Coupling of Enhancer and Insulator Properties Identified in Two Retrotransposons Modulates Their Mutagenic Impact on Nearby Genes

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We recently reported a novel transposition system in which two retroelements from Drosophila melanogaster, ZAM and Idefix, are highly mobilized and preferentially insert within intergenic regions. Among the loci where new copies are detected, a hot spot for their insertion was identified at the white locus, where up to three elements occurred within a 3-kb fragment upstream of the transcriptional start site of white. We have used these insertions as molecular entry points to throw light on the mutagenic effect exerted by multiple insertions of retrotransposons within intergenic regions of a genome. Analysis of the molecular mechanisms by which ZAM and Idefix elements interfere with the regulation of the white gene has shown that ZAM bears cis-acting regulatory sequences able to enhance transcription of the white gene in the eyes of the flies. This activation may be counteracted by Idefix, which acts as an insulator able to isolate the white gene from the upstream ZAM enhancer. In addition to revealing a novel insulator sequence with its own specific features, our data clearly illustrate how retroelements can act as epigenetic factors able to interfere with the transcriptional regulation of their host.

During the last few years, systematic sequencing of model genomes has yielded a huge amount of data. Today, a crucial complement is to identify sequences involved in the regulation of the identified genes and then to correlate these regulations with functions. The analysis of the nature, properties, and role of intergenic sequences with respect to regulation of the genome is thus an important goal.

Three classes of cis-regulatory sequences have been identified: enhancers and silencers, which activate and inhibit, respectively, gene expression, and insulators. Enhancers act in a distance- and orientation-independent manner to direct spatial and temporal patterns of expression. Most enhancers can activate transcription from a wide variety of promoters. By contrast, neighboring genes potentially influenced by the presence of the same enhancer within a defined chromosomal locus may display independent profiles of transcription. The second category of cis-regulatory sequences, i.e., silencers, was first implicated in the regulation of Saccharomyces cerevisiae mating type loci. More-recent studies have shown that localized patterns of Hox gene expression depend on polycomb response elements, which are thought to propagate long-range changes in chromatin structure (25). These observations suggest that mechanisms able to block the inappropriate action of an enhancer or a silencer on a neighboring gene are necessarily present in a cell. One of these mechanisms that enable the right enhancer or silencer to interact with the proper promoter implicates insulator elements.

Insulators play a fundamental role in genome organization and gene expression. They isolate independent transcriptional units from cross-reaction with neighboring regulatory sequences. Also, within complex genetic loci, insulators participate in refined modulations of internal enhancer-promoter interactions. The elements scs and scs’ identified in the flanking regions of the Drosophila melanogaster hsp70 locus were the first insulators identified (14, 15, 31). Additional insulator elements have since been described, including the suppressor of Hairy wing insulator within the gypsy retroelement, the Fab-7 and Fab-8 insulators within the bithorax complex of Drosophila, and, in vertebrates, the insulator present at the chicken β-globin locus (6, 7). All the insulators described so far block gene expression only when they are located between a distal enhancer and a target promoter. In addition, their action is not dependent on the distance between the enhancer and its promoter.

It has been reported that a large proportion of intergenic regions is made up of mobile element families. The site of their insertion within these regions may be responsible for the deregulation of a nearby gene if the insertion occurs within the endogenous cis-regulatory sequences of the affected gene. However, the sequence of the element itself may also contribute to the alteration of a gene regulation. Transposable elements are autonomous units of transcription and carry promoter regions and cis-regulatory elements able to interfere with the transcriptional regulation of genes located in the vicinity of the insertion. Among them, retrotransposons that are mobilized via an RNA intermediate are certainly essential factors able to imprint novel transcriptional regulations within the genome of their host.

