Mobilization of a *Minos* Transposon in *Drosophila melanogaster* Chromosomes and Chromatid Repair by Heteroduplex Formation

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ABSTRACT

Transposase-mediated mobilization of the element *Minos* has been studied in the *Drosophila melanogaster* genome. Excision and transposition of a nonautonomous *Minos* transposon in the presence of a *Minos* transposase gene was detected with a dominant eye color marker carried by the transposon. Frequencies of excision in somatic tissues and in the germ line were higher in flies heterozygous for the transposon than in homozygotes or hemizygotes. Transposition of a *X* chromosome-linked insertion of *Minos* into new autosomal sites occurred in 1–12% of males expressing transposase, suggesting that this system is usable for gene tagging and enhancer trapping in Drosophila. Sequence analysis of PCR-amplified donor sites after excision showed precise restoration of the original target sequence in ~75% of events in heterozygotes and the presence of footprints or partially deleted elements in the remaining events. Most footprints consisted of the four terminal bases of the transposon, flanked by the TA target duplication. Sequencing of a chromosomal donor site that was directly cloned after excision showed a characteristic two-base mismatch heteroduplex in the center of the 6-bp footprint. Circular extrachromosomal forms of the transposon, presumably representing excised *Minos* elements, could be detected only in the presence of transposase. A model for chromatid repair after *Minos* excision is discussed in which staggered cuts are first produced at the ends of the inverted repeats, the broken chromatid ends are joined, and the resulting heteroduplex is subsequently repaired. The model also suggests a simple mechanism for the production of the target site duplication and for regeneration of the transposon ends during reintegration.

TRANSPOSABLE elements are natural and ubiquitous components of genomes with a distribution ranging from bacteria to vertebrates (BERG and HOWE 1989). In spite of their mutagenic potential, transposable elements have been maintained in evolution, either due to their replicative ability or selection (FINNEGAN 1989; CHARLESWORTH *et al.* 1994). Whatever their origin, transposable elements represent an invaluable experimental tool for genetic manipulation and molecular genetic analysis. One class of eukaryotic transposable elements is characterized by the presence of inverted terminal repeats, usually flanking a single gene coding for a transposase function (FINNEGAN 1989). These Class II elements transpose via a DNA intermediate, probably by a cut-and-paste mechanism (KAUFMAN and RIO 1992). Class II elements can be grouped in three main families: the *P* family, composed by the *P* element from *D. melanogaster,* and a few other related transposons; the *hAT* (*hobo-Ac-Tam3*) family, represented by *hobo* from *D. melanogaster, Ac* from maize and *Tam3* from the snapdragon *Antirrhinum majus*; the *Tc1-mariner* family, including *Tc1* from *Caenorhabditis elegans* and *mariner* from *D. mauritiana* (ENGELS 1989; CALVI *et al.* 1991; HENIKOFF and HENIKOFF 1992).

Minos is a ~1.8-kb-long transposon of *Drosophila hydei,* belonging to the *Tc1-mariner* family of transposable elements (FRANZ and SAVAKIS 1991). Members of the *Tc1-mariner* family have been found in fungi (DABOUSSI *et al.* 1992; KACHROO *et al.* 1994; LANGIN *et al.* 1995), nematodes (COLLINS *et al.* 1989; HARRIS *et al.* 1990; ABAD *et al.* 1991), insects (BRIERLEY and POTTER 1985; BREZINSKY *et al.* 1990; CAIZZI *et al.* 1993; JEHLE *et al.* 1995; MERRIMAN *et al.* 1995) and vertebrates (HEIERHORST *et al.* 1992; RADICE *et al.* 1994; IZSVAK *et al.* 1995; LAM *et al.* 1996). *Tc1* shares similarity with the prokaryotic insertion element *IS630* (HENIKOFF 1992). The *mariner, Tc1,* and *IS630*-like transposases have been grouped, together with retroviral-retrotransposon integrases and *IS3*-like bacterial transposases, into a family characterized by the D,D35E motif, which is involved in the catalytic core of these enzymes (KULKOSKY *et al.* 1992; DOAK *et al.* 1994; VAN LUENEN *et al.* 1994; ROBERTSON and LAMPE 1995). The widespread occurrence of *Tc1/mariner*-like transposons in phylogenetically distant species (ROBERTSON and MACLEOD 1993) suggests that these elements might form useful germ-line transformation vectors in divergent species.

