Mobilization of Two Retroelements, *ZAM* and *Idefix*, in a Novel Unstable Line of *Drosophila melanogaster*

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We describe a novel transposition system in a line of *Drosophila melanogaster* called RevI in which two retroelements are mobilized. These elements are the retroelement *ZAM,* recently described in the literature, and a novel element designated *Idefix.* Like *ZAM, Idefix* displays the structural features of a vertebrate retrovirus. Its three open reading frames encode predicted products resembling the products of the *gag, pol,* and *env* genes of retroviruses. In situ hybridization and Southern analyses performed on the RevI genome revealed the presence of some 20 copies of *ZAM* and *Idefix,* whereas *ZAM* is absent and *Idefix* is present in only four copies on the chromosomal arms of the original parental line. From RevI, a series of mutations affecting eye coloration has been recovered. The genetic and molecular analyses of these mutations have shown that most of them affected the *white* locus through three rounds of mutational events. The first mutational event was previously shown to be caused by a *ZAM* insertion 3 kb upstream of the transcription start site of *white.* It confers a red-brick phenotype to the orange eye coloration of the parental line. The second event results from the insertion of an *Idefix* copy 1.7 kb upstream of the transcription start site of the *white* gene, which modifies the red-brick phenotype to orange. This second mutational event was recovered as a recurrent specific mutation in 11 independent individuals. The third event results from an additional *Idefix* located 1.7 kb upstream of *white* that is responsible for the full reversion of the orange phenotype to red-brick. The fact that such mutations due to recurrent appearances of both *ZAM* and *Idefix* at the *white* locus result in such a variety of phenotypes brings to light a new molecular system in which the interference of mobile elements with the correct expression of the host gene can be tested.

Introduction

Transposition of mobile elements plays an important role in the generation of genotypic and phenotypic diversity in eukaryotes. Their insertions account for a significant number of spontaneous mutations, changes in spatiotemporal gene expression, or formations of chromosomal rearrangements (Finnegan 1989; Lim and Simmons 1994). Thus, a tight regulation of their transposition is needed to prevent any deleterious effect that could result from such a potential source of constant damage of the genetic information. This important feature of transposable element regulation implies that the transpositions of mobile elements are usually rare events (Dominguez and Albornoz 1996). However, *Drosophila* offers several examples of genetic instability associated with massive mobilization of transposable elements. For instance, some transposable elements, such as *I, P,* and *hobo,* are dramatically activated in hybrid dysgenesis in which males with a particular active mobile element are crossed to females lacking the element (Yannopoulos et al. 1987; Engels 1989; Bucheton 1990). Another transposition system in *Drosophila melanogaster* depending on the retrotransposon *Stalker* differs from these cases of hybrid dysgenesis in that it does not depend on the direction of a cross (Georgiev et al. 1990). The possibility of simultaneous mobilization of multiple transposable elements has also been illustrated by several examples involving *Drosophila* in which certain genetically unstable strains exhibit elevated levels of mobilization of more than one type of element. A strain designated Uc has shown high levels of *hobo* and *gypsy* mobilization (Lim et al. 1983); similarly, both *copia* and *Doc* elements were found to be mobilized, albeit to a lesser extent, in an isogenic *D. melanogaster* stock identified by Pasyukova and Nuzdhin (1993). Finally, a system of hybrid dysgenesis has also been described for *Drosophila virilis* in which at least four unrelated transposable elements are mobilized following a dysgenic cross (Petrov et al. 1995). Although little is known about the regulatory systems governing transposable element mobilization, the occurrence of simultaneous mobilizations of more than one type of element suggests that these elements may share a common pathway of regulation in their host.

We previously reported the identification of a strain which suffered a recent and massive amplification of the retroviral element *ZAM* (Leblanc et al. 1997). This strain, which henceforth will be referred to as RevI, displays a high copy number of *ZAM* elements distributed in euchromatic regions; in contrast, all the other strains tested have low copy numbers and show very few, if any, signals on the chromosomal arms. RevI was isolated as a mutational insertion at the *white* locus that occurred in a line called w^{IR6} (from the name of its *white* allele). The w^{IR6} allele is a result of the insertion of the non-LTR retrotransposon *I factor* into the first intron of the *white* gene and results in a brown-orange eye phenotype (Lajoinie et al. 1995). The $w^{IR6RevI}$ allele generated by the insertion of the *ZAM* element at −3 kb from the transcription start site of the *white* gene displays a wild-type eye phenotype instead of the orange eye coloration of its parental line, w^{IR6}.

Key words: *Drosophila melanogaster,* retrotransposons/retrovirus, *ZAM, Idefix,* genetic instability.

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Here, we describe a series of unstable mutations affecting the eye coloration which arose as spontaneous mutants in the background of the $w^{IR6RevI}$ allele. We show that part of the phenotypic modifications are due to recurrent mutations affecting the *white* locus. These mutations have been analyzed at the molecular level and found to result from the insertion of a novel retrovirus-like element designated *Idefix* that belongs to the *Ty3-gypsy* family and inserted upstream of the transcription start site of *white.* By studying the genomic distribution of *ZAM* and *Idefix* elements, we show that the observed phenotypic changes arose in the course of their mobilization. In addition, our data indicate that both *ZAM* and *Idefix* remain active in our stocks for many generations after the initial $w^{IR6RevI}$ line isolation.

Materials And Methods

Fly Stocks

Fly stocks were maintained on cornmeal-glucose-yeast media at 20°C. The w^{IR6}, RevI, Cha, Canton S, w^{IR7}, and SV-att-X (wvl44,C(1)DX,yf) are from the INSERM 384 collection. Males containing X-chromosomal mutations were crossed to the SV-att-X females carrying attached X-chromosomes (att-X/Y). The mutated X-chromosome was maintained indefinitely in this way. The male containing the novel mutation located on the third chromosome was first crossed with the SV-att-X females, rendering homozygosity by selection for eye coloration (homozygotes are yellow while heterozygote mutants are orange). Mutations were localized by recombination analysis with closely located markers.

Drosophila DNA Preparation and Southern Blotting

Genomic DNA was prepared from strains of *Drosophila* according to the protocol of Udomkit et al. (1995). The DNA was then transferred to hybond N+ membrane by capillary action in 3.6 M NaCl, 0.2 M Na phosphate, and 0.02 M EDTA (pH 7.7). After hybridization at 42°C, filters were washed in 2 × SSC, 0.1% SDS at 42°C, and in 0.1 × SSC, 0.1% SDS at 42°C. Fragments used as probes were gel-purified and labeled with [α-^{32}P] dCTP by random priming (Stratagene).

PCR Amplification

PCR amplification was performed with the Expand Long Template PCR system of Boehringer. Conditions and procedures are those described in the manufacturer's protocol. Primers olPstI (5′-GCATTTACTGCAGGGGACAAC-3′) and olPvuII (5′-CTGCCGCCATCAGCTGTCCGGG-3′) were used to amplify the complete *Idefix* element at the *white* locus.

Inverse PCR Experiments

DNAs were treated with *Sau*3A restriction endonuclease, and the 5′ and 3′ ends of *Idefix* were identified by the inverse PCR method described by Gloor et al. (1983). Primers used were w1 (5′-CTCTCCGCACAGTCACACCTAC-3′) and w2 (5′-CAGCTCTTTCGCTGCTGCGACA-3′).

DNA Sequencing

The sequence of the *Idefix* insertion sites was determined by subcloning the *Pst*I/*Pvu*II PCR product in the SK pBluescript vector (Stratagene). Double-stranded templates were prepared using QIAGEN columns and sequenced by dideoxynucleotide chain termination using the DNA Dye Terminator Cycle Sequencing Kit (Perkin Elmer); The samples were loaded on an ABI377 sequence analyzer (ABI). Oligonucleotides used to prime sequencing were w2 (described above) and w3 (5′-GACCGCACTTCCTGTGACAATG-3′).

