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Drosophila Blimp-1 is a transient transcriptional repressor that controls timing of the ecdysone-induced developmental pathway

Running title: Unstable dBlimp-1 controls developmental timing

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Abstract

Regulatory mechanisms controlling the timing of developmental events are crucial for proper development to occur. *ftz-f1* is expressed in a temporally-regulated manner following pulses of ecdysteroid and this precise expression is necessary for the development of *Drosophila melanogaster.* To understand how insect hormone ecdysteroids regulate the timing of FTZ-F1 expression, we purified a DNA-binding regulator of *ftz-f1*. Mass spectroscopy analysis revealed this protein to be a fly homolog of mammalian B lymphocyte-induced maturation protein 1 (Blimp-1). *Drosophila Blimp-1* (*dBlimp-1*) is induced directly by 20-hydroxyecdysone, and its product exists during high ecdysteroid periods and turns over rapidly. Forced expression of dBlimp-1 and RNAi analysis indicate that dBlimp-1 acts as a repressor and controls the timing of FTZ-F1 expression. Furthermore its prolonged expression results in delay of pupation timing. These results suggest that the transient transcriptional repressor dBlimp-1 is important for determining developmental timing in the ecdysone-induced pathway.

Introduction

The steroid hormone ecdysone and its active metabolite 20-hydroxyecdysone (20E) (hereafter referred to collectively as ecdysone) is responsible for many essential developmental processes, including insect molting, metamorphosis, oogenesis and embryogenesis (25, 40). The insect ecdysone response provides an excellent model for studying hormone function, in which temporally regulated induction of multiple genes is required to control complex developmental events. For instance, at the onset of metamorphosis in *D. melanogaster*, a large pulse of ecdysone causes the third instar larval to prepupal transition. Based on the observation of puffs on polytene chromosomes in cultured salivary glands more than 30 years ago, it has long been known that there are at least four categories of ecdysone-inducible genes (1-4, 38). The early genes are induced directly by the ecdysone-receptor complex and are repressed by their product(s). The early-late genes are also induced directly by ecdysone, but require ecdysone-induced gene product(s) for maximal induction. The late genes are induced by the early gene product(s), and the mid-prepupal genes are induced only after ecdysone levels have declined. In the last two decades, many of the genes belonging to these four groups have been cloned and their regulated expression profile has been confirmed. These include multiple transcription factors, which constitute an ecdysone-induced gene cascade.

ftz-f1 is a mid-prepupal gene (29) that encodes a nuclear receptor-type transcription factor (30). The beta isoform of the *ftz-f1* gene product is expressed not only during the mid-prepupal period at the onset of metamorphosis, but also during late embryogenesis, just before larval ecdysis and eclosion (45, 51, 54, 55). All of these periods closely follow declines in

ecdysone levels. The importance of timing of *ftz-f1* expression has been shown by rescue of *ftz-f1* mutants by temporally specific expression of βFTZ-F1 as well as developmental arrest by premature expression of βFTZ-F1 (55). Expression and/or induction of *ftz-f1* after a decline in ecdysone levels has been reported in several insects besides *Drosophila* (17, 31, 46), suggesting that the temporally regulated expression of *ftz-f1* is crucial for insect development.

However, the mechanism by which *ftz-f1* is temporally regulated is still unclear; only two transcriptional regulators have been identified to date (20, 28, 52). One is the early-late gene product DHR3, a nuclear receptor-type transcription factor that is expressed from just before puparium formation to the mid-prepupal period. Several lines of evidence support it to be a transcriptional activator for the *ftz-f1* gene: (i) premature expression of DHR3 under the control of a heat shock promoter induces βFTZ-F1 (28, 52), (ii) βFTZ-F1 expression is reduced in a DHR3 mutant (27), and (iii) DHR3 binds to three sites downstream of the transcription initiation site of the *ftz-f1* locus (+150, +240 and +300), and mutations in these sites reduce expression of the *ftz-f1* promoter-*LacZ* fusion gene in transgenic flies (20).

The other transcription factor known to regulate *ftz-f1* is the early gene product E75B, which is also a member of the nuclear receptor superfamily, but lacks one zinc finger and thus cannot bind to DNA by itself. E75B is expressed around puparium formation, when ecdysone levels are high, and disappears after ecdysone levels decline during the mid-prepupal period. Because E75B binds directly to DHR3 and inhibits its activator function, DHR3 can activate *ftz-f1* only after ecdysone levels have declined (52). However, the temporal pattern of *ftz-f1* expression is preserved in DHR3 and E75B mutants (8, 27), and mutations in the DHR3 binding sites of the

ftz-f1 promoter-*lacZ* fusion gene have no effect on the timing of β-galactosidase expression in transgenic flies (20). Thus, all these results suggest that temporal regulation of *ftz-f1* expression is achieved by other factors.

