Regulation of *I*-Transposon Activity in Drosophila: Evidence for Cosuppression of Nonhomologous Transgenes and Possible Role of Ancestral *I*-Related Pericentromeric Elements

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ABSTRACT

We have previously shown that the activity of functional *I* retrotransposons (*I* factors) introduced into Drosophila devoid of such elements can be repressed by transgenes containing an internal fragment of the *I* factor itself and that this repressing effect presents the characteristic features of homology-dependent gene silencing or cosuppression. Here we show that the same transgenes can induce silencing of a nonhomologous reporter gene containing as the sole *I*-factor sequence its 100-bp promoter fragment. Silencing of the nonhomologous reporter gene shows strong similarities to *I*-factor cosuppression: It does not require any translation product from the regulating transgenes, sense and antisense constructs are equally potent, and the silencing effect is only maternally transmitted and fully reversible. A search for genomic *I*-like sequences containing domains with similarities to those of both the regulating and the reporter transgenes led to the identification of four such elements, which therefore could act as intermediates—or relays—in the cosuppression machinery. These results strongly suggest that ancestral transposition-defective *I*-related elements, which are naturally present in the Drosophila genome, may participate *per se* in the natural conditions of *I*-factor silencing.

THE *I* factor is a Drosophila LINE-like retrotransposon that transposes in a replicative manner, through the reverse transcription of an RNA intermediate (Jensen and Heidmann 1991; Pélisson *et al.* 1991). It is present in most *Drosophila melanogaster* strains that it invaded during the twentieth century, but there still exist some strains (called reactive strains) lacking functional *I* elements, mainly as a result of their sequestration in laboratories after they had been caught in the wild several decades ago. Such strains provide a remarkable *in vivo* model to analyze the effect of transposable elements on "virgin" genomes and the events leading to the final "taming" of the transposon. Actually, introduction of *I* factors by crossing into Drosophila genomes devoid of such elements results in high-frequency transposition of the incoming transposon, high mutation rate, chromosome nondisjunction, and female sterility, a syndrome referred to as *I-R* hybrid dysgenesis (Picard and L'Héritier 1971; reviewed in Brégliano *et al.* 1980; Brégliano and Kidwell 1983; Finnegan 1989; Bucheton 1990). However, high-frequency transposition is only transient, as the number of *I* elements reaches a finite value and transposition ceases after a few generations (Pélisson and Brégliano 1987). The physiology and underlying molecular events of this taming process are the subject of intense investigations, as it most probably involves features that are common to all transposon/host interactions. Since transposition can be highly mutagenic, mobile elements are actually severely repressed in all living species, possibly as a biological requisite to ensure stability of species and individuals.

Although the molecular mechanisms involved in this taming process are still far from being completely understood, *in vivo* genetic analyses have proven to be extremely potent tools for their study. For instance, it has been shown, in *Caenorhabditis elegans*, that some mutants for RNA interference (RNAi, see discussion) are defective for repression of their transposable elements (Tabara *et al.* 1998; Ketting *et al.* 1999), thus demonstrating a direct link between RNA interference and transposon taming. In Drosophila, it was previously shown that transgenes containing part of the *I* element confer to the corresponding transgenic Drosophila resistance to subsequent invasion by functional *I* elements introduced by crossing (Jensen *et al.* 1995, 1999a,b; Chaboissier *et al.* 1998; Gauthier *et al.* 2000; Malinsky *et al.* 2000). The protective effect is transgene copy number dependent, depends on the length of the *I* fragment, requires transcription of the regulating transgene (but see Chaboissier *et al.* 1998), and does not require any translatable sequence. The ability of the transgenes to repress *I*-element activity develops in a generation-dependent manner, via the germline transmission—only by females—of a silencing, still unidentified, effector. We also showed that "sense" and "antisense" con-

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structs identically trigger *I*-factor silencing, suggesting symmetry of the effector molecule (most probably double-stranded RNAs, see DISCUSSION; JENSEN *et al.* 1999b). Altogether, these results established that *I* elements are prone to homology-dependent gene silencing (HDGS) or cosuppression, a repressing process first discovered in plants (reviewed in VAUCHERET *et al.* 1998; WASSENEGGER and PÉLISSIER 1998; GRANT 1999; SELKER 1999) and then demonstrated in animals (PAL-BHADRA *et al.* 1997; reviewed in BIRCHLER *et al.* 2000).

In an attempt to characterize further these repressing effects and dissect the *in vivo* regulation of *I* elements, we have now used a reporter gene for the *I* element (I-CAT; UDOMKIT *et al.* 1996), composed of the self-transcribed *I* promoter driving the expression of the chloramphenicol acetyltransferase (CAT) gene, introduced as a transgene in Drosophila. Here we show that I-CAT expression is regulated by the *I*-containing transgenes in a manner similar to what was observed for the regulation of functional *I* factors, in a maternally transmitted, reversible, and transgene copy-number-dependent manner. However, a paradoxical feature of the observed cosuppression is that no sequence homology exists between the regulating and the reporter transgenes. These results are analyzed in relation to (i) the previous finding by PAL-BHADRA *et al.* (1999) that non-homologous transgenes can be mutually cosuppressed, provided that an endogenous sequence that discloses similarities to both transgenes exists, and (ii) the identification—via a systematic screening of the Drosophila genome database—of *I*-related elements acting as possible intermediates for the presently observed cosuppression. The role of these *I*-related sequences, which correspond to pericentromeric ancestral *I* elements present in all Drosophila strains (CROZATIER *et al.* 1988), is discussed. These elements are likely to be involved in the so-called "level of reactivity" of the Drosophila strains devoid of functional *I* factors and its modulation by epigenetic factors (*e.g.*, aging and temperature changes; BUCHETON 1979), as well as in the rate and extent of the taming process subsequent to invasion by functional incoming *I* factors.

MATERIALS AND METHODS

Drosophila strains: Flies were raised at 22° ± 1° on standard medium, and strains were maintained by using only young flies, as described in JENSEN *et al.* (1995). The w^{1118} (HAZELRIGG *et al.* 1984) and the reactive w^K (LÜNING 1981) strains were gifts from D. Coen and C. McLean. The "179" strain containing a single copy of an I-CAT reporter gene composed of the 100-bp *I* promoter (nucleotides 1–100 in FAWCETT *et al.* 1986) followed by the chloramphenicol acetyltransferase gene was a gift from D. J. Finnegan and is described in UDOMKIT *et al.* (1996). The hsp[i2Δ]pA and hsp[i2Δ*]pA, the control hsp-pA, and the promoterless pA′[i1-2Δ]pA constructs and the derived transgenic lines are described in JENSEN *et al.* (1999a); the hsp[i1-2Δ/S]pA sense and the hsp[i1-2Δ/AS]pA antisense constructs and the derived transgenic lines are described in JENSEN *et al.* (1999b). The hsp[i2Δ]pA, hsp[i2Δ*]pA, hsp[i1-2Δ/S]pA, and hsp[i1-2Δ/AS]pA constructs are also referred to as the hsp[. . .]pA constructs. The transgene copy number was assessed by Southern blots as in JENSEN *et al.* (1999a). The 2.5.1, 5.1.1, 5.2.1, 5.3.1, 5.5.1, 6.22.1*, and T43.1 lines derive by the loss of one transgene from the multiple-copy lines 2.5, 5.1, 5.2, 5.3, 5.5, 6.22*, and T43, respectively (JENSEN *et al.* 1999a,b). All transgenes are marked with the miniwhite gene as an indicator for transgenesis.

Measurements of the level of *I*-factor activity: The level of *I*-element activity was assessed as described in JENSEN *et al.* (1999a). Groups of 15 females were mated with 20 w^{1118} males (containing functional *I* elements), when <4 days old. The first 20 females and 20 males born from each batch of test crosses were collected and allowed to mate. When <4 days old, these flies were transferred to an egg collector. Sixteen hours later, five batches of 40 eggs were deposited as 4 × 10 matrices, thus allowing unambiguous counting (a further 48 hr later) of hatched and nonhatched (dead) embryos. The temperature was kept at 22° ± 1° throughout the experiments, as the intensity of the hybrid dysgenesis syndrome is influenced by temperature changes. The transgenic strains were controlled (in parallel to the CAT assays) for the absence of contamination by functional *I* elements as in JENSEN *et al.* (1995), by crossing transgenic males with reactive w^K females. Maternal/paternal transmission assays were carried out as in JENSEN *et al.* (1999b). The test for reversibility following transgene removal is described in JENSEN *et al.* (1995).

