Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*

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ABSTRACT

RNA interference (RNAi) designates the multistep process by which double-stranded RNA induces the silencing of homologous endogenous genes. Some aspects of RNAi appear to be conserved throughout evolution, including the processing of trigger dsRNAs into small 21–23-bp siRNAs and their use to guide the degradation of complementary mRNAs. Two remarkable features of RNAi were uncovered in plants and *Caenorhabditis elegans*. First, RNA-dependent RNA polymerase activities allow the synthesis of siRNA complementary to sequences upstream of or downstream from the initial trigger region in the target mRNA, leading to a transitive RNAi with sequences that had not been initially targeted. Secondly, systemic RNAi may cause the targeting of gene silencing in one tissue to spread to other tissues. Using transgenes expressing dsRNA, we investigated whether transitive and systemic RNAi occur in *Drosophila*. DsRNA-producing transgenes targeted RNAi to specific regions of alternative mRNA species of one gene without transitive effect directed to sequences downstream from or upstream of the initial trigger region. Moreover, specific expression of a dsRNA, using either cell-specific GAL4 drivers or random clonal activation of a GAL4 driver, mediated a cell-autonomous RNAi. Together, our results provide evidence that transitive and systemic aspects of RNAi are not conserved in *Drosophila* and demonstrate that dsRNA-producing transgenes allow powerful reverse genetic approaches to be conducted in this model organism, by knocking down gene functions at the resolution of a single-cell type and of a single isoform.

Keywords: *Drosophila melanogaster*; RNA interference; ecdysone receptor; batman

INTRODUCTION

RNA interference (RNAi) results in targeted down-regulation of gene expression (Fire et al. 1998). It is triggered by double-stranded RNAs (dsRNAs) that are processed into small 21–23-bp dsRNAs (siRNAs) by a dsRNA-specific RNase DICER (Bernstein et al. 2001). In subsequent steps, siRNAs act as guides for specific degradation of their complementary mRNA (Zamore et al. 2000). RNAi may be achieved upon injection of dsRNA in various organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster* (Fire et al. 1998; Kennerdell and Carthew 1998; Hammond et al. 2001b; Schmid et al. 2002). Expression of snap-back dsRNA using transgenes with inverted repeats of target

gene sequences (IR transgenes) has also been successfully employed to induce RNAi, first in plants (Chuang and Meyeroowitz 2000) and *C. elegans* (Tavernarakis et al. 2000), and more recently in *D. melanogaster* (Fortier and Belote 2000; Kennerdell and Carthew 2000; Lam and Thummel 2000; Martinek and Young 2000; Billuart et al. 2001; Keisman and Baker 2001; Piccin et al. 2001; Giordano et al. 2002; Kalidas and Smith 2002). The ability to control spatially and temporally the expression of such IR transgenes in *Drosophila* opens the possibility to inactivate any given single gene in a tissue- and/or stage-specific manner. However, two features of RNAi must be taken into account in using these new genetic tools.

First, an RNA-dependent RNA polymerase (RdRP) may be involved in an amplification step of RNAi (Cogoni and Macino 1999; Dalmay et al. 2000; Smardon et al. 2000). Using cell-free extracts of *Drosophila* embryos, Lipardi et al. (2001) showed that a synthetic siRNA may prime the 5' → 3' elongation of an antisense RNA using its target mRNA as a template. Degradation of dsRNA generated by such a mechanism may give rise to secondary

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siRNAs directed to sequences upstream of the initial trigger region on the target mRNA. Secondary siRNAs may thus target other mRNA species that contain these upstream sequences. Although this so-called transitive RNAi phenomenon was not observed in *Drosophila* cultured cells (Celotto and Graveley 2002), it clearly occurs in vivo in *C. elegans* (Sijen et al. 2001). Transitive RNAi is also observed in plants. In that case, siRNA are found both 5' and 3' of the initial trigger region, leading RNAi to spread from this region into both the adjacent upstream and downstream regions of the target gene (Vaistij et al. 2002). Spreading of siRNAs downstream from the initial target region may involve an unprimed RdRP activity.

