Inducible expression of double-stranded RNA directs specific genetic interference in *Drosophila*

Geanette Lam and Carl S. Thummel

Background: The introduction of double-stranded RNA (dsRNA) can selectively interfere with gene expression in a wide variety of organisms, providing an ideal approach for functional genomics. Although this method has been used in *Drosophila*, it has been limited to studies of embryonic gene function. Only inefficient effects have been seen at later stages of development.

Results: When expressed under the control of a heat-inducible promoter, dsRNA interfered efficiently and specifically with gene expression during larval and prepupal development in *Drosophila*. Expression of dsRNA corresponding to the *EcR* ecdysone receptor gene generated defects in larval molting and metamorphosis, resulting in animals that failed to pupariate or prepupae that died with defects in larval tissue cell death and adult leg formation. In contrast, expression of dsRNA corresponding to the coding region of the βFTZ-F1 orphan nuclear receptor had no effect on puparium formation, but led to an arrest of prepupal development, generating more severe lethal phenotypes than those seen with a weak *βFTZ-F1* loss-of-function allele. Animals that expressed either *EcR* or *βFTZ-F1* dsRNA showed defects in the expression of corresponding target genes, indicating that the observed developmental defects are caused by disruption of the genetic cascades that control the onset of metamorphosis.

Conclusions: These results confirm and extend our understanding of *EcR* and *βFTZ-F1* function. They also demonstrate that dsRNA expression can inactivate *Drosophila* gene function at later stages of development, providing a new tool for functional genomic studies in *Drosophila.*

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Background

The recent completion of the *Drosophila* genome sequence has provided a vast new resource for using this simple model organism to define conserved biological pathways. A critical step in exploiting these genomic resources, however, depends on methods that allow the investigator to move rapidly and efficiently from DNA sequence to gene function. Traditionally, these reverse-genetic approaches have been tedious and time-consuming, depending on fortuitous transposon insertions or classic genetic screens. Recently, however, Fire and colleagues showed that injection of double-stranded RNA (dsRNA) corresponding to the coding region of a particular gene will effectively and specifically interfere with the activity of that gene in *Caenorhabditis elegans* [1]. This method of RNA interference (RNAi), has provided a powerful new tool for functional genomic studies in a wide range of organisms, including planaria, hydra, trypanosomes, zebrafish and mice [2].

Injection of dsRNA into the early syncytial *Drosophila* embryo results in specific genetic interference during embryonic development [3–7]. Moreover, RNAi inactivates both maternal and zygotic gene contributions, circumventing more traditional and labor-intensive methods such as germ-line clonal analysis [8]. Efforts to use RNAi in *Drosophila* at later stages of development, however, have been less successful. For example, an attempt to phenocopy the *white* eye color mutation by injection of dsRNA into embryos resulted in an extremely low level of interference in the adult eye (<3%) [6]. As a result, the utility of this method has been limited to studies of embryonic gene function in fruit flies.

Here, we report a means of extending RNAi technology to later stages of *Drosophila* development, allowing the investigator to control when a specific gene will be inactivated. As a model for these studies, we focused on the remarkable biological transformations associated with metamorphosis, when the crawling *Drosophila* larva is transformed into a mature adult fly. *Drosophila* metamorphosis is initiated by two sequential pulses of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) [9]. A pulse of ecdysone at the end of the third larval instar triggers puparium formation, marking the onset of prepupal development. The larval midgut initiates steroid-triggered programmed cell death at this stage, as the leg and wing

imaginal discs evert and elongate to form rudiments of the adult appendages [10]. Another ecdysone pulse, ~12 hours after puparium formation, triggers eversion of the adult head and salivary gland cell death, marking the prepupal–pupal transition.

We have focused our studies of RNAi technology on two members of the nuclear receptor superfamily that play key roles during the onset of metamorphosis: the EcR ecdysone receptor and the βFTZ-F1 orphan nuclear receptor [11,12]. Here, we report that expression of dsRNA derived from the *EcR* or *βFTZ-F1* coding regions can effectively and specifically interfere with the functions of these genes. This method provides a useful new tool for functional genomic studies in *Drosophila*.