Measurements of the level of I-CAT activity: For standard and maternal transmission assays, 20 strain 179 males were crossed with 15 transgenic females (<4 days old) containing potentially silencing hsp[. . .]pA constructs, or control hsp-pA, or promoterless pA′[i1-2Δ]pA constructs. For paternal transmission assays, 15 strain 179 females (<4 days old) were crossed with 20 transgenic males containing the potentially silencing hsp[. . .]pA or control hsp-pA constructs. For a positive control of I-CAT downregulation, 20 strain 179 males were crossed with 15 w^{1118} females. Females from each batch of test crosses were then allowed to mate with their brothers and their ovaries were dissected to test CAT activity when <5 days old. For each sample, 25 pairs of ovaries were homogenized in 500 μl 0.25 M Tris-HCl, pH 8.0. The homogenate was passed through five freeze-thaw cycles and then heated to 60° for 15 min to inactivate proteins that interfere with the CAT assay. The extract was then centrifuged at 13,000 rpm, 4°, to pellet the debris. Protein concentration was measured by the BRADFORD (1976) method (Sigma-Aldrich). Three micrograms of extract was mixed with 0.5 μl D-threo-[dichloroacetyl-1,2-^{14}C]chloramphenicol (50 μCi/ml; New England Nuclear, Boston) and 0.25 M Tris-HCl, pH 7.6, to a final volume of 27.5 μl. To start the reaction, 12.5 μl 4 mM *N*-acetyl-coenzyme A was added prior to incubation at 37° for 1 hr. The reaction was stopped and extracted by vortexing with 200 μl of ethyl-acetate for 1 min. The organic phase was separated by centrifugation at 13,000 rpm, 4° for 5 min and was transferred to a new tube and the ethyl-acetate was allowed to evaporate for 45 min on the bench. Twenty-five microliters of fresh ethyl-acetate was added and vortexed, let stand for 10 min, and vortexed again. Acetylated and unacetylated forms of chloramphenicol were separated by silica gel TLC on Macherey-Nagel (Duren, Germany) TLC silica matrix. CAT activity was measured using PhosphorImager technology (FLA-3000 scanner) and normalized with respect to the mean value of two independent control assays, *i.e.*, CAT activity measured from ovaries of the F_1 progeny from crosses of strain 179 males with hsp-pA females. CAT activity was expressed as a percentage of activity of this mean control value.

For the reversibility assay of I-CAT downregulation, 15 females of the five tested hsp[. . .]pA strains were crossed with w^K males (the initial nontransgenic strain from which all the

transgenic lines are derived). From the F_1 progeny, 20 females were crossed with 20 of their brothers to generate the outcross line, 15 females were crossed with 20 w^{1118} males to test *I*-element activity in the hybrid progeny (see above), and 20 females were crossed with strain 179 males to test CAT activity in the ovaries of the female progeny as described above. In F_2 flies from the outcross line, nontransgenic white-eyed flies were obtained, from which 20 females and 20 males were crossed together to perpetuate the outcross line, while both transgenic (red- or orange-eyed) and nontransgenic (white-eyed) flies were separately tested for *I*-element activity and CAT activity. In F_3 and subsequent generations, only nontransgenic Drosophila were obtained, from which 20 females and 20 males were crossed together to perpetuate the outcross line, while others were tested for *I*-element activity and CAT activity. When transgenic and nontransgenic flies were obtained from the *I*-activity test cross, the percentages of dead embryos they laid were determined separately. And similarly, when from the CAT-activity test cross flies were born that contained or did not contain the hsp[. . .]pA transgene (they all contained the I-CAT transgene since the strain 179 fathers were homozygous for this transgene), they were analyzed separately when possible (no phenotypical difference could be detected for the 3.1 strain, because of the extremely low expression level of the transgenesis miniwhite marker gene).

Search for ancestral *I*-related sequences in the Drosophila genome database: Sequences homologous to the *I* factor were extracted from the Drosophila genome database (GenBank; Adams *et al.* 2000) by standard nucleotide-nucleotide BLAST search at the NCBI web page (http://www.ncbi.nlm.nih.gov), using the whole *I*-factor sequence from the $white^{IR3}$ insertion (deduced from Fawcett *et al.* 1986 and Abad *et al.* 1989). Occurrence in the w^K strain of the identified sequences (Ip2918, contained in GenBank accession no. AE002918; Ip3172, in GenBank accession no. AE003172; Ip2862, in GenBank accession no. AE002862; Ip3036, in GenBank accession no. AE003036), as well as the completeness of their promoter (Ip3172, Ip2862, Ip3036), was verified by PCR on w^K genomic DNA. The primers used for PCR were, for Ip2918, P30 (5′-ACG TTACAAGACGGACCCACTATC-3′) with P32 (5′-GTCAGTG GTTGCCACAATTAGGAC-3′) in the 5′- and 3′-flanking DNA, respectively; for Ip3172, P17 (5′-CAGTACCACTTCAACCTCC GAAGA-3′, nucleotides 1–24 in the *I* factor; Fawcett *et al.* 1986) with P37 (5′-CGTCCGGTCTTATCGTGGAGTTAG-3′, in the foreign insert of Ip3172), and P36 (5′-GAACTCATCT GAACGCGCATAGTC-3′, in the foreign insert of Ip3172) with P38 (5′-TGGTTAGCTGGAACTCTGGATCAC-3′, 3′-flanking DNA of Ip3172); for Ip2862, P17 with P40 (5′-ATACAGAGGC GACAACGAGGTGAC-3′, in the foreign insert of Ip2862); for Ip3036, P17 with P44 (5′-TACAGACAGACGGAAATAGAC AGT-3′, 3′-flanking DNA of Ip3036). The 3′ region of Ip2862 together with its 3′-flanking DNA was amplified by inverse PCR, following SpeI and PstI restriction of w^K genomic DNA and self-ligation, using P34 (5′-TGACCACAAGCACCTTATT CTGTT-3′) and P40. Fragments obtained by PCR were cloned in pGEM-T Easy Vector (Promega, Madison, WI) and sequenced (see accession numbers in Figure 6 legend). The percentage of similarity between the ancestral *I*-related sequences and the *I* factor was evaluated using the Wilbur-Lipman DNA alignment program. Multiple alignments were performed using the Clustal W multiple sequence alignments program (Thompson *et al.* 1994) at Infobiogen (http://www.infobiogen.fr).

RESULTS

Transgenic lines and rationale of the assay: We have previously shown that the transpositional activity of functional *I* elements introduced by crossing into Drosophila lacking such elements could be silenced by the prior introduction through transgenesis of transgenes containing transcribed internal parts of the *I* element, either translatable or not (Jensen *et al.* 1999a,b). We have now tested whether an I-CAT reporter transgene (Udomkit *et al.* 1996) containing the first 100 bp of the *I* factor, which correspond to the self-transcribed promoter of the *I* factor, followed by the chloramphenicol acetyltransferase gene, can be silenced by the same transgenes. The transgenes used for this study are schematized in Figure 1. The hsp[. . .]pA transgenes contain fragments of the *I* factor inserted between the *hsp70* promoter and the *Actin5C* polyadenylation signal: hsp[i1-2Δ/S]pA and hsp[i1-2Δ/AS]pA contain a 2318-bp fragment containing open reading frame (ORF)1 and a 969-bp fragment corresponding to the 5′ part of ORF2 inserted in the sense and antisense orientation, respectively; hsp[i2Δ]pA and