Another remarkable feature of RNAi in *C. elegans* and plants is that it involves a systemic response, the injection—or the expression—of a dsRNA into one tissue leading to gene silencing in other tissues (Palauqui et al. 1997; Fire et al. 1998; Voinnet et al. 1998; Winston et al. 2002). In contrast, tissue-specific expression of IR transgenes in *Drosophila* was shown to cause localized morphological defects in adults or localized cellular defects in larvae (Billuart et al. 2001; Giordano et al. 2002; Kalidas and Smith 2002). Although these studies suggested that spatially restricted expression of IR transgenes results in spatially restricted RNAi, clear conclusions concerning the absence of systemic RNAi in *Drosophila* awaited the direct demonstration that IR transgene expression patterns and inactivation patterns of targeted genes strictly overlap.

If systemic and transitive aspects of RNAi are conserved in *Drosophila*, they may considerably limit the use of IR transgenes to control specific gene inactivation: transitive RNAi may lead to inactivation of mRNA species that were not targeted by the initial trigger sequence of the IR, and systemic RNAi may cause the genetic inactivation in one tissue to spread to other tissues. We investigated these issues and demonstrated here that IR transgenes can target RNAi to specific regions of mRNA species without a transitive effect directed to sequences downstream from or upstream of the initial trigger region of the IR. Moreover, we provide strong evidences that RNAi mediated by *Drosophila* IR transgenes is as a cell-autonomous process.

RESULTS

RNAi mediated by IR transgenes remains restricted to the initial trigger region

The *batman* gene is expressed ubiquitously throughout *Drosophila* development and encodes a BTB/POZ domain transcription factor involved in the regulation of Hox genes (Fauchaux et al. 2003). To inactivate *batman* by RNAi, we cloned an inverted repeat of a 615-bp fragment of the *batman* cDNA downstream from the GAL4 UAS regulatory sequences (Fig. 1). A transgenic line carrying this UAS-IR[batman] construct was crossed with the *daughterless*-

FIGURE 1. Structure of the UAS-IR constructs. (A) Strategy for generation of transgenic RNAi. A portion of the coding sequence of a gene is dimerized in a head-to-head orientation and placed in the pUAST expression vector under the control of UAS transcription elements. (B) Maps of the *batman*, *batman-GFP*, and *EcR* (Talbot et al. 1993) genes depict the arrangement of the exons. Exons common to all three *EcR* isoforms are numbered 3–6. Specific exons of the *EcR* isoforms are designated by greek letters. The positions of the cDNA fragments cloned in the UAS-IR constructs for dsRNA expression are indicated by black arrows. The position of the probes used for the RNase protection assays are indicated by solid black bars.

GAL4 (*da-GAL4*) driver strain that expresses GAL4 in most tissues throughout development (Wodarz et al. 1995). A Northern blot analysis using either a sense or an antisense *batman* probe revealed the presence of 23-bp *batman*-specific siRNAs in mid-third instar larvae emerging from this cross (Fig. 2A, left panel). The *batman* siRNAs were also readily detected in an RNase protection assay using the sense *batman* probe (Fig. 2A, right panel). Although the measured size of siRNAs is subject to imprecision of a few nucleotides due to incomplete digestion of the unprotected probe by RNase (compare left and right panels in Fig. 2A), the RNase protection assay provided maximally sensitive detection of small RNAs. The *Batman* protein was undetectable in the UAS-IR[batman] *da-GAL4* larvae (Fig. 2B), which eventually died at the beginning of the pupal phase (not shown). Together, these results indicate that the *batman* gene can be readily inactivated by RNAi, using the UAS-IR[batman] construct.

To test whether transitive RNAi directed to sequences upstream of the IR trigger sequence may occur in *Drosophila*, we first took advantage of a transgenic line carrying a UAS-*batman-GFP* fusion gene that fuses the full-length *GFP* coding region to the last codon of the full-length *batman* coding region (Fig. 1B). We established new transgenic lines for a UAS-IR[GFP] construct carrying the *GFP* coding sequence in an inverted repeat orientation (Fig. 1B) and