To understand how *ftz-f1* expression is temporally regulated, we have analyzed the *cis*-regulatory region of the *ftz-f1* locus to identify developmentally regulated factors that bind to these regions (20). One factor, designated Factor I-4, binds to the region upstream of the transcriptional start site of *ftz-f1*, and is expressed during mid-embryogenesis and the early prepupal period. Here, we determined the binding site of Factor I-4, identified its gene based on the information on purified protein, and analyzed its biological function during development including the regulation of the *ftz-f1* gene.

Materials and Methods

Gel mobility shift assay

Binding was performed at 25^{o}C for 1 hour in 10 μl reaction buffer (15 mM Tris-HCl at pH 7.8, 150 mM NaCl, 0.1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.2% Igepal CA-630 (Sigma), 1 mM NaPO4 at pH 7.8, 10% glycerol), containing 20 fmol ^{32}P-labeled DNA probe, 2 μg poly(dI-dC)-poly(dI-dC) (Pharmacia), 100 ng salmon sperm DNA, 10 μg yeast tRNA, 50 μg bovine serum albumin fraction V (Sigma), and 1 μl nuclear extract or fraction. Complex and free probe were separated by agarose gel electrophoresis and detected as previously described (50). Site I-4 DNA was obtained by hybridization of synthesized oligonucleotides carrying 5'-GTTTCACTTTGGCTTTCCGTTTTGG and the complementary sequence. Site I-4m DNA was

similarly obtained using synthesized oligonucleotides carrying 5’-GTTTCACTTTAGATCTCCGTTTTGG and the complementary sequence. The mutant Site I-4m2 DNA was generated similarly, using the sequence 5'-AAAAGTCTGACTCTGGCTCTGCGTTTGGG. For the supershift assay, 1 μl of anti-dBlimp-1 or anti-βFTZ-F1 serum was added to the incubation mixture and further incubated for 30 minutes and loaded on the gel. Oligonucleotides carrying FTZ-F1 binding site in the *EDG84A* promoter, EDG84F1, was used to detect βFTZ-F1 (33).

Methylation interference

Methylation interference was performed as previously described by Kageyama et al. (20) using an *Eco52*I-*Hinc*II fragment carrying base pairs –70 to –470 and labeled at the *Eco52*I site by T4 polynucleotide kinase and [α-^{32}P]ATP.

Preparation of nuclear extracts and purification of Factor I-4

Staged nuclear extracts were prepared as previously described by Ueda et al. and Kageyama et al. (20, 51). Nuclear extracts from 8 to 16 hour embryos were prepared as previously described Ueda et al. (51), except that 0.2% Igepal CA-630 was added to Solution II and III. All purification steps were performed on ice or in the cold room. 25 ml nuclear extract from 130 g mid-stage embryos was loaded on S Sepharose columns. After washing with Buffer L (10 mM Hepes-NaOH at pH 7.9, 1 mM EDTA, 0.1% Igepal CA-630, 20% Glycerol, 1 mM DTT) containing 150 mM NaCl,

Factor I-4 was eluted by Buffer L containing 250 mM NaCl. 2.4 ml S Sepharose fraction containing high Factor I-4 activity was adjusted to 5.6 ml by L buffer after addition of 7 mg Site I DNA-latex resin, 112 μg shared salmon sperm DNA, 11.2 μg poly(dI-dC)-poly(dI-dC) and 112 μg yeast tRNA. The mixture was incubated for 30 minutes on ice, and then supernatant was removed after centrifugation at 15,000 rpm for 15 minutes. After the resin was washed three times with 1 ml L buffer containing 200 mM NaCl, Factor I-4 was recovered as supernatant by incubating with 230 μl L buffer containing 500 mM NaCl for 10 minutes. 200 μl of the supernatant was diluted with 470 μl L buffer containing 40 μg shared salmon sperm DNA, 4 μg poly(dI-dC)-poly(dI-dC) and 40 μg yeast tRNA, and mixed with 5 mg latex resin carrying mutated Site I-4 DNA. After incubation for 30 minutes and centrifugation, 660 μl supernatant was recovered, mixed with 3.5 mg wild-type Site I-4 DNA-latex resin, and then the mixture was incubated for 30 minutes. After the supernatant was removed, the resin was washed three times with 1 ml L Buffer containing 200 mM NaCl, and then the factor was eluted twice by 200 μl L Buffer containing 500 mM NaCl. Site I-4 DNA-affinity resin and mutated Site I-4 DNA-affinity resin were prepared as previously described (16) using latex beads as resin. Synthesized oligonucleotides, 5'-TTTCACTTTCGCTTTCCGTTTGGGGG and 5’-AAACGGAAAGCGAAAGTGAAACCCCC, and 5'-GATCCGTCTGACTCTGGCTCTGGCTCTGGCTCTGGCTCTGCGTTTGA and 5’-GATCTCAAACGCAGAGCCAGAGCCAGAGCCAGAGCCAGAGTCAGACG, were used for making wild-type and mutated Site I-4 DNA-latex resin, respectively.