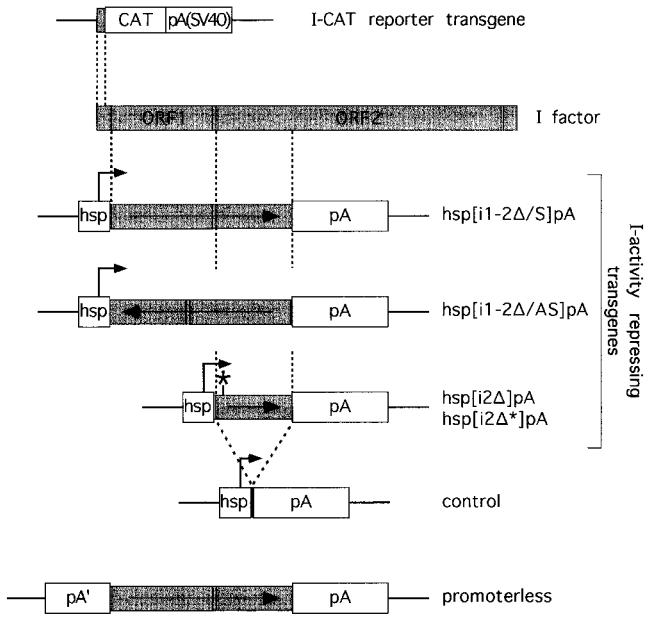

Figure 1.—Structure of the regulating and reporter *I*-containing transgenes. Structures of the full-length *I* factor with its two ORFs, of the hsp[i1-2Δ/S]pA and hsp[i1-2Δ/AS]pA constructs containing part of the *I* element in either sense or antisense orientation, and of the hsp[i2Δ]pA and hsp[i2Δ*]pA constructs containing part of the *I*-element ORF2, with an inserted 20-bp sequence with stop codons in all three reading frames (asterisk) for the latter construct are shown. The four *I*-fragment-containing constructs are referred to as the hsp[. . .]pA transgenes, with transcription (schematized with arrows) under the control of the *hsp70* promoter (hsp) and the *Actin5C* polyadenylation signal (pA). The control construct has the same structure as the hsp[. . .]pA constructs, with no *I*-element insert. In the promoterless construct, the *hsp70* promoter has been replaced by the *hsp70* polyadenylation sequence (pA′). The reporter I-CAT transgene (Udomkit *et al.* 1996) contains a 100-bp *I* fragment with promoter activity controlling expression of the chloramphenicol transferase gene (CAT), followed by the SV40 polyadenylation sequence [pA(SV40)]. All these constructs had been introduced into the reactive w^K strain of Drosophila (devoid of functional *I* elements) by *P*-mediated transgenesis (Udomkit *et al.* 1996; Jensen *et al.* 1999a,b).

hsp[i2Δ*]pA contain only the ORF2 fragment, with a linker containing stop codons in all three reading frames inserted downstream of the ATG initiation codon for the latter construct; the 2318-bp fragment was also inserted in a promoterless construct with a polyadenylation signal inserted in place of the promoter. These constructs, as well as a control construct without any insert, had been introduced into a reactive strain of Drosophila (the w^K strain, devoid of functional *I* elements) by *P*-mediated transgenesis, leading to several independent transgenic strains for each construct (Jensen *et al.* 1999a,b). The integrity of the transgenes and the transgene copy number (one to three copies per haploid genome) were assayed by Southern blots prior to the I-CAT silencing assays. Their ability to downregulate *I*-factor activity was verified for all transgenic lines used in the assay as described in Jensen *et al.* (1999a), by measuring the amount of dead embryos in a dysgenic cross. All hsp[. . .]pA transgenes repressed *I*-factor activity with variable efficiency consistent with our previous data (Jensen *et al.* 1999a,b), while the control and promoterless constructs had no effect on *I*-factor activity. The single-copy I-CAT transgene in strain 179 had no effect on *I*-factor activity (data not shown), most probably because of the short length of the *I*-homologous fragment (100 bp). Our previous unpublished data have also shown that even a 186-bp fragment encompassing the 5′ end of the *I* element was not sufficient to provoke a detectable protection against incoming functional *I* elements (assayed for transgene copy number up to four). Taking advantage of the absence of regulating effect for this short fragment, we have used the transgene of the 179 strain as a "neutral" reporter gene.

Silencing of the I-CAT reporter gene by *I*-element-derived transgenes: The ability of the *I* fragment containing hsp[. . .]pA transgenes, and of the control and promoterless constructs, to repress the I-CAT reporter gene was tested for several independent transgenic strains by introducing them maternally into the I-CAT 179 strain, thus generating individuals that are heterozygous for both the regulating transgene(s) and the I-CAT reporter gene (see scheme in Figure 2A). The F_1 females were dissected to isolate the ovaries and test CAT activity. The results in Figure 2B show that five strains among those tested are clearly able to repress the I-CAT reporter gene: Three strains correspond to transgenes with the 969-bp ORF2 *I* fragment (two of them containing the mutated untranslatable version with stop codons, *i.e.,* strains 6.8* and 6.10*), and two strains correspond to transgenes with the 2318-bp *I* fragment inserted in either sense or antisense orientation (strains 2.4 and 3.1, respectively). The other strains, containing the same transgenes but at different locations, disclose no or very limited repression of the I-CAT gene. Actually, the I-CAT-repressing strains correspond to those that have the strongest silencing effect on *I*-factor activity (see Jensen *et al.* 1999a,b; 5.2.1 being derived from the 5.2 strain), consistent with the notion that the threshold level for repression of I-CAT should be higher than that for repression of functional *I* factors. Along this line, it should be recalled that the silenced I-CAT transgene contains only a 100-bp-long *I* sequence on which the silencing machinery can exert its effect, while *I* factors are 5375 bp long. Finally, and as expected, the promoterless construct (at least for the 5/5 transgenic lines tested) and the control construct without any *I* fragment inserted (for the 4/4 transgenic lines tested) have no effect on the I-CAT reporter gene.

The silencing effect acting on the I-CAT transgene is maternally transmitted: We have previously shown that homology-dependent silencing of *I*-factor activity by *I*-fragment-containing transgenes was only maternally transmitted (Jensen *et al.* 1999a,b). We have therefore tested whether the silencing effect acting on the I-CAT reporter gene followed the same rule for its transmission. Silencing of *I*-factor activity and of the I-CAT reporter transgene was assayed in parallel, following either maternal or paternal introduction of the repressing hsp[. . .]pA transgenes. To test I-CAT silencing in maternal and paternal transmission assays, the five repressing hsp[. . .]pA strains and a control strain containing a transgene without *I* fragment were crossed with individuals from the I-CAT transgenic 179 strain, introducing the repressing transgenes or the control construct either maternally or paternally (Figure 3A, left). The ovaries of the resulting female progeny, containing both the repressing transgene(s) and the I-CAT reporter gene, were isolated to test CAT activity. The results (Figure 3B, left) clearly show that the I-CAT silencing effect is maternally transmitted for all five repressing strains, with no significant silencing effect in the paternal transmission assays. As expected, the control construct shows no I-CAT repression upon either maternal or paternal transmission. To assay the silencing of *I*-factor activity in parallel, the five repressing strains and the control strain were crossed with w^K individuals to introduce the transgenes either maternally or paternally, and the resulting heterozygous transgenic F_1 females were crossed with w^{1118} males, thus introducing functional *I* factors, the activity of which was then quantitated by measuring the percentage of dead embryos from the resulting F_2 females (Figure 3A, right). Embryo lethality was measured separately for transgenic and nontransgenic F_2 females. The results (Figure 3B, right) again clearly show, as has already been established (Jensen *et al.* 1999a,b), that *I*-factor silencing is essentially maternally transmitted for the five I-CAT-regulating transgenic lines. No silencing is observed for the control strain, as expected.

Reversibility of the silencing effect acting on the I-CAT reporter gene: We have previously shown that silencing of *I*-factor activity by homologous transgenes is fully reversible by crossing out the transgenes (Jensen *et al.* 1995, 1999b). To determine whether silencing of the