FIGURE 2. Specific inactivation of *batman* and *batman-GFP*. (A) Analysis of small RNAs from *da-GAL4/+* (control) and UAS-IR[batman]/*da-GAL4* (UAS-IR[batman]) third instar larvae. (Left panel) siRNAs were analyzed by Northern blot using the bat probe (see Fig. 1B) of sense or antisense polarity as indicated. (Right panel) siRNAs were analyzed by RNase protection assay using the bat probe of sense polarity. (B) Western blot analysis of Batman in *da-GAL4/+* (control) and UAS-IR[batman]/*da-GAL4* mid-third instar larvae. Immunodetection of the MBF-1 protein was used as a loading control. (C) Analysis of small RNAs from *da-GAL4*, UAS-*batman-GFP/+* (control) and *da-GAL4*, UAS-*batman-GFP*/UAS-IR[GFP] (UAS-IR[GFP]) third instar larvae. RNase protection assays were performed as indicated using the bat, gfp1, and gfp2 probes (all of sense polarity, see Fig. 1B). (D) Western blot analysis of late-third larval instar extracts from control *w¹¹¹⁸* larvae (WT), *da-GAL4*, UAS-*batman-GFP/+* larvae (+), and *da-GAL4*, UAS-*batman-GFP*/UAS-IR[GFP] (UAS-IR[GFP]) larvae using an anti-Batman specific antibody (several independent UAS-IR[GFP] transgenic insertions were tested). The Batman-GFP protein (39 kD) was undetectable or strongly reduced, but the levels of the Batman (14 kD) and MBF-1 (16 kD) proteins remained unchanged. (E) GFP fluorescence in *da-GAL4*, UAS-*batman-GFP/+* control larvae (left panel) and *da-GAL4*, UAS-*batman-GFP*/UAS-IR[GFP] larvae (right panel).

crossed them to a recombinant homozygous UAS-*batman-GFP*, *da-GAL4* strain. In the progenies of these crosses, the UAS-IR[GFP] insertions were expressed under the control of the *da-GAL4* driver and mediated genetic interference

with the *batman-GFP* fusion gene. GFP fluorescence was lost and the Batman-GFP fusion protein was undetectable in UAS-*batman-GFP*; *da-GAL4* larvae carrying the UAS-IR[GFP] insertions #1, #5, or #6 (Fig. 2D,E and data not shown). In contrast, expression of the Batman-GFP fusion protein was reduced but still detectable in UAS-*batman-GFP*; *da-GAL4* larvae carrying the UAS-IR[GFP] insertions #2, #3, or #4. The lower efficiency of the genetic interference mediated by these three insertions is likely to be due to position effects at the genomic insertion site of the UAS-IR[GFP] construct, as already discussed for other RNAi mediating *Drosophila* IR transgenes (Fortier and Belote 2000; Martinek and Young 2000).

An RNase protection assay using GFP sense probes gfp1 and gfp2 (Fig. 1B) showed that GFP-specific siRNAs were present in samples from UAS-*batman-GFP*; *da-GAL4*; UAS-IR[GFP]#1 larvae but not in UAS-*batman-GFP*; *da-GAL4* control larvae (Fig. 2C). In contrast, the batman sense probe bat did not detect any siRNA targeted to the *batman* sequences immediately upstream of the GFP sequences. Consistently, the level of the endogenous Batman protein remained unchanged in UAS-*batman-GFP*; *da-GAL4*; UAS-IR[GFP] larvae (Fig. 2D). These data indicate that RNA interference with the GFP sequences of the *batman-GFP* fusion gene is not associated with a significant spreading of RNA targeting to the upstream *batman* sequences.

The *Ecdysone Receptor* gene provided us with another model gene to explore the possibility of a transitive RNAi in *Drosophila*. It encodes three nuclear receptor isoforms, EcR-A, EcR-B1, and EcR-B2, which share a common C-terminal DNA-binding and ligand-binding region, but completely differ by their N-terminal domain (Talbot et al. 1993). We made two UAS-IR constructs with inverted repeat sequences designed to target the three 5' EcR-A-specific exons $\alpha 0$, $\alpha 1$, and $\alpha 2$ (UAS-IR[EcR-A]) and the 5' EcR-B1-specific exon $\beta 2$ (UAS-IR[EcR-B1]), respectively (Fig. 1B). Here we present a detailed analysis of a representative transgenic line for each of these constructs.

