

Efficient and heritable functional knock-out of an adult phenotype in *Drosophila* using a GAL4-driven hairpin RNA incorporating a heterologous spacer

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ABSTRACT

We have developed a modified RNA interference (RNAi) method for generating gene knock-outs in *Drosophila melanogaster*. We used the sequence of the *yellow* (*y*) locus to construct an inverted repeat that will form a double-stranded hairpin structure (*y-IR*) that is under the control of the upstream activating sequence (*UAS*) of the yeast transcriptional activator *GAL4*. Hairpins are extremely difficult to manipulate in *Escherichia coli*, so our method makes use of a heterologous 330 bp spacer encoding sequences from green fluorescent protein to facilitate the cloning steps. When the *UAS-y-IR* hairpin is expressed under the control of different promoter-*GAL4* fusions, a high frequency of *y* pigment phenocopies is obtained in adults. Consequently this method for producing gene knock-outs has several advantages over previous methods in that it is applicable to any gene within the fly genome, greatly facilitates cloning of the hairpin, can be used if required with *GAL4* drivers to avoid lethality or to induce RNAi in a specific developmental stage and/or tissue, is useful for generating knock-outs of adult phenotypes as reported here and, finally, the system can be manipulated to investigate the *trans*-acting factors that are involved in the RNAi mechanism.

INTRODUCTION

Drosophila melanogaster is undoubtedly one of the most versatile and useful organisms in molecular genetics research and presents some advantages over other models such as mouse, yeast or *Arabidopsis*. Yet until very recently, *Drosophila* suffered from an inadequacy not shared with some of its fellows: it was not suitable for reverse genetics because it is difficult to 'knock-out' a gene identified on the basis of its sequence or position. Although large collections of *P*-element mutagenised flies are readily available at various stock centres, insertions within the desired coding sequences are often absent. This drawback has become more of an issue given that

the recently completed *Drosophila* genome project has made available a wealth of new sequence information (1).

RNA interference (RNAi) is a powerful method for silencing genes at a post-transcriptional stage. Introduction of double-stranded RNA (dsRNA) triggers degradation of the mRNA bearing the same sequence in a variety of organisms (2–4). The mechanism by which this is achieved is as yet poorly understood but one of the earliest steps seems to consist of the degradation of the dsRNA into short fragments (5,6). Injection of specific dsRNA into the early *Drosophila* embryo results in interference persisting throughout the embryonic development (4,7,8) while interference of gene function at the adult stage seems to be greatly reduced (7). Apart from its transient nature, interference mediated by dsRNA injection is not heritable in a Mendelian fashion, so that each individual analysed in an experiment is necessarily the result of direct manipulation, making the collection of large data sets extremely labour intensive.

In order to overcome these hurdles we have developed an alternative method for administering dsRNA to *Drosophila*, which relies on transcription from an integrated construct consisting of two inverted repeats (IR), separated by a unrelated DNA sequence that acts as a spacer, to give a hairpin-loop shaped RNA. A similar method has been recently used to trigger RNAi in the nematode *Caenorhabditis elegans* (9) and in the fly (10,11). However, unlike these previous reports, we exploited the *GAL4*-upstream activating sequence (*UAS*) binary system (12) in order to drive expression of the transgene to produce knock-out of an adult phenotype. We describe here the generation of *yellow* (*y*) phenocopies in which the expression of the construct *UAS-y-IR* is driven by *GAL4* under the control of the *Actin5c*, *daughterless*, *timeless* or *heat-shock* promoters.