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Minos is characterized by 255-bp inverted terminal repeats and inserts at a TA dinucleotide, probably causing a target site duplication upon insertion (FRANZ and SAVAKIS 1991; FRANZ *et al.* 1994). *Minos* transposase can promote the integration of nonautonomous *Minos*-based transposons into germ line chromosomes of *D. melanogaster* (LOUKERIS *et al.* 1995a). *Minos* is the fourth transposon that can be used as a transformation vector in *D. melanogaster*, after *P* (RUBIN and SPRADLING 1982), *hobo* (BLACKMAN *et al.* 1989) and *mariner* (LIDHOLM *et al.* 1993). In addition, *Minos* is the first transposable genetic element that has been exploited successfully for the germ line transformation of a non-Drosophila insect species, the Mediterranean fruit fly *Ceratitis capitata* (LOUKERIS *et al.* 1995b).

Analysis of DNA sequences at the donor sites after transposable element excision has often provided an insight on the mechanism of transposition. Excision of eukaryotic class II transposable elements results in double-strand breaks that can be repaired by two alternative mechanisms: first by ligation of the broken ends, as in the plant transposable elements *Ac* and *Tam3* (SAEDLER and NEVERS 1985; COEN *et al.* 1989) and second by repair of the broken chromatid (gap repair) through gene conversion using the homologous chromosome as template; *P*-element excisions are repaired mainly by this last mechanism (ENGELS *et al.* 1990, 1994). Gap repair has also been shown to occur after excision of *Tc1* in *C. elegans* (PLASTERK 1991; PLASTERK and GROENEN 1992), *Tn7* in *Escherichia coli* (HAGEMANN and CRAIG 1993), and in *Tam3* excision in tobacco (HARING *et al.* 1991). The analysis of transposition mechanisms of the *Tc1*-like elements should be accelerated considerably after the recent demonstration that purified *Tc1* transposase catalyzes precise transposition of a *Tc1* transposon *in vitro* (VOS *et al.* 1996).

In this study, chromosomal donor sites have been analyzed after excision of a marked *Minos* element as a first step toward understanding the molecular mechanism of *Minos* transposase action. The structures of excision footprints suggest that both gap repair and direct ligation mechanisms may be involved in chromatid repair after *Minos* excision. Gap repair by gene conversion generates either precise restoration of the donor sites or partial deletions of the element, while direct ligation generates characteristic footprints that contain terminal nucleotides from the transposon.

MATERIALS AND METHODS

Fly strains, crosses and *in situ* hybridization: The following *Minos*-containing *D. melanogaster* strains were employed: (1) Line A10.1 (*y* w^{67C23} *Mi{*w^{+mC}*}*18F) contains a single *X*-linked insertion of *Miw1*, a nonautonomous *Minos* transposon marked with the *D. melanogaster white* gene (PIRROTTA 1988; LOUKERIS *et al.* 1995a); (2) Line C58 (*y* w^{67C23} *Mi{*w^{+mC}*}*17D) contains a tandem repeat of two *Miw1* transposons at 17D (LOUKERIS *et al.* 1995a); (3) Line M67 [*y* w^{67C23}*;TM3 Sb Ser/ry P{Hsp70:Mi;T}*ry^{+}*/*(87D)] contains a *P*-element insertion on the third chromosome, carrying a modified *Minos* element in which the left-hand repeat has been replaced by the promoter of the Drosophila *Hsp70* gene (FRANZ *et al.* 1994; LOUKERIS *et al.* 1995a); (4) Line M56 [*y* w^{67C23}*;TM3 Sb Ser/ry P{Hsp70:Mi;T}*ry^{+}*/*(76BC)] contains another insertion of the *Hsp70-Minos* fusion on the 3rd chromosome. Both M67 and M56 insertions are associated with recessive lethal mutations and are kept over a TM3 balancer chromosome. Single-pair crosses at 25° were used in all experiments for detection of *Minos* mobilization in the germ line. The mating schemes for scoring transposon loss or transposition events in the germ line are shown in Figure 1.

A homozygous-viable derivative of chromosome M56 (M56V) has been used both for the direct cloning of chromosomal donor sites after excision of the transposon and for the detection of *Minos* extrachromosomal copies. The reason for the lethality in the original M56 and M67 lines (or its loss in the M56V subline) is not known and is under investigation. Heat shock induction of *Minos* transposase was performed in plastic vials for 1 hr in a 37° incubator; flies were then returned to 25° for the indicated period before DNA extraction.

