss-of-function mutants of the early gene *E74A* or the *E74B* gene, although the timing of expression of FTZ-F1 did not change in these mutants (14). These results indicated that the product of the *E74* gene regulates expression of the *EDG84A* genes without influencing the expression of FTZ-F1. The presence of several possible E74 protein binding sites (48) within the 0.8-kb region of the *EDG84A* promoter raised the possibility that this interaction is direct. The ecdysone receptor complex and FTZ-F1β are other candidates responsible for the restriction of expression timing of the *EDG84A* gene, because they could bind to this promoter, as described above. It is also possible that some stage-specific coactivator is necessary for the activation of target genes by FTZ-F1. Recently, we identified two mediators which are necessary for the activation of the target gene by BmFTZ-F1 (25), a counterpart of FTZ-F1 in the silkworm *B. mori* (39).

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