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FB elements can promote exon shuffling: a promoter-less *white* allele can be reactivated by *FB* mediated transposition in *Drosophila melanogaster*

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Abstract Foldback (*FB*) elements are transposable elements found in many eukaryotic genomes; they are thought to contribute significantly to genome plasticity. In *Drosophila melanogaster*, *FB*s have been shown to be involved in the transposition of large chromosomal regions and in the genetic instability of some alleles of the *white* gene. In this report we show that *FB* mediated transposition of w^{67C23}, a mutation that deletes the promoter of the *white* gene and its first exon, containing the start codon, can restore expression of the *white* gene. We have characterized three independent events in which a 14-kb fragment from the w^{67C23} locus was transposed into an intron region in three different genes. In each case a local promoter drives the expression of *white*, producing a chimeric mRNA. These findings suggest that, on an evolutionary timescale, *FB* elements may contribute to the creation of new genes via exon shuffling.

Keywords Foldback elements · Transposition · Chimeric protein

Introduction

Transposable elements (TEs) are an intrinsic feature of the genome in all living organisms (Capy et al. 1998) and contribute substantially to genetic variation (Kidwell and Lisch 1997, 2001). They have a mutagenic effect on the host genome, and they can inactivate or deregulate gene expression when inserted in, or in the vicinity of, coding regions. Data obtained in the course of whole genome sequencing projects (Ashburner et al. 1999; Adams et al. 2000; Benos et al. 2000; International Human Genome Sequencing Consortium 2001; Venter et al. 2001) also suggest that TEs can actively contribute to genome evolution by reorganizing genetic material, usually by ectopic recombination between elements inserted at different genomic sites, a process which can result in several types of chromosomal rearrangements (Lim and Simmons 1994; Kim et al. 1998). Segmental duplications thus generated can provide the raw material required for the evolution of new or more specialized functions (Ohno 1973; Zhang et al. 2002).

Depending on their structure and mechanism of transposition, TEs can be grouped into two main categories (Berg and Howe 1989; Finnegan 1992). Class I elements move via an RNA intermediate and usually possess long terminal repeats, while Class II elements transpose from DNA to DNA and possess inverted repeats at their ends. Foldback (*FB*) elements are a particular type of mobile elements belonging to class II. Their transposition mechanism is not understood at present, and it is not known whether these elements use a transposase. Their inverted terminal repeats are composed of a variable number of short repeats in direct orientation, which can form extensive secondary structures (Potter et al. 1980). Such structures are thought to be highly recombinogenic, and to cause deletions, duplications and other chromosomal rearrangements at high frequency (Bingham and Zachar 1989).

The molecular mechanism underlying genetic instability associated with *FB* elements has been analyzed in detail in two unstable alleles of the *white* gene of *Drosophila melanogaster* (Green 1967; Levis et al. 1982b; Collins and Rubin 1984). Occasionally, *FB* s are found associated with a transposon-like element called *NOF* (Goldberg et al. 1982), which may encode a poorly

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characterized product required for transposition. Two composite *FB-NOF* sequences located several polytene bands apart can mediate the transposition of the chromosomal region that lies between them (Ising and Block 1981; Paro et al. 1983; Harden and Ashburner 1990; Lovering et al. 1991).

As far as we are aware, no evidence has yet been reported for a relationship between *FB* element-mediated ectopic recombination and transposition in *D. melanogaster*. It is not known, for example, whether some end product of ectopic recombination (for example, an excised segment of DNA such as could be produced by a recombination event leading to a deletion) can be used in a succeeding transposition event, and, if so, what evolutionary consequences the transposition of this genetic material elsewhere in the genome might have.

We show here that a defective *white* allele generated by *FB* insertion can be transposed to other locations in the genome, with concomitant restoration of a nearly wild type eye-color phenotype. We have analyzed the transposed DNA segments and their insertion sites at the molecular level in three such cases, and we have characterized the chimeric mRNAs responsible for the phenotypic reversion to w^+. The results show that *FB* elements can promote exon shuffling, thus contributing to the formation of new exon combinations on which natural selection can act.