In Situ Hybridization on Polytene Chromosomes

Larval salivary glands were dissected in saline solution (0.7% NaCl) and squashed in 45% acetic acid. ***ZAM*** DNA probes (clone EH8 encompassing the *pol* and *env* genes of *ZAM* and described in Leblanc et al. 1997) were labeled by nick translation using biotin-11-dUTP (Enzo) and detected by FITC-conjugated avidin (Vector Laboratories); ***Idefix*** DNA probes (clone BH described in fig. 7, see below) were labeled by nick translation with DIG-UTP (Boehringer Mannheim) and detected by a rhodamine-conjugated antibody (Boehringer Mannheim). Fluorescent in situ hybridization (FISH) and CCD camera analysis were carried out as described in detail elsewhere (Gatti, Bonascossi, and Pimpinelli 1994). Preparations of salivary gland chromosomes were stained with DAPI. Images were merged and analyzed by using the Adobe Photoshop 2.5 program. The accession number of *Idefix* is AJ009736.

Results

Eye Color Mutations Are Occasionally Recovered in the $w^{IR6RevI}$ Line

The $w^{IR6RevI}$ allele was identified in a line referred to as RevI and was shown to result from the insertion of the retroviral element *ZAM* 3 kb upstream from the w^{IR6} transcription start site (Leblanc et al. 1997). The mutation caused the full reversion of the w^{IR6} brown-orange phenotype to red-brick. RevI proved to be unstable, since it yielded spontaneous mutants with orange eyes among the red-eyed population. Over a period of 4 years, we were able to isolate 11 males with the orange-eye phenotype, which independently arose in three series of RevI stocks reared separately (fig. 1).

Each of these males was established as a line by crosses with attached-X-chromosome females (see *Materials and Methods*), and the cause of the lesion responsible for the eye color modifications was analyzed. Using a genetic approach with each of the 11 lines, we found that the orange eye color was associated with a mutation mapping close to the *white* gene on the X chromosome. These observations suggest that the mutational lesion could have affected the *white* locus again; in order to ascertain whether that was the case, the *white* locus organization was analyzed for all 11 lines by restriction mapping. Southern blot experiments using a battery of restriction enzymes revealed that no change had occurred throughout the *white* transcriptional unit in any of the 11 lines. In addition, the complete *I factor* and

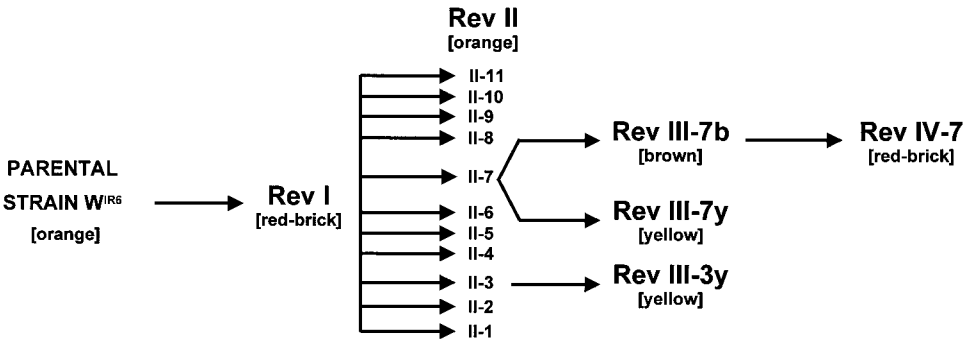

FIG. 1.—History of the unstable line.

the *ZAM* element, located in the first intron and upstream from the transcription start site of *white*, respectively, were still present with the same restriction map. However, in all 11 lines under examination, the locus had suffered a 7.4-kb insertion into the 1.4-kb *Bgl*II-*Bgl*II restriction fragment situated between the transcription start site of *white* and the *ZAM* insertion (see the 7.4-kb insertion in fig. 2*A*). Although the mutations had been recovered as independent events in three series of RevI stocks, all 11 lines were found to display the same structure. This new allele of *white* was called $w^{IR6RevII}$ to indicate that the DNA modification had affected the w^{IR6} allele in a second wave of mutation. The independent lines bearing this allele were called RevII-1, RevII-2, RevII-3, . . . , RevII-11, or, collectively, RevII (fig. 1).

Throughout the period during which we regularly isolated $w^{IR6RevII}$ alleles from the RevI line, we observed that the genetic instability detected in RevI was maintained in several established RevII lines. Indeed, flies with new eye color phenotypes were newly recovered among the progeny from RevII-3 and RevII-7. Two phenotypic classes from these new mutational events were found. One is characterized by a yellow eye color, and thus may be functionally regarded as an enhancer of the mutated phenotype, while the other yielded a stronger eye pigmentation, passing from orange to dark brown.

Two yellow-eye mutants were recovered from both the ReII-3 and RevII-7 lines and established as lines which were, respectively, called RevIII-3y and RevIII-7y. A brown-eye mutant was recovered from RevII-7, yielding the RevIII-7b line (fig. 1).

Genetic analysis placed the mutations responsible for the new phenotypes in genomic regions that were independent of the *white* locus, as they were separable by meiotic recombination. This was further confirmed by molecular analysis of the *white* loci in the RevIII-3y, RevIII-7y, and RevIII-7b derivative lines: in all three lines, the molecular organization of the *white* locus was identical to that of the parental line, i.e., to the $w^{IR6RevII}$ allele. Indeed, the *white* gene, the *I factor*, *ZAM*, and the novel insertion identified at the *white* locus in the RevII lines were still present, with the same restriction map (data not shown).

The mutational events responsible for the new eye colorations were genetically localized to the X chromosome in both RevIII-7b and RevIII-7y lines and

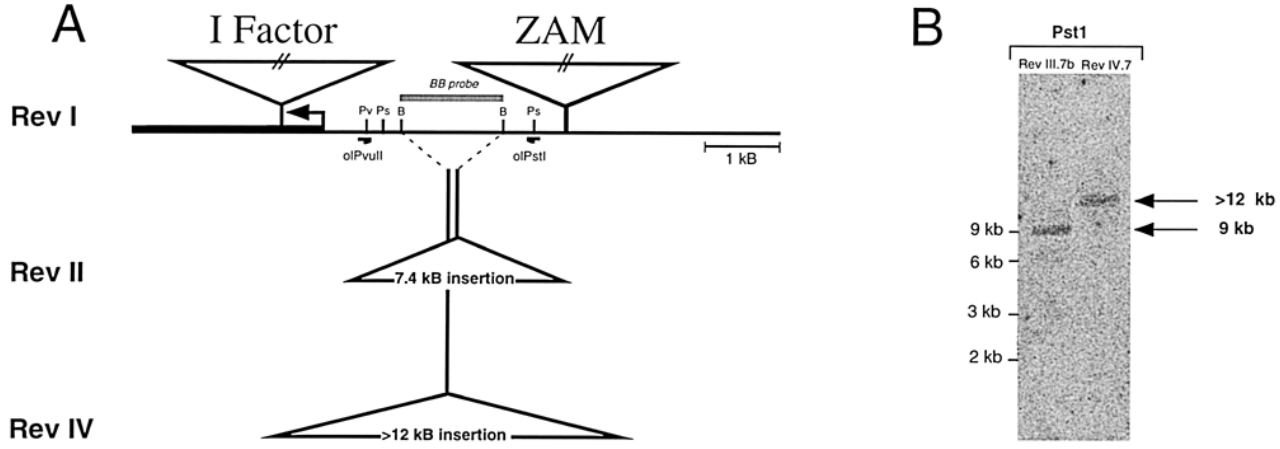

FIG. 2.—Molecular structure of the *white* loci in the RevI, RevII, and RevIV lines. *A*, The structure of the *white* alleles is represented as follows: the *white* gene transcription unit is indicated by a thick line and its transcription start site is indicated by an arrow. DNA upstream of the gene is shown as a thin line. The insertion sites of the *I factor* and *ZAM*, as well as the two novel insertions identified in RevII (7.4-kb insertion) and RevIV (>12-kb insertion), are indicated by triangles. *Pvu*II (Pv), *Pst*I (Ps), and *Bgl*II (B) are some of the restriction sites present at the *white* locus. Locations of the oligonucleotides olPvuII and olPstI and the fragment BB, used as a probe, are indicated. *B*, DNA from the RevIII-7b and RevIV-7 lines was digested with *Pst*I, and the blot was probed with the wild-type BB fragment of *white* presented in *A*. The heterogeneous migration of the *Pst*I fragments indicated that the corresponding 9-kb fragment of RevIII-7b has been submitted to an additional mutational event in RevIV-7.