Plasmid construction for forced expression of *dBlimp-1*

A cDNA clone (RE26660) containing the entire *dBlimp-1* coding region was obtained from Research Genetics. Double strand oligonucleotides obtained by hybridization of two synthesized oligonucleotides, 5’-AATTCTAGTCGCCATGCA and 5’-TGGCGACTAG and a 1.1 kb *Eco*T22I-*Sal*I fragment of RE26660 were inserted between the *Eco*RI and *Sal*I sites in pBluescript II. The established plasmid was digested with *Sal*I and *Apa*I, and a 2kb *Sal*I- *Apa*I fragment from RE26660 was inserted. After a *Not*I linker was inserted at the blunt-ended *Kpn*I site, an *Eco*RI and *Not*I digest of this plasmid was ligated into the *Eco*RI and *Not*I sites of pCaSpeR-HS plasmid to establish transgenic fly lines expressing *dBlimp-1* under control of the heat shock promoter. To construct a P-element expressing Flag-tagged dBlimp-1 protein, double strand oligonucleotides obtained by hybridization of two synthesized oligonucleotides, 5’-GATCATCGAATGCACGTAGATCTGGTAC and 5’-CAGATCTACGTGCATTCGAT, was inserted into the *Fba*I and *Kpn*I sites of RE26660, and then the *Eco*RI-*Not*I fragment of the obtained plasmid was inserted into the pCaSpeR-HS plasmid as described above.

Plasmid construction for *dBlimp-1* RNAi

A 550 bp DNA fragment spanning the beginning of the second exon to the beginning of third exon was obtained by PCR on genomic DNA using primer 5'-ATCAGATCTTGCATGGACATCACAACCACAACCAT, which contains a *Bgl*II site, and 5'-TAGAATTCGCTGCTCCAAACTCCTTCAGTCTGCAAG, which contains an *Eco*RI site. A 450 bp DNA fragment from the beginning to the end of the second exon was obtained by PCR on

genomic DNA using primer 5'-ATAGCGGCCGCTTGCATGGACATCACAACCACAACCATCT, which contains an *Eco*52I site, and 5'-AAGAATTCACATTTGGCGTTGAGTAGACCATGGA which contains an *Eco*RI site. After ligation of the two fragments using their *Eco*RI sites, ligated DNA was digested with *Bgl*II and *Eco*52I, and was inserted into the pUAST vector using the *Bgl*II and *Not*I sites.

Antibody preparation

An *Eco*RI-*Sal*I digest of *dBlimp-1* cDNA in pCaSpeR-HS was inserted into the *Eco*RI and *Sal*I sites of pET28b to express the N-terminal half (from 1 to 372 aa.) of dBlimp-1 in *E. coli*. The established plasmid was transformed into *E. coli BL21DE3* (*LysS*), and the N-terminal half of Blimp-1 was expressed according to the manufacturer's protocol and subjected to immunization after purification.

RNA extraction and Northern blotting

RNA was prepared using Sepasol-I super (Nakarai) according to the manufacturer's protocol. Northern blotting was performed as described in Molecular Cloning (44).

RT-PCR

For quantitative real-time RT-PCR, cDNA was synthesized using random 9-mer oligonucleotides and ReverTra Ace (Toyobo) and RNA was treated with RNase free DNase I (Takara), and used as a template for real-time PCR using a LightCycler system (Roche). The following synthetic

oligonucleotides were used for detecting reverse transcripts: 5'-CGCACCTCCAGAAGCATCAT and 5’-GGGCAGAGATCACAGGCATA were used for *dBlimp-1*, 5’-AGCCGCAGCAGCAAATG and 5’-ACCCGAGTGGTGCAGAT were for *E75A*, and 5’-CCACCAGTCGGATCGATATG and 5’-CACGTTGTGCACCAGGAACT were for *rp49* (23).

In vitro culture of salivary glands

Thirty pairs of salivary glands from middle stage of third instar larvae were cultured in Schneider medium in the presence or absence of 5 mM 20E or 70 mM cycloheximide.

Fly work

All flies used in the transformation study had a y^{1} *Df(1)*w^{67c1} background. Nuclear extracts were prepared from an *Oregon-R* strain. *dBlimp-1*^{P14751} was were kindly gifted from Dr. T. Aigaki and *Sgs-2* flies were from Dr. A.J. Andres. *hs-Gal4* lines were obtained from Genetic Stock Research Center in the National Institute of Genetics. Flies were raised at 25°C on 10% glucose, 8% corn meal, 4% ebios and 0.7% agar medium containing propionic acid and butyl-p-hydroxybenzoate as antifungal agents. Staging of mid to late third instar larvae was determined by observation of GFP signal in *Sgs-2* larvae (9) or by that of gut in larvae cultured in Bromophenol Blue (BPB)-containing food (21). Staging after puparium formation was done by incubating newly transformed white prepupae at 25°C. Transgenic fly lines were established by germ line transformation using the established plasmid.