Materials and methods

D. melanogaster strains

All stocks used were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, Ind.) and maintained at 24°C on standard culture media. The *y* w^{67C23} strain was used as the recipient in microinjection experiments; the w^{67C23} *white* allele is a deletion derivative of w^c in which the first exon and several kb of DNA at the 5′ end of the *white* gene have been deleted (Pirrotta and Brockl 1984; V. Pirrotta, personal communication, and our unpublished results).

osp^1 and *osp*^{rJ571} mutants and the deletions *Df* (2L) *A263* (breakpoints 34E5, 35C3–9), *Df* (2L) *A72* (breakpoints 35B2–3, 35B7–8) and *Df* (2L) *osp29* (breakpoints 35B1–3, 35E6) were used for complementation tests and deletion mapping. The deletions were balanced over *CyO*, a balancer for the second chromosome.

Plasmid construction and microinjection experiments

To obtain a *Bari1* construct marked with w^+, we used the plasmid p*28/47D*, which contains a complete copy of the *Bari1* transposon plus some adjacent sequences from the 47D region of the *D. melanogaster* genome (Caizzi et al. 1993). A 4.8-kb *Hin*dIII fragment from the CaSpeR vector, containing a *mini-white* gene (Pirrotta 1988), was inserted at the unique *Hin*dIII site in p*28/47D* (this *Hin*dIII site is located downstream of the putative *Bari1* transposase gene). The plasmid thus obtained, p *Bari1_47Dw*$^+$, was used at a concentration of 250 μg/ml for microinjection experiments.

A 7.3-kb *Eco* RI fragment containing the marked *Bari1* element was then recovered from *pBari1_47Dw*$^+$ and inserted into the *Eco* RI site of the vector Carnegie 4 (Rubin and Spradling 1983), thus creating the plasmid p *Ca4_47Dw*$^+$. This plasmid was coinjected with the *Pπ*25.7 helper plasmid into *y* w^{67C23} host embryos, following the procedure for *P* -mediated transformation described by Rubin and Spradling (1982).

Molecular techniques

Inverse PCR experiments were carried out on *Sal* I digests of DNA isolated from flies homozygous for the A5, B1 and C1 insertions (see below). Circular DNA molecules were amplified using the Expand Long Template PCR system (Roche) and two primers, Nof_Fa (5′-AGAAAAACCAAGGGGAAAA-3′) and Nof_R2 (5′-AAAAGCAAGAACCGAATAA-3′), corresponding to positions 3031–3049 and 645–663, respectively, of the *NOF* sequence with GenBank/EMBL Accession No. X15469.

To determine the sequence flanking the A5 transposed fragment at its 3′ end, PCR amplification was performed on DNA from homozygous A5 flies using the wV5_f5 primer from the *white* region (5′ CTGTGTGAGTGCGTTCCTA-3′; positions 148606–148624 in AE003425) and the osp_3up primer from the *osp* region (5′ CCACCAGAACCACGAGAAAT-3′; positions 248369–248388 in AE003410).

5′-RACE experiments were performed using poly(A)$^+$ mRNA prepared from adults with the QuickPrep micro mRNA Purification kit (Amersham) and the 5′/3′ RACE kit from Roche. Two nested primers based on the second and third exons of the *white* gene (Accession No. X02974) were used: w_race2 (5′-GGAGCCGATAAAGAGGTCAT-3′) and w_race3 (5′ CCAGGCATAGGTGAGGTTCT-3′), corresponding to the positions 11423–11442 and 11004–11023 of the X02974 sequence, respectively. The RACE products were cloned into the pGEM-T vector (Promega) and five independent clones were sequenced.

The DNA sequences corresponding to the chimeric mRNAs from A5, B1 and C1 are available under the Accession Nos. AJ537449, AJ537450 and AJ537451, respectively.