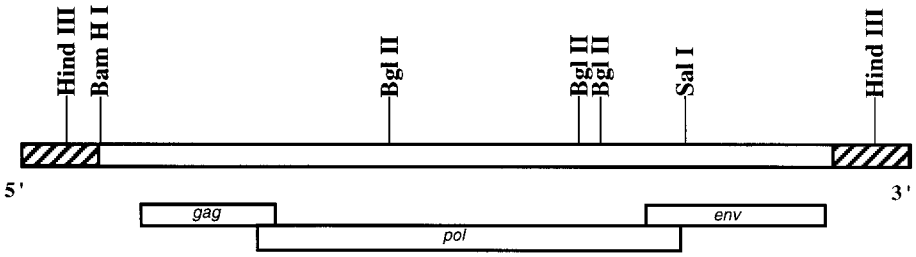

FIG. 3.—*Idefix* organization and molecular structure. Hatched boxes represent the long terminal repeats (LTRs); *Idefix* open reading frames (*gag, pol,* and *env*) are symbolized by rectangles below the restriction map.

mapped by genetic recombination within 1.5 and 9 map units of the *white* gene, respectively. The mutation responsible for the yellow phenotype of the RevIII-3y line was mapped by genetic recombination to the third chromosome, 32 map units from the *ebony* gene and 44 map units from the *Stubble* gene, suggesting that it is located at the tip of chromosome 3.

Furthermore, we isolated a fourth event of phenotypic variation in an individual that hatched in the RevIII-7b line and displayed a full reversion from the dark brown to the red-brick phenotype (fig. 1). The cause of this reversion was genetically mapped to the X chromosome linked to the *white* gene. A molecular analysis was undertaken on this new line, called RevIV-7, in an attempt to define the area of the *white* locus that had been affected in the revertant by comparison with the original $w^{IR6RevII}$ allele. The *white* gene, the *I factor,* and *ZAM* were found to be still present and structurally unchanged. However, when the DNA from the parental RevIII-7.b line was subjected to restriction digestion using the *Pst*I enzyme, which cuts outside the 7.4-kb insertion, and compared with the equivalent fragment from a RevIV-7 DNA digest, a clear difference in size was apparent between the fragments hybridizing to the *white*-derived BB probe (fig. 2*B*): the *Pst*I fragment generated in RevIV-7 was larger than 12 kb, indicating that a new modification of the *white* locus structure had occurred in the novel derivative line, possibly due to an additional insertion.

The *white* Locus Is Subjected to Recurrent Mutational Events Due to Successive Insertions of a Novel Retrovirus-like Element, *Idefix*

In order to better characterize the 7.4-kb insertion identified in RevII lines, two oligonucleotides (olPstI and olPvuII, presented in fig. 2*A*) encompassing both the *Pst*I and *Pvu*II sites at positions 4672 and 6816 of the *white* locus according to O'Hare et al. (1984) were used as primers in PCR amplification reactions. Restriction mapping of the amplified fragments from the 11 RevII lines was performed. These experiments enabled us to resolve the molecular organization of the 7.4-kb DNA insertion schematized in figure 3. Insertions of identical size and with the same restriction map were found at the same location in each of the 11 lines.

To gain further insight into the nature of the insert, PCR products obtained from one of the RevII lines (RevII-9) were cloned and sequenced. The insertion displays the typical structure of a *gypsy*-like retrotransposon (fig. 3), but clearly represents a family distinct from transposable elements described so far in *D. melanogaster*. We call this insertion *Idefix*. *Idefix* is flanked at both ends by LTRs that are 594 bp long and are identical in sequence. *Idefix* possesses a putative tRNA primer binding site (tRNA PBS); its 12-bp sequence, complementary to the 3′ end of *D. melanogaster* $tRNA_4^{Ser}$ (Cribbs, Gillam, and Tener 1987), overlaps the 5′ LTR by one nucleotide. *Idefix* sequence analysis with the DNA strider program (Marck 1988) showed that *Idefix* has a 393-bp noncoding sequence located upstream of the first open reading frame (ORF), followed by three ORFs able to encode polypeptides similar to the Gag, Pol, and Env proteins found in vertebrate retroviruses such as human immunodeficiency virus or Moloney murine leukemia virus (Shinnick, Lerner, and Sutcliffe 1981; Morrow, Park, and Wakefield 1994) or retrovirus-like elements such as *297, 17.6, gypsy, ZAM, tom,* and *TED* (Leblanc et al. 1997). In each case, these similarities extend over domains containing amino acids that are highly conserved among retroviruses.

Idefix ORF1 is 348 amino acids long. The predicted translation product of ORF1 exhibits a high context (11%) and uneven distribution of asparagine residues. *Idefix* ORF2 encodes a 1,151-amino-acid protein including sequences similar to other Pol proteins (Xiong and Eickbush 1990). Analysis with the BLAST-X program (Altschul et al. 1990) revealed four identifiable domains: the protease domain (prt) displays the short sequence Asp-Thr-Gly (dtg), which is thought to form the active site of aspartyl (or acidic) proteases; the reverse transcriptase (rt) characterized by the conserved motif called the YxDD box, which identifies its catalytic center; the Rnase H (rnh); and the integrase domain (int), which displays potential sites for binding of zinc ions (so-called ''zinc fingers'') and a DD35E motif characteristic of the active site of integrases (Polard and Chandler 1995). Figure 4 depicts partial alignments of these domains with those of several invertebrate retrovirus-like elements or vertebrate retroviruses.

Sequence analysis of ORF3 (484 amino acids) revealed similarities with Env-like proteins of *297, 17.6, gypsy, ZAM, tom,* and *TED.* A domain conserved in these retrovirus-like elements is presented in figure 4. It displays an Arg-x-Lys-Arg conserved domain (RxKR) which is considered to be a consensus proteolytic cleav-