In this study, we took advantage of recurrent insertions affecting a defined locus of the Drosophila genome to examine the novel cis regulations of a gene established after the successive appearance of three retroelements in its vicinity. We previously reported the structure of four alleles of the white...
First, the wIR6 allele is due to the insertion of an I factor within the first intron of the white gene (Fig. 1). This insertion is responsible for an alteration of the white gene splicing and thus a decrease in the wild-type transcripts of the gene. Since the white gene encodes a protein responsible for the brick-red color of the eyes, this mutation leads to flies with an orange eye phenotype. Second, the wIR6Rev allele, which derives from wIR6, is due to an insertion of ZAM 3 kb upstream of the white gene transcription start site (Fig. 1). The presence of this retrotransposon in the line successively called RevI induces a full reversion of the orange eye phenotype to brick-red (18). Third, the wIR6RevII allele present in the so-called RevII lines derives from the wIR6Rev allele through the additional insertion of a retrotransposon named Idefix, 1.7 kb upstream of the white gene transcription start site (Fig. 1). This new insertion leads to an orange eye phenotype. Finally, an allele called wIR6RevIV in a line called RevIV derives from wIR6RevII through an additional insertion of Idefix at the 3′ end of the first Idefix and in an opposite orientation (Fig. 1). Flies carrying this white allele display a full reversion of the wIR6RevII eye phenotype to brick-red. Although inserted upstream of the white gene, ZAM and Idefix are able to alter its expression. In this study, we have elucidated the molecular mechanisms responsible for the white gene deregulation upon ZAM and Idefix arrival and shown that ZAM bears an enhancer sequence able to activate the expression of the white gene in the eyes and that Idefix bears an insulator sequence that can disrupt this dialog between ZAM and white. This is the second insulator identified so far in a retrotransposon after the So(hw) insulator sequence described in Gypsy from D. melanogaster. In addition, our data cast new light on the level of complexity in the repertoire of interactions between transposable elements and the genome of their host.

**Materials and Methods**

**Plasmid constructions.** Different fragments of ZAM were amplified by PCR using primers Z1 (GGATCCTCAGCGCATTTGTTGACATZ) and Z2 (GTGGATCCATCCTCAGGGTTCCTCGCT) for its long terminal repeat (LTR) and Z3 (CGGCCGAAAACCTGAATGG) and Z4 (GGGATCCAAAACATA CGCTTTGGCGCT) for the 5′ untranslated region (5′ UTR) of the element (Fig. 2A). Both were inserted into the pGEM-T vector (Promega Corp., Madison, Wis.). They were then cloned between the flp recognition target (FRT) sites in the pKB345 plasmid (provided by Konrad Bassler, Zürich, Switzerland). The KpnI FRT-flanked fragments containing the 5′ UTR or the LTR of ZAM were inserted into the sole KpnI site of pCaSpeR4 (24) to give, respectively, the puU2 or plZU vector (Fig. 3). The NotI-BamHI fragment containing the 5′ UTR of ZAM was also inserted in the NotI-BamHI sites of the pCaSpeR4 to give a P transformation vector called puU2 and at the EcoRI site of the pCaSpeR4 to give puU2.

To construct the puU2W transformation vectors (Fig. 4A), the 5′ UTR of Idefix amplified by PCR using the primers I1 (GGCGAGCTGCTCGGATCCG) and I2 (CTGAGGTGATCTTGACCGTAC) (Fig. 2B) was cloned in pGEM-T. The BamHI-NotI fragment containing the 5′ UTR of Idefix was inserted between the FRT sites in the pKB345 plasmid. Then, the KpnI FRT-flanked 5′ UTR of the Idefix fragment was inserted into the unique site KpnI of the puU2 vector to give the puU2W vectors.

The LTR of Idefix amplified by PCR using the primers I1 (GGCGAGCTGCTCGGATCCG) and I2 (CTGAGGTGATCTTGACCGTAC) (Fig. 2B) was cloned in pGEM-T. Two different PCR products were obtained, the wild-type LTR and the LTR77, containing the mutation T→C at nucleotide (nt) 477 of Idefix. This mutation affects the sequence of the TATA-less promoter of Idefix, which is then unable to drive the expression of a downstream lacZ reporter gene (unpublished results). The NotI-BamHI fragments containing the LTR or LTR77 were then inserted between the FRTs in the pKB345 plasmid. The KpnI FRT-flanked LTR of the Idefix fragment was inserted into the KpnI sites of pU2 in order to construct the puU2W transformation vector (Fig. 4B). The KpnI FRT-flanked LTR77 of the Idefix fragment was inserted into the KpnI sites of pU2, pCaSpeR4, and puU2 to give, respectively, the puU2W77 (Fig. 4C). puU2W77 (Fig. 5A), and plZU77W (Fig. 5B) transformation vectors.