Western blotting

Western blotting was performed as described previously (33). Amounts of protein loaded in each lane were checked by staining the membrane again using anti-α-tubulin antibody.

Results

Determination of the Factor I-4 binding site in the *ftz-f1* promoter

To determine the precise binding site of Factor I-4 in the *ftz-f1* promoter, a methylation interference assay was performed using an *Eco52*I-*Hinc*II fragment (bases –470 to –70) that includes the restriction fragment to which Factor I-4 was previously shown to bind (20). As shown in Fig. 1A, four guanine residues located at positions –333, -337, -339, and -343 exhibited methylation interference upon Factor I-4 binding. To confirm this result, a gel mobility shift assay was performed using a 25 bp double-stranded oligonucleotide encompassing these positions (Site I-4) as a probe (Fig. 1B). As expected, a complex was clearly observed. This complex disappeared upon addition of cold Site I-4 DNA, but not by Site I-4m DNA carrying base substitutions, indicating that the factor binds with strong sequence specificity to the identified position. A developmental gel mobility shift assay revealed that the factor is present at high levels from 9-15 hours after egg laying (AEL), then rapidly disappears during the embryonic stage (Fig. 1C left). At the onset of metamorphosis, the factor is present from –3 hours after puparium formation (APF) to 4 hours APF, with a peak from 0 to 2 hours APF. It then reappears from 10 to 14 hours APF (Fig. 1C right). These observations corroborate previous results obtained using the larger

restriction fragment (20). These results indicate that Factor I-4 binds to the DNA sequence around 340 bp upstream of the *ftz-f1* transcriptional start site.

Purification of Factor I-4 from embryonic nuclear extract

To identify Factor I-4, we purified it from an embryonic nuclear extract, as outlined in Fig. 2A. Mid-embryonic stage nuclear extract was fractionated by S Sepharose column chromatography, and then the active fraction was further fractionated through a latex resin conjugated with multimeric wild-type Site I-4 DNA. After removing non-specific binding using a latex resin conjugated with polymerized mutated Site I-4 DNA, the factor was purified using the latex resin carrying wild-type Site I-4 DNA. Table 1 shows a summary of purification of the factor, and Fig. 2B shows the results of a gel mobility assay using typical fractions of the purification steps and silver staining after SDS-polyacrylamide gel electrophoresis using the same fractions. Even after purification by the second wild-type Site I-4 DNA affinity resin, several stained bands were observed in the eluted fractions (E3-1 and E3-2). However, the intensity of the 170 kDa band in the SDS-polyacrylamide gel correlated well with its binding to the Site I-4 probe in the gel mobility shift assay, suggesting that the 170 kDa protein is indeed Factor I-4.

Factor I-4 is a homologue of mammalian transcriptional repressor Blimp-1/PRDI-BF1

To identify the 170 kDa factor, protein in the final fraction was separated through a SDS-polyacrylamide gel and transferred to a PVDF membrane. The 170 kDa band was excised and subjected to Time of Flight Mass Spectroscopy (TOF-MS) analysis after Lysyl Endopeptidase

treatment. These data show that Factor I-4 is encoded by *CG5249*/*Blimp-1* (data not shown). The deduced amino acid sequence contains zinc fingers sharing strong homology with those of mammalian transcriptional repressor Blimp-1/PRDI-BF1 (22, 37, 49) (75% identity). Furthermore, it has been shown that Blimp-1/PRDI-BF1 binds to a similar sequence (22) as the identified binding site of Factor I-4. To confirm this, the zinc finger region of *Drosophila* Blimp-1 was expressed in *E. coli* and examined by gel mobility shift assay, using Site I-4 DNA as a probe. A complex produced by the recombinant protein was indeed observed. This complex disappeared by addition of cold Site I-4 DNA but not mutated Site I DNA (Fig. 3A, left panel). Similar results were obtained when nuclear extract containing Factor I-4 was used (Fig. 3A, right panel). In addition, anti-serum against the N-terminal region of *Drosophila* Blimp-1 produced a supershifted complex in nuclear extracts, while pre-immune serum did not (Fig. 3B). The supershift was not due to non-specific binding of the antibody as the anti-serum did not react to a complex by βFTZ-F1 (Fig.3C). From these results, we conclude that Factor I-4 is encoded by *CG5249*/*Blimp-1*, and hereafter refer to it as *Drosophila* Blimp-1 (dBlimp-1) to distinguish it from homologs in other species.

Besides the zinc finger motif, dBlimp-1 has another conserved motif at its N-terminal region, the PR domain (34, 48). The PR domain has strong sequence similarity to the SET domain, which is found in methyltransferase proteins. However, the PR domain in Blimp-1 is thought to lack methyltransferase activity, because it does not contain the NHSC(I) sequence which is conserved in other SET domain proteins with methyltransferase activity (24, 32). In addition to these two conserved motifs, dBlimp-1 and mammalian Blimp-1 share a central, proline-rich

region and a short, conserved N-terminal region that is not present in other SET domain proteins.

