RNAi Triggered by Symmetrically Transcribed Transgenes in Drosophila melanogaster

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ABSTRACT

Specific silencing of target genes can be induced in a variety of organisms by providing homologous double-stranded RNA molecules. In vivo, these molecules can be generated either by transcription of sequences having an inverted-repeat (IR) configuration or by simultaneous transcription of sense-antisense strands. Since IR constructs are difficult to prepare and can stimulate genomic rearrangements, we investigated the silencing potential of symmetrically transcribed sequences. We report that Drosophila transgenes whose sense-antisense transcription was driven by two convergent arrays of Gal4-dependent UAS sequences can induce specific, dominant, and heritable repression of target genes. This effect is not dependent on a mechanism based on homology-dependent DNA/DNA interactions, but is directly triggered by transcriptional activation and is accompanied by specific depletion of the endogenous target RNA. Tissue-specific induction of these transgenes restricts the target gene silencing to selected body domains, and spreading phenomena described in other cases of post-transcriptional gene silencing (PTGS) were not observed. In addition to providing an additional tool useful for Drosophila functional genomic analysis, these results add further strength to the view that events of sense-antisense transcription may readily account for some, if not all, PTGS-cosuppression phenomena and can potentially play a relevant role in gene regulation.

METHODS of gene silencing are presently attracting great interest, as they provide valuable approaches to the genome functional analysis. Double-strand RNA (dsRNA) is a powerful signal able to induce gene-specific silencing, a phenomenon known as RNA interference (RNAi). RNAi represents a powerful tool for obtaining targeted disruption of a given gene function, overcoming either the need for mutants or the knowledge of a complete and detailed gene structure. Initially observed in Caenorhabditis elegans (Fire et al. 1998; Montgomery et al. 1998), this method has been demonstrated to be effective in virtually any organism, from protozoa to plants and animals (reviewed by Fire 1999; Bosher and Labouesse 2000; Hammond et al. 2001). Although its overall pathway and its physiological role remain to be fully elucidated, it is well established that RNAi represses gene expression by eliciting specific degradation of the homologous target mRNA and thus represents a mechanism of post-transcriptional gene silencing (PTGS; reviewed by Fire 1999; Sharp 1999; Sharp and Zamore 2000; Sijen and Kooter 2000). Some events of cosuppression, a phenomenon in which the introduction of transgenes silences homologous chromosomal loci, similarly require an RNA effector molecule and have thus been classified as PTGS phenomena (reviewed by Montgomery and Fire 1998; Birchler et al. 2000). Links between cosuppression and RNAi have been envisaged at both the genetic and molecular level in both animals and plants (see Fagard et al. 2000; Ketting and Plasterk 2000), supporting the suggestion that some examples of PTGS-cosuppression may rely on the production of dsRNA molecules via read-through transcription starting from a fortuitous promoter flanking the site of insertion (Montgomery and Fire 1998). This hypothesis implies that a transgene able to produce dsRNA molecules by means of sense-antisense transcription might be able to trigger the silencing of the endogenous homologous loci. We have investigated this possibility, with the aim of establishing whether this type of transgene could provide an additional tool useful for functional genetic studies. In fact, although RNAi can rapidly and simply be induced by injections of specific dsRNA into living organisms, this experimental approach has a number of disadvantages, given that the interference with gene expression may be transient and genes expressed in later stages of development cannot be inactivated (Kennerdell and Carthew 1998; Montgomery et al. 1998; Misquitta and Paterson 1999; Wianny and Zernicka-Goetz 2000). In organisms for which transgenic technology is available, these problems have recently been circumvented by using heritable transgenes having an inverted-repeat (IR) configuration, which are able to produce dsRNA molecules in vivo as extended hairpin RNA. These transgenes have already been used to generate efficient RNAi

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in several organisms, such as C. elegans (Tavernarakis et al. 2000), Trypanosoma (Bastin et al. 2000; Shi et al. 2000), Drosophila (Fortier and Belote 2000; Kennerdell and Carthew 2000; Lam and Thummel 2000; Martinek and Young 2000), and plants (Chuang and Meyerowitz 2000; Smith et al. 2000). However, some IR constructs may be difficult, if not impossible, to clone. Moreover, genomic IR sequences are mitotically unstable and stimulate rearrangements in eukaryotic cells (Leach 1994; Bi and Liu 1996; Lobachev et al. 2000), raising the possibility that IR transgenic lines may be characterized by genetic instability. High rates of structural instability have in fact been reported for palindromic transgenes in mice, where mitotic mosaicism and rearranged versions of the transgene have been detected in the progeny (Collick et al. 1996; Akgun et al. 1997; Lewis et al. 1999; Waldman et al. 1999). To avoid these problems, we have investigated the silencing potential of constructs designed to generate Gal4-dependent simultaneous transcription of both sense-antisense strands. We report here that, in Drosophila, these transgenes are capable of repressing gene activity in vivo, in the transformed adult flies. According to the expression pattern of the Gal4 line used as driver, RNAs can be induced ubiquitously, or in selected tissues at specific developmental times, and the silencing effect is not significantly spread to the neighboring cells. In addition, to extend the potential of RNAi’s applications, our results add further strength to the view that spontaneously occurring transcription of sense-antisense strands can potentially play a relevant role in the regulation of gene expression and support the hypothesis that unintended symmetrical transcription causing aberrant dsRNA formation may readily account for some, if not all, PTGS-cosuppression phenomena.