In situ hybridizations to polytene salivary gland chromosomes were performed by standard technique (ASHBURNER 1989).

Molecular analysis of chromosomal donor sites: Chromosomal donor sites resulting from germ line excision in females heterozygous or homozygous for the A10.1 insertion were amplified by PCR from exceptional white-eyed male progeny of the crosses shown in Figure 1, b and c. Donor sites from the hemizygous state were PCR-amplified from white-eyed male progeny recovered from crosses of A10.1 ; TM3/M67 males with compound-X [C(1)*D y w f*] females. During the experiments, it was discovered that *X*0 males were recovered at unusually high frequencies among the progeny of heterozygous and homozygous A10.1 females. Because of this, the white-eyed male progeny from crosses b and c in Figure 1 were composed of two classes: males carrying an A10.1 chromosome that has lost the white-marked *Miw1* transposon and *X*0 males that had inherited the *yw* chromosome from their fathers. For this reason, only *XY* males, distinguished from *X*0 males by a diagnostic PCR assay for the *Y*-linked dynein gene (GEPNER and HAYS 1993; data not shown), were analyzed molecularly and germ line transposon loss was scored only in the female progeny (Figure 1). Increased rates of *X* chromosome nondisjunction have been described previously for other transposable elements (KIDWELL *et al.* 1977; PICARD *et al.* 1978).

General DNA manipulations were performed according to standard procedures (SAMBROOK *et al.* 1989). Fly DNA for the PCRs was prepared from single flies (GLOOR and ENGELS 1992). Five microliters of DNA (one-tenth of the preparation) was used in 50-μl PCRs essentially as described (SAIKI *et al.* 1988). Dynein sequences were amplified (30 cycles: 94° for 1 min, 55° for 1 min, 72° for 30 sec) using primers Dyn-F 5′GGGCGAGCTTTGGGTATGAT3′ and Dyn-R 5′CAACGGTTGTGCGCAAAGCA3′ (bp 97–116 and 346–327, respectively, in EMBL entry accession No. L23199). Only flies showing the 250-bp diagnostic band for dynein were used for further analysis of donor sites. Donor sites were amplified (30–40 cycles, 94° for 1 min, 50° for 1 min, 72° for 30 sec) using the following oligonucleotide primers in the region flanking the insertion: A10.1L 5′GATCATATCTGGATGTATAG3′ and A10.1R2 5′CGATCCTATAAAAACATTCG3′. Longer extension times, (90 sec) were necessary for the amplification of some donor sites (*G4* and *G9* of Figure 3). PCR products were gel purified (KOENEN 1989), subcloned into the *Eco*RV site of the pBCKS(+) plasmid vector (Stratagene) and sequenced with the A10.1L primer.