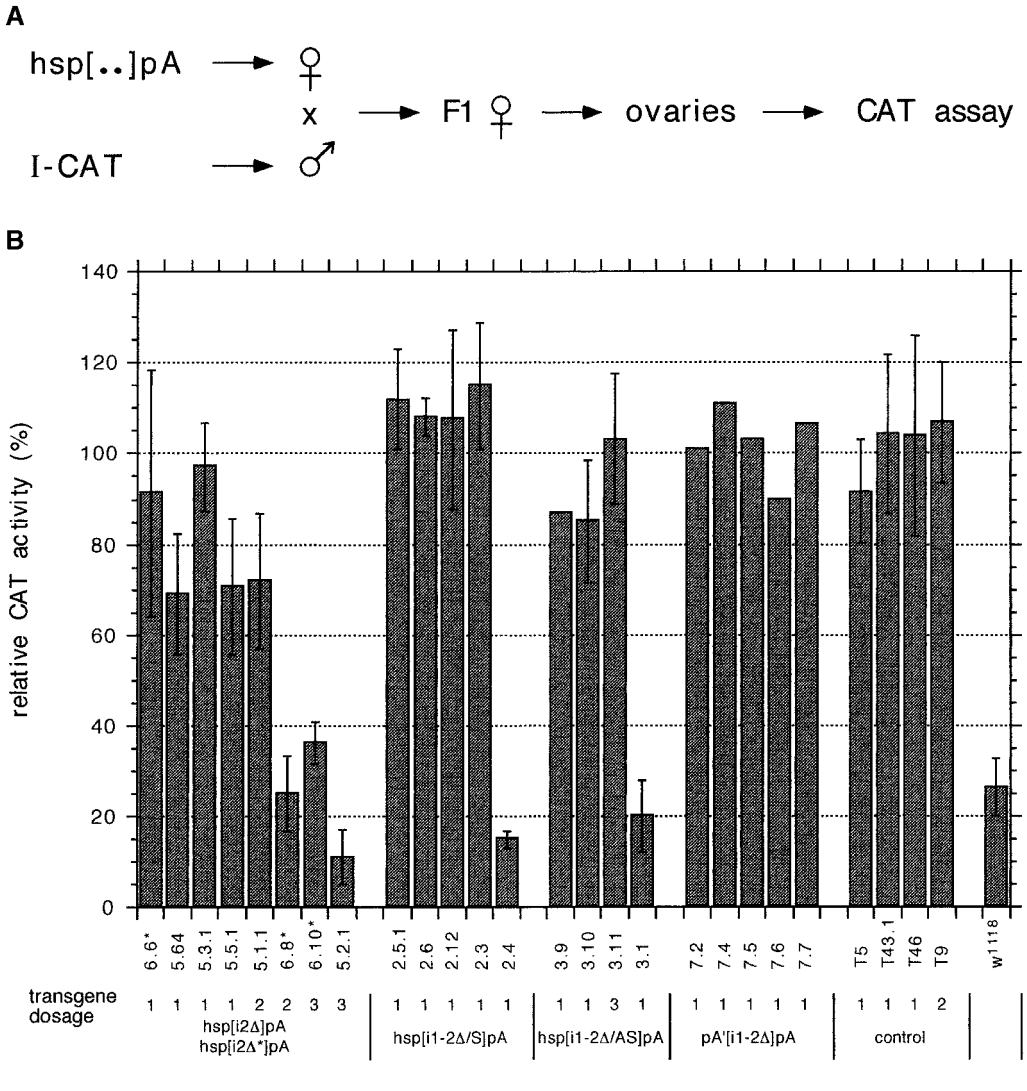

FIGURE 2.—Transcribed *I*-fragment-containing transgenes repress the I-CAT reporter. (A) Mating scheme and rationale of the assay. To assay downregulation of I-CAT activity by the hsp[. . .]pA, control, or promoterless transgenes, females of the corresponding transgenic strains (or of the w^{1118} inducer strain as an additional control) were crossed with males from the I-CAT strain 179 (UDOMKIT *et al.* 1996). The F_1 females from these crosses were allowed to mate and their ovaries were isolated to extract proteins for CAT assays. (B) CAT activities are expressed relative to the mean value of two control assays performed systematically in the same series of assays (100%). The transgenic lines used in the test cross, the names of the corresponding transgenes, and the transgene dosages are indicated. Strains containing stop codons in the *I* sequence of the transgene are marked by an asterisk. The data are the mean values for 2–16 assays (±SD when more than two assays were performed), corresponding to at least two independent crosses.

I-CAT reporter gene by nonhomologous I-related transgenes is similarly reversible, repressing transgenes were crossed out following the scheme depicted in Figure 4A. Females of these strains were first crossed with males of the initial, nontransgenic w^K strain from which all of the transgenic strains are derived. At each generation, silencing capacity of the I-CAT gene was tested by crossing the resulting F_1 females with strain 179 I-CAT transgenic males and assaying CAT activity from the ovaries of the resulting female F_2 progeny. In parallel, we assayed *I*-factor silencing capacity by crossing other F_1 females with w^{1118} males and measuring embryo lethality of the female F_2 progeny from these crosses. Results are given in Figure 4, B and C, for silencing of the I-CAT reporter and for repression of *I*-factor activity, respectively. As observed for the repression of *I*-factor activity, repression of the I-CAT gene by nonhomologous *I*-fragment-containing transgenes is fully reversible. It is noteworthy that, while *I*-factor silencing may persist for up to three generations in nontransgenic females from outcrosses, no I-CAT silencing is observed as soon as the repressing transgene is absent in the tested females (even if it was still present in their mothers). This difference is clearly visible when looking at the levels of repression for hsp[. . .]pA-containing G1 females *vs.* their sisters devoid of the hsp[. . .]pA transgene(s), with the former still repressing I-CAT activity but not the latter. A simple explanation for this difference might be that the threshold for repression of I-CAT is much higher than that for repression of *I*-factor activity, as already mentioned and consistent with the observation in Figure 2B that not all strains regulating *I*-factor activity regulate I-CAT (but only those with the highest repressing effect).

Search for endogenous sequences homologous to both the I-CAT reporter gene and the repressing *I*-fragment-containing transgenes: Repression of the I-CAT reporter by the *I*-containing transgenes, following rules that are common to the previously characterized cosuppression of functional *I* factors by the same transgenes is, actually, paradoxical: Indeed, as illustrated in Figure 1, there is no sequence identity between the I-CAT reporter and the *I*-containing regulating transgenes. Interestingly, PAL-BHADRA *et al.* (1999) have shown that even nonhomologous transgenes can silence each other in