MATERIALS AND METHODS

Plasmid construction: To prepare the Sym-pUAST-w construct, the Drosophila transformation pUAST vector (Brand and Perrimon 1993) was modified by sequential insertion of three genetic cassettes. In a first cloning step, we duplicated the SV40 polyadenylation region, by inserting a pUAST-derived 871-bp BamHI-SV40-BamHI polyadenylation cassette into the BamHI site of the partially digested pUAST vector, upstream of the UAS array, and with opposite orientation with respect to the SV40 polyadenylation sequence originally present in the vector. In a second step, we duplicated—with opposite orientation—the five-copy array of the UAS activating sequence, by directionally inserting a pUAST-derived 364-bp Xhol-UAS-Xhol cassette into the unique Xhol/Xhol sites of the previously obtained construct. After the insertion of these two genetic cassettes, the pUAST cloning polylinker carried on both sides two identical but oppositely oriented regulatory regions, each composed of a five-copy array of the UAS activating sequence coupled with an inversely oriented SV40 polyadenylation site. In a third step, a 1.4-kb EcoRI-white-EcoRI cassette, containing an exon-rich genomic fragment of the Drosophila white (w) gene, was ligated into the unique EcoRI site between the two inserted UASSV40 sequences. The BamHI-SV40-BamHI, Xhol-}

US-Xhol, and EcoRI-w-EcoRI (from nucleotides 10886 to 12222 of GenBank accession no. X002974) cassettes represent PCR-amplified fragments obtained using, respectively, the following pairs of primers: BamHI-5′-SV40 (5′-TACGATCCTCACTTTGTAAGAAA-3′) and BamHI-3′-SV40 (5′-ATCGAGGCTCACTAGTTGGAAC-3′); Xhol-5′UAS (5′-TATCTAGAATCTGAGGCTGAC-3′) and Xhol-3′UAS (5′-TATCTAGAGCTTGTCTCTTGATGTTT-3′); EcoRI-5′w (5′-TTCTAAGATTCGCATCTCCACCC-3′) and EcoRI-3′w (5′-ATTGACCGCCGACTTTGGTT-3′). All PCR-amplified fragments were cloned in pBluescript or pUC18 in appropriate orientation for subsequent manipulations. To obtain the pUAST-IR-w and pUAST-IR-w constructs, inverted repeats of the w cassette were initially generated in pBluescript and then cloned in the pUAST vector according to the following steps. First, a pBluescript plasmid containing the 1.4-kb EcoRI-w-EcoRI cassette (pBS1.4w clone) was digested with BamHI and HindIII and the resulting w fragment was cloned into the corresponding restriction sites of pUC18 vector, to give the pUC1.4w clone. This clone was digested with KpnI and HindIII, and the 1.4-kb w fragment was inserted into the corresponding sites of the pBS1.4w clone, obtaining a construct with two copies of the 1.4-kb w fragment arranged as tail-to-tail repeats (pBS1IRw clone). A second construct (pBS-IRw clone) was subsequently generated by inserting a 200-bp PCR-amplified fragment of the Escherichia coli lacI gene into the EcoRV restriction site of the pBS-IRw clone. The lacZ DNA spacer was amplified using the primers EcoRV-lacZ5′ (5′-TTTACGATTCTGTATGAACGGTCTGGTC-3′) and EcoRV-lacZ3′ (5′-ATTGATATCAGCCGACCACATCCAGTGC-3′). Finally, the pUAST-w and pUAST-IR-w transgenes were generated by individually inserting the spaced or perfect wIRs (from the pBS-IRw and the pBS-IRw clone, respectively) as KpnI/Xhol fragments into the corresponding restriction sites of the pUAST vector. The transformation constructs used to transcribe in vivo only the sense (pUAST-w) or the antisense strand (pUAST-w) of the w cassette were obtained by inserting the 1.4-kb EcoRI-w-EcoRI DNA fragment into the unique EcoRI site of the pUAST vector and subsequently recovering recombinant clones in both orientations.

The Sym-pUAST-mfl construct was obtained by substituting the 1.4-kb EcoRI-w-EcoRI cassette of the Sym-pUAST-w transgene with a 1.7-kb PCR-generated EcoRI-mMinify-EcoRI cassette. This fragment, corresponding to nucleotides +619 to +2297 of the mfl gene (GenBank accession no. AF097834), was obtained using as primers the EcoRI-5′ mfl (5′-ATGAGATCTCAAGTAGGACAGCGCAAAATTTCC-3′) and EcoRI-3′ mfl (5′-TAGATTCTGTTTTTATTGGG-3′) oligonucleotides. Structure of each cloned construct was verified by a combination of PCR amplification, restriction mapping, and DNA sequencing. In all the experiments involving construction of clones containing inverted repeats, the E. coli SURE strain (Stratagene, La Jolla, CA) was used as a bacterial host capable of tolerating IRs; the DH5α strain was utilized in the other cases.