The U3 part of Idefix (LTRU3) was amplified from nt 1 to 470 to the according to the Idefix sequence by PCR experiments using the primers I1 and I5 (CTGACGGATTATTCTGATTTGGTTGAC) (Fig. 2B) and cloned in pGEM-T. Two different PCR products were obtained, the wild-type LTR and the LTR77, containing the mutation T→C at nucleotide (nt) 477 of Idefix. This mutation affects the sequence of the TATA-less promoter of Idefix, which is then unable to drive the expression of a downstream lacZ reporter gene (unpublished results). The NotI-BamHI fragments containing the LTR or LTR77 were then inserted between the FRTs in the pKB345 plasmid. The KpnI FRT-flanked LTR of the Idefix fragment was inserted into the unique site KpnI of the puU2 vector to give the puU2W vectors.

**Transformation vector.** Each construct was introduced into the Drosophila germ line by injection into w1218 embryos as described previously (27). At least five independent transformatant lines bearing either construct were established and analyzed.

**Fly strains and heat shock regimens.** Fly stocks were maintained on cornmeal-glucose-yeast media at 20°C. The hsFLP flies (w1218 70FLP; cu kar2 Sh/TM6, Ubx e) kindly provided by Kent Golic, express the flp recombinase under the heat shock promoter. Virgin females hsFLP were crossed with transgenic males for 24 h on cornmeal-glucose-yeast media at 20°C. Heat shocks of embryos less than 24 h old were performed as described by Ahmad and Golic (1). For each recombinant P element transformation vector injected, five independent transgenic lines were heat shocked to compare the eye colorations of the heat-shocked flies with those of the non-heat-shocked flies.

**Pigment determination.** Transgenic females were crossed to males that were hs-FLP or w1218. One-hour-old eggs of each cross were grown at 37°C for 1 h and then placed at 25°C. Transgenic males from the two resulting categories of crosses, i.e., hs-FLP and w1218, were compared. Pigment concentration was determined by extraction of red eye pigments of 20 heads and measurement at 485 nm as described by Evans and Howells (10).
ZAM has an enhancer effect on the white gene expression in the eyes. The insertion of ZAM 3 kb upstream of the white gene leads to a reversion of the eye color toward a wild-type eye color. In a first approach, we wondered whether the transcripts synthesized from the w^F0 allele were different in the RevI line bearing the w^F0revI allele. In Northern blot experiments, the 11-kb transcript resulting from the nonsplicing of the w^F0s first intron and the 2.6-kb transcripts were detected in the two lines. Additionally, reverse transcription-PCR experiments confirmed that two transcripts were present within the band migrating around 2.6 kb. As reported by Lajoinie et al. (16), one of them corresponds to the wild-type transcript of white and the second corresponds to a chimeric transcript with an 89-bp additional exon created by the recognition of cryptic splice sites within the I factor (data not shown). One explanation for the increase in the eye coloration of RevI could then be that the whole transcription of white is qualitatively unchanged but varies quantitatively. Although differences in the amount of white transcripts detected in w^F0s and RevI were not obvious on the Northern blots, ZAM might supply some enhancer sequences able to activate the white gene expression.

To seek potential enhancer sequences borne by the 8.4-kb ZAM element, transgenic experiments were performed. The ZAM sequence is composed of two LTRs flanking a long 5′ UTR (1.317 bp long) and three open reading frames (Fig. 2A) (18). Since the 5′ UTRs of diverse retroviral sequences have been found to be responsible for the deregulation of genes located close to their insertion, we first tested the potential involvement of the 5′ UTR of ZAM in its effects on the white gene.