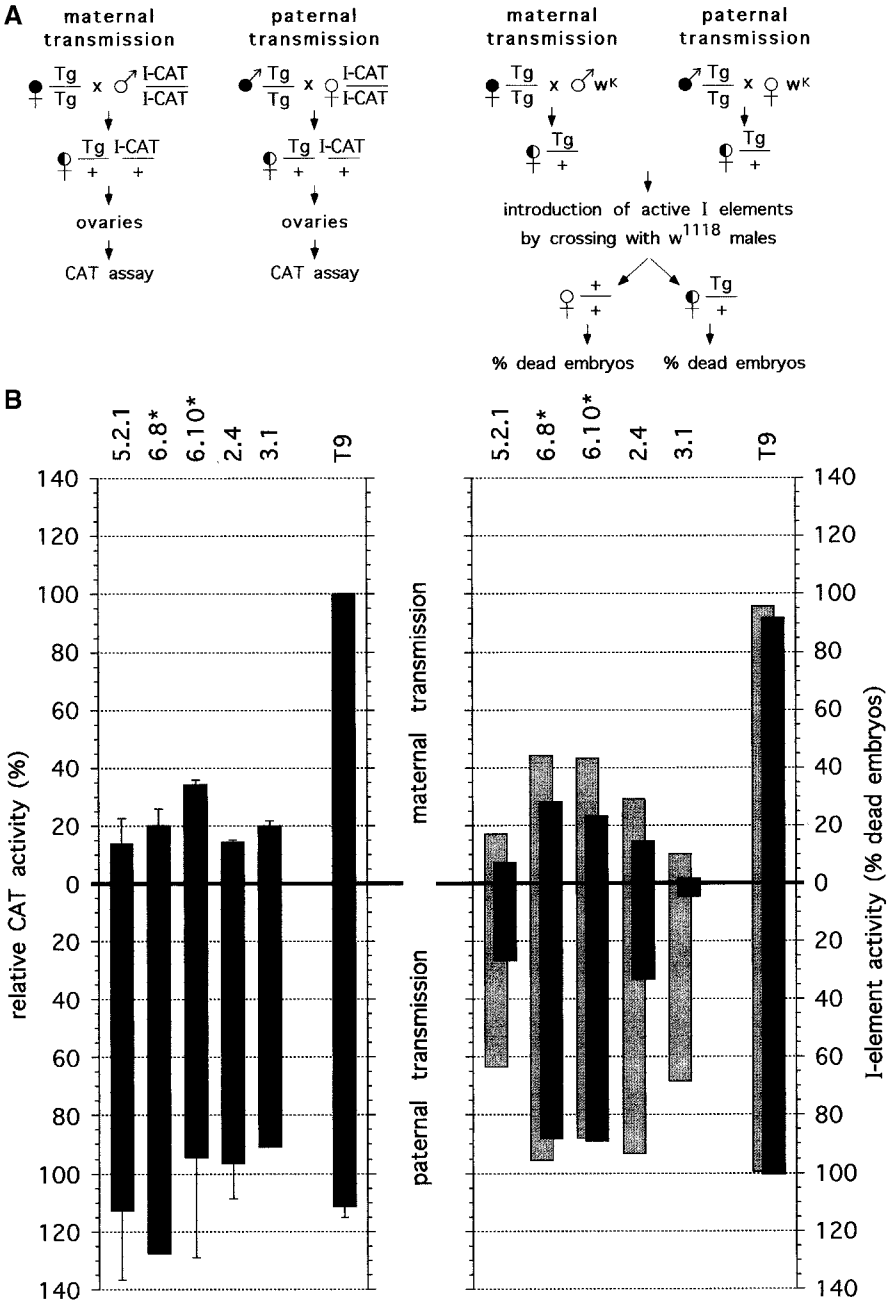

FIGURE 3.—Maternal inheritance of I-CAT repression. (A) Mating schemes for the maternal and paternal transmission of the regulating and control transgenes and rationale of the assay for transmission of I-CAT silencing (left) and *I*-element activity repression (right). Left: hsp[. . .]pA or control transgenes were introduced maternally or paternally by crossing the corresponding transgenic females or males (solid symbols) >30 generations after transgenesis, with strain 179 I-CAT flies (open symbols); the resulting heterozygous females (half-solid symbol) were allowed to mate with their brothers and their ovaries were isolated to extract proteins for CAT assays. Right: Females or males containing hsp[. . .]pA or control transgenes (solid symbols) were crossed with w^K flies (open symbols); the resulting heterozygous females were crossed with w^{1118} males to introduce active *I* elements. *I*-element activity was quantitated by measuring the percentage of dead embryos laid by the transgene-containing (half-solid symbol) or transgene-free (open symbol) female progeny. (B) Results for maternal (top) and paternal (bottom) transmission of the hsp[. . .]pA or control transgenes. Strain names are indicated at the top (with an asterisk for strains containing a mutated transgene). Left: I-CAT activities are expressed relative to that for maternal transmission of the control transgene in the T9 strain (100%). Standard deviations are indicated when two assays from independent crosses were performed. Right: I-element activity (percentage of dead embryos), with values for transgene-free F_1 females indicated with shaded bars and those for transgene-containing F_1 females with solid bars.

Drosophila, provided that an endogenous sequence homologous to both transgenes exists in the genome; this endogenous sequence then plays the role of a relay between the mutually nonhomologous transgenes. Accordingly, we searched for endogenous sequences containing domains common to both the I-CAT and *I*-containing transgenes (see Figure 5), which then could act as a relay in the cosuppression process as observed in PALBHADRA *et al.* (1999) in the case of the endogenous Adh gene and mutually exclusive, Adh fragment-containing transgenes. A search of such homologs in the Drosophila genome database revealed six full-length *I* factors (and possibly two additional, not fully sequenced, copies), seven 5′-truncated *I* elements of various length disclosing 99–100% homology to the *I* factor, and 24 sequences of varying length disclosing <95% similarity to the *I* factor, but still giving a BLAST score >300. Eight of the latter *I*-related sequences are located in or near pericentromeric—most probably heterochromatic—regions, four are in euchromatic regions, and for 12 of them the localization is unknown. The results of this search are consistent with the fact that the Drosophila strains used in the Drosophila genome sequencing program are "inducer" *I*-factor-containing strains, thus accounting for the sorting out of functional *I* elements (and of 5′-truncated *I* elements). Then, the 24 *I*-related sequences most probably correspond to previously identified "ancestral" *I* elements, localized by *in situ* hybridization of salivary gland chromosomes in the pericentromeric heterochromatin (BUCHETON *et al.* 1984). These elements were found in

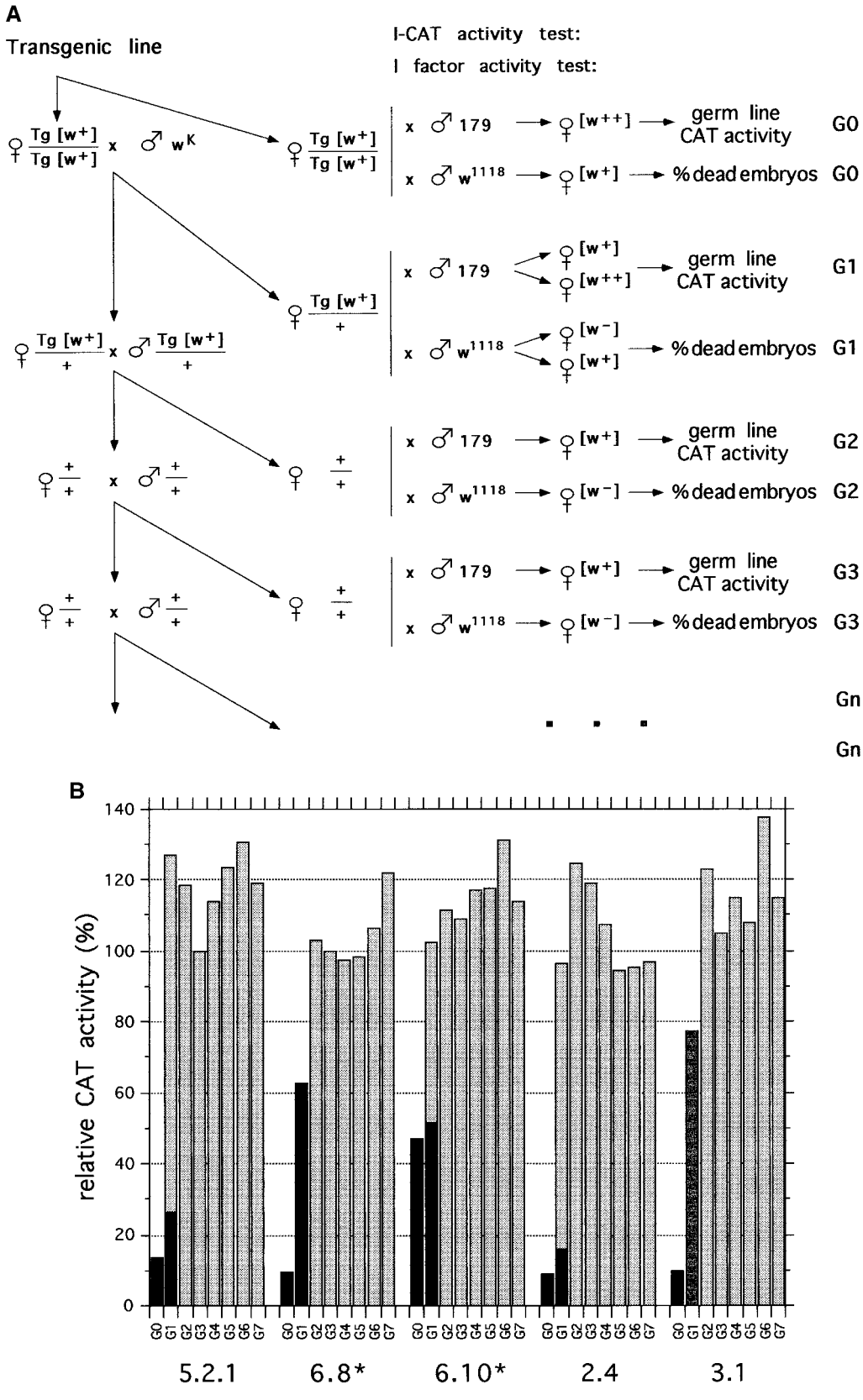

FIGURE 4.—I-CAT silencing is fully reversible. (A) Mating schemes. Homozygous hsp[. . .]pA transgenic females were crossed with w^K males to generate heterozygous individuals from which were derived nontransgenic flies. I-CAT activity and *I*-factor activity silencing were determined at each generation (G0–G7) by crossing the resulting females with either homozygous strain 179 males or w^{1118} males and measuring in the corresponding female progeny either the germ-line CAT activity or the percentage of dead embryos, respectively; light-red- or orange-eyed females, containing only one type of transgene, either I-CAT (in the I-CAT activity test) or hsp[. . .]pA (in the *I*-factor activity test), are noted [w^+]; dark-red-eyed females, having both types of transgenes, are noted [w^{++}]; white-eyed (nontransgenic) females are noted [w^-]. (B) I-CAT activity for dark-red-eyed females containing both the haploid I-CAT and the hsp [. . .]pA transgenes (solid bars) and for orange-eyed females that contain only the I-CAT reporter gene and no hsp[. . .]pA transgene (lightly shaded bars). The hsp[. . .]pA transgene in strain 3.1 confers only an extremely light orange eye color, making it impossible, in the presence of the 179 transgene, to distinguish between G1 individuals with or without this transgene (results for this mixed population are indicated with a dark shaded bar). (C) *I*-factor activity as measured by the percentage of dead embryos from the transgenic, hsp[. . .]pA-containing, female progeny of the test cross (solid bars) and from the nontransgenic female progeny (shaded bars).