MATERIALS AND METHODS

Plasmid construction

A 1120 bp fragment from the coding sequence of the *y* gene was amplified using the pair of primers 5'-CTTTGACTT-GACCACGGATAC-3' and 5'-ATGATGCCACCACCCA-GATTG-3' and cloned in the T vector pDK101 (13) to yield pDK-*y*. A 950 bp subfragment was then excised using the

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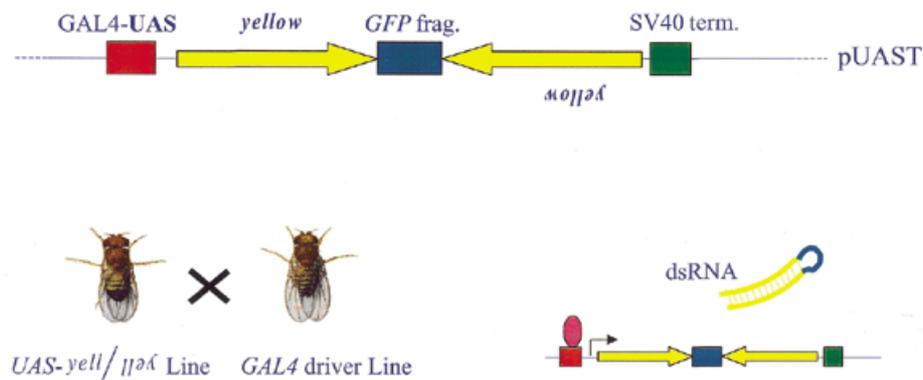


Figure 1. Scheme for the generation of transgenic, hairpin-mediated RNAi. A coding fragment of the *yellow* gene was cloned as an inverted repeat into the *Drosophila* transformation vector pUAST, using a *GFP* fragment to separate the repeats and therefore facilitate the cloning. The resulting transformants were then crossed to various *GAL4* strains to drive expression of the hairpin-encoding transgene.

endogenous *EcoRI* and *PstI* sites and cloned at the *EcoRI*–*PstI* sites of the pBC KS+ vector (Stratagene). In parallel, the primer pair 5′-ACGGCCTGCAGTGCTTCAGC-3′ and 5′-GAGCTGCAGGCTGCCGTCCT-3′ was used to amplify a fragment of the green fluorescent protein (*GFP*) coding sequence, resulting in a 330 bp DNA sequence with *PstI* sites at both ends. pDK-*y* was then linearised by digesting with *PstI* (from the *yellow* fragment) and *SalI* (from the vector polylinker) and re-circularised together with the 330 bp *PstI*–*PstI* *GFP* fragment and the 950 bp *y* fragment excised from pBC using *SalI* and *PstI*. Finally, the 2.2 kb *EcoRI*–*EcoRI* fragment containing the two *y* IR separated by the *GFP* spacer was cloned into the *Drosophila* transformation vector pUAST (12) to give *UAS*–*y*–*IR*.

P-element mediated transformation

Transformation of *Drosophila* embryos was carried out according to Spradling (14). Several transformant lines were obtained with autosomal insertions, two of which (5F and 7A) were analysed for their ability to trigger dsRNAi upon activation of transgene transcription by crossing to *daughterless*–*GAL4* (*da*–*GAL4*), *Actin5c*–*GAL4* (*Act5c*–*GAL4*), *timeless*–*GAL4* (*tim*–*GAL4*) and *heat-shock*–*GAL4* (*hsp70*–*GAL4*) driver strains. In the latter case, expression of the driver was triggered by placing vials, containing *Drosophila* at different developmental stages, in a 37°C water bath for 1 h.

The chromosome harbouring the *UAS*–*y*–*IR* insert(s) was determined with standard genetic crosses and the insert was then balanced with dominantly marked, multiple inverted chromosomes and later made homozygous. *In situ* hybridisation on third instar salivary gland chromosomes was used to pinpoint the map position and detect the number of inserts in each line using the *y*–*IR* probe (the two *y* IR separated by the *GFP* spacer), by means of the DIG-DNA Labeling and Detection Kit (Boehringer Mannheim) (15).

RESULTS

In situ hybridisation on polytene chromosomes revealed that transgenic line 5F was homozygous for an insert mapping at position 98–99 (chromosome 3R), while line 7A carried two homozygous inserts at map positions 69F (3L) and 97–98 (3R).