Both UAS-IR[EcR-A] and UAS-IR[EcR-B1] lines driven by *da*-GAL4 developed normally during the larval period. However, the UAS-IR[EcR-B1] third instar larvae did not pupariate normally. They occasionally everted their spiracles but failed to shorten and never formed a tanned pupal case (Fig. 3A). This phenotype is very similar, if not identical, to the phenotype of EcR-B1 null mutant larvae (Bender et al. 1997). In contrast, the UAS-IR[EcR-A] animals entered into the pupal phase and died as pharate adults (Fig. 3A). Although no EcR-A-specific alleles have been isolated yet, it should be noted that the previously described EcR^{k06210} allele that results both in the down-regulation of EcR-B1 and the absence of detectable EcR-A isoform leads to a phenotype identical to that of UAS-IR[EcR-A] larvae (D'Avino and Thummel 2000).

The presence of specific siRNAs in UAS-IR[EcR-A] and UAS-IR[EcR-B1] mid-third instar larvae was analyzed in an RNase protection assay using different probes. In UAS-IR[EcR-A] larvae, a strong siRNA signal was observed with a probe A specific for the EcR-A IR trigger sequence (Fig. 3B, lane 2). In contrast, siRNAs were not detected with a probe C specific for the immediately downstream common EcR sequence (Fig. 3B, lane 3) or with a probe A-UP specific for the upstream EcR-A sequence (Fig. 3B, lane 1). Symmetrically, a probe B1 specific for the EcR-B1 trigger sequence revealed a strong siRNA signal in UAS-IR[EcR-B1] larvae (Fig. 3B, lane 5), but siRNAs were not detected in these larvae using either the probe C specific for the downstream common EcR sequence or a probe B1-UP specific for the upstream EcR-B1 sequence (Fig. 3B, lanes 4,6). As UAS-IR[EcR-A] and UAS-IR[EcR-B1] transgenes do not induce the presence of detectable siRNAs corresponding to the common EcR region downstream from the targeted regions, they should not induce transitive RNAi directed to the untargeted isoform. Indeed, the EcR-A isoform was undetectable in UAS-IR[EcR-A] larvae whereas the level of the EcR-B1 isoform remained unchanged in these animals (Fig. 3C). Conversely, the EcR-B1 isoform was strongly reduced in UAS-IR[EcR-B1] larvae, whereas the level of the EcR-A isoform remained unchanged.

Taken together, these results provide strong evidence that *Drosophila* IR transgenes can specifically target unique exonic sequences without transitive RNAi directed to sequences either upstream of or downstream from the initial trigger IR sequences.

UAS-IR transgenes induce cell-autonomous RNA interference

We next asked whether IR transgenes induce a systemic RNAi or whether RNAi remains localized to the cells where dsRNA had been expressed. We took advantage of the ubiquitous expression of *batman* throughout development (Faucheux et al. 2003) and set up an experiment to visualize *batman* inactivation by the UAS-IR[batman] construct

FIGURE 3. Specific inactivation of EcR isoforms. (A) Pharate adult from a cross of the *da*-GAL4 driver line with the UAS-IR[EcR-A] construct line (left panel) and late-third instar larvae from a cross of the *da*-GAL4 driver line with the UAS-IR[EcR-B1] construct line (right panel). Arrowheads point to necrotic tissues. (B) Analysis of small RNAs from *da*-GAL4/+ (control), *da*-GAL4/UAS-IR[EcR-A] (UAS-IR[EcR-A]), and *da*-GAL4/UAS-IR[EcR-B1] (UAS-IR[EcR-B1]) third instar larvae. RNase protection assays were performed using the indicated sense probes (see Fig. 1B). (C) Western blot analysis of mid-third larval instar extracts from control *da*-GAL4/+ (+) and transgenic lines expressing the UAS-IR[EcR-A] and UAS-IR[EcR-B1] constructs. MBF-1 level was unchanged in the samples.

when expressed under the control of cell-specific drivers. The IR[batman] line was crossed with a strain homozygous

for both an *engrailed*-GAL4 driver (*en*-GAL4) and a UAS-GFP transgene reporter for GAL4 activation. The *en*-GAL4 driver induced GFP expression in the posterior compartment of wing discs of third larval instar progeny from this cross, whereas no expression was detected in the anterior compartment (Fig. 4A, panels a). Immunostaining revealed that the Batman protein was completely absent from the posterior compartment of the wing disc, but still present in the anterior compartment. Confocal microscopy showed that the cellular boundary of the area of *batman* inactivation perfectly colocalized with that of UAS-IR[*batman*] expression, as visualized by GFP fluorescence. No spreading of *batman* inactivation was detected in the anterior compartment of the wing disc or other larval or imaginal tissues where the *en*-GAL4 driver is not active (Fig. 4A, panel a, and data not shown). Consistently, adults flies emerging from the UAS-IR[*batman*] × *en*-GAL4; UAS-GFP cross all harbored a strong morphological defect restricted to the posterior compartment of the wing.