We constructed a P transformation vector pUzW in which the potential enhancer present within the 5′ UTR of ZAM (Uz) would direct the expression of a downstream mini-white gene (W). Additionally to these sequences, the 5′ UTR of ZAM was flanked by FRT elements, which are targets for the flp recombinase (Fig. 3A and B) This construct was introduced by microinjection in flies carrying the w^1118 null mutation, and transgenic lines identified by their eye color due to the mini-white gene present in the transformation vector were isolated. These lines, called pUzW from the name of the injected plasmid, were then subjected to the flp recombinase action. To do this, they were crossed with flies expressing the flp recombinase under the control of a heat shock promoter. Expression of flp in the descendants results in recombination between the two FRTs and deletion of the intervening 5′ UTR of ZAM. This yielded flies called pUzW-flp. This strategy permits an assessment of the white gene activity in the presence (pUzW flies) and absence (pUzW-flp flies) of ZAM UTR to avoid any complications in interpretation due to position effects. PCR experiments were also performed on DNA extracts from heat-shocked and non-heat-shocked flies in order to control the excision of the FRT-flanked fragment in all the subsequent experiments (data not shown). Two series of pUzW plasmids were tested in these assays: (i) pUzW1, which bears the 5′ UTR of ZAM in the 5′ to 3′ orientation according to the mini-white gene expression (Fig. 3A), and (ii) pUzW2, which bears the 5′ UTR of ZAM in the opposite orientation (Fig. 3B). All the assays were performed on five independent transgenic lines. The pUzW lines exhibited an intense eye color when the 5′ UTR of ZAM was positioned upstream of the mini-white gene. This color was observed whatever the orientation of the ZAM fragment. In addition, this color was strongly reduced to yellow upon removal of this fragment of ZAM in independent pUzW-flp transgenic lines (Fig. 3A and B). Eye pigment dosage confirmed these data (Table 1).

Since enhancer sequences are often found in retroelement LTRs, lines bearing a transgene in which the ZAM LTR is located upstream of the mini-white gene were also established. When subjected to the flp recombinase action, the five trans-
genic lines tested did not display any variation in their eye color (Fig. 3C).

These results indicate that the LTR sequence of ZAM does not exert an enhancer effect on the mini-white gene expression. However, the 5' UTR of ZAM acts as an enhancer of the white gene, being able to direct its expression in the eyes.

**Idex LTR counteracts the effect of ZAM at the white locus.** An insertion of Idex 1.7 kb upstream of the white gene leads to a fading of the eye color, and the resulting RevII line passes from a brick-red to an orange eye phenotype (Fig. 1) (9). Identification of the exact Idex insertion site at the white locus of RevII revealed that Idex inserted at nt −1624 upstream of the transcription start site of white. Thus, its insertion occurred outside the described sequences involved in the eye expression of white that have been identified between nt −1084 and −1465 relative to the transcription start site of the gene (Fig. 1) (20). This indicates that Idex does not break cis-regulatory sequences necessary for the white gene expression.

In an attempt to elucidate the molecular bases of its mutagenic effect on wRIR6RevII, we examined the eye phenotypes of transgenic lines established with P-transformation vectors comprising different fragments of Idex inserted between the 5' UTR of ZAM and the mini-white gene.

Idex is 7.4 kb long and contains a 393-bp-long 5' UTR and three open reading frames, gag, pol and env, surrounded by two LTRs (Fig. 2B). We first tested the impact of the 5' UTR of Idex on the white gene expression. To do this, the 5' UTR of Idex (Ui) was inserted in either orientation between the 5' UTR of ZAM (Uz) and the white gene (W) in constructs subsequently called pUzUiW1 and pUzUiW2 (Fig. 4A). The 5' UTR of Idex was also flanked by FRT sequences as explained above for ZAM. We found that the flp-mediated excision of the 5' UTR of Idex did not modify the eye color of five independent transgenic lines obtained from the injection of both pUzUiW1 and pUzUiW2 (Fig. 4A). These experiments indicate that the 5' UTR of Idex has no effect on the white gene expression or on the enhancer effect of ZAM exerted on white.

Similar constructs were then tested with other portions of Idex such as its LTR (Li). The FRT-flanked LTR of Idex (Li) was inserted in either orientation in P transformation vectors called pUzLiW1 and pUzLiW2 (Fig. 4B). The FRT-flanked LTR of Idex was excised in five independent transgenic lines for each construct. All the transgenic flies bearing a transgene with the Idex LTR display paler eye color than do flies in which it was removed (Fig. 4B). This observation was confirmed by analysis of the pigment levels measured in 10 transgenic lines tested (data not shown). Two remarks can be made. First, this effect is due to a specific property conferred by the LTR of Idex, since no modification of the eye color is observed when another DNA fragment is used as the Idex UTR in the pUzUiW1 or pUzUiW2 constructs (see above). Second, a stronger modification of the eye color is always noted in the pUzLiW2 lines than in pUzLiW1 (Fig. 4B).

Thus, regulatory sequences able to counteract the eye enhancer of ZAM are present within the LTR of Idex. In addition, their effect is more efficient when Idex is inserted in a 5' to 5' orientation with respect to the mini-white gene transcription than when the reverse is done.