Additionally, both lines showed the presence of another copy of the *UAS*–*y*–*IR* insert, mapping at 101A–B (chromosome 4) in line 5F and at 59 (2R) in line 7A. These additional copies must have hitch-hiked during the various crosses performed in order to make the two transformant lines homozygous for chromosome 3 and therefore must be present in the transgenic populations at a somewhat low frequency. In case of the insert mapping at position 101A–B, there is a high probability that this transgene falls in a heterochromatic portion of the genome and is therefore not expressed.

Transgenic flies carrying the *UAS*–*y*–*IR* construct were crossed to the *GAL4* drivers in order to activate transcription from the hairpin-encoding transgene (Fig. 1) and the progeny were inspected for the colour of cuticle, wings and bristles. In order to estimate the efficiency of the system in promoting gene silencing, we set up individual crosses for each line using single homozygous *UAS*–*y*–*IR* flies, to homozygous (*da*–*GAL4*, *tim*–*GAL4*, *hsp70*–*GAL4*) or balanced (*Act5c*–*GAL4/TM6c*, *Tb*) driver lines. We estimated the silencing efficiency by examining the percentage of progeny that gave a *y*-like phenotype. Crosses *UAS*–*y*–*IR* × *Act5c*–*GAL4/TM6c*, *Tb* resulted in a 100% of progeny [after removal of the *Tubby* (*Tb*) pupae, devoid of the *Act5c*–*GAL4* driver] mimicking *y*¹-type mutants, displaying *yellow*-like cuticle, wings and bristles (Fig. 2), irrespective of the *UAS*–*y*–*IR* line used for the cross. We retrieved 109 *y*-like males and 127 *y*-like females from crossing *Act5c*–*GAL4* with line 5F, and 90 and 74 *yellow* males and females, respectively, with *UAS*–*y*–*IR* 7A (Table 1). As expected, all *Tb* flies displayed a wild-type pigmentation.

When *da*–*GAL4* was used to drive *UAS*–*y*–*IR* expression, the resulting phenotype resembled *y*²-type mutants (16), with *yellow*-like cuticle and wings but wild-type bristles (Fig. 2) and overall interference efficiency was significantly reduced (down to ~37%, with similar values in both *UAS*–*y*–*IR* lines, Table 1) compared to *Act5c*–*GAL4*. A high proportion of the progeny also displayed a yellowish cuticle, especially visible on the pale areas of abdomen and thorax, what we refer to as an ‘incomplete *y* phenotype’. The degree of variation of this yellow hue is rather broad, ranging from almost *y*-like to almost wild-type. For this reason we classified those ‘incomplete *y*’ flies as wild-type when computing the knock-out

Table 1. Summary of data relative to the crosses between *UAS-y-IR* transgenic lines and *GAL4* driver strains

Parental genotype	Wild-type male	Male ^a	y male	% y males	Wild-type female	Female ^a	y female	% y females	% y total
<i>UAS-y-IR</i> 5F × <i>da-GAL4</i>	33	138	119	41	33	122	95	38	40
<i>UAS-y-IR</i> 7A × <i>da-GAL4</i>	38	192	138	37	51	166	141	39	38
<i>UAS-y-IR</i> 5F × <i>Act5c-GAL4</i>	–	–	109	100	–	–	127	100	100
<i>UAS-y-IR</i> 7A × <i>Act5c-GAL4</i>	–	–	90	100	–	–	74	100	100
<i>UAS-y-IR</i> 5F × <i>hsp70-GAL4</i>	–	44	32	42	–	71	21	23	32
<i>UAS-y-IR</i> 7A × <i>hsp70-GAL4</i>	–	–	171	100	–	–	154	100	100

The number of wild type progeny are indicated, together with that of *yellow* phenocopies. *y*²-type for crosses with *da-GAL4* and *y*¹-type when the *UAS-y-IR* is activated by the *Act5c-GAL4* driver. Percentage of *y* phenocopies is computed taking into account only the number of *y*-like flies and considering the incomplete *yellow* phenocopies as wild-type. Figures indicated for the *UAS-y-IR* × *hsp70-GAL4* crosses refer to progeny obtained after reiterated heat treatments.