Results and discussion

Expression of *EcR* dsRNA leads to defects in puparium formation and ecdysone-regulated gene expression

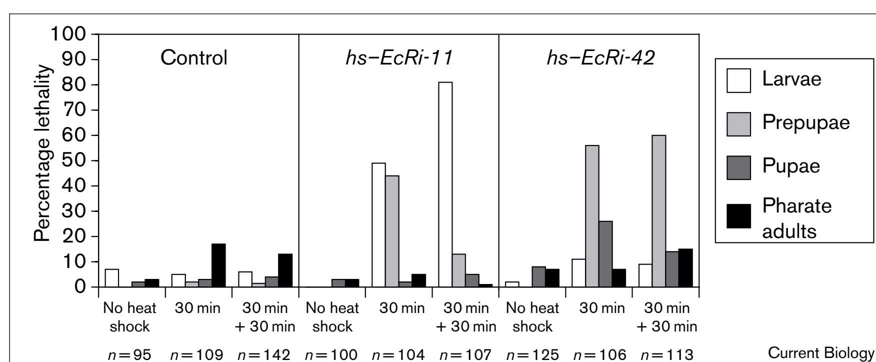
EcR encodes three protein isoforms, designated A, B1, and B2, which can function together with the *Drosophila* RXR homolog, USP, as receptors for ecdysone [13–17]. Some isoform-specific *EcR* mutations result in a small number of escapers that arrest development at the onset of metamorphosis, indicating that this receptor plays a key role in transducing the ecdysone signal at this stage [18,19]. To assess null mutant phenotypes for *EcR* during the onset of metamorphosis, we established *P*-element transformants that use the heat-inducible *hsp70* promoter to drive expression of a snapback dsRNA corresponding to the coding region shared by all *EcR* isoforms.

Two lines, *hs–EcRi-11* and *hs–EcRi-42*, were examined in detail. As expected, these transformants survived as well as controls in the absence of heat treatment (Figure 1, no heat shock). A single 30 minute heat treatment in mid-third instar larvae however resulted in a high degree of lethality in both lines. An additional heat treatment 6 hours later increased the severity of this phenotype in *hs–EcRi-11* transformants, resulting in predominantly larval lethality (Figure 1).

To understand how *EcR* dsRNA could exert these effects on viability, we assayed the levels and stability of *EcR* dsRNA induced by heat treatment as well as the effects of this expression on EcR protein levels at puparium formation. A single 30 minute heat treatment induced high levels of *EcR* dsRNA, which was then rapidly degraded (Figure 2a). Only very low levels were detectable by 6 hours after heat treatment. This observation is consistent with recent studies indicating that dsRNA is rapidly broken down into 20–25 nucleotide RNA fragments that may then guide degradation of the target mRNA over an extended period of time [20,21]. Consistent with this model, we found that the peak of EcR protein accumulation, which normally occurs at the end of larval development, was blocked in *hs–EcRi-11* animals (Figure 2b). The levels of USP protein, however, were unaffected, indicating that this response is specific to EcR (Figure 2b). The observation that EcR protein levels were significantly reduced 12–18 hours after heat-induced dsRNA synthesis, in spite of the rapid turnover of *EcR* dsRNA, is consistent with the long-term effects of RNAi reported in both *C. elegans* and *Drosophila* [1,6,7].

Two sequential heat treatments, at ~18 and 12 hours before puparium formation, generated the most severe lethal phenotypes in *hs–EcRi-11* animals (Figure 1). We therefore performed a more detailed characterization of this transformant line using the double 30 minute heat treatment regime. The majority of *hs–EcRi-11* animals (81%; $n = 107$) arrested development as stationary late third instar larvae (Figure 3b). Although these animals stopped moving, as did wild-type late third instar larvae, they failed to shorten their body to form the characteristic shape of a prepupa (Figure 3a) and died several days later. Some animals attempted to pupariate (13%), but formed long prepupae that occasionally failed to tan, and died 1–2 days later (Figure 3c,d). A small number of animals (6%) died as pupae with defects in leg elongation (Figure 3e, arrow). Dissection of *hs–EcRi-11* prepupae revealed normal larval midguts in newly-formed prepupae