Neuronal tissues in *C. elegans* and stomata in plants are excluded from systemic spread of RNAi. Therefore, the absence of *batman* RNAi spreading outside from the posterior compartments of discs could reflect a particular property of these compartments. To test this possibility, we first repeated a similar *batman* inactivation experiment, using a *distal-less*-GAL4 driver whose expression is specified in the presumptive distal axis of appendages in leg and antennal imaginal discs (Diaz-Benjumea et al. 1994; Gorfinkel et al. 1997) and extends across the dorsoventral border in the wing imaginal discs. The UAS-IR[*batman*] line was crossed with a *distal-less*-GAL4, UAS-GFP line. As expected, the *distal-less*-GAL4 driver induced GFP expression along both sides of the wing margin in the wing discs and in the central part of the antennal and leg discs (Fig. 4A, panels b, c, d, respectively). Induction of the UAS-IR[*batman*] construct in these territories completely eliminated the Batman protein. However, no spreading of *batman* inactivation was detected outside from the *distal-less*-GAL4 expression areas. In good agreement with these observations, adult flies emerging from the cross harbored morphological defects restricted to the antennae, the legs, and the wings (Fig. 4A, right panels). In a second experiment, we used the “flip-out” GAL4 driver Act5C>>GAL4 to coactivate expression of the UAS-GFP and UAS-IR[*batman*] transgenes (Fig. 4B). This technique employs heat-shock induction of the FLP recombinase to fuse an Act5C promoter to GAL4, generating random clones of GAL4-expressing cells. First and second instar larvae emerging from a cross between hs-FLP; Act5C>>GAL4, UAS-GFP males and UAS-IR[*batman*] females were heat-shocked and allowed to develop until the end of the third larval instar. Tissues from late third instar larvae were dissected and clones of cells expressing GAL4 were visualized by monitoring induction of the GFP fluorescence under confocal microscopy. The Batman protein was undetectable in all GAL4-expressing clones in imaginal

discs and larval tissues immunostained with the Batman antibody (Fig. 4B and data not shown). In striking contrast, spreading of *batman* inactivation was never detected outside from the GAL4 expressing clones.

In a last set of experiments, we tested whether UAS-IR transgenes can induce RNAi with other genes than *batman* with the same absence of detectable systemic effect. Using a fat-body specific *Lsp2*-GAL4 driver and the UAS-IR[EcR-B1] transgenic construct, we were able to specifically inactivate the EcR-B1 isoform in the fat body of late third instar larvae. No spreading of EcR-B1 inactivation was observed in the salivary glands attached to the fat body or in other larval or imaginal tissues (Fig. 5A and data not shown). Likewise, a UAS-IR[Pcaf] transgenic construct mediated a strictly tissue-specific inactivation of the ubiquitously expressed histone-acetyltransferase *Pcaf* gene (Smith et al. 1998), when driven by the *distal-less*-GAL4 driver (Fig. 5B). Together, our results provide strong evidence that RNAi induced by UAS-IR transgenes is cell autonomous and does not spread outside from the tissues where the transgene was expressed.

DISCUSSION

Genetic analysis has demonstrated that RNA-dependent RNA polymerases are required for RNAi in plants (Dalmay et al. 2000; Mourrain et al. 2000), fungi (Cogoni and Macino 1999), and nematodes (Smardon et al. 2000). Evidence for the involvement of RdRP activities in RNAi came from the observation that RNAi targeting a specific region of an mRNA is accompanied by the production of secondary siRNA corresponding to sequences of the transcript upstream of (Sijen et al. 2001) or even downstream (Vaistij et al. 2002) from the region originally targeted. This RdRP-dependent siRNA spreading is reflected in the transitivity of RNAi in these organisms: The secondary siRNA target other RNAs with sequences contained within the spreading area (Sijen et al. 2001; Vaistij et al. 2002).