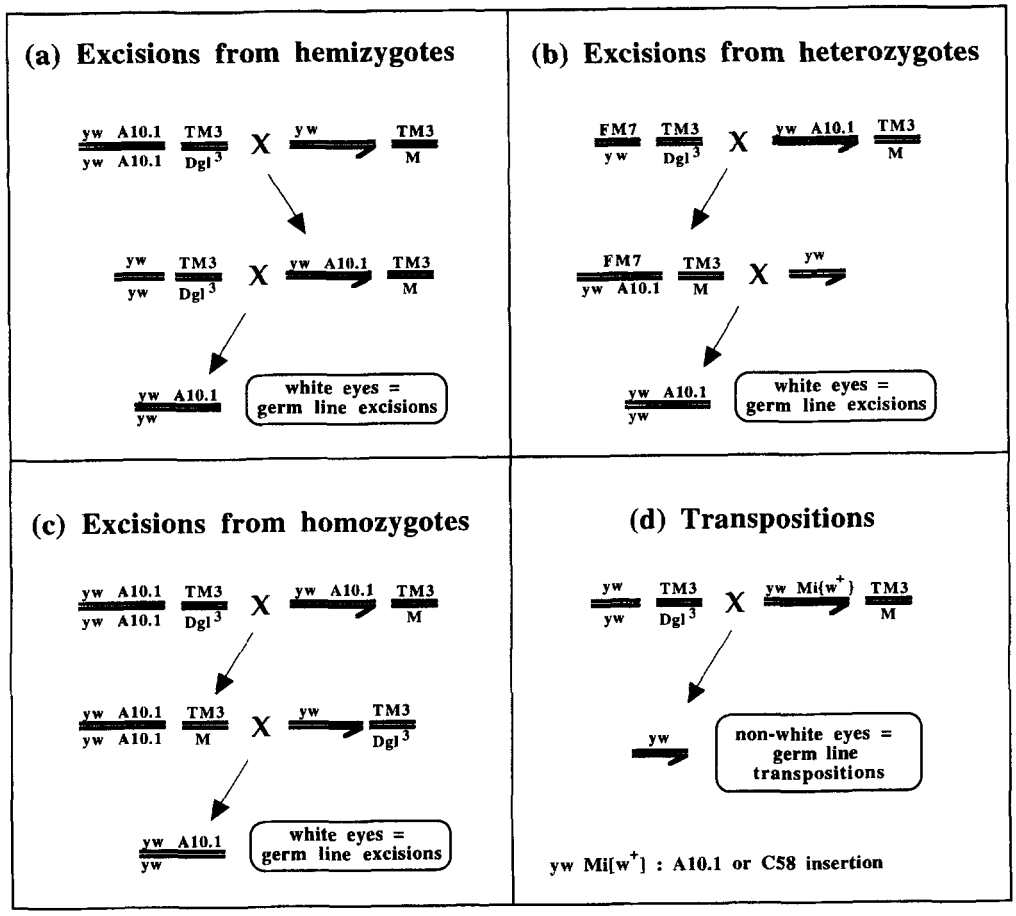

FIGURE 1.—Mating schemes for detection of loss and transposition of the *Minos* transposon *Miw1* in the germ line. Germ line excisions were scored in the female progeny (as shown in a–c). The molecular analysis of excisions was done in males from a mating scheme similar to that shown in a, in which compound-X females were used instead of the *yw* females (not shown; see MATERIALS AND METHODS for details of phenotypes and chromosomes used). M is M56 or M67.

Direct cloning of donor sites after somatic excisions: Homozygous A10.1 virgins were crossed to homozygous M56V males. A10.1;M56 males were subjected to heat shock for 1 hr at 37° to maximize expression of *Minos* transposase then left to recover for 1 hr at 25°. Genomic DNA was extracted (ASHBURNER 1989) and digested with *SalI* and *BclI* that cut *D. melanogaster* genomic DNA 86 bp to the left and 137 bp to the right of the *Miw1* insertion in A10.1 (see EMBL accession No. Z48626 for the sequence of the A10.1 donor site). The digest was size-fractionated on a 1% agarose gel, and DNA fragments of the size expected for precise or almost precise transposon loss (~220 bp) were gel purified and ligated to *SalI-Bam*HI-digested pBCKS(+) vector. The ligated DNA was used for transformation of *E. coli* XL1-blue cells (Stratagene) and ~30,000 clones were screened by colony hybridization using the PCR amplified empty donor site as probe.

Extrachromosomal copies of the *Minos-white* transposon: Homozygous A10.1 virgin females were crossed to homozygous M56V males; the progeny was heat shocked at 37° for 1 hr, followed by a 20-min recovery at 25°. Genomic DNA was deproteinated by phenol extraction (ASHBURNER 1989) and size fractionated on 12-ml 10–40% sucrose gradients as described (AUSUBEL *et al.* 1989); ~250 mg of DNA was loaded on each gradient, and 0.75-ml aliquots were collected from the top. For Southern analysis, fractions were diluted twofold, and DNA was ethanol precipitated. A 1.7-kb *HhaI* fragment containing most of *Minos* (FRANZ *et al.* 1994) was used as probe.

Approximately 1.5 mg of DNA was size fractionated for the diagnostic restriction analysis shown in Figure 6B. Fractions enriched in extrachromosomal copies were identified by Southern hybridization, pooled, dialyzed against 10 mM Tris-Cl, pH 8.0, 1 mM EDTA and ethanol precipitated. Half of the material recovered was digested with *SalI* and the other half was used as uncut control. The blot was hybridized with the 914-bp *Hin*dIII-*Eco*RI fragment of *Minos* and, after stripping, reprobed with a ~4.1-kb *Eco*RI fragment containing the *white* gene.