Drosophila strains and Pmediated transformation: The genetic markers and chromosomes used in our experiments are described in Lindsey and Zimm (1992); most of the stocks were from Bloomington Drosophila Stock Center. In the Gal4 expression-defective Act5C-Gal4 strain, the P(Act5C-Gal4) transposon was inserted on the second chromosome and carried a functional copy of the yellow (y) gene, flanked by two FRT target sites of the FLP recombinase, between the Actin5C promoter and the Gal4 gene (Ito et al. 1997). The Gal4-producing Act5C-Gal4 strain was obtained from the Act5C-Gal4 stock by in vivo FLP-mediated excision of the y gene (Ito et al. 1997). Pelemon-mediated germline transformation and insertion mapping were performed as previously described (Giordano et al. 1999). In all Drosophila transgenic lines, integrity and in vivo stability of the IR-containing constructs were verified by Southern blot experiments.
Cloning techniques, DNA and RNA analysis, and in situ hybridization: Basic cloning techniques, DNA and RNA extraction, PCR amplifications, labeling, and sequencing techniques were carried out essentially according to Sambrook et al. (1989). For Southern blot genomic analysis, 5 μg of BamHI-digested DNA was electrophoresed, transferred to Hybond-NX (Amersham, Arlington Heights, IL) filters, and hybridized using the 1.4-kb w cassette as probe. For each transgenic line, four DNA samples, each derived from a pool of 30 adult flies, were examined. For Northern blot analysis, 3 μg of poly(A)+ or 5 μg of total RNA were electrophoresed and transferred to Hybond-NX (Amersham) filters for hybridization. Single-stranded probes spanning w exons 2–4 were generated by asymmetrical PCR reactions utilizing forward or reverse M13 primers and the pBS1.4w clone as template. Endogenous w mRNA was analyzed by poly(A)+ Northern blot experiments using as probe a w genomic fragment external to the 1.4-kb w cassette, spanning the w exons 5 and 6 (from coordinates 12490 to 13230 of GenBank accession no. X02974). In situ hybridizations were performed as previously described (Gior-dano et al. 1999). RNA quantitative analyses were carried out with ImageQuaNT software and the Molecular Dynamics (Sun-nysale, CA) PhosphorImager.

Eye pigment determination: Eye pigment determination assays were performed on the F1 heterozygous females of straight-wing phenotype, derived, respectively, from the crosses between w+; P(Act5C-Gal4)/CyO or w+; P(Act5C-Gal4)/CyO virgin females and males of generic w1118; P(UAS)/CyO genotype, where the P(UAS) symbol indicates the appropriate UAS construct, as described in Figure 3B. To quantify the eye pigment, virgin females of the appropriate phenotypic class were collected, aged for 5 days after eclosion, and frozen in liquid nitrogen. Heads were manually dissected and pooled, and the pigments were extracted according to Ashburner (1989b). For each genotypic class, the extraction was performed from 5 heads if flies displayed red eyes or from 15 heads if eyes were orange or light yellow. Pigment absorption was measured at 450 nm; five extractions were performed for each genotypic class, and the mean values of the absorption per head were calculated.

RESULTS AND DISCUSSION

Construction and effectiveness of the Sym-pUAST-w transgene: To investigate the potential of symmetrical transcription in producing RNAi we constructed Drosophila heritable transgenes able to generate dsRNA molecules by simultaneous transcription of both strands. To take advantage of the yeast Gal4/UAS binary system to modulate the expression of these transgenes, we modified (see MATERIALS AND METHODS) the previously described pUAST vector (Brand and Perrimon 1993) in a way that its cloning polylinker was flanked on both sides by two identical but oppositely oriented regulatory regions, each composed of a five-copy array of the UAS activating sequence coupled with an inversely oriented SV40 polyadenylation site. The obtained construct was thus able to drive simultaneous transcription of both sense-antisense strands of a given DNA insert from two convergent arrays of the Gal4-responsive UAS regulatory elements. The silencing potential of this type of construct was first tested by preparing Sym-pUAST-w (symmetrically transcribed pUAST-w), a transgene containing a 1.4-kb genomic segment spanning exons 2–4 of the Drosophila white (w) gene. This transgene, whose structure is depicted in Figure 1A, would symmetrically transcribe a genomic segment of the w gene, reproducing an event of read-through transcription whose occurrence has been invoked as a possible cause of PTGS-cosuppression events (Montgomery and Fire 1998).