Expression pattern of *dBlimp-1* mRNA

To determine the expression pattern of *dBlimp-1*, we performed Northern blot analysis using total RNA prepared from animals around prepupal stage. As shown in Fig. 4A (upper panel), a strong 5.5 kb band was detected between 0 to 2 hours APF. The bands were detected from 3 hours before puparium formation and disappeared at 4 hours APF. A 6.3 kb band was detected at 10 to 14 hours APF, which corresponds to the increase of ecdysteroid that leads to head eversion and the completion of the prepupal stage. This result is consistent with developmental profiles of the dBlimp-1/Factor I-4 binding activity detected by gel mobility shift assays (Fig. 1C), indicating that temporal regulation of *dBlimp-1* occurs at the level of mRNA expression. The coincidence of *dBlimp-1* mRNA expression with the ecdysone peaks raises the possibility that *dBlimp-1* mRNA is induced by ecdysone. Interestingly, the expression profile of the transcript did not completely coincide with that of the *E75A* early gene transcript detected by RT-PCR method using the same staged RNA preparation; appearance and disappearance of dBlimp-1 mRNA was delayed slightly (Fig. 4A, lower panel), suggesting that regulation mechanism is slightly different between these two genes.

Properties of d*B*limp-*1* transcript

To examine whether *dBlimp-1* mRNA is induced by ecdysone like *E75A* mRNA, salivary glands from late third instar larvae (more than 10 hours before puparium formation) were

cultured for 1.5 hours in the presence or absence of 20-hydroxyecdysone (20E), and the expression level of *dBlimp-1* transcript was detected using quantitative RT-PCR (Fig. 4B). While no *dBlimp-1* expression was detected when salivary glands were cultured in the absence of 20E, transcript was observed when 20E was added in the culture medium, even in the presence of cycloheximide. These results suggest that *dBlimp-1* transcript is directly induced by 20E. Interestingly, the level of induction in the presence of both 20E and cycloheximide was four times higher than that in the presence of 20E alone. This difference was less prominent for *E75A* mRNA. As cycloheximide is known to stabilize some mRNA species (15, 19, 39), these results suggest that the normal turnover rate of *dBlimp-1* mRNA is much faster than that of *E75A* mRNA.

Knock down of *dBlimp-1* results in prepupal lethality and altered timing of βFTZ-F1 expression

To elucidate the function of *dBlimp-1* during *Drosophila* development, we knocked it down by RNAi. We established transgenic *UAS-dBlimp-1i* fly lines that express ds-*dBlimp-1* RNA under the control of GAL4, and observed the phenotype after mating with an *Act5C-GAL4* strain expressing the GAL4 activator ubiquitously under the control of the *Actin5C* promoter. As shown in Fig. 5A, five out of eight established *dBlimp-1i* lines showed lethality at pupal stages in most of the observed animals. Many of them eclosed but died shortly thereafter (eclosed) or died during eclosion (incomplete eclosion). The rest of the lines showed prepupal lethality in more than half of the observed animals (Fig. 5A). Level of the dBlimp-1 transcript in *Act5c-GAL4>UAS-dBlimp-1i7* with the strongest phenotype was reduced about one fourth

compared to that in the control line, as revealed by quantitative RT-PCR (Fig. 5B) at 2h APF. To test if these phenotypes were caused by reduction of dBlimp-1 function, we observed the RNAi phenotype in the presence of a hypomorphic *dBlimp-1*^{P14751} mutation, which carrys a *P* element insertion in the first intron of the gene. *dBlimp-1*^{P14751} homozygous mutants show disintegration of the tracheal network, closely resemble to that of the deficiency strain (34), and die before hatching. Introduction of this mutation into *ActGAL4>UAS-Blimp-1* flies clearly enhanced the RNAi phenotype (Fig. 5A, lower panel), strongly suggesting that observed RNAi phenotype was caused by specific effect on the dBlimp-1 gene and that *dBlimp-1* is required for metamorphosis to be completed, and may be involved in regulating the prepupal to pupal transition.

Because dBlimp-1 is thought to bind to the *cis*-regulatory region of *ftz-f1*, we analyzed the effect of RNAi on βFTZ-F1 expression during the prepupal period by Western blotting (Fig. 5C). Two independent *Act5c-GAL4>UAS-dBlimp-1i8* lines that showed prepupal lethality were collected every 2 hours and subjected to Western blot analysis to obtain reliable results. In the animals from the control line, high-level expression of βFTZ-F1 was detected from 8 to 10 hours APF, as previously reported (33), and very low-level expression was occasionally detected at 6 hours APF. However, in *dBlimp-1* knock down animals, high-level expression was detected even 6 hours APF (lanes 3 and 4). Although the penetrance of this phenotype was not 100%, the higher-level expression at 6 hours APF was observed in 60% of animals in duplicated experiments using two independently established RNAi lines. Furthermore, the high-level expression in RNAi animals persisted only until 8 hours APF; by 10 hours APF, the expression level was greatly reduced. These results suggest that dBlimp-1 prevents premature expression of the *ftz-f1* gene by

acting as a transcriptional repressor during the high ecdysone periods.