Although Lipardi et al. (2001) provided evidence for an RdRP activity in *Drosophila* embryonic extracts, a number of observations suggested that such an RdRP activity is not necessarily involved in the *Drosophila* RNAi pathway. To date, the use of crude or fractionated *Drosophila* extracts only pointed to essential roles of DICER and RISC complexes with endonuclease activities for efficient in vitro RNAi (Bernstein et al. 2001; Hammond et al. 2001a; Nykanen et al. 2001) and no member of the RdRP family has been found by BLAST searching of the *D. melanogaster* genome. An experiment designed to monitor the in vitro degradation of a labeled mRNA did not reveal any cleavage upstream of the target sequence present in the trigger dsRNA, as would have been expected if an RdRP mediated the spreading of siRNAs (Zamore et al. 2000). Moreover, the integrity of the siRNA 3' hydroxyl group is not required for RNAi in *Drosophila* embryo lysates or in cultured human cells, indicating that RNAi may occur in these organ-

FIGURE 4. UAS-IR[batman] induces cell-autonomous inactivation of the *batman* gene. (A) Confocal analysis of GFP (green) and Batman (red) expression in wing discs from *en*-GAL4, UAS-GFP/UAS-IR[batman] larvae (a) or in wing discs (b), eye-antennal discs (c), and leg discs (d) from *dll*-GAL4, UAS-GFP/UAS-IR[batman] larvae. Adult structures in control and inactivated flies are shown in the right panels. The size of the posterior compartment of the wings is reduced in *en*-GAL4, UAS-GFP/UAS-IR[batman] flies and contains a bubble indicative of abnormal adhesion between dorsal and ventral epithelial sheets (a). In *dll*-GAL4, UAS-GFP/UAS-IR[batman] flies, wings are abnormally curved (b), third antennal segments are reduced in size, unpigmented, and devoid of bristles (c, black arrow), the size of aristas is greatly reduced (c, white arrow), and the size of the bristles is greatly reduced on the legs (d). (B) Confocal analysis of GFP (green) and Batman (red) in FLP/FRT mediated cell clones coexpressing the UAS-GFP and UAS-IR[batman] transgenes. Clones are shown in a wing disc (a), the fat body (b), the gut (c), and the malpighian tubules (d).

FIGURE 5. UAS-IR[Ecr-B1] and UAS-IR[Pcaf] induce cell-autonomous RNAi. (A) Fat body specific inactivation of Ecr-B1 in late-third instar larvae. A *Lsp2*-GAL4 driver line that expresses GAL4 specifically in the third larval instar fat body was crossed with the *w¹¹¹⁸* control line (upper panel) or a UAS-IR[Ecr-B1] transgenic line (lower panel). Ecr-B1 antibody staining (brown) of late-third larval instar tissues showed that Ecr-B1 isoform was undetectable in the fat body (FB) but remained at the same level in salivary glands (SG) as well as other larval tissues (not shown). (B) Confocal analysis of GFP (green) and P/CAF (red) expression in leg discs (a), and wing discs (b) from *distal-less*-GAL4, UAS-GFP/UAS-IR[Pcaf] larvae.

isms independently from an RdRP activity (Chiu and Rana 2002; Schwarz et al. 2002). Finally, a recent report demonstrated that transfection of cultured Schneider cells with a dsRNA corresponding to an alternatively spliced exon only mediates the degradation of mRNA isoforms that contain this exon (Celotto and Graveley 2002). This suggested that spreading of RNAi along the target mRNA and resulting transitive RNAi do not occur in vivo in *Drosophila*.

Using IR transgenes to target three distinct mRNA sequences (GFP, Ecr-A, and Ecr-B1), we showed that RNAi is accompanied by the readily detectable accumulation in animals of siRNAs complementary to the initial trigger regions. However, in all three cases, siRNAs complementary to sequences immediately upstream of these regions were not detected. This suggests that RNAi in *Drosophila* is not associated with a significant spreading of secondary siRNAs upstream of the initial target sequence. The finding that the targeting of RNAi to the GFP moiety of the *Batman*-GFP fusion transgene had no transitive effect directed to the endogenous *batman* gene strongly support this conclusion.

RNAi targeting of the Ecr-A and -B1 isoforms also offered the opportunity to test the possibility of siRNA spreading downstream from the trigger IR sequences. In both these cases, we did not detect the accumulation of siRNAs complementary to immediately downstream exons shared by all Ecr isoforms. This absence of significant siRNA spreading downstream from the target sequence was confirmed by the finding that UAS-IR[Ecr-A] and UAS-IR[Ecr-B1] mediates specific RNAi with Ecr-A and -B1 isoforms, respectively, without any detectable transitive effect directed to the other isoform.