Fluorescent In Situ Hybridization (FISH)

Probes were labeled by nick-translation with Cy3-dCTP or FluorX-dCTP (Amersham). The whpla probe containing the *white* promoter was a gift from V. Pirrotta. Polytene chromosomes prepared as described by Pardue (1986) were stained with DAPI (4′, 6′-diamidine-2′-phenylindole-dihydrochloride). Digital images were obtained using an Olympus epifluorescence microscope equipped with a cooled CCD camera. Gray scale images, recording Cy3, FluorX and DAPI fluorescence were obtained separately using specific filters, and were pseudo colored and merged for the final image using the Adobe Photoshop software.

Results

Transposition of w^{67C23} can restore w^+ expression

The first observed case of reactivation of *white* expression from the w^{67C23} allele occurred during transformation experiments designed to determine whether the *Bari1* transposon (Caizzi et al. 1993) is capable of autonomous integration into a host genome. *Bari1* is a member of the *Tc* 1-*mariner* superfamily, which is widely distributed in many different taxa and has been well studied for its potential use as a universal transformation vector (Plasterk et al. 1999). We injected the plasmid p *Bari1_47Dw*$^+$, a construct carrying a *mini-white*

Table 1 Recovery of w^{67C23} transposition events that restore wild type function

Experiment No. [a]	Number of fertile G_0 flies	Number of w^+ transpositions	Name of w^+ strain
1	12	1	A5
2	87	1	B1
3	136	1	C1

[a]The plasmids microinjected were *pBari1_47Dw*$^+$ in experiments 1 and 2, and *pCa* 4_*47Dw*$^+$ in experiment 3 (see Materials and methods for details)

gene inserted at the *Hin*dIII site in the complete *Bari1* element, into host embryos that were homozygous for the w^{67C23} *white* allele. A single fly with almost wild-type eye color was found among the progeny of 12 successfully injected flies. The homozygous line established from this fly was named A5. In situ hybridization experiments showed that in the A5 line an insertion of *white* material had occurred in the 35B region. However, in this region no hybridization signal was observed using either a *Bari1* probe or a probe encompassing the *white* promoter. On the other hand, the 35B band was labeled by a probe derived from the first *white* intron, which is present in w^{67C23} but not in the *mini-white* gene carried by *pBari* 1_*47Dw* $^+$. Thus, the A5 w^+ phenotype was apparently due to *white* expression driven by a local promoter after transposition of the w^{67C23} allele into the 35B region.

An independent w^+ line (named B1) was recovered in a second microinjection experiment using the *pBari* 1_*47Dw* $^+$ construct and host embryos from a w^{67C23} strain independently maintained in a different laboratory. In this line w^{67C23} material appeared to have been transposed to the 67B region, since a probe from the first *white* intron labeled this polytene band; however, the 67B band did not hybridize either with *Bari1* or with sequences from the *white* promoter.

Finally, in a third microinjection experiment, a w^+-labeled *Bari1* construct was injected into w^{67C23} hosts under conditions allowing *P*-mediated transformation (i.e., using p *Ca*4_*47Dw* $^+$ and a *P*π25.7 as a helper, see Materials and methods). Among 17 recovered w^+ flies, 16 were due to *P* -mediated insertions. However, on the basis of in situ hybridization experiments, the w^+ strain named C1 seemed to have arisen by an event similar to those that had produced the A5 and B1 strains (Table 1). The *white* insertion in C1 was mapped cytologically to the 6B–C region.

The eyes of A5, B1 and C1 homozygotes are similar in color. This is easily distinguished from wild-type and can best be described as ''Ferrari'' red. B1 and C1 homozygotes are fully viable and show no obvious phenotypic defect; A5 homozygotes have a curved wing phenotype similar to mutants for the *outspread* (*osp*) gene, which resides in the 35B region. In fact, heterozygotes carrying the A5 insertion and either *osp* ^{1} and *osp* ^{rJ571} *osp* mutants, or deletions that uncover the 35B region [i.e., *Df* (2L) *A263*, *Df* (2L) *A72* and *Df* (2L) *osp* ^{29}], show a typical *osp* phenotype.