The w gene was chosen because the UAS- and Gal4-bearing transposons both included a copy of the w coding sequence (Figure 1A), usually referred to as mini-white (mini-w), whose expression level can easily be followed phenotypically, and can thus provide an ideal target for the silencing effect. mini-w elements that are transcribed from their own promoter are in fact routinely utilized as selectable gratuitous transformation markers (see Ashburner 1989a), since in a w mutant background their expression represents the unique source of the eye pigmentation and directly establishes the eye color of the fly. In a first set of experiments, we thus utilized strains having a w1118 (a white partial deletion) genotype, so that their eye pigmentation was entirely dependent on the expression of the mini-w reporters. mini-w elements are known to generate different eye-color phenotypes depending on the specific transposon’s insertion site in the genome. In our experiments, we obtained three independent transformed lines carrying a single-copy insertion of the Sym-pUAST-w transgene on the second chromosome; as a consequence of the position effect, all had faintly pigmented eyes. These lines were then mated to the Act5C-Gal4 strain, which expresses the Gal4 activator gene under the control of the constitutive Actin5C promoter (Ito et al. 1997), to activate ubiquitously the symmetrical transcription of the transgene in the F1 progeny. In this cross, the two parental strains had markedly different eye-color phenotypes, that is, pale yellow for Sym-pUAST-w and orange for Act5C-Gal4 (see Figure 1B). Given that the simultaneous presence of two independent mini-w elements is known to have additive effects on eye color (Levis et al. 1985), the F1 progeny were expected to have more intense eye pigmentation than each parental, unless the transcriptional activity of the Sym-pUAST-w transgene was able to silence the expression of the mini-w reporters. We thus analyzed the F1 progeny, comparing for the eye-color phenotype the Act5C-Gal4/Sym-pUAST-w trans-heterozygous females to their daughters of Sym-pUAST-w/CyO and Act5C-Gal4/CyO genotypes, which carried, respectively, only one of the two parental transposons. Only the F1 females were carefully compared, to avoid any effect related to dosage compensation of the mini-w elements. Interestingly, phenotypic analysis showed that all the Act5C-Gal4/Sym-pUAST-w F1 females had light yellow eyes (see Figure 1B) and thus exhibited a strong silencing of the mini-w expression. No additional or abnormal phenotype was observed in these silenced flies, suggesting that the silencing was sequence specific. The effect was also stably maintained during the entire adult life and identically reproduced in hybrids having
Figure 1.—Transcriptional activation of the Sym-pUAST-w transgene triggers phenotypic silencing of the mini-w reporters. (A) Diagram of the genetic crosses performed to test the silencing potential of the Sym-pUAST-w transgene, whose schematic organization is depicted on the right. Sym-pUAST-w transgenic lines (on the right) were mated to the Act5C-Gal4 strain, which expresses the Gal4 activator gene under the control of the constitutive Actin5C promoter, to induce ubiquitous symmetrical transcription of the transgene in the F1 progeny. Crossing to the Act5C-γ'-Gal4 line, which carries a Gal4-defective transposon, was used as a negative control. As shown in the scheme, the Sym-pUAST and the Gal4-producing or nonproducing transposons all carry a copy of the mini-w gene, whose expression can easily be followed at the phenotypic level in a w mutant background. (B) The transcriptional activation of the Sym-pUAST-w transgene in the Sym-pUAST-w/Act5C-Gal4 F1 trans-heterozygous females (middle) causes phenotypic silencing of the mini-w reporters. Phenotypes of daughters of Sym-pUAST-w/CyO and Act5C-Gal4/CyO genotypes, respectively, are shown at left and right. (C) Absence of silencing is instead observed in Sym-pUAST-w/Act5C-γ'/H11001-Gal4 trans-heterozygous females (middle), in which the transcription of the transgene is not activated. In fact, phenotypic comparison of these individuals with their daughters of Sym-pUAST-w/CyO and Act5C-γ'/H11001-Gal4/CyO genotypes (left and right) indicates that they exhibit the expected additive effect on the eye pigmentation. (D) Northern blot analyses of total RNA preparations confirm that sense and antisense strands of the w cassette are both actively transcribed in the silenced (Sym-pUAST-w/Act5C-Gal4), but not in the unsilenced trans-heterozygotes (Sym-pUAST-w/Act5C-γ'Gal4). A probe corresponding to the gene encoding the Drosophila rp49 protein was also utilized to control the amount of RNA (5 μg) loaded in each lane.

Different genomic insertions of the Sym-pUAST-w transgene.

Sym-pUAST-w-mediated gene silencing is dependent on Gal4 transcriptional activation: A crucial point was that of establishing whether the silencing effect exerted by Sym-pUAST-w was induced by homology-dependent
DNA/DNA interactions (reviewed by Selker 1999; Birchler et al. 2000) or instead was directly dependent on the transcriptional activation of the transgene. To assess this point, we crossed the Sym-pUAST-w lines to Act5C-y-Gal4 flies, which carry a Gal4-defective transposon. The Act5C-y-Gal4/Sym-pUAST-w heterozygous females provided an ideal control of the eye-color phenotype in the absence of Sym-pUAST-w transcriptional activation, since the Act5C-Gal4 line was originally obtained from the Act5C-y-Gal4 strain after FRT/FLP-mediated excision of y+ sequences (Ito et al. 1997). This excision event rescued Gal4 function without altering the level of expression of the transposon’s mini-w reporter, so that the Act5C-y-Gal4 and Act5C-Gal4 flies exhibited identical eye-color phenotype (see Figure 1, B and C). Strikingly, Sym-pUAST-w/Act5Cy-Gal4 transheterozygotes all showed an additive effect on the eye pigmentation, as expected in the absence of any silencing effect (see Figure 1C). This observation indicated that the transcriptional activation of the Sym-pUAST-w transgene was actually responsible for the mini-w silencing. Northern blot analysis of total RNA preparations confirmed that the transgene was actively expressed in the silenced flies, where both sense and anti-sense transcripts accumulated at high levels, whereas it was completely inactive in the Sym-pUAST-w/Act5Cy-Gal4 controls (Figure 1D). Finally, when the Act5C-Gal4 element was combined with transgenes transcribing only the sense (pUAST-w, construct) or the antisense (pUAST-wa, construct) strand of the 1.4-kb w insert, no appreciable silencing was observed (data not shown; see below, Figure 3B). Hence, all the data are compatible with the view that the mini-w phenotypic silencing is triggered by the active synthesis of w dsRNA molecules, which might possibly be recruited by the RNA interference pathway.