**The repressive effect of Idex is independent of its transcription.** Since the promoter of Idex is contained within its LTR, we then wondered whether the phenotypes observed in the transgenic lines were related to the transcriptional activity of the element within the transgene. From preliminary results of our group, we knew that Idex transcription starts at nt 475 within the LTR and that a mutated LTR, called LTR477 or Li477 when fused to a lacZ reporter gene, is unable to drive Idex transcription in transgenic lines (unpublished results). Using this mutated LTR as a tool, we then verified that the repressive effect of Idex was (or was not) independent of its transcription.

Li477 was inserted in either orientation between the UTR of ZAM and the mini-white gene in constructs called pUzLi477W1 and pUzLi477W2 (Fig. 4C). Again in 10 independent transgenic lines, a darker eye color was observed with the removal of the LTR whatever its orientation, and with a stronger effect in the pUzLi477W2 lines (Fig. 4C and Table 1). This orientation dependence was assessed by eye pigment coloration determination, which confirmed the observations. Consequently, the repressive effect of Idex is not due to its transcription but rather to regulatory sequences present in its LTR. This LTR is able to decrease the white gene expression when localized between the 5' UTR of ZAM and the mini-white reporter gene independently of its transcription. In addition, its impact is stronger in one orientation than in the other (Table 1).

**The LTR of Idex acts as an insulator.** As mentioned in the introduction, three classes of cis-regulatory elements have been identified: enhancers, silencers, and insulators. The presence of a silencer within the Idex sequence or an insulator able to block the enhancer of ZAM could potentially explain the phenotype of the RevII line.

A first series of experiments in which the silencing activity of Idex was assayed was conducted. The FRT-flanked LTR was placed upstream of the white reporter gene in a P transformation vector called pLi477W (Fig. 5A). The flp-mediated excision of the Idex LTR induced no modification of the eye color in 10 transgenic lines tested whatever its orientation (Fig. 5A). Therefore, the LTR of Idex is not able to repress the white gene promoter activity and thus does not contain a silencer sequence.

The above results are consistent with a model in which the
Idefix sequence could act as an insulator able to block the enhancer-promoter communication. Insulators are known to block gene expression only when positioned between a distal enhancer and its target promoter (reviewed in references 3, 13, and 30). This is the structure encountered in the RevII line, in which Idefix is indeed inserted between the ZAM enhancer and the white transcription start site and in the first series of transgenic constructs pUzLiW. To confirm the existence of an insulator, the LTR of Idefix was inserted upstream of both the 5′ UTR of ZAM and the mini-white gene in constructs successively called pLi/H11032 and pUzLi/ZAMW, in which the U3 part of Idefix was inserted between the 5′ UTR of ZAM and the mini-white gene, were constructed (Fig. 5B). No modification of the eye color was observed between pLi/H11032 and pLi/ZAMW-flp lines whatever the orientation of the LTR (Fig. 5B). This result indicates that the LTR of Idefix has an enhancer-blocking activity only when it is inserted between the enhancer and the target promoter. To conclude, these results show that the ability of Idefix to counteract ZAM enhancer is related to the presence of an insulator in its LTR.

The U3 part is known to contain most of the regulatory sequences of the retrotransposon LTR. The impact of the U3 part of Idefix, located between nt +1 and +470 of the element, was tested (Fig. 2B). Vectors called pUzLiL3W1 and pUzLiL3W2, in which the U3 part of Idefix was inserted between the 5′ UTR of ZAM and the mini-white gene, were constructed (Fig. 5C). Ten pUzLiL3W lines displayed darker eyes than the pUzLiL3W-flp lines did (Fig. 5C). As previously reported, a stronger difference was observed between these lines when U3 was inserted in the 3′ to 5′ orientation with regard to the white gene transcription. Pigment assays on transgenic flies with and without Li/L3 fragment confirmed that this difference in the amount of pigment is repeatedly greater when U3 is inserted in the 3′ to 5′ orientation with regard to the white gene transcription (data not shown).

Taken together, these results are consistent with the presence of an insulator located within the U3 region that is 470 nt of the 594 nt making up the Idefix LTR. This insulator acts in a preferred orientation-dependent manner.