all Drosophila strains, either inducer or reactive (*i.e.*, devoid of functional *I* factors, as is the w^K strain from which all transgenic strains used in this work are derived), and most probably derive from an "ancient" invasion of Drosophila by *I*-like factors distinct from the present-day *I* factor. These elements diverge from functional *I* factors in that they are highly mutated and often rearranged and can be distinguished by specific

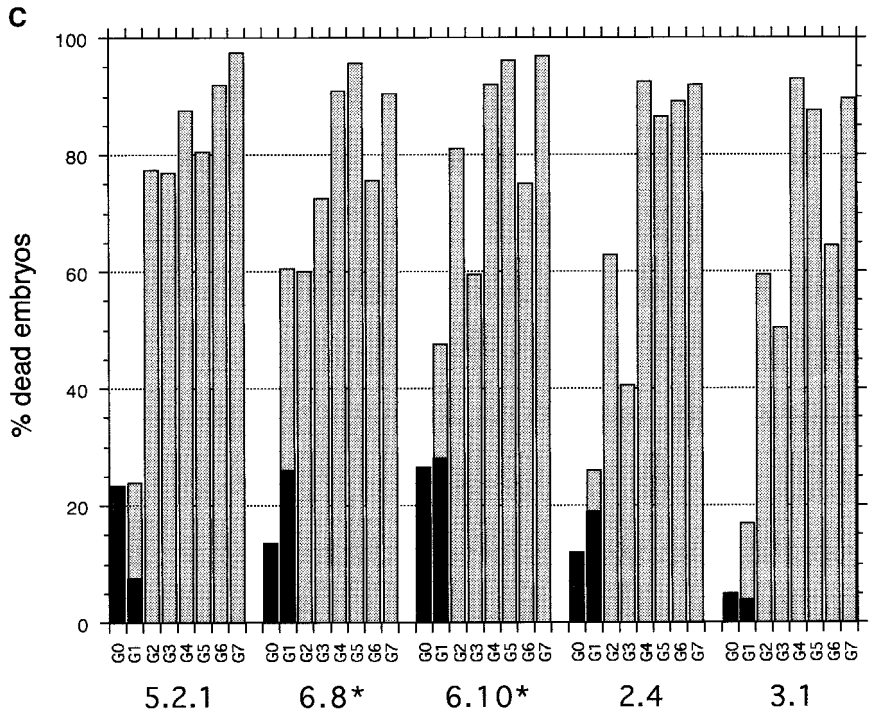

FIGURE 4.—*Continued.*

signatures (VAURY *et al.* 1990, and see below and Figure 6). Among the numerous ancestral *I*-related elements found in the database, four were possible candidates for acting as relays in the observed cosuppression (Figure 6A). These four elements contained sequences similar to the i2Δ region, which is common to all the presently used regulating transgenes; one of them was already fully sequenced and also contained a sequence similar to the promoter region present in I-CAT (Ip2918); for the others (Ip3172, Ip2862, Ip3036), the database disclosed only partial sequences, their 5′ ends—including the promoter region—being not sequenced. To ascertain (i) that these four sequences indeed exist in the w^K genome and (ii) that Ip3172, Ip2862, and Ip3036 also possess the 100-bp promoter region, a PCR search was performed using w^K genomic DNA and appropriate primers indicated in Figure 6A, leading to the cloning and complete sequencing of these elements (Figure 6). Two of these elements, Ip2862 and Ip3036, disclose large (423 and 534 bp) deletions in the ORF1 region, and two, Ip3172 and Ip2862, have foreign inserts (288 and 240 bp long, respectively) in the i2Δ region (Figure 6A). Sequence alignments show that they all contain signatures of ancestral *I* elements (conserved nucleotidic positions that are common to all ancestral *I*-like elements and different from the *I*-factor sequence), both in the 100-bp promoter and the i2Δ regions (Figure 6B), and similarly in the ORF1 region (data not shown). Similarity with the *I* factor is in the 91–95% range for the promoter region and in the 80–94% range for the i2Δ region (values after exclusion of the foreign inserts; for detail see Figure 6A and legend). According to the database, Ip2918 is localized in the pericentromeric heterochromatin (cytogenetic localization 40B–40D), while the localizations of Ip2862, Ip3172, and Ip3036 are unknown.

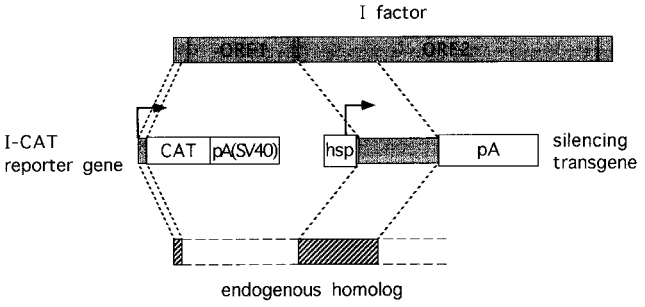

FIGURE 5.—Minimal sequence requirements for an endogenous *I*-related homolog potentially acting as an intermediate for I-CAT silencing by the hsp[. . .]pA transgenes. The putative endogenous intermediate for I-CAT silencing should contain domains with sequence similarities to the 100-bp 5′ end of the *I* factor present in the I-CAT reporter transgene and with the i2Δ internal *I* fragment (or at least part of it) present in all the hsp[. . .]pA regulating transgenes. The *I* factor and transgenes are schematized as in Figure 1, with the hatched domains in the putative endogenous homolog representing regions with required sequence similarities.

DISCUSSION

Silencing of the I-CAT reporter gene by nonhomologous *I*-related transgenes: role of ancestral *I*-like sequences: In this study, we show that hsp[. . .]pA transgenes containing short (969 bp) or long (2318 bp) *I* fragments, in either sense or antisense orientation, translatable or not, are able to repress a nonhomologous I-CAT reporter gene