Prolonged expression of *dBlimp-1* results in reduced βFTZ-F1 expression and delayed pupation

To test the possibility that dBlimp-1 functions as a transcriptional repressor of *ftz-f1*, we established transgenic *hs-dBlimp-1* lines that express *dBlimp-1* under the control of the heat shock promoter, and analyzed the effect of forced *dBlimp-1* expression on the expression of βFTZ-F1 during the prepupal period by Western blotting. When prepupae of the *hs-dBlimp-1* line were treated at 34^{o}C for 1 hour at 5 hours APF, the expression level of βFTZ-F1 was significantly reduced in prepupae at 8 hours APF, although the same treatment did not cause any effect on βFTZ-F1 expression in the control animals (Fig. 5D). This result supports the idea that dBlimp-1 acts as a repressor for the *ftz-f1* gene. To further explore the effect of prolonged *dBlimp-1* expression, heat-treated animals were observed at later developmental stages. The *hs-dBlimp-1* animals exhibited a delay in pupation (Fig. 5E), suggesting that dBlimp-1 has an important role not only in controlling the timing of βFTZ-F1 expression, but also in pupation.

dBlimp-1 protein is unstable

As the timing of *dBlimp-1* expression is important for temporal regulation of the ecdysone-induced pathway, and our results suggested that *dBlimp-1* mRNA might be unstable, we examined the stability of dBlimp-1 directly. Transgenic fly lines expressing flag-tagged dBlimp-1 under the control of the heat shock promoter were established, and prepupae were heat shocked at

0 hour APF for 1 hour at 37^{o}C and examined by Western blot using anti-Flag antibody (Fig. 6). Strong expression of dBlimp-1 was detectable soon after the heat shock, and the protein level rapidly decreased and became undetectable 3 hours after heat induction. A similar turnover profile was observed using heat shock induced dBlimp-1 without Flag tag (data not shown). In contrast, such rapid turnover was not observed for heat shock induced βFTZ-F1, which was detectable at least 6 hours after heat shock. This prolonged βFTZ-F1 protein expression is not caused by the stability of its mRNA, because induced βFTZ-F1 mRNA disappeared within 3 hours after heat shock (data not shown). These results indicate that dBlimp-1 protein is less stable than βFTZ-F1.

Discussion

Over thirty years ago, the existence of a factor that is directly induced by ecdysone and that represses early genes at the onset of metamorphosis was proposed based on observations of chromosomal puff patterns in cultured salivary glands (3). Here, we show that the expression profile of *dBlimp-1* mRNA mirrors that of a typical early gene, and that *dBlimp-1* transcript is directly induced by 20E in cultured salivary glands. Similar findings from cultured organs in a recent independent report corroborate our results (7). Moreover, we demonstrate that dBlimp-1 acts as a repressor, making it a good candidate for the factor predicted by Ashburner and colleagues. Intriguingly, although there is no direct evidence showing that dBlimp-1 can repress early genes, the 5' upstream region of the early gene *br* contains putative dBlimp-1 binding sites. On the other hand, the map position of *dBlimp-1* was not identified as an early puff locus. This might be due to the low level of *dBlimp-1* expression compared to that of other early genes.

Further study is necessary to examine the effect of *dBlimp-1* on early genes.

Although the dBlimp-1 transcript is directly induced by 20E, its expression profile is slightly delayed compared to that of the *E75A* transcript. The delay in its disappearance after puparium formation and rough coincidence between the time of its disappearance and decline of ecdysone level suggest that the gene may not be repressed by early gene product as are known early genes. Rather its transcription may require the continual presence of 20E so that it is reduced when the ecdysteroid titer falls. Since the ecdysteroid titer is already quite high 3 hr before puparium formation, the reason for the delay in the appearance of *dblimp-1* mRNA is not clear. These questions require further study.

The discovery of an ecdysone-inducible repressor provides new insights into the regulatory mechanisms of *ftz-f1*, which is induced by pulses of ecdysone. Although the ecdysone-inducible transcription factors DHR3 and E75B were previously identified (8, 20, 27, 52), they cannot entirely account for the regulatory mechanism for βFTZ-F1 expression. Our results show that the timing of βFTZ-F1 expression is altered in prepupae in which *dBlimp-1* is knocked down or expressed for a longer period of time, indicating that the timing of *dBlimp-1* expression is crucial for temporal control of the *ftz-f1* gene. In our RNAi experiment, however, we observed only 2 hours earlier expression. This might be due to the incomplete knock down of *dBlimp-1* (see below), or other redundant repression mechanisms, such as DHR3 and E75B. In spite of these unresolved questions, our results provide clear evidence that dBlimp-1 plays a key role in determining the timing of *ftz-f1* expression by acting as a repressor during the high ecdysone period at the onset of metamorphosis.