Two Idefix insertions in opposite orientations result in the loss of insulator activity. As indicated in the introduction, we recovered an individual with a brick-red phenotype in the ZAM line, in whose eye the orange RevII phenotype was counteracted by a second insertion of Idefix (Fig. 5C). As previously reported, these data show that the Idefix insulator does not act by inactivating the enhancer or the promoter of white since they are still active in the wRevII allele. In addition, they indicate that two juxtaposed Idefix may interact with each other, leading to the suppression of their insulator function. These data observed from a spontaneous allele recovered in our stock of flies supply evidence as to the potential role of two Idefix insulators at their genomic site. They indicate that Idefix insertions can either allow or restrict interactions among neighboring regulatory elements, such a modulation depending on their copy number and their respective positions toward the regulatory sequences of a transcriptional unit (see Discussion).

**DISCUSSION**

A large proportion of intergenic regions is made up of mobile element families in humans and plants. The latter exhibit some degree of insertion site preference (17, 28), and in particular, retrotransposons have been frequently described as being present in the intergenic or silent regions of genomes (12, 29). What the impact is of these elements that are not excised during successive rounds of replication is thus a fundamental question in estimating their potential role in the evolution of eukaryotic genomes. Here, we analyzed the repertoire of interactions set up when several insertions of retroelements occur in the vicinity of a euchromatic gene of D. melanogaster. We previously reported that insertions of the two retroelements ZAM and Idefix upstream of the white gene were responsible for modifications of eye color (8, 9). Regulatory sequences present in ZAM and Idefix have been searched, and the molecular mechanisms responsible for the modifications of the eye phenotypes upon their insertion have been elucidated.

**Evolutionary implications of intergenic insertions of ZAM.** Through transgenesis and flp recombinase assays we have shown that ZAM brings an enhancer able to activate the white gene expression in the eyes of flies. This activity offers an explanation for the phenotype of the RevI line. The white transcription of the wRevI allele increases in the RevI line because of the presence of ZAM and thus leads to a full reversion of the mutated w allele. This type of change induced by a retroelement when located within intergenic regions might be expected to be of particular importance because alterations in the regulation of gene expression are likely to be less deleterious and thus more important for morphological evolution than alterations in the coding regions of genes. When inserted within intergenic regions, such an element can provide a novel repertoire of transcriptional regulatory sequences and confer novel spatial and temporal patterns of expression on host genes close to where it inserts. Since ZAM is a mobile genetic entity able to affect the germ line of its host (19), this implies that altered expressions of its neighboring genes are predicted to be stochastic and inheritable and thus can ultimately be-

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**FIG. 4.** The LTR of Idefix counters the enhancer effect of ZAM independently of its transcription. (A) The 5′ UTR of Idefix has no effect on the white gene expression. The 5′ UTR of Idefix flanked by FRT recombination sites is inserted between the 5′ UTR of ZAM and the mini-white reporter gene in P transformation vectors. The mini-white gene and the FRT sites are indicated as in Fig. 3. The 5′ UTR of ZAM is represented by a stippled box (Uz). The insertion of the 5′ UTR of Idefix is shown by a triangle. Arrowheads below the construct indicate the orientations of the Idefix 5′ UTR. (B) The LTR of Idefix decreases the white gene expression driven by the enhancer effect of ZAM. The P transformation vectors assayed for the effect of Idefix LTR on the white gene expression are the same as for panel A except that the 5′ UTR of Idefix is replaced by its LTR. (C) The mutated LTR of Idefix decreases the white gene expression driven by the 5′ UTR of ZAM. The P transformation vectors assayed for the effect of the LTR of Idefix on the white gene expression are the same as for panel B except that the wild-type LTR of Idefix is replaced by the mutated LTR. For all panels, the eye colors of the flies bearing these P transformation vectors before or after the flp recombinase action are shown on the right.
A

LTR^{177} of ldefix

\[
\begin{align*}
&\text{pLi}^{477}\text{W1} \\
&\text{pLi}^{477}\text{W2}
\end{align*}
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B

LTR^{177} of ldefix

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\begin{align*}
&\text{Uz} \\
&\text{pLi}^{477}\text{UzW1} \\
&\text{pLi}^{477}\text{UzW2}
\end{align*}
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C

LTR^{U3} of ldefix

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\begin{align*}
&\text{Uz} \\
&\text{pUzLi}^{U3}\text{W1} \\
&\text{pUzLi}^{U3}\text{W2}
\end{align*}
\]
come material for evolutionary change. It has been shown that divergence in the cis-regulatory regions serves as an important basis for the evolution of distinct developmental functions as gene duplications (20). The fact that ZAM insertions are frequently found within intergenic regions combined with the fact that it is able to provide enhancer sequences may serve as a potential genomic tool promoting the acquisition of novel spatiotemporal control functions.