Altogether, our results provide direct evidence that RNAi mediated by IR transgenes in *Drosophila* does not involve the RdRP-dependent synthesis of secondary siRNAs. Cosuppression in *Drosophila* resulting from the transcription of multiple transgene copies dispersed in the genome has been shown to be a RNAi-related mechanism that involves the production of siRNAs homologous to the silenced gene (Pal-Bhadra et al. 2002). However, the mechanism by which antisense strands of siRNAs are generated in this case remains to be elucidated. One possibility is that the RdRP activity identified by Lipardi et al. (2001) is involved in cosuppression rather than in IR transgene-mediated RNAi in *Drosophila*.

We show, using both specific GAL4 drivers and a somatic recombination system, that *batman* gene inactivation remains strictly localized to the cells in which *batman* RNAi has been triggered. Likewise, we were able to inactivate both *Ecr-B1* and *Pcaf* in a tissue-specific manner without any detectable RNAi spreading. Therefore, our results demonstrate for three distinct genes the cell autonomy of RNAi in *Drosophila*. This situation is in striking contrast with the situation encountered in *C. elegans* and plants, in which a remarkable feature of RNAi is its ability to spread over long distances throughout the organism (Palauqui et al. 1997; Fire et al. 1998; Voinnet et al. 1998; Winston et al. 2002). How may this divergence be explained?

Systemic RNAi requires a system to pass a specific signal from cell to cell (Hamilton and Baulcombe 1999; Klahre et al. 2002). Although the nature of this system is currently unknown, dsRNAs or siRNAs themselves constitute obvious candidates for the role of molecules acting as specific signals. Systemic RNAi may involve additional components such as transporters to export the signal from cells undergoing RNAi process and receptors for importing the signal in other cells. A first possibility is that one or several of these components are lacking in *Drosophila*. The lack in *Drosophila* of an homolog of the *sid-1* gene that encodes a transmembrane protein involved in systemic RNAi in *C. elegans* supports this hypothesis (Winston et al. 2002). Systemic RNAi may also involve a system to amplify the signal and generate the de novo synthesis of silencing RNA molecules in distant cells (Sijen et al. 2001; Klahre et al. 2002; Vaistij et al. 2002). Therefore, it is tempting to propose that the absence of detectable systemic RNAi in *Drosophila* sim-

ply reflects the absence of the de novo synthesis step of RNA silencing molecules found in plants and *C. elegans*. In this respect, it is interesting to note that the lack of systemic RNAi in *Drosophila* correlates with the lack of detectable transitive RNAi, another feature that involves the de novo synthesis of silencing RNAs molecules.

Together our results show that dsRNA-producing IR transgenes offer the opportunity to perform reverse genetic studies in *Drosophila* by controlling gene inactivation in single tissues or cells, at the resolution of a single isoform. This exquisite precision opens a new possibility to analyze gene functions in specific tissues or at specific developmental stages. Studies designed to determine whether systemic and transitive RNAi do or do not operate in other higher organisms such as vertebrates will be required because similar reverse genetic approaches based on the use of DNA vectors to induce in vivo the expression of siRNAs have started to emerge in these organisms (Tuschl 2002, and references therein).

MATERIALS AND METHODS

IR constructs

cDNA fragments were amplified by using PCR with primers containing restriction sites and cloned in the pUAST vector (Brand and Perrimon 1993) in two consecutive steps, first in a reverse orientation between *Bgl*III and *Kpn*I, then in a direct orientation between *Eco*RI and *Bgl*III. Recombinant UAS-IR constructs were transformed in Sure[®] (Stratagene) competent bacteria to minimize DNA recombination, and screened using appropriate restriction enzyme digestions. Transgenic flies for UAS-IR constructs were generated as previously described using a w¹¹¹⁸ strain as a recipient stock (Rubin and Spradling 1982).