We also obtained unexpected evidence suggesting that the turnover rate of *dBlimp-1* mRNA is quite rapid. Whereas *dBlimp-1* mRNA levels increased upon addition of cycloheximide in cultured organs, other ecdysone-induced early genes, including *br* (6), *E74A* (7, 47), *E75A*, and *E75B* (43) did not show significant increases in mRNA levels during the two hours of culture. The instability of *dBlimp-1* mRNA may have affected our RNAi experiment, in which we were able to reduce the level of *dBlimp-1* mRNA only to one fourth of the normal level at 2 h APF.

Furthermore, we found that transgenic dBlimp-1 protein expressed under the control of the heat shock promoter disappeared rapidly. In contrast, BR-C proteins, which are early gene products, have been shown to persist as long as βFTZ-F1 when expressed under the control of the heat shock promoter (13, 26). Furthermore, the *dBlimp-1* mRNA peak detected by Northern blot and protein activity peak detected by gel mobility shift assay coincided well. In contrast, the protein peaks for other ecdysone-induced transcription factors, such as E74A (10) and E75B (8, 52) were roughly 2 hours later than their mRNA peaks. These observations support the idea that the degradation rate of endogenous dBlimp-1 is also more rapid than that of many other ecdysone-inducible transcription factors. The degradation rate of each protein is controlled by signals within its own sequence. For example, PEST sequences are proline, glutamic acid, serine and threonine-rich sequences that target proteins for degradation (36, 41). Indeed, dBlimp-1 contains a proline-rich PEST sequence that may be responsible for its instability, since removal of this region stabilizes protein expressed under the control of the heat shock promoter (M. S. and H.U., unpublished data). Whatever the mechanism of the instability, our results indicate that instability of *dBlimp-1* mRNA and protein plays a crucial role in determining the timing of

βFTZ-F1 expression and pupation.

In strongly affected *dBlimp-1* RNAi lines, most animals arrested development at the prepupal stage and expressed βFTZ-F1 prematurely. We have previously shown that premature expression of βFTZ-F1 during the prepupal period causes developmental arrest at the prepupal stage (55). Thus, developmental arrest in the *dBlimp-1* RNAi animals might be mediated through the premature expression of βFTZ-F1. On the other hand, we found that forced expression of *dBlimp-1* caused delays in both the timing of βFTZ-F1 expression and pupation. Thus, the timing of pupation might be controlled by the timing of βFTZ-F1 expression.

Recently, it has been reported that *dBlimp-1* expression in the tracheal system in *Drosophila* embryos is important for development of this tissue (34). In addition, *dBlimp-1* is expressed in a spatially-restricted manner in other regions during early embryogenesis, although the functions of these early expression domains remain unknown. *Blimp-1* is similarly expressed in many different tissues in vertebrates, where it is known to play important roles in embryogenesis, germ cell determination, specification in nerve and muscle cells, linage determination in epidermis, and B cell maturation (5, 11, 12, 14, 18, 35, 42, 49, 53). Thus, *dBlimp-1* may be involved in many other developmental events in the fly.

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Figure legends

Fig. 1. Determination of the binding site and developmental expression pattern of Factor I-4. (A) Methylation interference using a 400 bp *Eco52*I-*Hinc*II fragment. Positions of nucleotides showing methylation interference are represented by asterisks with distance from the transcription start site. G+A Maxam & Gilbert-sequencing reaction was used as a marker. (B) Confirmation of sequence-specific binding to the identified site by a gel mobility shift competition assay. ^{32}P-labeled Site I-4 DNA was used as a probe, and the indicated amounts of Site I-4 or Site I-4m competitor DNA compared with the probed Site I-4 DNA were added to the binding reactions. Nucleotide sequences of site I-4 and site I-4m DNA are indicated at the bottom. Positions of introduced mutations are indicated by dots. (C) Confirmation of Factor I-4 binding by gel mobility shift assays using developmentally staged nuclear extracts at embryonic stages (left) and at the onset of metamorphosis (right). ^{32}P-labeled site I-4 DNA was used as a probe.

Fig. 2. Identification of Factor I-4 as a *Blimp-1*/*CG5249* encoded protein. (A) Purification scheme of Factor I-4. Nuclear extract (NE) derived from AEL 8-16 hour embryos was loaded onto an S Sepharose column. Fractions eluted between 0.15 and 0.25 M NaCl concentration (SE) were subjected to affinity purification. The first affinity purification was performed using Site I-4 DNA-conjugated latex beads. Eluate of the first affinity chromatography (E1) was then incubated with mutated Site I-4 DNA-conjugated beads to perform subtraction. Supernatant of subtraction was subjected to a second affinity purification. Eluate of the second affinity chromatography (E3-1 and E3-2) was obtained. (B) Detection of the binding activity and proteins in typical

fractions during purification by a gel mobility shift assay (upper panel) and SDS-PAGE (lower panel). 1 μl of the fractions in the purification step was used for the gel mobility shift assay and 10 μl of the same fraction was reserved for SDS-PAGE except for NE and SE, which were loaded only 0.5 μl. Proteins were detected by silver staining.