**Specific properties of a novel insulator identified in the retrotransposon Idefix.** In the RevII line, the insertion of Idefix 1.7 kb upstream of the white gene transcription start site and outside its regulatory sequences induces an orange eye phenotype very similar to the eye color phenotype of the wIR6 line (Fig. 1). We have shown that this modification of the eye phenotype is due to an insulator sequence that we identified in the U3 part of the Idefix LTR (corresponding to the first 470 nt of its 594-nt full-length LTR). In a transgene bearing the ZAM-Idefix LTR-mini white gene, the Idefix sequence is able to counteract the enhancer effect of ZAM on the downstream reporter gene.

This novel insulator sequence displays various interesting features. One of them is that its action is more efficient in one orientation than in the other. The strength of its blocking effect was found to be greater when the enhancer was located at the 3' end of the LTR sequence bearing the insulator than at its 5' end.

Second, the presence of two adjacent copies of Idefix inserted in the opposite orientation nullifies the action of the insulator as observed in the RevIV line where two Idefix inserted between the white gene and ZAM lead to a brick-red instead of an orange phenotype when a single copy of Idefix is present (Fig. 1) (9). This property recalls recent results reported in the literature for the Su(Hw) insulator. Cai and Shen (5) and Murayowa et al. (21) have shown that two copies of the Su(Hw) insulator are ineffective in blocking various enhancers from a downstream promoter. This phenomenon is explained by the fact that two insulators may interact with each other through protein complexes bound to them, forming chromatin loop domains able to facilitate enhancer-promoter communication.

Two main models have been proposed to explain the mechanism by which insulators interfere with enhancer-activated transcription. Either they segregate DNA into distinct chromatin loop domains (15, 31) or they act as transcriptional attenuators (4) or decoys (11), which more directly interfere with transcriptional processes. Concerning the mechanism used by the insulator identified in Idefix, the former model is supported by the fact that two copies of Idefix, instead of a single one, are ineffective in blocking the upstream enhancer from the downstream promoter of white. As suggested above, formation of loops due to interactions between two copies of Idefix could explain the RevIV phenotype. If an interaction between the two juxtaposed Idefix elements exists at the white locus in RevIV, this suggests that other interactions can potentially occur between two Idefix insertions present at different genomic sites. Preferential interaction of two juxtaposed copies would then exclude other interactions necessary to sequester enhancers and promoters into distinct chromatin domains. At this point, it must be noticed that the Idefix insulator is located within the LTR of the element, and is present as two direct repeats in each Idefix insertion, i.e., in the 5' LTR and the 3' LTR. This suggests that the insulator is neutralized not when two copies are present and in the same orientation within a single element but when it is present in two independent Idefix elements, as in the wIR6Rev IV allele.

Although data obtained on this allele are consistent with the idea that the Idefix insulator is involved in loop formation, the second model corresponding to the transcriptional attenuators or decoy model cannot be eliminated. Indeed, the insulator of Idefix is localized within a 470-bp fragment corresponding to the U3 region of the LTR. The 3' end of this fragment is thus in close proximity to the transcription start site of the element located at nt 475. Although we have shown that the insulator does not depend on the transcriptional activity of the down-

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**TABLE 1. Quantitation of the amount of eye pigments in transgenic lines**

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Line</th>
<th>Amount of eye pigment a</th>
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<tr>
<td></td>
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<td>flp+</td>
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<tr>
<td>pUzW1</td>
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a Transgenic lines were subjected (flp+) or not subjected (flp−) to the flp recombinase action.