Figure 1



Lethal phases of animals expressing *EcR* dsRNA. Control w^{1118} animals, w^{1118}; *hs–EcRi-11* animals, and w^{1118}; *hs–EcRi-42* animals were staged as mid-third instar larvae, ~18 h before puparium formation. These animals were either maintained at 25°C (no heat shock), given a single 30 min 37°C heat treatment (30 min), or given a 30 min 37°C heat treatment followed by a second 30 min heat treatment 6 h later (30 min + 30 min). Animals that died as larvae, prepupae, pupae, or as pharate adults were counted and are depicted as the percentage of total animals studied.

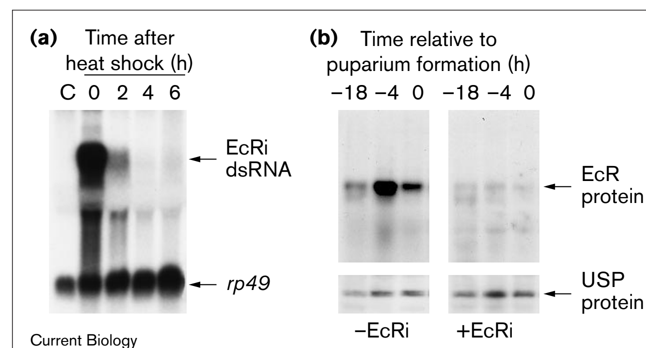
(Figure 3h), but the midguts failed to progress beyond the stage normally seen in prepupae that are 2 hours old [22]. The midgut failed to contract and undergo cell death, resulting in persistence of the gastric caeca and proventriculus (Figure 3i, arrows). In addition, the adult midgut, which would normally surround the dying larval cells by 12 hours after puparium formation, did not appear to form [22]. Similarly, larval salivary glands were still present 24 hours after puparium formation in *hs–EcRi-11* animals, indicating a block in their normal cell-death response (Figure 3j). Therefore, expression of *EcR* dsRNA at ~18 and 12 hours before puparium formation has a profound effect on the ability of animals to enter metamorphosis in response to the late larval pulse of ecdysone.

Ecdysone exerts its effects on development through a well-defined cascade of ecdysone-regulated gene expression. The hormone directly induces a small set of primary-response genes, including the *E74* and *E75* early genes [23,24]. These genes encode transcription factors that regulate downstream secondary-response late genes. In the salivary glands, the late larval pulse of ecdysone triggers a switch in late gene expression, repressing the glue genes and inducing the *L71* late genes, along with more than 100 late puffs visible in the giant polytene chromosomes [25–27].

To understand the molecular basis of *EcR* interference by dsRNA, we examined the patterns of ecdysone-regulated gene expression in staged *hs–EcRi-11* late larvae and early prepupae (Figure 4). As expected, the levels of *EcR* mRNA were significantly reduced in these animals, consistent with the reduced levels of EcR protein detected by western blot analysis (Figure 2b). Ecdysone-induced *E74A* and *E75A* transcription was significantly reduced in *hs–EcRi-11* late third instar larvae (Figure 4). Repression of *E74B*, which is linked to *E74A* induction [28], also failed to occur in these animals. Consistent with these defects in early ecdysone-induced regulatory gene expression, the switch in salivary gland late genes is effectively blocked. The *Sgs-4* glue gene is not repressed at puparium formation and the *L71-6* late gene is not induced. Moreover, *reaper* mRNA failed to accumulate to normal levels in *hs–EcRi-11* mid-prepupae, consistent with the observed defects in ecdysone-triggered cell death.