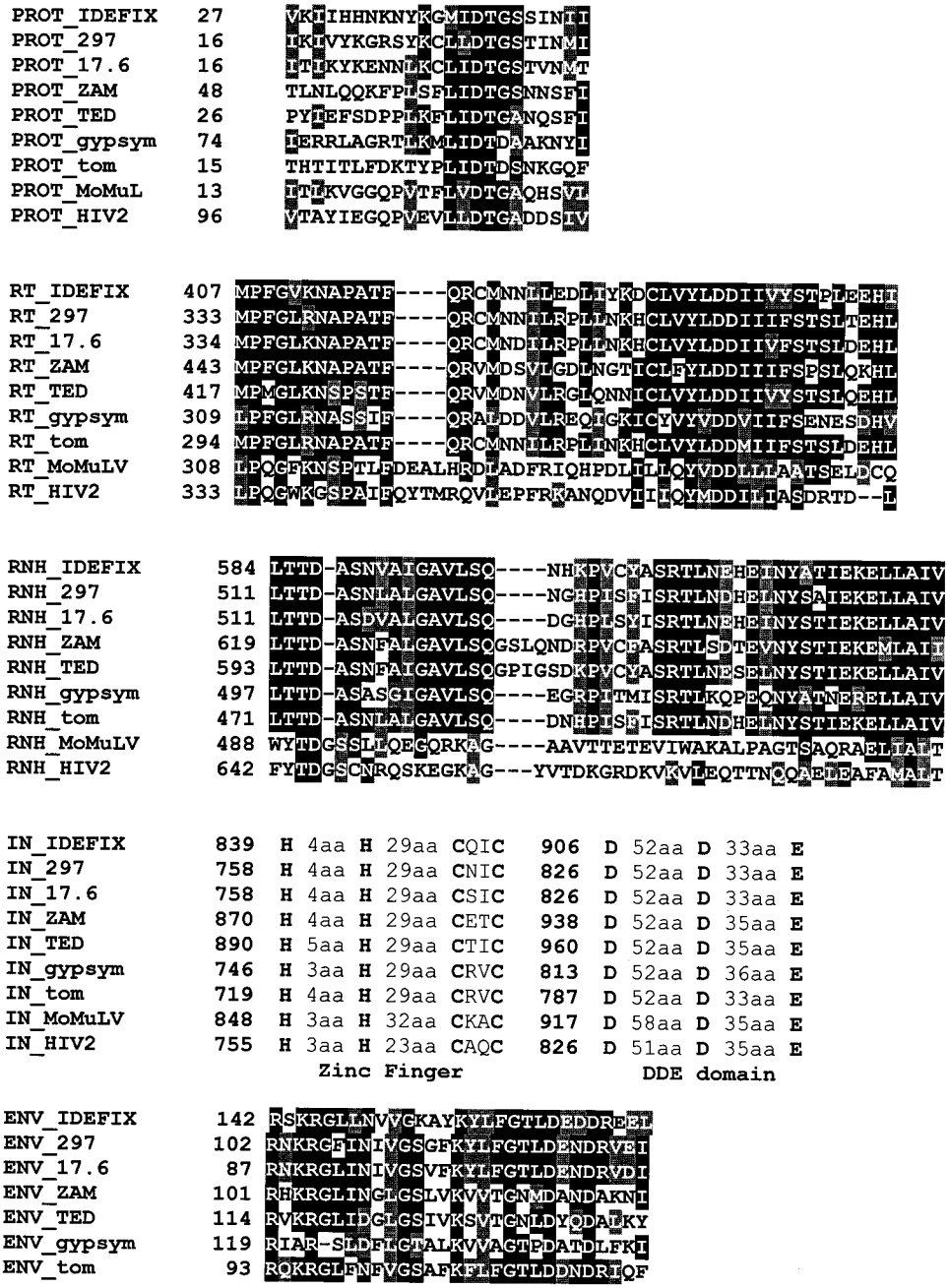

FIG. 4.—Multiple alignment of the deduced amino acid sequence of the *pol* regions. Comparisons of the partial amino acid sequences of conserved regions of Protease (PROT), reverse transcriptase (RT), Rnase H (RNH), and integrase (IN) of the *pol* ORF and a conserved region (ENV) of the env-like ORF. Amino acids that are conserved between *Idefix* and the other members analyzed are shaded black and in bold type, whereas amino acids which are similar are shaded gray. Dashes indicate gaps introduced to preserve the alignment. MoMuLV = Moloney murine leukemia virus; HIV2 = human immunodeficiency virus type 2. Accession numbers (for Pol and Env, respectively) are as follows: *297*, B24872 and ENV2 DROME; *17.6*, GNFF17 and Y172 DROME; *tom*, S34639; *gypsym*, GNFFG1 and ENV1 DROME; *ZAM*, AJ000387; *TED*, B36329 and C36329; MoMuLV, POL MLVMO; and HIV2, 1072794.

age site (Klenk and Garten 1994). A hydrophobic region of 23 amino acids from residues 447–469 is located at a position expected for a transmembrane domain (Coffin 1990). Putative N-glycosylation sites conforming to the consensus sequence Asp-x-Ser or Thr (N-x-S/T), as well as cysteines residues similar to those known in retroviruses to mediate attachment between the surface and the transmembrane proteins via disulfide bonds, are found upstream of the transmembrane domain.

Idefix Insertions Are Occurring Within an AT Repeat of the *white* Locus

In order to determine the exact position of *Idefix* upstream of the *white* transcriptional unit in the 11 RevII lines, *Idefix*/*white* junctions were sequenced on both sides of the element in 7 lines and on one side in the remaining 4 lines. PCR products were obtained from amplification reactions using the [olPstI] and [olPvuII] oligonucleotides (positions are shown in fig. 5*A*, and

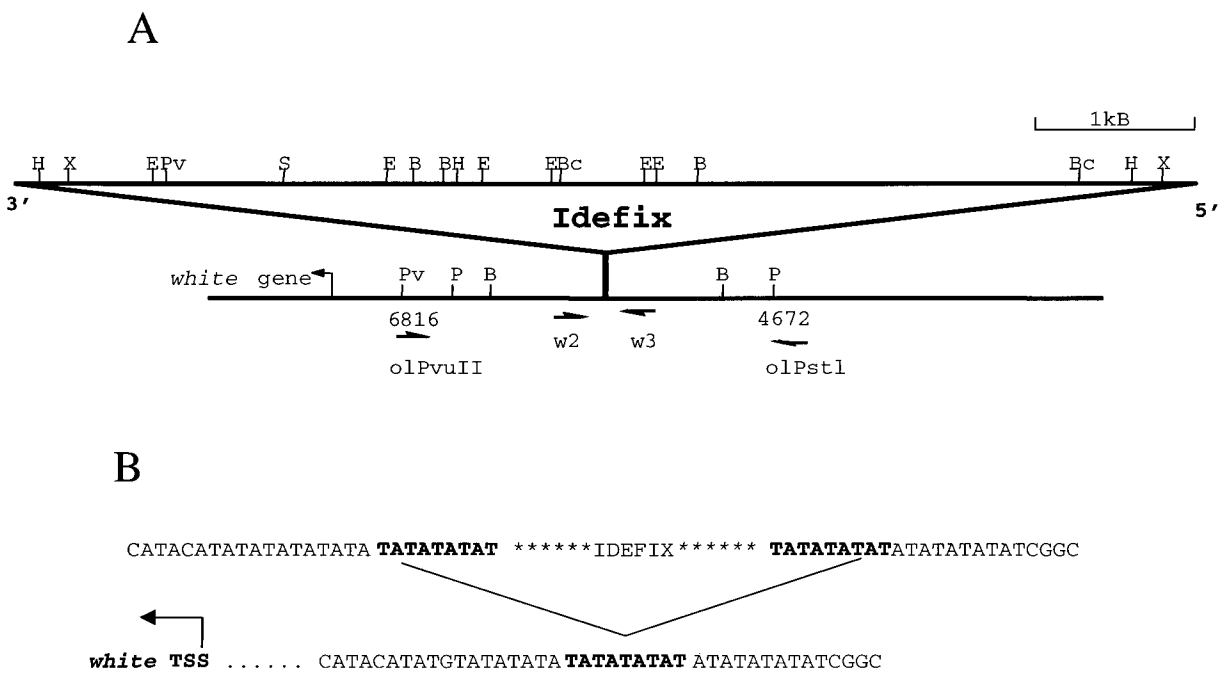

FIG. 5.—*A, Idefix* location at the *white* locus; only part of the *white* locus is shown. Restriction enzyme symbols are as follows: Bc = *Bcl*I; B = *Bgl*II; E = *Eco*RI; H = *Hin*dIII; P = *Pst*I; Pv = *Pvu*II; S = *Sal*I; X = *Xho*I. Oligonucleotides used for PCR amplification of the complete insertion ([olPstI] and [olPvuII]) or to prime sequencing of the insertion sites ([w2] and [w3]) are indicated by arrowheads. *B,* Nucleotide sequences of a segment of the wild-type *white* locus and the host/*Idefix* junction of RevII. The transcription start site of *white* is denoted TSS. *Idefix* was located within the 16 AT repeats upstream of *white.* Bold letters indicate the 9-bp genomic DNA duplicated upon *Idefix* insertion.

sequences are listed in *Materials and Methods*); amplified fragments were then treated with *Pst*I and *Bcl*I restriction enzymes, and the resulting fragments were cloned in pBluescript (see *Materials and Methods* for details). As expected from the restriction map of the insertion, two clones of 1.7 and 4.8 kb were isolated which hybridized with the BB probe of the wild-type *white* locus. The sequence of each *Idefix*/*white* junction was determined for these clones by DNA sequencing using two *white*-derived oligomers [w2] and [w3] (listed in *Material and Methods*) to prime synthesis. Surprisingly, each of the 11 *Idefix* insertions was located precisely at the same site in each independent RevII line, at coordinate 5460 of the sequence map of O'Hare et al. (1984). The target site was revealed to be a stretch of the dinucleotide AT present in 16 copies at the *white* locus. Eleven copies of AT are present at the end closer to *white* (corresponding to the 3′ end of *Idefix*), and nine copies of AT plus one T are present at the end closer to ZAM (5′ end of *Idefix*) figure 5*B.* These data are consistent with a direct duplication of the sequence 5′-ATATATATA-3′ flanking the 11 *Idefix* insertions.