In summary, the results reported in this section indicate that in no case was *Bari1* able to integrate autonomously into the genome autonomously, but *white* expression could be restored by the concomitant transposition of material from the w^{67C23} region to another location. In the A5 transformant the insertion of material from the w^{67C23} region disrupted the function of the *osp* gene, whereas in B1 and C1 the insertions occurred in different, functionally uncharacterized, genes.

The structure of the transposed fragments and their insertion points

We first wanted to establish the size of the transposed fragments. Therefore, a series of probes from a region covering 40 kb and spanning the *white* gene (see Fig. 1) was used for in situ hybridization experiments to determine which probes labeled the transposed material in its new locations. By this criterion, in all three w^{67C23} transposition events studied the transposed fragment encompasses the first intron of *white* and extends downstream for approximately 14 kb. It should be noted that in this region an *FB* element was postulated to exist by Collins and Rubin (1984) to explain the origin of some w^- derivatives of the w^c allele.

Because it is known that the w^{67C23} deletion originated by recombination between a composite *FB-NOF* element present in the w^c allele and an *FB* sequence located some kb upstream (Levis et al. 1982b; Pirrotta and Brockl 1984), we also tested for the presence of *NOF* sequences at the new insertion sites. As shown in Fig. 2, such sequences are in fact present at the site of insertion of the transposed material in all three w^{67C23} revertants analyzed, while in the original w^{67C23} stock no hybridization with a *NOF* probe is observed at any of these three sites (data not shown).

On the assumption that a *NOF* sequence should be present at least at one end of the transposed fragments, we used divergent primers based on this sequence for Inverse PCR experiments to detect, isolate and sequence the chromosomal regions flanking the transposed DNA fragments. The diagram shown in Fig. 3 summarizes the strategy used to clone the sequence flanking the A5 insertion in the 35B region at its 5′ end. Figure 3 also shows the physical map of the transposed w^{67C23} fragment in which an *FB-NOF* element is located, which was obtained from an inverse PCR experiment using the primers Nof_Fa and Nof_R2 (see Materials and methods) and a circularized *Sal* I digest of A5 DNA. As shown in the lower part of the Fig. 3, a specific 3.6-kb band was produced. This band was eluted from the gel and sequenced. The same strategy was used to obtain the sequences flanking the B1 insertion in the 67B region and the C1 insertion in the 6B–C region. The 3′ end of the A5 insertion was also determined using two divergently oriented primers derived from the *white* locus and

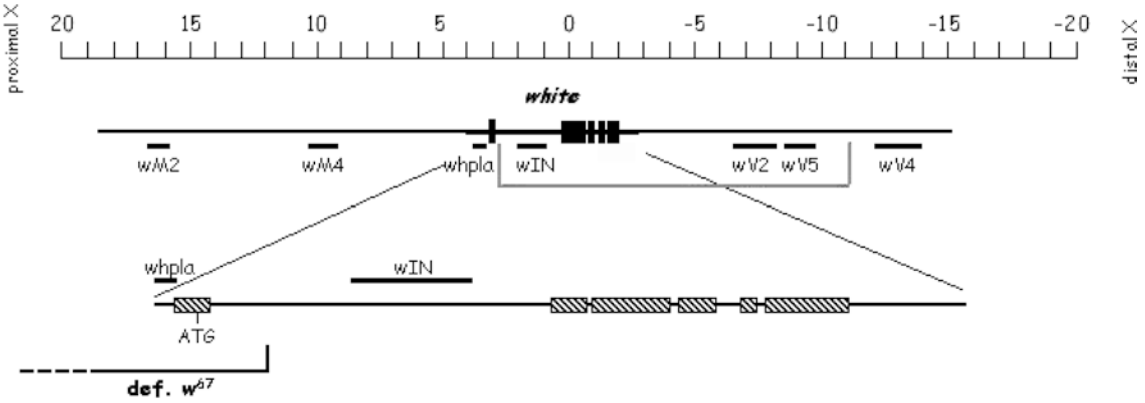

Fig. 1 Extents of the fragments from the *white* region transposed in the *FB*-mediated events described in this work. A schematic representation of the wild type *white* region is shown at the *top*, and part of this is expanded to indicate the positions of the ATG start codon and the distal breakpoint of the w^{67C23} deletion. The *filled* and *hatched boxes* represent the exons of the *white* gene. The 0 coordinate is the position of the *copia* insertion in the w^{a} allele (Levis et al. 1982a). The *solid line segments* represent the probes used for in situ hybridization experiments. The *bracket* marks the boundaries of the transposed material

from the *osp* gene, respectively (see Materials and methods).