To test whether the Sym-pUAST-induced RNAi could appropriately be modulated by means of the Gal4/UAS system, we analyzed the phenotype of the progeny generated by crossing the Sym-pUAST-w lines to strains expressing Gal4 under the control of various promoters. In these crosses we also wished to check whether the Sym-pUAST-w transgene could effectively disrupt the expression of a wild-type genomic copy of the w gene, so we kept the Sym-pUAST-w transgenic lines in w+/w+ genetic background, to generate a female progeny having a w/w+ heterozygous genotype. Despite the increased dosage of the target, we observed a dramatic reduction of the eye pigment level in Sym-pUAST-w/β-tub-Gal4 transheterozygotes, which expressed the Gal4 gene under the control of the strong ubiquitous β-tubulin promoter (see Figure 2). Two strains in which Gal4 expression was restricted to specific cell types were also tested as drivers. In these strains, Gal4 expression was under the control of the elav or the sevenless (sev) promoter, respectively. The elav promoter, which directs persistent Gal4 expression in ommatidial photoreceptor cells (Lin and Goodman 1994), triggered an appreciable silencing of the w gene in the Sym-pUAST-w/elav-Gal4 heterozygotes. In contrast, the sev promoter, which drives transient Gal4 expression in a subpopulation of ommatidial precursor cells (Brunner et al. 1994), was unable to induce a detectable reduction of the wild-type red color. However, we noted that Sym-pUAST-w/sev-Gal4 heterozygotes had red/brown-mottled eyes (Figure 2; see inset), a phenotype highly reminiscent of that of individuals showing position-effect variegation at the w locus (Birchler et al. 1994), whose eyes are characterized by small flecks of red- and brown-pigmented ommatidia. This variegated phenotype is expected if w silencing occurred in a restricted population of photoreceptor progenitor cells and thus fits nicely with the pattern of Gal4 expression in these heterozygous flies. Moreover, it reveals that even a transient expression of the Sym-pUAST-w transgene is capable of interfering appreciably with gene expression and thus generates noticeable adult phenotypes. This is consistent with the long-term effects of RNAi observed in several organisms (Fire 1999). Taken together, the results obtained support the conclusion that symmetrically transcribed transgenes can effectively be utilized to block the expression of endogenous genes and may thus represent a useful tool for the functional analysis of the Drosophila genome.

Phenotypic comparison of flies silenced by IR or symmetrically transcribed w transgenes: A method to express dsRNA molecules as extended hairpin RNA has recently been reported to be capable of generating efficient RNAi in Drosophila (Fortier and Belote 2000; Kennerdell and Carteth 2000; Lam and Thummel 2000; Martinek and Young 2000; Piccin et al. 2001). According to this method, dsRNA can be produced from
transgenes exhibiting dyad symmetry. However, this approach is complicated by the fact that IR constructs may be difficult to clone in *E. coli* (Collins 1981). Also, in eukaryotes, from yeast to mammals, IR sequences are unstable and undergo genetic rearrangements (see Leach 1994; Waldman et al. 1999; Lobachev et al. 2000). In mice, palindromic transgenes show high rates of structural instability, which is associated with the presence of an inverted repeat within each transgene (Collick et al. 1996; Akgun et al. 1997; Lewis et al. 1999; Waldman et al. 1999). Rearranged versions of IR transgenes are generated at high frequency, and this aspect might complicate the analysis of knock-out or knock-down experiments. Symmetrically transcribed transgenes are instead expected to be prepared more easily and maintained more stably and can thus provide a convenient alternative approach, if it is established that they can induce RNAi at a comparable efficiency. With the aim of carrying out a quantitative comparison between the silencing effect induced by IR and symmetrically transcribed transgenes, we prepared two types of IR *w* constructs. In a first type, named *pUAST-IR*-w, two inverted repeated copies of the same 1.4-kb *w* segment carried by the Sym-*pUAST*-w transgene were inserted downstream of the UAS regulatory elements, with 200 bp spacing. In a second type, named *pUAST-IR*-w, the same IRs were inserted without spacing, to produce a perfect snap-back RNA (Figure 3A; see MATERIALS and METHODS).

Several independent lines carrying a single-copy insertion of each IR construct on the second chromosome were then obtained. Among them, we selected for each type of IR transgene two lines having an eye-color phenotype similar to that displayed by those carrying Sym-*pUAST*-w, to facilitate the phenotypic comparison of *F*1 flies resulting by individually combining each transgene with the Gal4-producing or nonproducing transposons. To enhance the sensitivity of the assays, the phenotypic and quantitative analyses were again performed on *F*1 females having the *w*/*w* heterozygous genotype, who thus carried, in addition to the two mini-*w* transposons, a wild-type genomic copy of the *w* gene. When in combination with the Act5C-Gal4 active element, the Sym-*pUAST*-w, *pUAST-IR*-w, and *pUAST-IR*-w transgenes all induced an appreciable reduction of the eye pigmentation compared to that of their Gal4-defective sibling controls (Figure 3A, compare Gal4+ and Gal4− lanes in each panel). Flies carrying either of the two types of IR transgenes appeared to be more strongly silenced, although those carrying the *pUAST-IR*-w construct were remarkably heterogeneous at the phenotypic level (Figure 3A, compare top and bottom). Moreover, we noted that a fraction of these flies exhibited a variegated eye-color phenotype, mainly characterized by the presence of darker red patches (Figure 3A, see arrow) on a less pigmented, orange background. Size and position of the patches varied among individuals, suggesting that *w* mosaicism occurred throughout development.