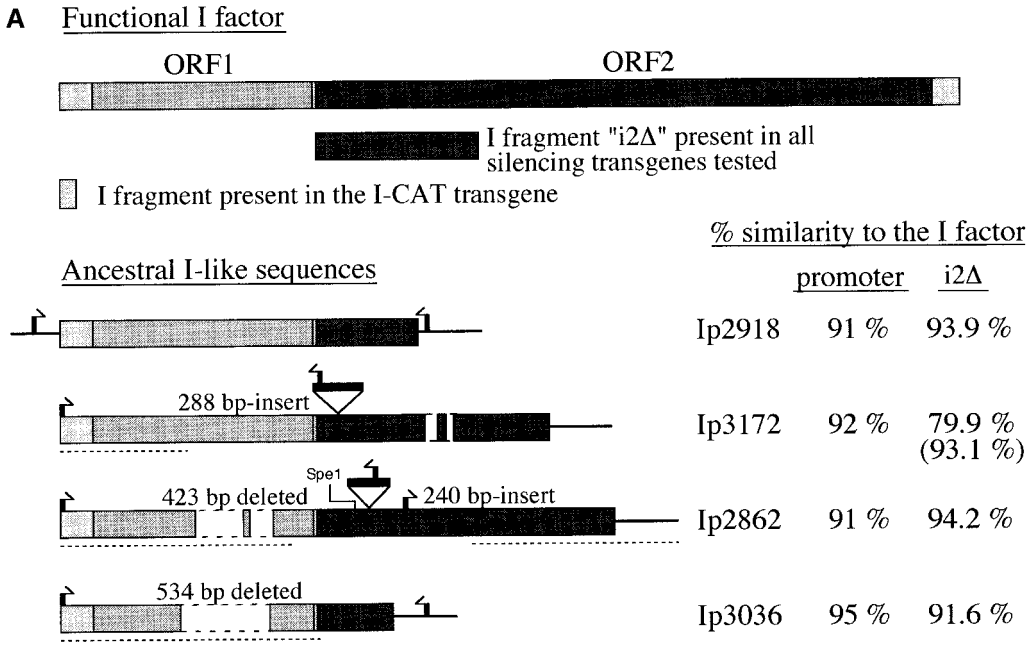

FIGURE 6.—Characterization of ancestral *I*-like sequences disclosing sequence requirements for being potential intermediates in I-CAT silencing by the nonhomologous hsp[. . .]pA transgenes. (A) Structure of four identified ancestral *I*-like sequences with sequence similarities to the *I* fragment present in the I-CAT reporter gene and to the i2Δ fragment present in all the silencing hsp[. . .]pA transgenes. Four possible intermediates in the I-CAT silencing process were identified by BLAST search in the Drosophila genome database. They contain a substantial part of i2Δ and correspond to ancestral *I*-like sequences. Their presence in the w^K strain was verified by PCR. Sequencing of the regions that were not in the genome database (dotted lines) established that they also contain the entire 100-bp *I* fragment from I-CAT. Primers for PCR amplification and subsequent cloning are indicated, as well as the SpeI restriction site that was used to amplify (by reverse PCR) the 3′ part of Ip2862. Ip2862 and Ip3036 disclose 423- and 534-bp deletions, respectively (dashed lines), and Ip3172 and Ip2862 have non-I-related 288- and 240-bp inserts (triangles). On the right are indicated the percentages of similarity to the *I* factor in the regions corresponding to the 100-bp promoter fragment present in the I-CAT transgene and to the 969-bp i2Δ fragment present in the silencing transgenes (values after exclusion of the foreign inserts). Ip3172 discloses only 79.9% similarity with the whole i2Δ region, while the 545-bp region between the 288-bp insert and the deleted part of i2Δ shows 93.1% similarity (in parentheses). (B) Sequence alignments of the identified ancestral *I*-like sequences and the *I* factor. Two domains are shown, nucleotides 1–100 (FAWCETT *et al.* 1986), present in the I-CAT transgene, and the i2Δ region common to all silencing transgenes (delineated by brackets), *i.e.*, nucleotides 1516–2484. The positions of the non-*I*-related 288- and 240-bp inserts in Ip3172 and Ip2862 are shown. Nucleotides that are not identical to the *I*-factor sequence, as well as insertions and deletions, are indicated in gray. Nucleotides that are identical in the four ancestral *I*-like sequences but different from the *I*-factor sequence (signatures of ancestral *I*-like elements) are indicated by an asterisk below the sequences. Sequence of primer P17 (nucleotides 1–24), which served for PCR amplification of the 5′ ends of Ip3172, Ip2862, and Ip3036, is in italic. GenBank accession numbers for newly sequenced parts are as follows: Ip3172, AY135216; Ip2862, AY135213 and AY135214; Ip3036, AY135215.

containing a 100-bp *I* fragment with promoter activity, in a genetic background devoid of functional *I* elements. As observed for the homology-dependent silencing of *I*-factor activity, (i) no protein from the *I* element is required for silencing of I-CAT; (ii) the control construct without any *I* fragment inserted and the promoterless *I*-fragment-containing construct have no silencing effect on I-CAT, indicating that the presence of a transcribed *I* fragment is required; and (iii) the repressing effect acting on the I-CAT reporter gene is maternally transmitted and fully reversible upon transgene removal. In addition, the I-CAT-silencing strains correspond to the strains that also have the strongest silencing effect on *I*-factor activity. Altogether, these data strongly suggest that the repression of the I-CAT reporter gene is related to that of the *I*-factor activity and relies on the same mechanism, *i.e.*, homology-dependent gene silencing. However, the I-CAT and regulating transgenes have no *I* sequence in common. This situation is reminiscent of that in PAL-BHADRA *et al.* (1999, 2002), who demonstrated cosuppression of nonhomologous Adh-related transgenes involving the endogenous Adh gene, which played the role of a relay or intermediate in the cosuppression process. In our study, we identify in the w^K strain—from which the transgenic strains are derived—at least four endogenous *I*-related sequences that could play the role of intermediates between the I-CAT and the hsp[. . .]pA transgenes. These ancestral *I*-like sequences disclose sequence similarities to both the silenced I-CAT reporter gene and the silencing hsp [. . .]pA transgenes, which range from 91 to 95% for the 100-bp promoter region and for at least 450 bp of the i2Δ region. Homology-dependent gene silencing or cosuppression has been shown to be genetically linked—at least in part—to RNAi (CATALANOTTO *et al.* 2000; KETTING and PLASTERK 2000) and to be triggered by small interfering double-stranded RNAs (siRNA, 21–25 nucleotides long; HAMILTON and BAULCOMBE 1999), resulting from the degradation of long double-stranded RNA molecules by specific enzymes (Dicer; BERNSTEIN *et al.* 2001). BOUTLA *et al.* (2001) have further shown that the silencing machinery responsible for

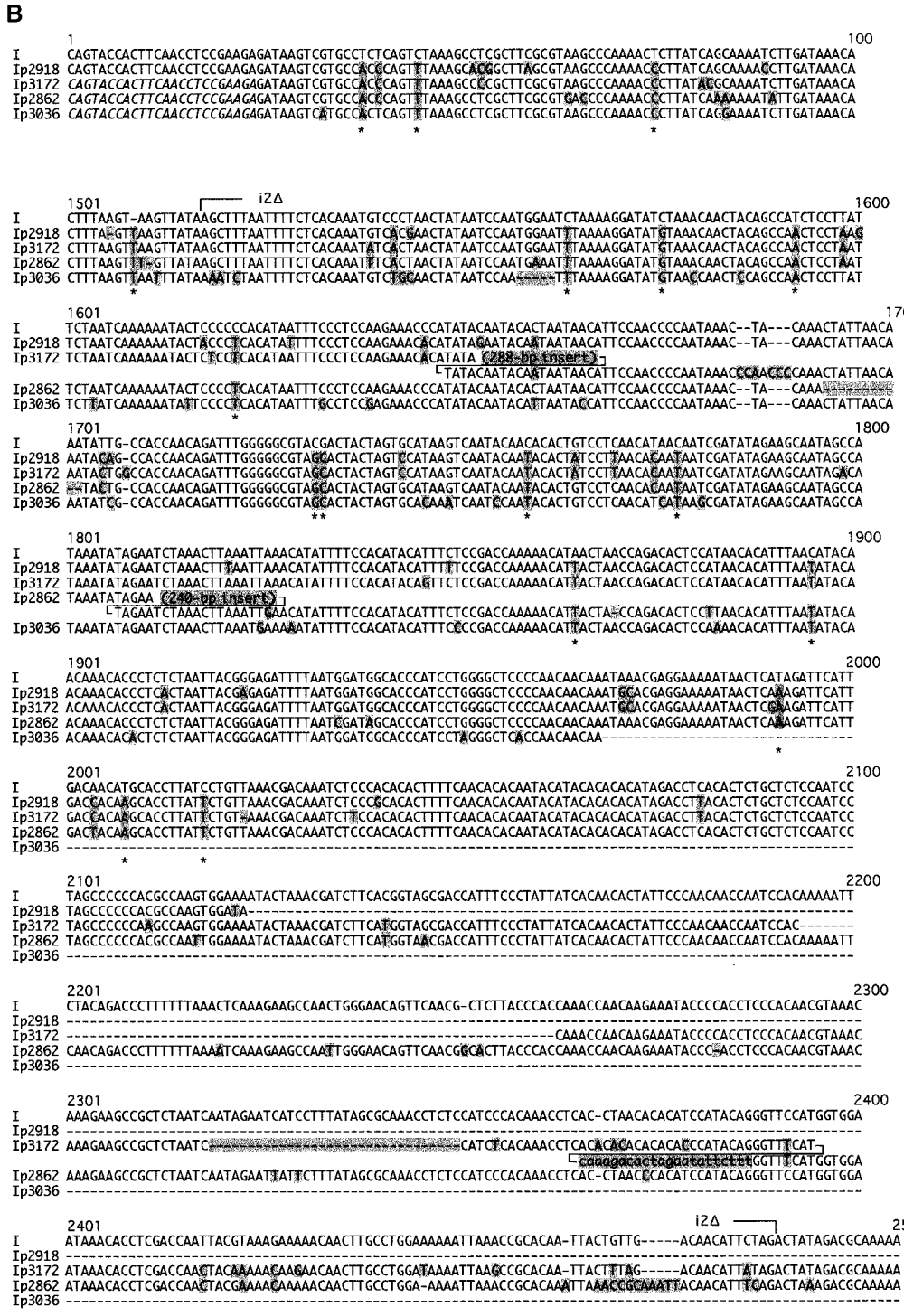

FIGURE 6.—*Continued.*

RNAi does not require perfect sequence identity, since introduction of point mutations in siRNA had only moderate effect. Analysis of the sequence similarities between the *I* factor and the four identified ancestral *I*-related elements (Figure 6B) shows that many of the siRNAs that could be generated from either sequence would display perfect identity or would differ only by single-point mutations. Accordingly, homology between the transgenes and the ancestral *I*-like sequences should be sufficient to account for I-CAT repression.