Taken together, these phenotypes correspond closely to those described for loss-of-function *EcR* mutations. A small number of null mutants that are missing only the *EcR-B* isoforms survive to the third larval instar and attempt to initiate metamorphosis, forming stationary larvae that resemble the predominant lethal phenotype of *hs–EcRi-11* animals [19] (Figure 3b). *EcR-B* null mutants, however, undergo larval cuticle apolysis, a process that failed to occur in *hs–EcRi-11* animals. This observation is consistent with the proposal that *EcR* dsRNA would inactivate all *EcR* isoforms,

Figure 2



The *hs−EcRi-11* animals express high levels of heat-induced *EcR* dsRNA and display reduced levels of EcR protein. **(a)** High levels of *EcR* dsRNA were expressed following heat treatment. Total RNA was extracted from either control w^{1118} mid-third instar larvae or from w^{1118}; *hs−EcRi-11* mid-third instar larvae that had been exposed to a 30 min 37°C heat treatment and allowed to recover at 25°C for 0, 2, 4, or 6 h. Equal amounts of RNA were analyzed by northern blot hybridization using a radioactive probe for *EcR* mRNA. A probe to detect *rp49* mRNA was also included as a control for loading and transfer. **(b)** EcR protein, but not USP protein, was significantly reduced following expression of *EcR* dsRNA. Control w^{1118} mid-third instar larvae (−EcRi) or w^{1118}; *hs−EcRi-11* mid-third instar larvae (+EcRi) were heat-treated at ~18 and 12 h before puparium formation, and protein samples were analyzed by western blotting using either the DDA27 EcR monoclonal antibody [13] or AB11 USP monoclonal antibody [37], as described [38]. Approximately equal amounts of protein were present in each lane, as determined by Coomassie blue staining of a second gel run in parallel.

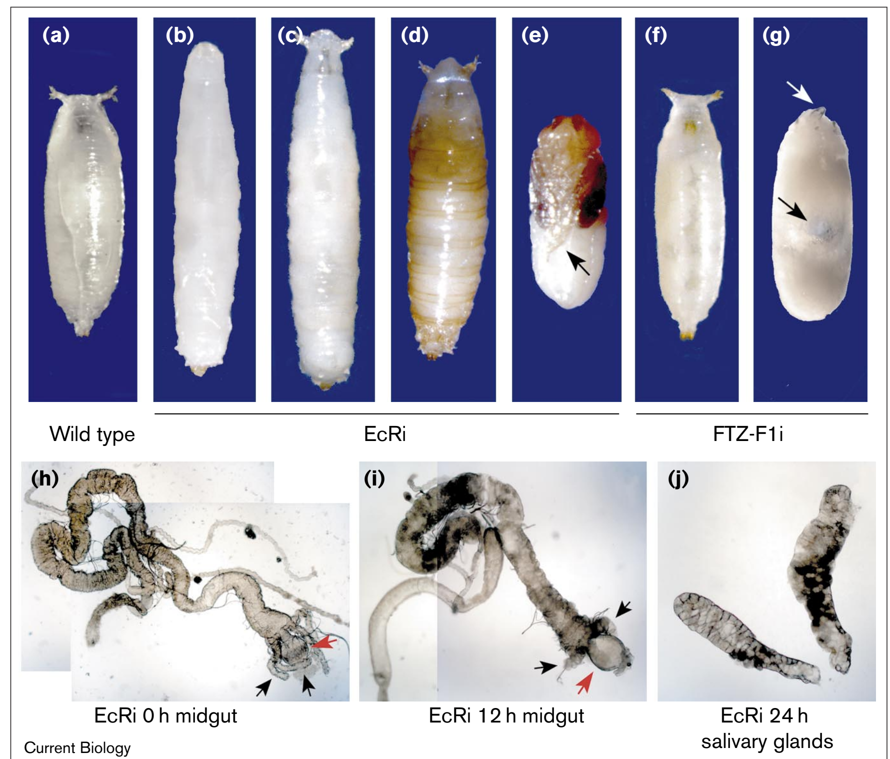
and would therefore generate more severe phenotypes than those seen in the absence of only the B isoform.