^aThe incomplete *yellow* phenotype observed with the *da-GAL4* driver and those resulting from crosses with *hsp70-GAL4*.

frequency, thereby generating an extremely conservative estimate of efficiency.

Crosses with the *tim-GAL4* driver did not generate *y* individuals, consistent with the reported expression of *tim* within internal organs that have circadian function (17).

UAS-y-IR expression was also driven with an *hsp70-GAL4* transgene. A once-in-a-lifetime, 1 h heat pulse could trigger RNAi with a low efficiency when administered at the late third instar/early pupa stage, resulting in ~5–10% of the flies displaying a *y*¹-like phenotype while for a higher proportion (~30%) *y* silencing was only restricted to the bristles (these flies had yellow-brown bristles but wild-type wings and body). No *y* silencing was observed when the heat treatment was given to embryos, first or second instar larvae, older pupae or adults. The frequency of interference was dramatically enhanced in the double transformants *hsp70-GAL4; UAS-y-IR* line 7A (originated in the cross *hsp70-GAL4* × *UAS-y-IR* 7A; 100%, Table 1) when *Drosophila* were treated with a 1 h heat pulse per day from the late third instar stage until hatching. The same treatment on *hsp70-GAL4; UAS-y-IR* 5F resulted in a high frequency of flies with a body colour reminiscent of the incomplete *y* obtained in the *da-GAL4* crosses (yellowish body and dark pigmented areas) readily discernible from their untreated siblings and *y* bristles (~60%), while the remaining flies also had a mosaic pigment pattern of the tergites.

In summary, the overall results indicate that the presence of a heterologous 330 bp fragment separating the two inverted repeats does not obstruct the ability of the construct to trigger RNAi.

DISCUSSION

We have obtained a knock-out of the *y* gene by expressing a transcript in *Drosophila*, consisting of two IRs which presumably fold to give a dsRNA. These flies, although genotypically *y*⁺, mimic amorphic mutations of the *y* locus, resulting in a yellow-brown cuticle colour (16). The difference in the phenotypes obtained with *Act5c*, *da*, *tim* and *hsp70* promoters will presumably reflect not only their intrinsic expression patterns but also their promoter strength and insert positions. The *hsp70* promoter may drive a high but transient expression of *GAL4* (and therefore of the *UAS-y-IR* hairpin), that results in a weak

silencing effect. On the other hand, reiterated heat treatments may maintain the expression level of the *y-IR* transcript above a certain threshold necessary to evoke RNAi in line 7A. In line 5F the average *y-IR* transcript level may be lower since this line carries a single copy of the *UAS-y-IR* construct (albeit some individuals may carry an extra copy on chromosome 4), although a position effect cannot be ruled out. It is noteworthy that a dose/position effect can be observed with the *hsp70-GAL4* driver but not when *UAS-y-IR* transcription is activated by *da-GAL4*. Such dose/position effects in modulating the outcome of RNAi can have practical advantages when trying to silence lethal genes, as they could allow survivors to display unexpected phenotypes.

Since previous reports indicate that a very limited number of dsRNA molecules are sufficient to induce interference (4,18), we speculate that the *hsp70-GAL4* result could mean that the hairpin RNA is rapidly degraded in *Drosophila* and possibly does not undergo an amplification step as reported in *C.elegans* (19), *Arabidopsis* (20) and *Neurospora* (21). Absence of an RNA amplification step is in agreement with findings that RNAi in *Drosophila* is independent of RNA synthesis (22).