The *white* Allele Generated in the RevIV-7 Line Is Due to an Additional *Idefix* at the *white* Locus

We next undertook a molecular analysis of the *white* allele identified in the RevIV-7 line (see above). In a first set of experiments, we localized the lesion at the end closest to *white,* i.e., the 3′ end of *Idefix.* As an example of the experimental approach that was followed, *Sau*3A digests of both the parental RevIII-7b and the RevIV-7 DNAs are presented in figure 6*A* and *B.* When probed with the BB fragment of *white* spanning the mutated fragment, the blot revealed a 500-bp-long fragment from the 5′ end of *Idefix* in both lines. The identities of these two 500-bp fragments from RevIII-7b and RevIV-7 were confirmed by cloning and sequencing (data not shown). In contrast, the 3′ end of *Idefix* differs in RevIII-7b and RevIV-7 lines, as visualized by the appearance of two fragments of 900 and 1,100 bp, respectively. The 1.1-kb-long fragment from RevIV-7 was amplified by an inverse PCR experiment using primers w1 and w2 targeting the *white* sequence adjacent to the 3′ end of *Idefix* and sequenced. This 1.1-kb fragment was found to contain the *white* sequence from position 5150 upstream of w2 to the AT stretch at position 5452. This sequence of the *white* locus was linked to an *Idefix* 5′ end, whereas a 3′ end was expected according to the orientation of *Idefix* at the *white* locus in RevII lines. This *Idefix* was revealed to be inserted 14 bp inside the AT repeat of the *white* locus, while *Idefix* insertion identified in the parental line, RevIII-7b, was localized 22 bp inside this repeat. These results indicate that a second *Idefix* (called *Idefix* 2) inserted close to the 3′ end of the previously identified *Idefix* (which will be referred to as *Idefix* 1) and in an opposite orientation. After Southern blot analysis and partial sequencing of the region, we established that *Idefix* 2 is a full-length element displaying the same restriction map as *Idefix* 1. Thus far, our attempts to read the sequence between the two elements have been unsuccessful, presumably because of the (AT) nature of their target. However, if the target duplication of *Idefix* 2 is identical to

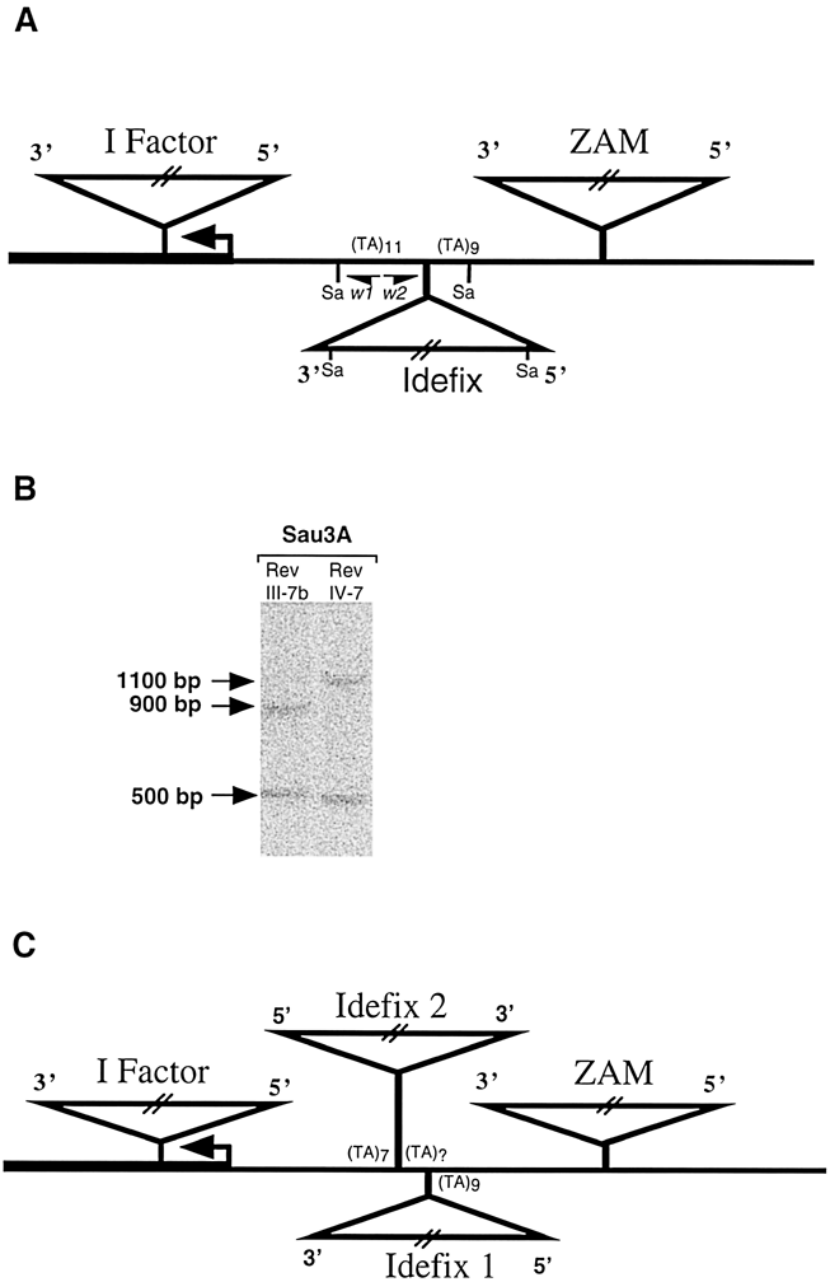

FIG. 6.—*A,* Structure of the *white* locus in RevIII-7b: the location of the w1 and w2 oligonucleotides as well as the *Sau*3A (Sa) sites used for the inverse PCR experiment are indicated. *B,* Southern analysis of *Sau*3A restriction fragments adjacent to the *Idefix* element at the *white* locus in the RevIII-7b and RevIV-7 lines. DNA from RevIII-7b and RevIV-7 was digested with *Sau*3A, and the blot was probed with the BB *white* fragment presented figure 2. The heterogeneous migration of the 900-bp *Sau*3A fragment to 1,100 bp indicates that the left end of *Idefix* is modified, while the 500 bp at the right end is not. *C,* Structure of the *white* allele in the RevIV-7 line.

that of *Idefix* 1, it may be expected that some 10 AT repeats are separating them. Taken together, these results indicate that the *white* locus in RevIV-7 has integrated four transposable elements: the *I factor*, *ZAM*, and two *Idefix* elements in opposite orientations (fig. 6*C*).