The results of the experiments reported above allowed us to map the insertion site of the transposed fragment in A5 at the nucleotide level; the insertion occurs within the first intron of the *osp* gene, at position 248479 of the sequence with Accession No. AE003410. A direct repeat of 9 bp is present on both sides of the insertion, representing the target site duplication usually found after *FB* transposition events (Collins and Rubin 1983). The B1 and the C1 insertion points were mapped to the second intron of the *CG6487* gene (position 62329 in AE003547) and the third intron of the *CG3973* gene (position 97770 in AE003438), respectively. Figure 4 summarizes the mapping of the insertion sites of the transposed fragments in the three cases analyzed (see also the Electronic Supplementary Material).

The formation of chimeric mRNAs

To study the mechanism by which *white* expression was restored following the transposition events in A5, B1 and C1, we performed 5′-RACE experiments on poly(A)$^{+}$ mRNA from the three homozygous stocks, using a primer based on the sequence of the second and third exons of the *white* gene. The PCR amplification products were sequenced and compared with the structure of the genomic insertions. In all three cases, a chimeric mRNA was detected, which included the second exon of the *white* gene and the exon(s) of the target genes upstream of the insertion point (Fig. 4). In all three cases an in frame ATG starting codon was provided by the disrupted gene. The polypeptides produced by these fused coding structures would be chimeric proteins including 658 out of the 687 amino acids of the *white* product and an N-terminal extension encoded by the upstream exons of the disrupted genes. In the A5, B1 and C1 insertions the N-terminal extensions consist of 71, 61 and 56 amino acids, respectively.

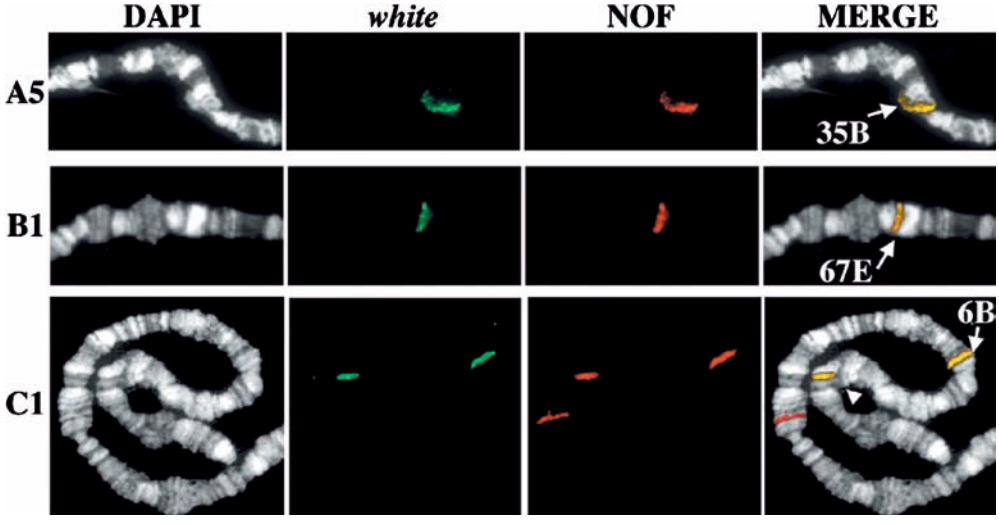

Fig. 2 *NOF* and *white* material are present at the insertion sites following the transposition events. Polytene chromosomes from homozygous A5, B1 and C1 flies were co-hybridized using *NOF* (*red* signal) and wIN (representing the first *white* intron; *green* signal) probes. The merged signals show that the two sequences co-map at all three insertion points (*arrows*). The *arrowhead* in C1 indicates the 3C region, at which the original w^{67C23} allele is located