Quantitative estimate of the silencing effect induced by IR or symmetrically transcribed *w* transgenes: To estimate the silencing efficiency of each type of transgene, we determined the eye pigment content in the three types of silenced and control hybrid females. Although the eye color was only slightly reduced in the Sym-*pUAST*-w transgenic lines, the red pigment content was reduced to ~10% with respect to the controls (Figure 3B). This discrepancy is apparent only because, as reported in the literature, variation in the eye pigment level cannot be easily appreciated visually (Ziegler-Gunder and Haldorn 1958; Green 1959; Mackenzie et al. 1999). The pigment amount was reduced even further in flies homozygous for a third chromosome Sym-*pUAST*-w insertion (Figure 3B, line 7b), indicating that the silencing effect is reinforced by increasing the transgene dosage. In contrast, the transgenes transcribing only the sense (*pUAST*-w, construct) or the antisense (*pUAST-IR*-w, construct) strand did not induce any significant reduction of the pigment amount, consistent with the view that the genetic silencing is dependent on the production of dsRNA. As already indicated by the phenotypic analysis, the eye pigment content was more drastically reduced in the *pUAST-IR*-w and *pUAST-IR*-w silenced flies, where it was found, respectively, to be ~2 and 3% of the controls. We then checked, by Northern blot analysis, whether the gene silencing triggered by the Sym-*pUAST*-w transgene was paralleled by depletion of the homologous target mRNA. To detect specifically the endogenous *w* mRNA, we used a probe derived from *w* mRNA sequences not included in the Sym-*pUAST*-w transgene. As shown in Figure 3C, the level of the endogenous *w* mRNA was reduced to ~24% of the controls in these silenced flies and, more drastically, to ~9% in those expressing the *pUAST-IR*-w transgene. Thus, all the results obtained indicate that Sym-*pUAST*-w can actively silence the endogenous copy of the *w* gene, although it is less effective (approximately threefold) than the corresponding IR transgenes. One of the factors limiting the efficiency of the Sym-*pUAST*-w construct might be the collision of polymerases during simultaneous transcription on both strands. However, we noted that Sym-*pUAST*-w sense and antisense transcripts were produced at a quite appreciable level (see Figure 1D). Thus, it is more likely that only a minor fraction of the sense-antisense RNA molecules produced by this transgene anneal to form dsRNA and that this may limit its silencing efficiency. Accordingly, our findings that the Sym-*pUAST*-w effectiveness can be modulated by the strength of the Gal4 promoter, and reinforced by increasing the transgene dosage, support the hypothesis that the amount of dsRNA molecules produced may be a limiting factor. It is noteworthy that these effects have been reported also for IR transgenes (Piccin et al. 2001), in spite of the fact that a very limited number of dsRNA molecules are sufficient to induce RNAi when injected in Drosophila embryos (Kennerdell and Carthew 1998). It is
Figure 3.—Comparison of the silencing effect induced by symmetrically transcribed or IR w transgenes on target gene expression. (A) At the top, schematic structure of three transgenes expressing dsRNA is shown. The SympUAST-w transgene produces dsRNA by symmetric transcription, while the two IR constructs carry the same segment of the w gene arranged as a dimer with dyad symmetry, spaced (pUAST-IR-wsp) or not (pUAST-IR-w) by a 200-bp segment of nonpalindromic DNA. Below, phenotypes of w/H11001 heterozygous females carrying each type of transgene, either in combination with the Act5C-Gal4 inactive transposon (lane Gal4/H11002) or activated by the Act5C-Gal4 driver (lane Gal4/H11001), are shown. Within each line, the silenced flies carrying the Symp-pUAST-w or the pUAST-IR-wsp transgene showed a substantially uniform phenotype (compare the two individuals of the same genotype shown in the top and bottom), whereas those carrying the pUAST-IR-w construct are remarkably heterogeneous at the phenotypic level, with many exhibiting a variegated eye color (see arrow). (B) Quantitative estimate of the eye pigment content accumulated in each of the three types of silenced (shaded bars) and control hybrid females (solid bars). In the transgenic line 7b, which carries a third chromosome insertion of the Symp-pUAST-w transgene, the silencing effect has been checked in both heterozygosity (transgene dosage 1) or homozygosity (transgene dosage 2). Note that constructs transcribing only the sense (pUAST-ws) or the antisense strand (pUAST-was) did not induce any significant reduction of the pigment amount. For each transgene, phenotypic and quantitative analyses were performed in at least two independent transformed strains. (C) Northern blot analysis of poly(A)+RNA preparations obtained from pUAST-IR-wsp or Symp-pUAST-w silenced flies. To detect specifically the level of the endogenous w mRNA, the probe used was derived from w mRNA sequences not included in the Symp-pUAST-w transgene (see MATERIALS AND METHODS). In both types of silenced flies, the level of endogenous w mRNA was strongly reduced with respect to the controls (w+/w). A probe corresponding to the gene encoding the Drosophila rp49 protein was also utilized to control the amount of RNA loaded in each lane.

therefore possible that both types of transgenes produce, in vivo, a significantly lower level of active dsRNA species than expected on the basis of their transcriptional activity.

Structural stability of IR or symmetrically transcribed w transgenes: Flies with variegated eyes were detected in both the pUAST-IR-w lines examined, indicating that this construct can generate mitotic mosaicism. We excluded the possibility that the eye mosaicism could be caused by a position effect, as w mosaics were present in both transgenic lines. In addition, it has recently been shown that heterochromatin-mediated transcriptional silencing of a Gal4-dependent promoter is efficiently counteracted by Gal4 binding (Ahmad and Henikoff 2001). To check whether w variegation was due to structural instability of the pUAST-IR-w palindromic construct, we examined the two pUAST-IR-w-transgenic lines by Southern blot genomic analysis. As a control, we analyzed also flies carrying either the pUAST-IRs or the Symp-pUAST-w construct. Flies of each genotype were all homozygous for the w118 allele (a white partial deletion). Two independent lines were examined for each transgene. For each line we analyzed four BamHI-digested DNA samples, each derived from a pool of 30 adult flies, by hybridization to the 1.4-kb w cassette used as probe. This probe should detect three bands, two of...
which derived from the transposon (see the restriction map in Figure 4), while a third, of largest size (marked by the arrow in Figure 4), derived from the endogenous \( w^{1118} \) allele. One of the transposon’s bands was expected to be of 2.8, 2.9, or 3.0 kb, depending on the specific transgene (the position of these bands is marked by the arrowhead in Figure 4), while the second, derived from the mini-\( w \) reporter, was expected to be a different size (\( \geq 4.7 \) kb) in each independent line. In fact, one end of this fragment was located within the transposon, with the other being located in the flanking genomic sequence. Surprisingly, novel bands, whose intensity was less than that of single-copy sequences, were detected in some of the samples derived from each of the two \( pUAST-IR-w \) lines. These bands might presumably derive from rearrangements occurring at the \( pUAST-IR-w \) locus. With only one exception, these bands were < 2.8 kb (the position of most of these additional bands is marked by the bracket in Figure 4). It is therefore plausible that they might involve large deletions in the region spanning the mini-\( w \) reporter or partial deletions occurring in the IR region. In that case, they might represent heterogeneous truncated forms of the transgene, readily accounting for the phenotypic heterogeneity of \( pUAST-IR-w \)-silenced flies. In mice carrying perfect palindromic transgenes, rearrangement of the inverted repeat has been reported to occur frequently in either somatic or germline cells (Collick et al. 1996), being detectable in some cases in 15–56% of the transgenic progeny (Akgun et al. 1997). Most frequently, these events produce small asymmetric deletions at the center of the palindrome that lead to a stabilized structure (Collick et al. 1996; Akgun et al. 1997). Although we have not determined the precise nature of rearrangements occurring at the \( pUAST-IR-w \) transgenic locus, it is plausible that it might similarly undergo the same types of events described in mice.