UAS-IR constructs include the following cDNA fragments repeated in a head-to-head arrangement (Fig. 1). UAS-IR[Batman]: a 615-bp fragment between positions 104 and 719 relative to the *batman* cDNA sequence (GenBank accession number AF308476); UAS-IR[EcR-B1]: a 597-bp fragment between positions 1110 and 1707 relative to the EcR-B1 cDNA sequence (GenBank accession number M74048); UAS-IR[EcR-A]: a 646-bp fragment between positions 424 and 1070 relative to the EcR-A-specific exon sequence (GenBank accession number S63761); UAS-IR[GFP]: a 604-bp fragment between positions 28 and 632 relative to the translation start codon in the EGFP cDNA (Clontech); and UAS-IR[Pcaf]: a 850-bp fragment between positions 1203 and 2053 relative to the *Pcaf* cDNA sequence (GenBank accession number NM140329).

Fly strains

The *da*-GAL4 driver line refers to GAL4^{daG32} as described in Wodarz et al. (1995). The *hs*-GAL4 and *en*-GAL4 driver lines were gifts from Andrea Brand, Wellcome/CRC Institute, University of Cambridge, U.K., and the UAS-GFP reporter line was provided by Jean-Paul Vincent, National Institute for Medical Research, Medical Research Council, London. The *distal-less*-GAL4 driver line was

obtained from the Bloomington *Drosophila* Stock Center. The *Lsp2*-GAL4 driver line was a gift from Bassem Hassan, Department of Human Genetics, Flanders Interuniversity Institute of Biotechnology, Leuven, Belgium. The UAS-*batman*-GFP transgene is a fusion between the complete *batman* ORF to the EGFP ORF (Clontech).

Generation of GAL4 expressing clones

GAL4-expressing clones were induced by the FRT "flip out" method (Struhl and Basler 1993; Pignoni and Zipursky 1997) by crossing *hs*-FLP, *Act5c*>>*CD2*>>*GAL4*, UAS-GFP flies (Neufeld et al. 1998) with the UAS-IR[*batman*] line. Larvae were heat-shocked 2 h at 37°C during first and second larval instar. Dissected discs and larval tissues were fixed and immunostained as described below.

Detection of siRNAs

Total RNAs were extracted from larvae using Trizol (GIBCO BRL) and treated with RNase-free DNase I. Northern blot analyses of siRNAs were performed as described (Hamilton and Baulcombe 1999). RNase protection assays were chosen to provide maximally sensitive detection of small RNAs and were performed using the Roche RNase Protection kit in accordance with the manufacturer's instructions. Our ³²P-labeled RNA probes were generated by in vitro transcription with T7 polymerase using PCR-amplified fragments as templates. Probes used were: *bat* (365–653 relative to the *batman* cDNA sequence), *gfp1* and *gfp2* (1–300 and 293–518 relative to the translation start codon in the EGFP cDNA, respectively), *a-up* and *a* (108–395 and 478–786 relative to the EcR-A-specific exon sequence, respectively), *b1-up*, *b1*, and *c* (762–1026, 1203–1523, and 1768–2004 relative to the EcR-B1 cDNA sequence, respectively).

Western blotting and immunohistochemistry

Larvae were staged (Andres and Thummel 1992) and reared at 25°C. Western blot analyses were conducted as described in Brodu et al. (1999) using the monoclonal anti-EcR-B1 AD4.4 and anti-EcR-A 15G1a (a gift of C. Thummel, University of Utah, Salt Lake City, UT, USA) antibodies, the affinity-purified rabbit anti-Batman polyclonal antibody (Fauchaux et al. 2003), and the rabbit polyclonal anti-MBF-1 (a gift of M. Jindra, Institute of Entomology, CAS, Ceske Budejovice, Czech Republic) directed against the constitutively and ubiquitously expressed *Drosophila* Multiprotein Bridging Factor 1 (M. Jindra, pers. comm.). The polyclonal PCAF antibody was prepared against the bacterially expressed *Drosophila* PCAF bromodomain and affinity purified.

For immunofluorescence assays, imaginal discs were dissected from mid-third instar larvae, fixed in the Brower fixation buffer (Sullivan et al. 2000) for 2 h, and antibody stained as described in Dequier et al. (2001). Anti-Batman, Anti-P/CAF, and fluorescent Cy3-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:500, 1:1000, and 1:300 dilutions, respectively. Imaging was carried out using a Leica TCS-SP confocal microscope. Immunostainings of larval tissues were performed as described (Brodu et al. 1999).

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