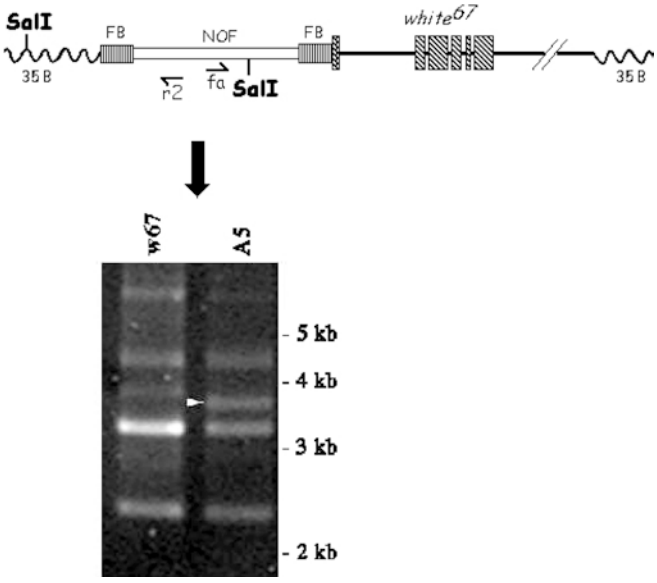

Fig. 3 Cloning of the *FB-NOF* -containing end of the fragment transposed in the A5 line. The diagram shows the structure of the end of the insertion where the *FB-NOF* sequences are located. The *wavy line* represents the flanking regions from 35B. The positions of the *Sal* I sites and the NOF_Fa and NOF_R2 primers used for the inverse PCR experiments that allowed the cloning of the region are also indicated. The results of PCR using w^{67C23} and A5 DNA are shown *below* the map: the *arrowhead* indicates the 3.6-kb band specific to A5 DNA; other bands are derived from the multiple *NOF* elements present at other locations in the genome

Discussion

What induces the transposition of w^{67C23}?

Although the $y\ w^{67C23}$ strain (together with w^{1118}, which carries a more extensive, *FB* -generated deletion) is one of the *white* deficiency alleles routinely used in many laboratories as a host in transformation experiments, no reversion to the w^{+} phenotype has been reported so far. Yet, our results show that transposition to a new genomic location of the remaining *white* exons of the w^{67C23} allele may restore *white* function. To date, the mechanism of *FB* transposition is not completely understood. The most detailed information available relates to the giant TEs of Ising (Ising and Block 1981; Goldberg et al. 1982; Paro et al. 1983; Harden and Ashburner 1990; Lovering et al. 1991) and the unstable *white* alleles w^{c} and w^{DZL} (Green 1967; Levis et al. 1982b; Collins and Rubin 1984). According to the results of these studies, *FB* elements are not capable of autonomous transposition, yet they can mediate mobilization of adjacent DNA segments. The *NOF* elements present in some, but not all, members of the Ising TE family have been proposed to promote recombination between or within *FB* elements (Collins and Rubin 1984; Harden and Ashburner 1990). In the three w^{+} revertants studied here, the composite *FB-NOF* element is found at a new location, but retains the structure present in the w^{67C23} allele. Thus, while the *FB* sequences appear