noticeably, no trace of rearrangements was found in flies carrying the \( pUAST-IR_{sp}-w \) transgene (Figure 4), nor were phenotypic mosaics observed among these transgenic individuals, suggesting that a spacing length of 200 bp can be sufficient to allow a stable mitotic inheritance of IR transposons. This observation is in good agreement with a recent report that indicated that the recombinogenic potential of long, perfect genomic IRs is strongly reduced by increasing the distance between the repeats (Lobachev et al. 2000).

Gal4-dependent gene silencing can be restricted to a localized body domain of the fly: To assess the general effectiveness of symmetrically transcribed transgenes and to test their ability to silence gene expression in specific body regions only, we attempted to interfere
with the expression of a Drosophila gene involved in an essential, ubiquitous metabolic process. For this purpose we chose minifly (mfl, also called Nop66B; see Phillips et al. 1998), a gene involved in ribosome biogenesis whose defective mutations have been characterized in our laboratory (Giordano et al. 1999). mfl provides an ideal target for RNAi, given that mfl-defective alleles are characterized by quantitative alterations of the gene product. Functional analysis of these alleles, all due to P-element insertions outside the coding sequence, showed that the level of mfl transcription was critical, so that a dose-effect rule modulates the mutant phenotypes. In fact, strong loss-of-function mutations cause larval lethality, while partial loss-of-function alleles produce a viable hypomorphic phenotype characterized by pleiotropic defects, such as small body size; developmental delay; hatched abdominal cuticle; reduction in the number, length, and thickness of bristles; and reduced female fertility (Giordano et al. 1999).

To interfere with mfl expression we then prepared Sym-pUAST-mfl, a transgene allowing symmetrical transcription of a 1.7-kb segment derived from the mfl gene (see MATERIALS AND METHODS), and a second transgene, named pUAST-IRmfl, in which two inverted repeated copies of the same 1.7-kb mfl segment, spaced by 200 bp, were introduced into the pUAST vector. Both transgenes were then ubiquitously activated by the Act5-C-Gal4 driver and the corresponding silenced flies were compared phenotypically. Ubiquitous activation of the Sym-pUAST-mfl transgene led to developmental delay and reduced female fertility, with a large percentage (>90%) of flies showing hatched abdominal cuticle and reduction in the number, length, and thickness of bristles, all features consistent with a partial loss-of-function mfl phenotype. In contrast, pUAST-IRmfl/Act5-C-Gal4 heterozygotes showed late-larval or pupal lethality, indicating that in these flies mfl expression was reduced below the critical threshold. To disrupt the mfl activity only in selected cell types, we then crossed the Sym-pUAST-mfl and pUAST-IRmfl transgenic lines to pnr-Gal4, a strain expressing Gal4 under the control of the promoter of the pannier (pnr) gene. As shown in Figure 5A, this promoter drives Gal4 expression specifically along a mid-dorsal band (Caldeira et al. 1996). Remarkably, Sym-pUAST-mfl/pnr-Gal4 heterozygotes exhibited a strict localization of the defects typical of the mfl hypomorphic phenotype, showing a reduction in the number, length, and thickness of bristles only along the dorsal midline (Figure 5B). These traits, which reproduced with fidelity the phenotypic defects typical of the mfl' allele within the pnr dorsal domain, indicated that mfl function was partially disrupted within this specific region only. This observation substantially confirms that symmetrically transcribed transgenes can be of general validity in inducing RNAi and that the silenced phenotypes are those expected for a partial loss-of-function of the target gene. As expected on the basis of the previous results, the phenotype of pUAST-IRpnr-mfl / pnr-Gal4 silenced flies was instead more extreme, being characterized by a nearly total depletion of bristles within a body region that, although essentially centered along the dorsal midline, appeared to extend slightly beyond the pnr domain (Figure 5C, line 2c). The phenotype of one of the two independent transformed lines (2c and 3a) analyzed was even more strongly affected, since the silenced flies were further characterized by the presence of a cleft in the middle of the thorax (Figure 5C, line 3a). This cleft presumably results from a failure of the two halves of the thorax to fuse correctly at the dorsal midline during metamorphosis, suggesting the occurrence of localized cell death during the late larval or early pupal stages. As mfl is an essential gene, its restricted inhibition is indeed expected to cause cell death within the specific body region. In fact, apoptotic cell death has been described in the ovaries of females homozygous for a mfl partial loss-of-function mutation (Giordano et al. 1999).