RESULTS

Mobilization of a defective *Minos* transposon depends on the expression of transposase and chromosomal configuration: To study transposase-dependent *Minos* mobilization, we used two insertions of *Miw1* on the *X* chromosome (A10.1 and C58) in combination with two helper 3rd chromosomes (M67 and M56), each carrying a *Hsp70-Minos* fusion that expresses active transposase. The *Miw1* transposon was mobilized by bringing together the A10.1 or C58 chromosome and a helper chromosome, using the mating schemes shown in Figure 1. The A10.1 chromosome only was used for studies involving loss of the transposon; both A10.1 and C58 chromosomes were used for transpositions. In these crosses, the transposon was followed in the soma and in the germ line through the dominant marker gene it contains, which gives a yellow-orange phenotype on a w (white eyes) background.

Somatic mobilization events were scored as patches of white or darker ommatidia or as adjacent dark and

TABLE 1

Frequency of somatic mobilization of insertion A10.1 in males and in females, using chromosomes M67 and M56 as sources of transposase

	M67			M56		
Configuration	Total	Mosaic	Percent mosaic	Total	Mosaic	Percent mosaic
ywA10.1/yw	1175	50	4.3	946	96	10.1
ywA10.1/Y	827	9	1.1	597	11	1.8

white patches (twin spots) in the eyes of the progeny from the crosses A10.1;TM3/D gl^3 × *yw/Y*;TM3/M67, and A10.1;TM3/D gl^3 × *yw/Y*;TM3/M56. White patches represent clones of cells without pigmentation due to loss of the transposon in their founder cell. Darker patches could result either from transpositions to new locations that cause a darker phenotype or from duplications of the *white* gene. Twin spots may result from events involving transposon excision and loss from one chromatid in the G2 followed by integration into another chromosome. After such events, one of the two daughter cells may inherit two copies of the transposon while the other will get none, giving rise to a twin spot.

As shown in Table 1, the frequency of somatic events varies between ~1 and 10%, depending on the helper chromosome and on the configuration of the chromosome carrying the transposon. Events are four- to fivefold more frequent in heterozygous females compared with males; in either of the configurations, the M56 helper is about twofold more effective than M67. No eye mosaicism was detected in the absence of *Minos* transposase, among 2185 flies that were screened.

Table 2 shows the frequencies of germ line transposon loss in A10.1 flies that express transposase detected as exceptional female progeny that had reverted to the *w* phenotype. The highest frequencies were detected among the progeny of females that were heterozygous for the transposon; ~20% of A10.1/FM7 females carrying the M67 chromosome and 58% of A10.1/FM7 females carrying the M56 chromosome had at least one exceptional daughter among their progeny. Lower frequencies of events were detected in the germ line of males and of females homozygous for the A10.1 insertion. In these configurations, the frequencies of flies showing at least one exceptional progeny varied from 4.26 to 13.79%. Higher reversion frequencies in heterozygotes have been described in heterozygotes for a *P* transposon inserted into the *X*-linked *w* gene of *D. melanogaster* (ENGELS *et al.* 1990) and for *Tc1* in *C. elegans* (PLASTERK 1991). The higher reversion rates in heterozygotes can best be explained by the double-strand break repair model, according to which gaps are repaired by gene conversion using the homologous chromosome, the sister chromatid or ectopic copies as templates (ENGELS *et al.* 1990, 1994). According to this model, the wild-type sequence can be restored in heterozygotes, resulting in precise transposon loss and phenotypic reversion. In homozygotes, transposon loss and reversion frequencies are much lower because a new copy of the transposon is restored by gene conversion.

Although the frequencies of parents giving exceptional progeny are relatively high, the frequencies of

TABLE 2

Frequencies of germline excision events of insertion A10.1 using chromosomes M67 and M56 as sources of transposase

	M67		M56	
Configuration	Total	Excisions[a]	Total	Excisions[a]
Hemizygous				
Crosses[b]	94	4 (4.26)	89	10 (11.24)
Progeny[c]	2967	4 (0.13)	2365	14 (0.59)
Homozygous				
Crosses[b]	58	8 (13.79)	36	3 (8.33)
Progeny[c]	1980	9 (0.45)	1016	3 (0.30)
Heterozygous				
Crosses[b]	71	14 (19.72)	43	25 (58.14)
Progeny[c]	1582	19 (1.2)	858	41 (4.77)

[a] Excision events were detected as white-eyed A10.1 daughters from the crosses shown in Figure 1. Values in parentheses are percentages.

[b] Excisions expressed as number of crosses showing at least one progeny which has lost the w^+ marker on the A10.1 chromosome.

[c] Excisions expressed as fraction of total progeny that have lost the w^+ marker on the A10.1 chromosome.