Fig. 3. Confirmation of Factor I-4 as dBlimp-1. (A) Gel mobility shift competition assay using *in vitro* expressed dBlimp-1 (left panel) and Factor I-4 in the nuclear extract (right panel). The indicated amounts of Site I-4 or Site I-4m2 DNA compared with the probed Site I-4 DNA were added to the reaction mixtures of the gel mobility shift assay. Six point nine ng of purified recombinant proteins or 1 μl of nuclear extract was used in each binding reaction. (B) Supershift of Factor I-4 by anti-dBlimp-1. Anti-dBlimp-1 or preimmune serum was added to the reaction mixture for the gel mobility shift assay to detect dBlimp-1 in the complex. (C) Specificity of anti-dBlimp-1. βFTZ-F1 was detected by gel mobility shift assay. Anti-dBlimp-1 or anti-βFTZ-F1 serum was added to the reaction mixture for the gel mobility shift assay to examine specificity.

Fig. 4. Characterization of *dBlimp-1* transcripts. (A) Detection of *dBlimp-1* mRNA by Northern blotting using staged total RNA at the onset of metamorphosis. High ecdysone periods are indicated at the top by trapezoids. Positions of *dBlimp-1* mRNA are indicated by arrows. Middle panel detected *rp49* mRNA using the same membrane. Bottom panel shows level of *E75A* transcript detected by RT-PCR using the same staged RNA. (B) Induction of *dBlimp-1* (left) and

E75A (right) mRNA by 20-hydroxyecdysone (20E) in cultured salivary glands. Expression levels were measured by quantitative real-time RT-PCR using total RNA from salivary glands cultured for 90 minutes in the presence of 20E and/or cycloheximide (CHX). The value of each transcript was normalized by that of *rp49* transcripts, with the level obtained with 20E and cycloheximide set as 1 for each transcript. The same template was used to measure the amounts of *dBlimp-1* and *E75A* mRNA.

Fig. 5. dBlimp-1 has a repressor activity and controls the timing of βFTZ-F1 expression and pupation. (A) Phenotype of *dBlimp-1* RNAi lines. Eight independent *UAS-dBlimp-1i* lines were mated with the *Act-Gal4* line, and prepupae of their progeny were collected and their lethal phases were scored from the prepupal stage to adult. Effect of mutation in the *dBlimp-1* gene by P element insertion, $dBlimp\text{-}1^{P14751}$, was examined for *UAS-dBlimp-1i1* and *5* lines. Numbers in parentheses represent the number of scored animals. Typical examples of arrested animals are shown on the right. (B) Reduction of dBlimp-1 transcript level in RNAi animals. Expression levels were measured by quantitative real-time RT-PCR using total RNA from prepupae at 2 hours APF in the indicated lines. The same template was used to measure the amounts of *dBlimp-1* and *E75A* mRNAs. (C) Premature expression of βFTZ-F1 by RNAi of *dBlimp-1*. The expression of βFTZ-F1 in *dBlimp-1* RNAi (*Act5c-Gal4>UAS-dBlimp-1i8*) and its control animals (+/*UAS-dBlimp-1i8*) from 4 to 10 h APF was estimated by Western blotting. Anti-α-tubulin antibody was used to confirm amount of loaded protein in each lane. (D) Delay of βFTZ-F1 expression by induction of *dBlimp-1*. Prepupae at 5 hours APF of *hs-dBlimp-1* or host strain were

heat shocked at 34^{o}C for 1 hour and then reared at 25^{o}C. The expression level of βFTZ-F1 in two individual prepupae at the indicated times was estimated by Western blotting. Anti-α-tubulin antibody was used to confirm the amount of loaded protein in each lane. (E) Delay of pupation timing by forced induction of *dBlimp-1*. Prepupae at 5 hours APF of *hs-dBlimp-1* or host strain were heat shocked at 34°C for 1 hour and then reared at 25°C. Numbers of newly pupated animals were counted every hour and the percentage of pupated animals in each period was plotted. Numbers in parentheses represent the number of scored animals. Four out of 26 animals in *hs-dBlimp-1* failed to pupate.

Fig. 6. dBlimp-1 is a labile protein

dBlimp-1 or βFTZ-F1 was expressed under the control of the heat shock promoter at 0 hour APF, and the level of induced protein was detected by Western blot using either an anti-Flag antibody or anti- βFTZ-F1 serum. Samples from two independent animals of *hs-Blimp-1* or *hs-βFTZ-F1* line were examined every hour. Non-heat shock control animals were examined at 0 hour APF.