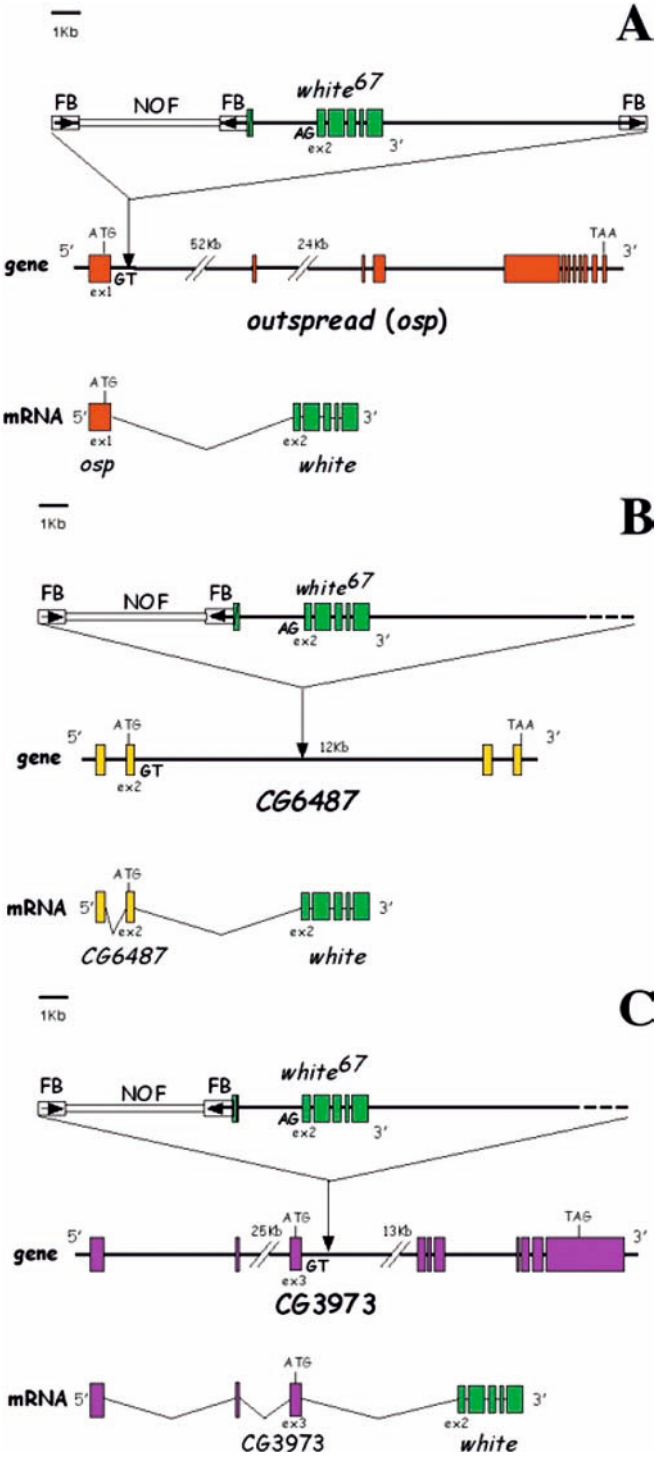

Fig. 4A–C Sites of insertion of the transposed w^{67C23} fragments and structures of the chimeric mRNAs produced. In each panel the *top two* diagrams illustrate the structure of the transposed fragment and of the disrupted gene, respectively. At the *bottom* the structure of the chimeric mRNA produced, as deduced from RACE experiments, is shown. In each panel the positions of the GT-AG splicing sites involved and the ATG translation start codon are indicated. The insertions into the genes *osp*, *CG6487* and *CG3973* are shown in panels **A**, **B** and **C**, respectively

to be a necessary *cis* condition for the transposition, at present no role can be postulated for the associated *NOF*. It is plausible that the inverted *FB* repeats may serve as the substrate for an as yet unidentified transposase.

Although we lack direct evidence in this respect, it should be pointed out that the three transposition events reported in this study were detected in three independent experiments that involved the *Bari1* transposon. Intriguingly, *FB* can also be associated with *HB1* (Brierley and Potter 1985), a non autonomous member of the *Tc1* family which shares partial homology with *Bari1* (Caizzi et al. 1993). Indeed, the *FB* sequences we found in the transposed fragments are very similar in structure and composition to those associated with *HB1*. Thus it is possible to imagine that the injected *Bari1* plasmid DNA renders the host genome unstable, either on its own or because it gives rise to a transient byproduct after delivery to the embryos. Of course, until further studies clarify the role of *Bari1* elements in *FB* -mediated transposition, alternative explanations of the origin of the instabilities observed are possible. For example, the traumatic stress associated with microinjection could in some way induce the transposition of the w^{67C23} region, although this appears unlikely because we routinely use w^{67C23} embryos as hosts in *P* -mediated transformation and have never observed similar events in experiments not involving *Bari1*. Also, an intrinsic instability of the w^{67C23} allele in the *y* w^{67C23} strain cannot be excluded; in any case, the lack of reports of phenotypic reversions in the stock at least point to a very low frequency of such events under normal circumstances.