Consistent with this proposal, null mutants for all three *EcR* isoforms that are rescued to the third larval instar by ectopic expression of an *EcR-B2* cDNA not only fail to pupariate properly, but also display a block in larval cuticle apolysis similar to that in *hs–EcRi-11* animals [29]. These partially rescued *EcR* mutants also show little or no expression of ecdysone-inducible early proteins, consistent with a block in the late larval genetic cascade. These effects resemble the transcriptional defects we observed in *hs–EcRi* animals (Figure 4). It is interesting to note that heat treatment of unstaged *hs–EcRi-11* larvae resulted in molting defects that resemble those seen in *EcR* loss-of-function mutants, indicating that this construct can also be used to define *EcR* functions during larval development (data not shown). Taken together, the phenotypes of *hs–EcRi-11* animals are entirely consistent with a specific and effective block in *EcR* function during later stages in the life cycle.

Expression of *βFTZ-F1* dsRNA results in stage-specific defects during metamorphosis

To test the generality of this method, we also attempted to interfere with *βFTZ-F1* function during the onset of metamorphosis. There are two reasons why *βFTZ-F1*

Figure 3



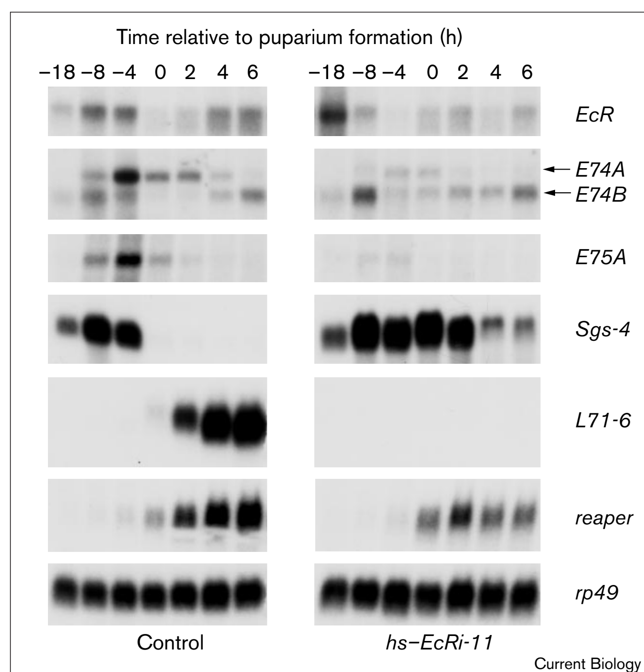
Lethal phenotypes of animals expressing *EcR* or *βFTZ-F1* dsRNA. **(a)** A newly-formed w^{1118} prepupa (wild type) is depicted along with **(b–e)** *hs–EcRi-11* animals in which *EcR* dsRNA was expressed at ~18 and 12 h before puparium formation. (b) The majority of animals expressing *EcR* dsRNA arrested development as stationary non-pupariating late third instar larvae. Fewer animals formed elongated prepupae that were either (c) untanned or (d) tanned. (e) A minority of these animals died as pharate adults with defects in leg elongation. The legs at this stage normally extend to the posterior tip of the animal, whereas the legs in these mutant animals were short and misshapen (arrow). **(f)** When *βFTZ-F1* dsRNA was expressed at ~18 and 12 h before puparium formation, normal prepupae were formed but they arrested development shortly thereafter. **(g)** A similar lethal phenotype was seen in animals in which *βFTZ-F1* dsRNA was expressed at 0 and 6 h after puparium formation. These animals had a prominent gas bubble that failed to translocate to the anterior end (black arrow). Head eversion also failed in these animals and the larval mouthhooks remained attached (white arrow). The animals in (e,g) have been removed from the pupal case. **(h–j)** Larval midguts and salivary glands were dissected from *hs–EcRi-11* animals in which *EcR* dsRNA was expressed at ~18 and 12 h before puparium formation. Midguts were dissected from larvae (h) that had just become stationary or (i) 12 h after the stationary phase. Red arrows, the proventriculus; black arrows, the gastric caeca. (j) Larval salivary glands were detected in these animals 24 h after the stationary phase.

provides a valuable additional test of this method. First, unlike *EcR*, *βFTZ-F1* exerts a stage-specific function at the onset of metamorphosis, with no apparent function at puparium formation and an essential role in providing competence for the ecdysone-triggered prepupal–pupal transition [30]. Second, only hypomorphic *βFTZ-F1* mutants have been studied during the onset of metamorphosis because null mutants die during early stages of development [30]. Thus, more severe phenotypes associated with *βFTZ-F1* RNAi might provide new insights into the function of this receptor.