TABLE 3

Frequencies of transposition of the *X*-chromosome insertions (A10.1 and C58) to autosomes, using chromosomes M67 and M56 as sources of transposase

Configuration	M67		M56	
	Total	Jumps[a]	Total	Jumps[a]
A10.1				
Crosses[b]	94	4 (4.26)	89	1 (1.12)
Progeny[c]	3014	4 [1] (0.13)	2433	1 [1] (0.04)
C58				
Crosses[b]	82	5 (6.10)	84	10 (11.90)
Progeny[c]	2694	6 [3] (0.22)	2329	11 [7] (0.47)

[a] Numbers in brackets indicate transpositions that were mapped cytologically. Values in parentheses are percentages.
[b] Transpositions expressed as number of crosses showing at least one exceptional progeny.
[c] Transpositions expressed as number of exceptional progeny.

exceptional progeny vary between 0.13 and 4.77%. In most of the crosses, only one and, occasionally two or three, events were detected. For example, of the 25 heterozygous A10.1 females carrying M56, 12 showed one, 10 showed two, and three showed three marker-loss events among their female progeny. The small size of clusters is consistent with either independent excision events or with single premeiotic events that took place late in development.

The frequency of transposon loss is several-fold higher in the germ line than in somatic cells. This difference may be due to a bias inherent in the detection of marker loss in somatic tissues. In the germ cells, any single excision event may result in a white-eyed progeny, while only relatively early somatic events can produce clones large enough to be detected in the adult eye.

Germ line transposition events of the *Miw1* transposon from the *X* chromosome into the autosomes were detected as non-*white* sons among the progeny of males carrying an insertion and a source of transposase. For these experiments, each of two different insertions of *Miw1* (A10.1 and C58) were mobilized by the *Minos* transposase-producing third chromosomes M67 and M56. As shown in Table 3, the frequencies of males showing germ line transposition events varied between ~1 and 12%, while the corresponding progeny frequencies varied between 0.04 and 0.47%. The cytological locations of a number of transpositions were determined by *in situ* hybridization and are shown in Table 4. Of the 20 crosses that gave transposition events 18 had one exceptional progeny each and two showed two progeny with transpositions. In the last two cases, the two siblings from each cross carried an insertion at the same position (Table 4). The two different transposase sources (M56 and M67) do not show any appreciable difference in their ability to induce transposition. On the other side the higher transposition rates shown by the C58 insertion compared to A10.1 (~10-fold using the M56 transposase source) may be due to the former consisting of two tandemly repeated transposons inserted at the same site (LOUKERIS *et al.* 1995a).

Excision of *Minos* leaves characteristic footprints at the donor site: To determine the molecular structure of donor sites after *Minos* excision, we used a PCR-based strategy. Starting from the DNA sequence around the insertion site of A10.1 (LOUKERIS *et al.* 1995a), oligonucleotide primers were designed that are flanking the point of insertion (Figure 2A). Donor sites were amplified by the PCR using as template DNA from A10.1 homozygous females or males also carrying the M56 or M67 chromosome (for somatic events) or from male progeny carrying an A10.1 chromosome that has lost the white-marked transposon (for germ line events). As shown in Figure 2, the wild-type (empty) donor site can be amplified by PCR yielding the expected 257-bp product (lane 1); the 6-kb *Miw1* insertion cannot be amplified under the conditions used (Figure 2B, lane 2). Representative amplification products of donor sites after somatic and germ line excisions are shown in lanes 3–14. With very few exceptions (*e.g.*, lane 11), the size of the obtained products is compatible with a precise or nearly precise transposon loss. Moreover, the PCR

TABLE 4

Localization of new insertions of *Miw1* into autosomes

Cross number	Donor parent	Site
14	A10.1;M67	94C
56	A10.1;M56	64BC
19	C58;M67	29E
19	C58;M67	29E
41	C58;M67	96F
5	C58;M56	$85E_{3\text{-}5}$
5	C58;M56	$85E_{3\text{-}5}$
14	C58;M56	99E
29	C58;M56	92E
36	C58;M56	97F
54	C58;M56	$85D_{15\text{-}16}$
84	C58;M56	41F