Evidence for Multiple Mobilization of *Idefix* and *ZAM* in the RevI Line and its Derivatives

In a previous work, we reported that the first derivative of w^{IR6} due to a *ZAM* insertion upstream of the *white* transcription start site had been produced in the course of an amplification of *ZAM* copy number within the w^{IR6} genome (Leblanc et al. 1997). Therefore, we wondered whether a similar invasion of the w^{IR6} genome by *Idefix* could explain the occurrence of the new recurrent mutations identified at the *white* locus in successive generations of flies. To that end, the genomic distribution of *Idefix* was studied for several *D. melanogaster* strains by Southern blotting. Typical results obtained by probing equal amounts of *Bgl*II/*Bcl*I-digested DNA with the ES probe are shown in figure 7*A* (depicted below the figure). In all tested strains, a complex

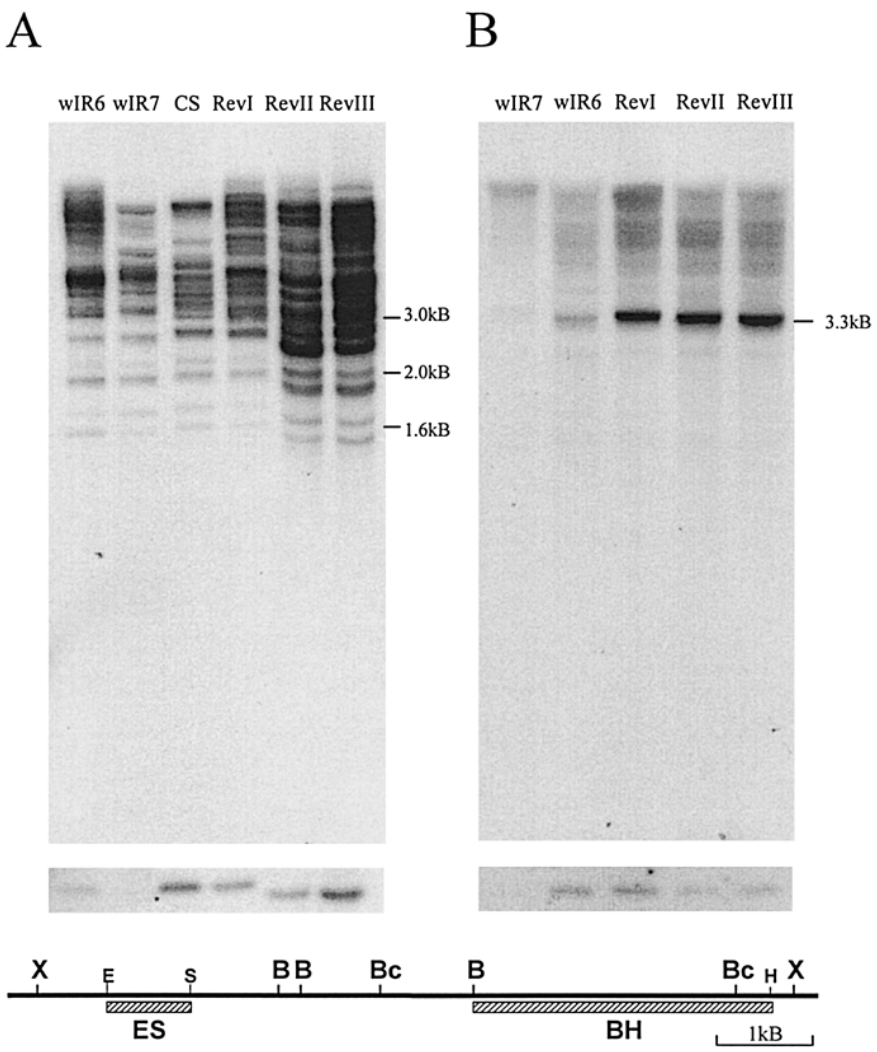

FIG. 7.—Genomic distribution of *Idefix*. *A,* Southern blotting experiments showing the degree of heterogeneity in the distribution of the *Idefix* elements in the genomes of *D. melanogaster* strains. DNAs were digested with *Bgl*II/*Bcl*I and probed with the ES fragment from *Idefix*. *B,* A similar experiment, showing the degree of conservation of the *Idefix* element characterized within the *white* locus in *D. melanogaster* genomes. DNAs were digested with *Bgl*II/*Xho*I and probed with the internal *Bgl*II/*Hin*dIII (BH) fragment of *Idefix*. Strains used in these experiments are marked at the top of the autoradiograph. The amount of DNA visualized by hybridization of the same blot with a nonmutated fragment from the *white* gene as a control of the DNA loaded in each lane is presented below. The lower part of the panel is a restriction map of *Idefix,* with the locations of probes ES and BH, used in these experiments. Restriction enzyme symbols are as follows: Bc = *Bcl*I; B = *Bgl*II; E = *Eco*RI; H = *Hin*dIII; S = *Sal*I; X = *Xho*I.

pattern of heavily hybridizing bands was visualized, indicating that *Idefix* is highly repeated in the *Drosophila* genome. In a *Bgl*II/*Xho*I genomic digest experiment, a more prominent band was revealed using a BH internal probe of *Idefix*. This band corresponds to the 3.3-kb *Bgl*II/*Xho*I fragment present in the *Idefix* element that was identified because of its recent insertion upstream of the *white* gene in RevII lines (see fig. 7*B*). The fragment was present at a low copy number in w^{IR6} and in a battery of other tested strains, yet its intensity was much higher in RevI and its derivatives. This result suggests that the number of *Idefix* copies increased as early as the time of RevI isolation and was still mobile in the RevII, RevIII, and RevIV genomes in which it was identified.

Both the mobility and the increase in the copy number of *Idefix* were analyzed by in situ hybridization experiments on polytene chromosomes from salivary glands of third-instar larvae. We detected a strong hybridization signal in the chromocenters of all tested strains, regardless of their origin. This signal is illustrated in figure 8*A*, which depicts the distribution of *Idefix* in the w^{IR6} line. In addition to the heterochromatic localization, the parental strain contains a few (four) hybridization sites dispersed on chromosomal arms. In contrast, *Idefix* copies were found to be abundant within the euchromatic region in each of the RevI, RevII, RevIII, and RevIV unstable lines. This increase in *Idefix* copy number can be clearly appreciated in figure 8*B,* in which about 20 copies can be counted on the arms of a RevI genome taken as an example.

The X-chromosomal distributions of both *Idefix* and *ZAM* elements were subsequently analyzed with the aim of assessing whether mobilization of either element