Expression of *βFTZ-F1* dsRNA ~18 and 12 hours before puparium formation, which is identical to the double heat-shock regime used with *hs–EcRi-11*, resulted in normal puparium formation (Figure 3f), although 37% of these animals (n = 70) failed to evert usually one anterior spiracle (data not shown). The ability of these animals to pupariate is consistent with the absence of *βFTZ-F1* expression in third instar larvae as well as the absence of any effects of *βFTZ-F1* mutations on puparium formation [30,31]. The majority of animals expressing *βFTZ-F1* dsRNA, however, failed to progress through the early stages of metamorphosis and died as prepupae. Sequential heat induction of *βFTZ-F1* dsRNA at 0 and 6 hours after puparium formation led to a similar phenotype, with all animals arresting development at the prepupal stage (Figure 3g). Although these animals displayed normal gas bubble formation, they failed to translocate the bubble to the anterior end, and died after several days with a prominent bubble in the middle of the body (Figure 3g, black arrow). In addition, eversion of the adult head was completely blocked and the larval mouthhooks that are normally expelled at head eversion remained attached at the anterior end of the animal (Figure 3g, white arrow). Although *βFTZ-F1* hypomorphic mutants also show defects in adult head eversion, this phenotype was more severe and more penetrant in animals that express *βFTZ-F1* dsRNA. Most *βFTZ-F1* hypomorphic mutants die as pupae with defects in head eversion and leg elongation, with some animals surviving to adulthood [30]. The fully penetrant prepupal lethality associated with *βFTZ-F1* RNAi is likely to be due to a severe reduction in *βFTZ-F1* function, and indicates that *βFTZ-F1* is absolutely required for progression through the mid-prepupal stage.

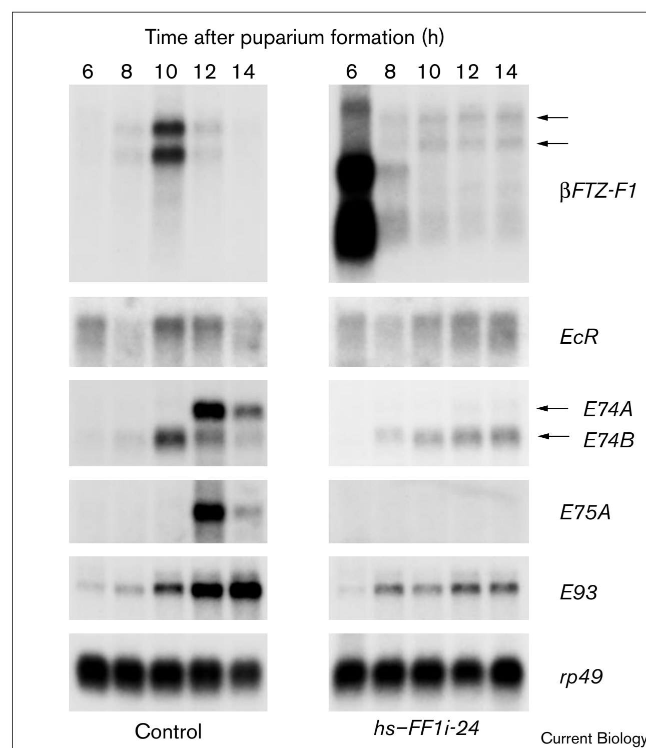
We also examined the effects of *βFTZ-F1* dsRNA on ecdysone-inducible gene expression (Figure 5). Similar

Figure 4



Expression of *EcR* dsRNA inhibits genetic responses to the late larval pulse of ecdysone. Third instar w^{1118} larvae (control) and *hs–EcRi-11* larvae were heat-treated at ~18 and 12 h before puparium formation and then collected at different stages of larval and prepupal development. Total RNA was extracted and analyzed by northern blot hybridization using probes to the indicated genes. The abundant RNA detected by the *EcR* probe in *hs–EcRi-11* larvae at −18 h is an uncharacterized transcript derived from the heat-induced transgene. Hybridization with *rp49* was used as a control for loading and transfer.