Finally, it should be kept in mind that a *FB-NOF* element is found at the distal breakpoint of the w^{67C23} deletion, and another *FB* element is present 14 kb downstream. As in situ hybridization experiments on polytene chromosomes identify at least 20 *FB* and at least 4 *NOF* elements dispersed throughout the genome (data not shown), the genetic background of the *y* w^{67C23} strain could generate genetic instability similar to the well known instability associated with the giant TE elements of Ising, and with the w^{c} and w^{DZL} *white* alleles. It has been suggested by Harden and Ashburner (1990) that, in order to prevent the potentially harmful effects of *FB* -element-induced rearrangements, a specific mechanism exists that inhibits recombination between *FB* elements. If this is the case, our observations could be explained by assuming that our experimental conditions somehow perturb this control mechanism.

FB elements as inducers of genome plasticity

The presence of foldback elements has been reported in a wide range of species including *Caenorhabditis elegans* (Yuan et al. 1991), *Ciona intestinalis* (Simmen and Bird 2000) and several plants (Ananiev et al. 1998; Cheng et al. 2000; Windsor and Waddell 2000; Wicker et al. 2001). Much evidence points to these elements as possible inducers of genome plasticity in evolution (Kidwell and Holyoake 2001). For example, besides the well known *FB* -mediated genomic rearrangements and the genetic instability associated in *D. melanogaster* with very large transposed *X* chromosome segments and with unstable *white* alleles, *FB* elements have been shown to be responsible for a massive reshuffling of the genome over a short period of evolutionary time in *D. buzzati* (Caceres et al. 2001).

The transposition of large genomic segments, such as can be mediated by *FB* elements, can play a crucial role in evolution (Kidwell and Lisch 2001; Paro et al. 1983) by generating paralogs of existing genes that will potentially be able to evolve, at least initially, without major functional constraints. The evolutionary generation of new genetic functions would be surely facilitated if separate segments of existing genes, in particular exons, could be independently transposed into new genomic contexts and so placed under the control of new, local promoters. For example, *Tpn1* , a Class II mobile element present in the Japanese morning glory, appears to have captured several host exon sequences, and hybrid transcripts have been detected in the flower buds of some plant lines (Takahashi et al. 1999). Interestingly, the subterminal repetitive regions of the *Tpn1* element (Hoshino et al. 1995) share a similar organization with the *FB* transposons of Drosophila. In this work we present the first experimental evidence that, in *D. melanogaster*, *FB* -element mediated transposition can originate new exon combinations, a mechanism long assumed to be important in gene evolution and known as exon shuffling and transduction transposition (Gilbert 1978; Moran et al. 1999), both of which are suggested to be important contributors to genome evolution (Brosius 1999; Pickeral et al. 2000; Kidwell and Lisch 2001).

A number of genes generated by exon shuffling have been identified and studied. In most cases, new genes were apparently created by the integration of a retrotranscribed mRNA, which originated from a preexisting gene, into or near a second transcriptional unit (see, for example, Wang et al. 2000, 2002). So, formation of new genes by this mechanism could be a special case of the process believed to generate the many pseudogenes found in all eukaryotic genomes, and its success would depend not only on the nature of the integration site, but also on several other conditions, e.g. retrotranscription of a sufficient amount of the starting mRNA, formation of a new splicing acceptor site after integration in an intron, formation of a new promoter if integration occurred in an intergenic region. In comparison, formation of a new functional transcriptional unit should be less problematical after *FB* element mediated transposition of one or more exons together with the adjacent introns, because the transposed segment already contains properly positioned splicing signals. In fact, in all three w^{+} revertants reported in this work, a donor splicing site in the target gene reacts

with an acceptor site present in the transposed w^{67C23} material.

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