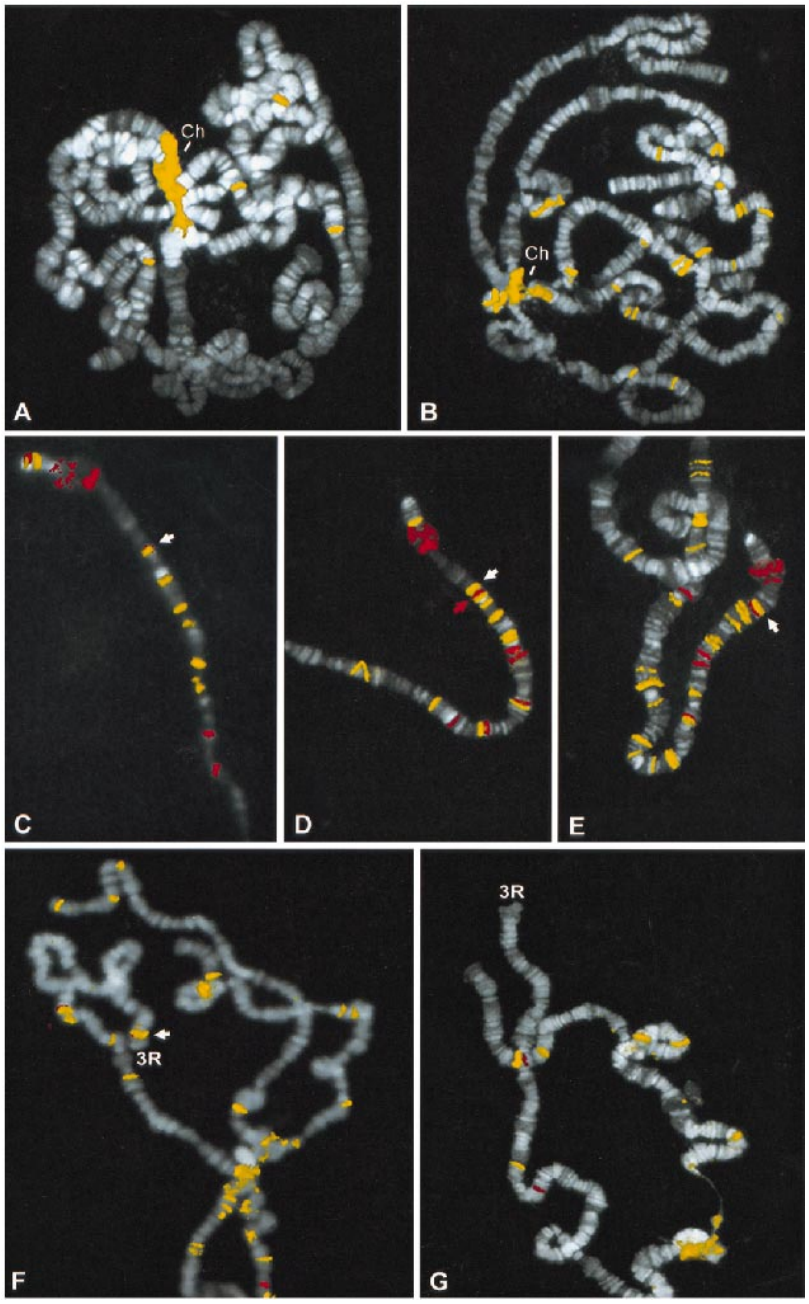

FIG. 8.—FISH mapping of the *Idefix* and *ZAM* sequences in salivary gland chromosomes of unstable lines. *Idefix* hybridization signals (yellow) are present in the chromocenters of all *Drosophila* strains. *ZAM* hybridization signals (red) are only presented in panels *C, D,* and *E.* The signals are merged with the DAPI staining (blue) of salivary gland chromosomes. *A, Idefix* distribution in the parental w^{IR6} genome. *B, Idefix* distribution in the first unstable line identified: RevI. *C , D,* and *E, ZAM* and *Idefix* distribution on the X chromosomes of RevII-7, RevIII-7b, and RevIV-7. The *white* arrow points to the *white* locus at which *ZAM* and *Idefix* probes colocalize; the red arrow points to the additional *ZAM* site on RevIII-7b. Ch = chromocenter. *Idefix* and *ZAM* distribution on the third chromosomes of RevIII-3y and RevII-3 are presented in panels *F* and *G. F, Idefix* (yellow) and *ZAM* (red) hybridization signals colocalize to the tip of chromosome 3R in RevIII-3y; the *white* arrow points to the region in which *ZAM* and *Idefix* probes colocalize. *G,* In RevII-3, *Idefix* and *ZAM* hybridization signals are absent on the tip of chromosome 3R.

still occurred in the mutant lines. The X chromosome represents a good candidate for monitoring novel insertions of *Idefix* and *ZAM*, since each derivative strain was obtained from a single male and, thus, from a single X chromosome. This X chromosome had been maintained through generations in the absence of crossing over by crosses with females carrying attached X chromosomes (attX/Y). These crosses were performed for all lines displaying a mutation on the X chromosome, such as RevII, RevIII-7y, RevIII-7b, and RevIV-7. The only line bearing a mutation on the third chromosome (RevIII-3y) was also established from a single male (and hence from a single X chromosome), crossed with attached-X-chromosome females, and, finally, rendered homozygous by

selection for the phenotype, since this allele is dose-dependent; i.e., a stronger effect on eye coloration is observed in a homozygotic than in a heterozygotic genetic context for the mutation (yellow coloration of the eyes toward orange).

Idefix insertion sites were analyzed for lines derived from the same X chromosome, such as RevII-7 and its RevIII-7b and RevIV-7 derivatives (see fig. 8*C–E*). Multiple insertions of *ZAM* and *Idefix*, which differ among the three lines, are distributed all along the X chromosomes. The finding that the distribution of *Idefix* and *ZAM* varies among strains derived from a single X chromosome suggests that their mobilization is still active, at least in some derivative lines.

We then asked whether mutations modifying the eye-color phenotype isolated as RevIII lines could be due to *Idefix* or *ZAM* insertions. We compared *ZAM* and *Idefix* distributions in RevII-7 and RevIII-7b in order to detect any potential insertion close to the *white* gene, where the mutation responsible for the brown phenotype had been genetically localized (see above). An additional *ZAM* element (indicated by a red arrow in fig. 8*D*) has been found close to *white* in RevIII-7b, while it is absent in RevII-7.

Additionally, in situ hybridization of *Idefix* and *ZAM* elements to polytene chromosomes of the RevII-3 and RevIII-3y strains were compared in order to visualize potential insertions at the tip of chromosome 3, to which the origin of the yellow phenotype had been mapped (see above). The presence of both *ZAM* and *Idefix* is detected in chromosomal subdivision 100 C-D in the RevIII-3y line, while it is absent in the RevII-3 line (see fig. 8*F* and *G*).

Discussion

The present work describes a new genetic instability detected in a line called RevI, characterized by the active transposition of two mobile elements. This instability differs from those previously described for *D. melanogaster* in that the two relevant elements in this mobilization system have been identified as two novel retrovirus-like elements from the *Ty3*/*gypsy* family: *ZAM*, recently described by Leblanc et al. (1997), and *Idefix*, which was previously unknown.

Idefix Is Similar in Structure to Mammalian Retroviruses

Idefix displays all the structural characteristics of an invertebrate retrovirus. Its structure is composed of three ORFs corresponding to the *gag*, *pol*, and *env* genes, flanked by two LTRs. Each of the genes encodes putative products homologous to retroviral proteins. The predicted translation product of *Idefix* ORF1 displays some similarities to the *gag* product of the retrovirus-like elements *297*, *17.6*, and *tom*. It has numerous basic amino acids that have been hypothesized to play a role in the interaction with genomic RNA (Mesnard and Carrière 1995)

The Pol-like ORF of *Idefix* is the most similar to other retrovirus-like elements, and includes regions identified with the retroviral enzymes protease (prt), reverse transcriptase (rt), RNase H (rnh) and integrase (int) (for a review, see Varmus and Brown 1989). These domains contain the same or conserved amino acids at sites that have been shown to be invariant among many retroviruses and retroviral elements (Xiong and Eickbush 1990). In addition, the organization of these different domains is prt-rt-rnh-int, which is the order found in retroviruses and the *gypsy-Ty3* group of elements and which differs from the prt-int-rt-rnh order found in the *copia-Ty1* group of retrotransposons.

Idefix possesses an additional ORF similar in position and structure to the retroviral *env* gene responsible for the infectious properties of a virus. The active retroviruses are composed of two polypeptides: an external, glycosylated hydrophilic polypeptide (SU) and a membrane-spanning protein (TM). They are synthesized in the form of a polyprotein precusor that is proteolytically cleaved during its transport to the cell surface (Hunter and Swanstrom 1990). The predicted protein encoded by *Idefix* ORF3 displays all the structural characteristics of retroviral Env proteins, i.e., a consensus proteolytic cleavage site, N-glycosylation sites, cysteine residues, and a transmembrane domain, strongly suggesting that *Idefix* is capable of extracellular transmission.

ZAM and *Idefix* Copy Number Is Increasing in the Unstable Line

ZAM and *Idefix* copy number is highly variable among *Drosophila* strains. All strains have a low copy number, with very few or no signals of either element on chromosomal arms. While *ZAM* appears to be mainly absent from the chromocenter, *Idefix* displays multiple heterochromatic copies in all strains. Both elements have invaded the genome of the RevI line, which presently displays some 20 sites with *ZAM* and *Idefix* insertions distributed on its chromosomal arms. As already reported for *ZAM*, it is worth noting that the parental strain w^{IR6} from which RevI was derived belongs to strains originally carrying a low copy number of *ZAM* and *Idefix*, indicating that *ZAM* and *Idefix* have been recently amplified.