A model for *I*-factor cosuppression: The data presented strongly suggest that homology-dependent silencing of *I*-factor activity could be achieved along two different pathways, as illustrated in Figure 7: a pathway where the repressing transgene would act directly by homology-dependent gene silencing on the *I* factors and an indirect pathway where the transgene would have an effect on homologous relay sequences, leading in turn to the silencing of *I* factors and/or *I*-related sequences (*e.g.*, the I-CAT transgene). These intermediate sequences

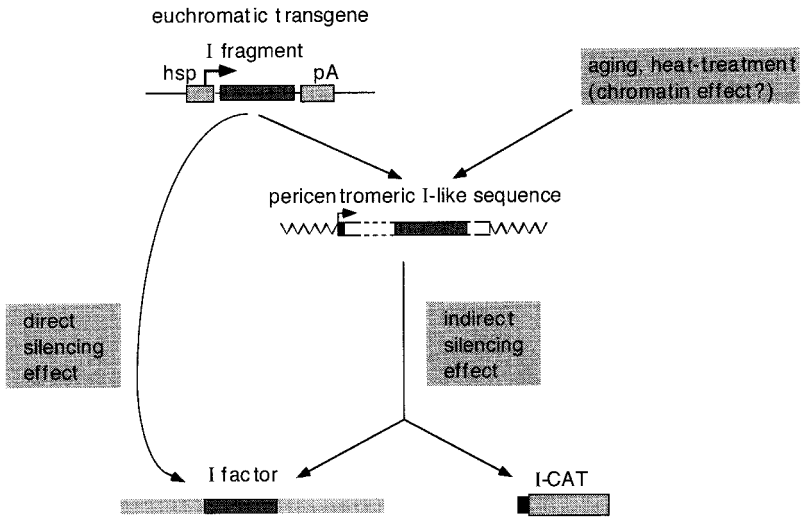

FIGURE 7.—Model for *I*-factor and I-CAT silencing. The *I*-fragment-containing hsp[. . .]pA transgenes silence *I* factor and I-CAT reporter gene activity by a homology-dependent process: by a direct silencing effect when the transgene is homologous to the gene to be silenced (*e.g.*, the *I* factor) and/or by an indirect silencing effect when a pericentromeric *I*-like sequence, with sequence similarities to both the silencing and the silenced (*e.g.*, the I-CAT reporter) transgenes, plays the role of an intermediate or relay. Transcriptional activation and/or chromatin changes at the level of such ancestral *I*-like sequences might be responsible for silencing of the *I* factor and I-CAT reporter transgene, as well as, possibly, for the effect of aging and heat treatments on the level of reactivity (BUCHETON 1979) of strains devoid of functional *I* factors.

would correspond to the ancestral *I*-related sequences present in all *D. melanogaster* strains. These elements are located essentially in heterochromatic regions. Ancestral *I*-like sequences are transcribed in the soma and seem to be silent in the adult germ line (*cf.* data in CHABOISSIER *et al.* 1990), and thus at least some of them should have an active and an inactive state. Among the four ancestral *I*-like sequences that we identified as possible intermediates in I-CAT silencing, one (Ip3172) is 100% homologous to an expressed sequence tag (EST) from adult head RNA (EST GH20531, RUBIN *et al.* 2000) and thus is clearly transcribed in the soma.

In the proposed model, a euchromatic *I*-containing transgene would activate some of the pericentromeric ancestral *I*-like elements in the adult germ line (or maintain an activated state possibly existing early in development). Once activated, these elements would in turn be responsible for a silencing effect acting on euchromatic *I*-containing sequences, *e.g.*, the *I* factor or the I-CAT reporter gene in our experiments. Activation of the ancestral *I*-like sequences most probably involves an RNA molecule since we presented evidence that transcription of the regulating transgenes is required and thus could be due to RNA-DNA interactions resulting in chromatin remodeling. Actually, chromatin changes might be directed by homologous RNAs and/or siRNAs, as suggested by the data of WASSENEGGER *et al.* (1994) and METTE *et al.* (2000; reviewed in MATZKE *et al.* 2001). In contrast to the resulting silencing effect observed by these authors on the promoter sequences of euchromatic genes, in the case of the ancestral *I*-like elements, chromatin remodeling would result in an activation, leading to "ectopic" transcription of the relay sequence as proposed by PAL-BHADRA *et al.* (2002) for the cosuppression of nonhomologous Adh-related transgenes. Activation instead of silencing might be due to the fact that RNA-DNA interactions apply to nonpromoter sequences and/or to the heterochromatic location of the *I*-related relay sequences. Along these lines, data on expressed heterochromatic genes such as *rolled* and *light* in Drosophila indicate that these genes have fundamentally different regulatory requirements compared to those typical of euchromatic genes (HEARN *et al.* 1991; LU *et al.* 2000). Clearly, investigation of the transcriptional status of the ancestral *I*-like sequences and/or of their chromatin state specifically in the germ line should now be undertaken. Finally, as illustrated in Figure 7 (left), silencing by cosuppression not requiring pericentromeric *I*-like sequences might also take place, provided that "direct" sequence similarities exist between the *I*-containing transgenes and/or *I* factors. This direct silencing, as well as the second step in the "indirect" pathway, is likely to be mediated by dsRNA and siRNA production, leading posttranscriptionally to degradation of homologous mRNAs, as now classically demonstrated in RNA interference (FIRE *et al.* 1998; reviewed in AMBROS 2001; HAMMOND *et al.* 2001), although transcriptional silencing cannot be definitely excluded.

An important feature of *I* regulation, which concerns both *I*-factor and I-CAT activities and has to be accounted for by the model, is the cumulative, maternally transmitted, generation dependence of the repressing effect. As suggested above, one possible explanation involves the progressive increase of ectopic transcription of genomic *I*-containing sequences (*e.g.*, the pericentromeric *I*-like sequences and/or the *I*-containing regulating transgenes) from one generation to the next, via chromatin changes that would be transmitted to the next generation like "imprinting" in mammals—in the present case only by females. These chromatin changes could be mediated by dsRNA (or siRNA) molecules produced by the transgene itself, which would in turn potentiate dsRNA production along a positive feedback loop. The possible involvement of such progressive chromatin remodeling is further strengthened by the fact that aging, which has been suggested to be correlated with a net loss in heterochromatinization and to be at the origin of significant changes in gene expression

(reviewed in Villeponteau 1997), is also known to induce changes of the so-called "reactivity level" of Drosophila; *i.e.*, it reduces the activity of functional *I* elements introduced by crossing. This age-dependent effect has the same characteristic features as those presently observed: It is generation dependent upon repeated crosses of aged Drosophila, it is fully reversible, it follows maternal inheritance, and this is in the absence of any functional *I* factor or *I*-related transgene in the aging Drosophila (Bucheton 1978, 1979).

Pericentromeric *I*-related elements as "memory effectors" for resistance to invading *I* factors: In conclusion, pericentromeric ancestral *I* elements appear to be necessary relays in the cosuppression of definite *I*-element-derived constructs. They also most probably act as regulators/enhancers of the repressing effect to invading functional *I* elements, being as such master genes for the regulation of the reactivity level of Drosophila. Accordingly, and to extend the already noted homology between cosuppression and the immune response (Voinnet 2001), one might speculate that these ancestral elements, which are remnants of "old" invasions, play a role closely related to that of the memory cells of the adaptive immune system: The latter are generated upon an initial encounter of the host with a parasitic element and allow the triggering of a very efficient and rapid answer upon a subsequent invasion by the same—or a related—parasite. Ancestral *I*-related elements might play a similar role—a sort of genetic vaccination—allowing an enhanced/regulated rate and extent of the host response to—not necessarily strictly identical—invading *I* elements.

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