Interestingly, the finding that both types of mfl silenced flies showed highly localized defects indicates that RNAi triggered by both types of transgenes is not substantially spread to the neighboring cells. This contrasts with that previously reported for worms (Fire et al. 1998) and Planes.
ria (Sanchez Alvarado and Newmark 1999), where RNAi can spread from the site of interfering RNA application, and with the systemic spread of transgene-induced cosuppression states observed in plants (Palauqui et al. 1997; Voinnet and Baulcombe 1997). Although it is presently unclear whether this reflects a peculiar difference distinguishing the process of RNAi occurring in Drosophila from that of other organisms, it certainly indicates that, at least in flies, the Gal4/UAS binary system can productively be utilized to trigger RNAi in restricted body domains.

**Nuclear localization of transgenic transcripts:** We wished to investigate the possibility that the transcripts derived from symmetrically transcribed or IR transgenes might have any specific subcellular localization in vivo. Interestingly, in situ hybridization experiments revealed that both the sense and antisense transcripts accumulated at high levels within the nuclei of ovary follicle cells of flies silenced for both w (Figure 6) and mfl (data not shown), regardless of the specific type of construct they carried. While the mfl gene is expressed constitutively (Giordano et al. 1999), the w gene is inactive in follicle cells. This indicates that the transgenic transcripts accumulate within the nuclei regardless of the presence of the homologous target mRNA. Although further experiments are required to assess the functional significance, if any, of this unusual localization, it is possible that it may reflect the existence of a nuclear step of the RNAi process. To this regard, it is interesting to note that in situ experiments performed in C. elegans cells have similarly revealed the presence of a strong nuclear signal after RNAi (Montgomery et al. 1998). More recently, the biochemistry of RNAi has been intensively investigated by using Drosophila embryo extracts (Tuschk. et al. 1999). The results obtained by several authors have indicated that short sense and antisense 21- to 22-nucleotide (nt) RNAs are produced from both dsRNA and the target mRNA during a two-step process (Hammond et al. 2000; Ketting and Plasterk 2000; Zamore et al. 2000). In a first step, a RNaseIII-type protein, called Dicer-1, degrades dsRNA into ~21-nt dsRNA intermediate active forms [short interfering RNAs (siRNAs)], forming a siRNA-Dicer complex (Bernstein et al. 2001), while in a second step this complex might recruit additional proteins, recognizing and cleaving the target RNA. Although it is well established that the RNAi process leads to a substantial loss of the cytoplasmic target mRNA, it cannot presently be excluded that one of these steps might, at least in part, take place in the nucleus. Compartmentalization of separate dsRNA-processing pathways in the nucleus and cytoplasm has in fact been hypothesized (Matzke et al. 2001). Moreover, it has recently been suggested that the active degradation complex might cleave the target RNA at the nuclear pores, as it exits the nuclear compartment (Waterhouse et al. 2001). Finally, a candidate Dicer homolog in Arabidopsis has been noted to contain two bipartite nuclear localization signals (Jacobsen et al. 1999; Bernstein et al. 2001).

**CONCLUSIONS**

Our results show that symmetrically transcribed transgenes, activated by the Gal4/UAS-inducible expression system, can successfully be utilized to trigger RNAi in Drosophila and that their use can be particularly valuable when partial loss-of-function mutant phenotypes are desirable. Symmetrical transcription of transgenes was reported to induce effective RNAi also in Trypanosoma (Wang et al. 2000), indicating that this approach might be of general validity and possibly be extended to other organisms. Our data also provide direct evidence that endogenous dsRNA formed by simultaneous transcription of sense-antisense strands can actually represent a cellular regulatory signal able to trigger a genetic repressed state. Intriguingly, in vivo sense-antisense transcription has been invoked as a possible mechanism for the regulation of several genes (Lipman 1997; Eddy 1999), and many biological phenomena, as, for example, X chromosome inactivation or imprinted gene expression in mammals, are known to involve transcription of both strands at specific loci (see Heard et al. 1999; Sleutels et al. 2000 for reviews). In Drosophila, both strands of the Suppressor of Stellate [Su(St)] locus are transcribed, and the production of Su(St) dsRNA has been implicated in the silencing of the paralogous Stellate (Ste) gene (Aravin et al. 2001). Given the strong silencing effect exerted by dsRNA molecules in a large variety of organisms, it is possible that symmetrical transcription of endogenous genomic sequences might play a wider role in the regulation of gene expression than previously suspected.

Concerning the functional analysis of the Drosophila genome, our results might also furnish a useful clue toward a better comprehension of the mechanisms accounting for the unexpected high efficiency displayed by a recently developed gene search (GS) mutagenesis approach. This method makes use of P element vectors containing copies of the UAS sequence oriented to direct outward transcription bidirectionally (Toba et al.
2006). With the principal aim of eliciting over- or ectopic expression of the target gene, this would lead to the collection of gain-of-function mutations, favoring the identification of genes that are not uncovered by loss-of-function phenotypes. The efficiency of the GS method, much higher than that of classical P-element mutagenesis schemes, was unexpected and so far substantially unexplained (Toba et al. 1999). We suggest that its high efficiency is based on the potential ability of this vector to interfere with the expression of the target gene by producing dsRNA. In fact, the capability of triggering outward transcription bidirectionally makes the transposon able to induce either loss- or gain-of-function mutations, depending on the transcriptional polarity of the target gene. On this basis, we surmise that, despite the expectation, a significant portion of the mutations isolated by this method might be loss-of-function instead of the expected gain-of-function alleles.

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