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FIG. 5. Idefix does not act as a silencer but does act as an insulator through the U3 part of its LTR. (A) The LTR577 of Idefix does not contain a silencer. The LTR577 of Idefix flanked by FRT sites is inserted upstream of the mini-white reporter gene. The yellow color of the transgenic lines is due to the presence of a weak eye enhancer located close to the transcription start site of the mini-white reporter gene. (B) The LTR577 of Idefix does not block the enhancer effect of ZAM when inserted upstream. The LTR577 of Idefix flanked by FRT sites is inserted upstream of the 5' UTR of ZAM and the mini-white reporter gene. (C) Idefix insulator is located in the U3 part of its LTR. The U3 part of the FRT-flanked Idefix LTR is inserted between the 5' UTR of ZAM and the mini-white reporter gene. For all three panels, the arrowheads below the constructs show the orientation of the LTR577 or the LTRU3 of Idefix with the corresponding names of the P transformation vectors below the figure. The eye colors of the flies bearing these P transformation vectors in which Idefix LTR577 or LTRU3 has been removed or not by the flpase are shown on the right.
stream white gene, a connection between the presence of a core promoter and a juxtaposed insulator cannot be excluded. Determining the exact location of the insulator function present within the still-large 470-bp fragment will certainly yield complementary information relative to such a possible connection.

**Coupling of an insulator and a promoter may act as a novel level of gene regulation.** The insulator of Idefix is located in the U3 part of its LTR, upstream of the transcription start site of the element. An insulator in this region can potentially protect itself against any position effect due to neighboring sequences and reflect how a retrotransposon with its own pattern of expression can defend itself from its host. However, since U3 parts of retrotransposons are known to contain enhancers necessary for their transcription, this raises the question of how enhancers bypass the insulator to activate Idefix transcription. One answer may reside in the orientation-dependent property of the Idefix insulator. As described above, the strength of the insulator is greater when the enhancer is located at its 3′ end. Thus, Idefix enhancers located upstream of the insulator should be able to bypass the insulator and activate its transcription. A similar situation has already been described for Drosophila for another insulator identified just upstream of the even-skipped (eve) promoter. The authors have shown that enhancers located 3′ of the eve promoter were unable to interact with neighboring genes located 5′ of eve (23). It is tempting to suppose that such promoters might be implicated in the functional organization of gene regulation by preventing the transcriptional activation of a promoter located upstream by enhancers located downstream. On the other hand, enhancers located upstream would be able to bypass the insulator and thus activate their own promoter.

The insulator activity of the eve promoter can be uncoupled from the core promoter elements. However, it depends on the binding of GAGA proteins between the TATA box and the transcription start site (23). We have found that the LTR of Idefix contains a putative GAGA binding site located 100 bp upstream of its transcription start site, suggesting that GAGA could also be implicated in the insulator activity of Idefix. Arkhipova has shown that almost 15% of 250 Drosophila promoters tested contain at least one optimal GAGA element within 50 bp 5′ of the transcription start site (2). An earlier study of one of these promoters, α-tubulin, indicates that GAGA helps to insulate tubulin expression from position effects (22). These results show that many promoters probably possess an intrinsic enhancer-blocking activity essential for their proper regulation. In addition, if insulator activity and promoters were to be found combined more generally, this might add another level in the regulation of gene transcription and in the mutagenic impact of any mobile element insertion within the genome.

In conclusion, it is possible to ascribe specific and essential functions to retrotransposons within a genome. Due to their stochastic mobilization and insertion, they can certainly be considered as epigenetic factors able to interfere with the proper regulation of genes located in their vicinity. Several ways by which these elements can act on the genome regulation are clearly identified in our study and can be summed up as follows: (i) their enhancer sequences can drive endogenous genes to be misexpressed or overexpressed in some defined tissues; (ii) their insulator sequences can be involved in partitioning the genome in functionally independent domains; (iii) these insulators behave as nonstatic barriers, but rather because of their mobilization and/or potential accumulation at some preferred loci as modulatable regulatory elements limiting or facilitating the range of action of nearby enhancers; and (iv) their insulators may help to decrease the general mutagenic impact of retrotransposons within a genome. The presence of an insulator associated with any novel insertion can prevent interactions between regulatory sequences of the element and regulatory sequences of neighboring host genes and thus leave the regulation of the endogenous genes unchanged. This effect on mutagenic impact exerted on genes located in the vicinity of a retrotransposon insertion ought certainly to be taken into account to estimate the overall influence of retrotransposons on genomic regulations. These findings therefore indicate that retroelements can certainly be considered creative forces directly contributing to the regulation and ultimately the evolution of their host.

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