Figure 5



Expression of *βFTZ-F1* dsRNA inhibits genetic responses to the prepupal pulse of ecdysone. Control w^{1118} prepupae and prepupae carrying the *hs–FF1i-24* transgene were heat treated at 0 and 6 h after puparium formation, and collected at 2 h intervals from 6–14 h after puparium formation, spanning the prepupal pulse of ecdysone. Total RNA was extracted and analyzed by northern blot hybridization using probes to the indicated genes. High levels of *βFTZ-F1* dsRNA were detected at the 6 h timepoint, as well as subsequent reduced levels of endogenous *βFTZ-F1* mRNA (arrows). Hybridization with *rp49* was used as a control for loading and transfer.

to the kinetics of *EcR* dsRNA, *βFTZ-F1* dsRNA was expressed at high levels in response to heat treatment and then turned over very rapidly (compare Figures 2a and 5). Furthermore, the levels of endogenous *βFTZ-F1* mRNA were significantly reduced in these animals, consistent with their selective degradation by RNAi. Ecdysone-induced *E74A* transcription was significantly reduced in animals expressing *βFTZ-F1* dsRNA, *E74B* was not repressed, *E75A* failed to be expressed, and *E93* was only weakly induced. The levels of *EcR* mRNA are similar to those of control animals although there is a slight decrease at 10 hours after puparium formation. It is likely that this reduction reflects a requirement for *βFTZ-F1* in directing this prepupal peak in *EcR* activity. The levels of *usp* mRNA were unaffected by the expression of *βFTZ-F1* dsRNA (data not shown). Importantly, all of these effects on ecdysone-regulated gene expression were virtually identical to those seen in *βFTZ-F1* mutant prepupae [30], indicating that *βFTZ-F1* dsRNA acts as an effective and specific block to the activity of this competence factor.

Conclusions

Three lines of evidence argue that induced expression of dsRNA results in specific genetic interference in *Drosophila*. First, the late larval peak of *EcR* mRNA and protein accumulation was significantly reduced in the presence of *EcR* dsRNA, whereas the level of its heterodimer partner, USP, remained unaffected (Figures 2,4). Second, puparium formation was effectively blocked when *EcR* dsRNA was expressed in mid-third instar larvae, whereas this transition occurred normally when *βFTZ-F1* dsRNA was expressed at the same stage in development. This stage specificity is consistent with the known stage-specific functions of these two nuclear receptors [29,30]. Third, expression of *EcR* or *βFTZ-F1* dsRNA resulted in distinct lethal phenotypes and effects on gene expression—effects that are very similar to those defined through loss-of-function genetic studies. Taken together, these observations argue that expression of dsRNA is highly specific in its effects on gene activity, similar to the

specificity observed with injection of dsRNA. Moreover, recent studies in *C. elegans* have shown that expression of dsRNA can specifically and effectively interfere with the activity of a wide range of genes, indicating that this approach may be applicable to other organisms that are amenable to genetic transformation [32].

Heat-induced expression of dsRNA from a transgene provides a means of controlling when RNAi will occur, significantly expanding the usefulness of this approach. This method also provides a new way to study the later functions of genes which, when mutated, result in early lethality. In effect, this provides a means of generating dominant temperature-sensitive alleles for any coding region in the *Drosophila* genome. In the past, these mutations have only been obtained as fortuitous alleles in open-ended genetic screens. An additional advantage of this method is that it provides uniform interference throughout the animal, circumventing the mosaic effects often seen with embryonic injection of dsRNA [7]. It should be possible to modify this method in order to direct stage- and tissue-specific expression of dsRNA using the GAL4 system in *Drosophila* [33]. This refinement should allow investigators to control precisely when and where gene interference will occur.