In fact it is conceivable that dsRNA degradation may explain why the *y*²-like bristle pattern is obtained with the *da-GAL4* construct, even though *da* is expressed in the cuticle and peripheral sense organs (23,24). After differentiation of the proneural cluster, *da* transcription may be switched off, leading to a rapid degradation of the *y-IR* transcript before the late third instar stage, when its expression would be required to silence cuticular *y* mRNA.

In order to facilitate the cloning of the *yellow* inverted repeat construct, we used a spacer between the two repeats, consisting of an unrelated DNA sequence, a 330 bp *GFP* fragment. As reported in the literature (25) and also in our experience, inverted repeats are cloned in *Escherichia coli* at an extremely inefficient rate and the introduction of this spacer greatly increased the efficiency. The spacer's length appears to be a critical factor since the use of a shorter spacer (150 bp) did not result in any improvement of the cloning efficiency. We attempted to remove the *GFP* fragment from the pDK-*y-IR* (the complete construct cloned in pDK101 rather than in the *P*-element vector pUAST), an operation requiring a simple *Pst*I digest followed by electrophoretic separation of the



Figure 2. Effect of *UAS-y-IR*-encoded dsRNA transcription on the pigmentation of male and female adult *D. melanogaster*. y^+ flies (w^{1118} ; $da-GAL4/+$) and y^1 -type mutants are shown for comparison. Expression of *UAS-y-IR*-encoded dsRNA mediated by a *daughterless-GAL4* driver blocks melanin pigment deposition in cuticle and wing structures but not in bristles, mimicking the colour pattern of y^2 -type mutants. *Act5c-GAL4* driven dsRNA transcription triggers effective RNAi also in bristles, producing y^1 -like phenocopies.

resulting two fragments and re-circularisation of the 5 kb band. However, even from this sterically advantaged ligation reaction, we were unable to recover any transformant colony.

We can confirm that the difficulty in cloning the two IRs was not a peculiarity of the y sequences we used for the work described here, since we met the same problems when preparing two analogous constructs for performing RNAi of the *tim2* and *cry* genes of *Drosophila* (A. Piccin and E. Rosato, unpublished observations). Also in those cases, the use of a ~300 bp spacer dramatically increased the cloning efficiency. Given the high levels of knock-out recovered after induction of the hairpin-encoding transgene, we can infer that the presence of a relatively long spacer (approximately one-third of the repeat length) does not significantly obstruct the ability of the

dsRNA in mediating interference, while greatly facilitating the IR cloning steps.

This method of producing virtual knock-out of a given gene appears to be more practical than the homologous recombination recently obtained at the y locus by Rong and Golic (26). A difficulty with the latter technique is that homologous recombination may occur via a double-strand breakage-induced replication mechanism, therefore limiting the efficacy of the method to genes located near the tip of the chromosome (27). A similar strategy adopted by a different group indeed failed to produce any recombinants at the less distal *white* locus (28). In any case, our method is more flexible since the appropriate choice of the *GAL4* driver can limit gene silencing to the tissue and/or timing chosen by the investigator, thus circumventing problems of lethality. Moreover, the relatively small constructs needed for transgenic RNAi integrate within the fly genome with high efficiency, therefore making this technique a rather suitable approach to functional genomic studies.

A possible downfall of transgenic RNAi as a mean of generating knock-outs is that dsRNA fragments may mediate post-transcriptional silencing of an undesired gene which happens to fortuitously share sequence homology with the chosen target. Future experiments should assess the correlation between degree of homology of dsRNA and mRNA and effectiveness of interference. Meanwhile, careful designing of the IR construct should reduce this problem.

Finally, *UAS-y-IR* transgenic flies can be used as a valuable model system in the identification of *trans*-acting factors involved in establishing dsRNA-mediated genetic interference, by subjecting it to a mutagenesis screening and selecting for individuals resistant to RNAi.

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^aThe incomplete *yellow* phenotype observed with the *da-GAL4* driver and those resulting from crosses with *hsp70-GAL4*.