We have not yet identified the molecular basis of the mechanism that induced or allowed the amplification of both elements simultaneously within the w^{IR6} genome. The amplification of other mobile elements, i.e., *P*, *1731*, *gypsy*, *412*, and *17.6*, has been tested and found to be conserved in each substock (data not shown). The copy number of the five tested elements, which belong to different classes of transposable elements (Finnegan 1992), did not increase between the original w^{IR6} strain and its derivatives, indicating that these mobile elements were stable.

Crossing the RevI strain with other laboratory strains containing a few euchromatic *ZAM* and *Idefix* copies did not activate their transposition, indicating that crosses are not involved in induction of mobility, in contrast to the mechanism underlying the genetic instabilities involving the *P* element (Engels 1989), the *I factor* (Bucheton 1990), *hobo* (Yannopoulos et al. 1987), and

Stalker (Georgiev et al. 1990). It is unlikely that the recent appearance of active copies of *ZAM* and *Idefix* results from recombinations between inactive copies. Two independent recombination events leading to two new active elements in this line can be expected to have a very low probability of occurring. Indeed, Southern blot analyses and partial sequencing suggest that active copies of both *ZAM* and *Idefix* were already present in the genome of the parental w^{IR6} strain. These considerations more or less favor the view that *ZAM* and *Idefix* share a common pathway of regulation in the host that may be disrupted in the unstable strain.

Over a period of 4 years, the RevI line gave rise to 11 independent sublines, called RevII, identified on the basis of recent insertions of *Idefix* at the *white* locus. From the RevII lines, further *Idefix* and *ZAM* mobilizations were newly detected in derivative lines which were called RevIII and RevIV thereafter. These data suggest that both elements are still active in our stocks and that the genetic determinant(s) responsible for activation of their transposition has been maintained for dozens of generations.

Phenotypic Variations Are Recovered After Recurrent Insertions of *ZAM* and *Idefix* at the *white* Locus

A major finding in the present study concerns the mutagenic events detected in RevI, RevII, RevIII, and RevIV lines in the course of *ZAM* and *Idefix* mobilizations. Indeed, both elements were identified in our search for the molecular cause of the recurrent variations in the eye color of our fly stocks. The RevI line was identified as a full reversion of the brown-orange phenotype in the line bearing the w^{IR6} allele. This first mutational event at the *white* gene was found to be due to a *ZAM* insertion 3 kb upstream of the transcription start site of *white* (Leblanc et al. 1997). The second mutational event that affected the expression of *white* in the ensuing RevII lines was caused by *Idefix* insertions 1.7 kb upstream of *white.* These insertions modify the red-brick phenotype to orange. RevIII lines displaying either yellow or dark brown phenotypes carry mutations that are independent of the *white* locus but again affect eye coloration. Finally, a third event of mutation within the *white* locus occurred in the RevIV line, which displays a novel *Idefix* insertion linked to the original one but in the opposite orientation, 1.7 kb upstream of *white,* that is responsible for the full reversion of the mutant phenotype to red-brick.

At present, we have determined the nature of the mutagenic events responsible for the phenotypic changes affecting the *white* locus, at least in the RevI, RevII, and RevIV lines. However, the mechanism(s) through which these successive insertions cause phenotypic variations remains to be elucidated. Mutagenesis by another retrovirus-like element, *gypsy,* has brought fundamental information on the interaction established between that repeated sequence and the host genome: it was shown that insertions of *gypsy* into various *Drosophila* genes cause mutant phenotypes that can, in turn, be altered by second-site mutations in a variety of modifier loci (Geyer and Corces 1992). One of these loci is *suppressor of hairy wing,* which encodes a DNA-binding protein capable of interacting with specific sequences in the *gypsy* element. It was shown that Su(Hw) protein participates in delineating boundaries of higher-order chromatin domains that determine levels of gene activity by creating a chromatin insulator (Roseman, Pirrotta, and Geyer 1993). A similar type of interaction between *ZAM*/*Idefix* and putative proteins encoded by the host might explain the observed variations in *white* gene expression. It will be easy to trace such an interaction by searching second-site modifiers of the RevI, RevII, or RevIV phenotypes. Therefore, the phenotypic variations of the eye color reported here may provide an interesting paradigm, which may bring insight into the mutagenic effects of mobile elements on the host gene expression and may also lead us to identify specific genes involved in the modulation of this expression.

Idefix Displays a High Target Specificity

Very surprisingly, our data reveal that the *white* locus has been subjected to recurrent identical mutations due to *Idefix* insertions at a specific site within the AT repeat upstream of *white.* This puzzling result cannot be due to recurrent contamination events that might have occurred in our stocks of flies. Indeed, these *Idefix* insertions were recovered in three independent stocks of flies with different genotypes, which excludes any undetected contamination. In addition, the 11 mutations have been recovered over a period of 4 years and, thus, through more than 100 generations of flies. A mutation affecting the sexual chromosome such as the one observed in RevII could not have been present yet hidden in our stocks for more than two generations of flies. Finally, we analyzed *Idefix* distribution on polytene chromosomes between different RevII lines and clearly identified different distributions of *Idefix* from one RevII strain to another. However, this result cannot be taken as real proof of independence, since it may also be interpreted as de novo mobilizations of *Idefix* having occurred in these strains from the time they were established.

Previous results indicate that retroelements, including retrotransposons and retroviruses, are not randomly distributed in eukaryotic genomes (Sandmeyer, Hansen, and Chalker 1990), suggesting that the cDNA intermediates of retroelement replication preferentially integrate within specific genomic sites. That conclusion is supported by data obtained for the *Ty3* retrotransposon of *Saccharomyces cerevisiae* that targeting is determined by interactions between the *Ty3* integration complex and components of the polIII transcription apparatus (Kirchner, Connolly, and Sandmeyer 1995). Interactions between retroelements and chromosomal proteins necessary for integration were also demonstrated for *Ty5* in *S. cerevisiae* and were further suggested for the human immunodeficiency virus (HIV) (Kalpana et al. 1994; Zou and Voytas 1997). Based on these observations, an interesting question is whether the DNA sequence per se is critical for the target choice of *Idefix* or whether that choice is determined by the assumption of a specific chromatin organization possibly constituing a domain

around the AT-rich sequence. Several other *Drosophila* retrotransposons, such as *297* (Inouye, Yuki, and Saigo 1986) and *17.6* (Inouye, Yuki, and Saigo 1984) in *D. melanogaster* and *tom* in *D. ananassae* (Tanda et al. 1988), have been reported to preferentially insert within an AT repeated sequence, yet none of them has been detected thus far within the AT repeat upstream of *white*. As mentioned above, mobilizations of transposable elements are rare events in a genome. Thus, *297* and *17.6* may be expected to have a low probability of inserting upstream of *white*. In addition, even if such elements do insert near or within the *white* locus, they may still remain unidentified if the phenotypic variation generated by *Idefix* specifically requires the $w^{IR6RevI}$ allele context. Nevertheless, such a high coincidence of two independent events, represented by the mutation on one hand and the appearance of *Idefix* on the other hand, in 11 individuals is very unlikely to be due to chance; hence, the question arises as to the specificity in *Idefix*-mediated mutagenesis in other alleles. It is well documented that retroelement mobilization may have detrimental effects on the host due, at least in part, to integration into coding sequences. In that context, it is interesting to note that despite the large increase in both *ZAM* and *Idefix* copy numbers in Rev genomes, no other obvious mutations were detected besides the eye color changes. The lines displayed no sterility or any decrease in viability, suggesting that the recent and massive mobilizations have no deleterious effects. Do *ZAM* and *Idefix* avoid most coding regions by preferentially integrating into regions of silent chromatin, as reported for other retrotransposons (Ke, Irwin, and Voytas 1997)? A high-resolution characterization of *Idefix* and *ZAM* insertion sites might help us to gain information about their target preferences. Thus, the search for other *Idefix* and *ZAM* insertions at other chromosomal loci may be expected to provide a useful tool with which to detect and tag specific regions of the chromosomes.

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