Materials and methods

P-element vectors and germ-line transformation

Two pairs of oligonucleotides were used as primers to amplify a 1092 bp portion of the *EcR* common region by PCR. One reaction used two primers: 5′-CGGAATTCCGGAATGCGTCGTCCCG-3′ and 5′-GAATGCGGCCGCCAATGCCGGCGGTAAT-3′. This resulted in *EcoR*I and *Not*I sites at either end of the 1092 bp fragment. The other reaction used two related primers: 5′-CGGGATCCCGGAATGCGTCGTCCCG-3′ and 5′-GCTCTAGACAATGCCGGCGGTAAT-3′. This resulted in *BamH*I and *Xba*I sites at either end of the 1092 bp fragment. Each of these fragments was then sequentially inserted between the corresponding restriction sites in the pCaSpeR-hs-act *P*-element vector [34]. This resulted in two copies of the 1092 bp region, arranged as a head-to-head repeat, located downstream from the *hsp70* promoter and upstream from the *actin 5C* transcription termination and polyadenylation signals.

The *βFTZ-F1* sequences were amplified and cloned in a similar manner. One reaction used two primers: 5′-CGGAATTCGTTCGAGCGGATAGAAT-3′ and 5′-GAAGATCTAGTATTCCGTGTCACG-3′. This resulted in *EcoR*I and *Bgl*II sites at either end of a 1392 bp *βFTZ-F1* fragment. The other reaction used two related primers: 5′-CGGGATCCGTTCGAGCGGATAGAAT-3′ and 5′-GCTCTAGAAGTATTCCGTGTCACG-3′. This resulted in *BamH*I and *Xba*I sites at either end of the 1392 bp fragment. Each of these fragments was then sequentially inserted between the corresponding restriction sites in the pCaSpeR-hs-act *P*-element vector, as described above. Both the *EcR* and *βFTZ-F1* clones were transformed into DH5α and their structures were confirmed by restriction enzyme mapping. We refer to the *EcR* construct as *hs–EcRi* and the *βFTZ-F1* construct as *hs–FF1i*. The 1092 bp region of the *EcR* gene corresponds to nucleotides 2059–3150 from the *EcR-B1* cDNA (GenBank accession number M74078). The 1392 bp region of the *βFTZ-F1* gene corresponds to nucleotides 1082–2473 (GenBank accession number M98397). Neither region contains repetitive sequences.

P elements were introduced into the germ line of w^{1118} flies using standard methods [35]. Eight independent lines of *hs–EcRi* and three independent lines of *hs–FF1i* were established as stocks. Further characterization was conducted on two *hs–EcRi* lines: *hs–EcRi-11* (a homozygous viable insertion on the second chromosome) and *hs–EcRi-42* (a homozygous viable insertion on the third chromosome); as well as three *hs–FF1i* lines: *hs–FF1i-12* (a homozygous viable insertion on the second chromosome), *hs–FF1i-24* (a homozygous viable insertion on the second chromosome), and *hs–FF1i-27* (an insertion on the X chromosome). As described in the text, *hs–EcRi-42* had weaker effects than *hs–EcRi-11*. Similarly, *hs–FF1i-12* and *hs–FF1i-27* displayed only weak activity while *hs–FF1i-24* effectively inhibited *βFTZ-F1* function. This latter stock was used for the studies described here. All fly stocks were maintained at 25°C on standard cornmeal molasses medium.

Developmental staging and heat induction of dsRNA

Mid- and late third instar larvae were staged by maintaining them on food containing 0.5% bromophenol blue, and prepupae were staged in hours after puparium formation, as described [36]. At the appropriate stage, animals were transferred to 1.5 ml microcentrifuge tubes with a perforated cap, incubated for 30 min in a 37°C water bath, transferred into new vials, and maintained at 25°C. Any subsequent heat treatments were performed by incubating the vial in a 37°C water bath for 30 min. Mutant phenotypes were characterized in either intact animals or animals dissected out of the pupal case after incubation at 95°C for 2 min (Figure 3e,g).

Northern blot hybridization

Total RNA was extracted from staged late third instar larvae and prepupae as described [36]. Equal amounts of RNA were fractionated by formaldehyde agarose gel electrophoresis, transferred to nylon membranes and crosslinked by UV irradiation. Filters were hybridized, washed and stripped as described [28]. DNA probes were prepared and labeled as described [31], except for the *reaper* probe [22].

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