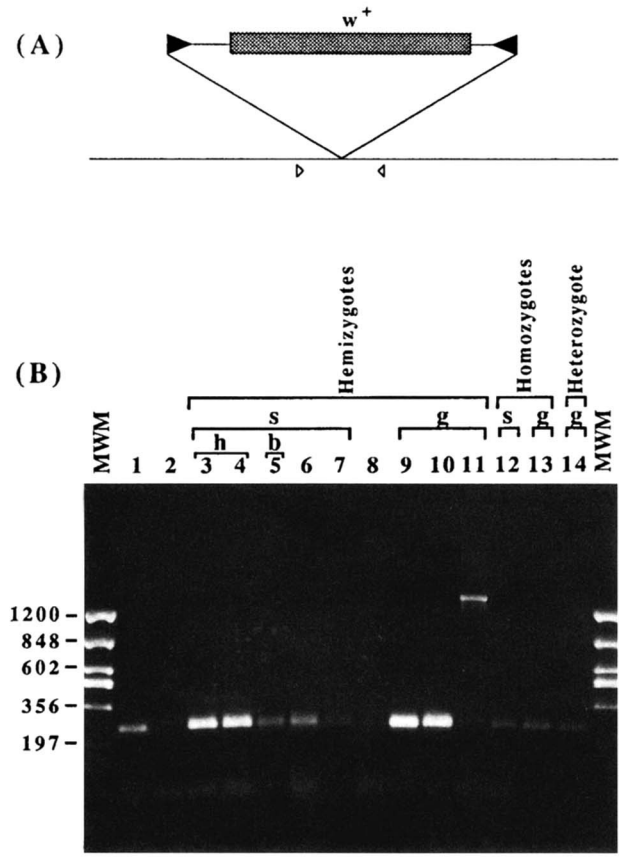

FIGURE 2.—PCR amplification of donor sites after *Miw1* excision. (A) Schematic representation of the A10.1 insertion: *Minos* inverted repeats are shown as filled arrowheads, the *white* minigene is represented as a stippled bar. Open arrowheads indicate the position of the PCR primers in the genomic regions flanking the insertion. (B) Representative amplification products of donor sites after somatic and germ line *Minos* excision. s, somatic excision; g, germ line excision; MWM, molecular weight marker. The DNA templates used in the PCR reactions were from single male flies except: h, head; b, body; 12, single female. Genotypes: lane 1, yw^{67C23}; lane 2, A10.1/Y; lanes 3–7, A10.1/Y;TM3/M56; lanes 8–11, A10.1/Y;TM3/*D* gl^3; lane 12, A10.1/A10.1;TM3/M56; lanes 13–14, A10.1/Y;TM3/+. Eye phenotypes: lanes 1, 9–11, 13 and 14, *w*; lanes 2 and 6–8, w^+; lanes 3–5 and 12, w^+ mosaics.

analysis of somatic events in males shows that in A10.1;M56 flies, *Minos* is highly active in the soma; excisions could be detected by PCR in all the flies examined, independently from the presence (lanes 3–5) or the absence (lanes 6–7) of eye color mosaicism. No excisions were detectable in siblings carrying the A10.1 insertion but no source of transposase (lane 8). In total, 50 A10.1;M56 males were tested by PCR for somatic excisions and all were found positive, irrespective of the presence of eye color mosaicism.

The structure of 52 donor sites, from 33 germ line and 19 somatic events that were independently recovered was analyzed by cloning and sequencing the amplified DNA products. The results of this analysis, shown in Figure 3, can be summarized as follows.

First, precise excisions, *i.e.*, events resulting in restoration of the wild-type donor site, were observed only in the progeny of heterozygous flies, as would be expected from the gap repair model. Data for somatic events from heterozygous flies could not be obtained because of the simultaneous presence of wild-type target sequences.

Second, the majority of donor sites recovered after *Miw1* excision in homozygous flies and in males (~85%) contained characteristic 6-bp footprints. These footprints consist of 4 bp, which are identical to either the 5′ (CGAG) or the 3′ (CTCG) end of the transposon, flanked by the target site duplication (A. . .T). The same kind of footprints were also detected in 25% (four of 16) of the germ line events from heterozygous flies. Other kinds of small footprints, such as the 4-bp footprint of *Hos3* or the 26-bp footprint of *S9*, were recovered at much lower frequency. The footprint in *S9* consists of an 18-bp sequence of unknown origin flanked by CGA and TCG, which correspond to the three terminal nucleotides of the transposon. Extraneous sequences such as the 18 bp sequence have been found at donor sites of several transposable elements after excision (COEN *et al.* 1989; MOERMAN and WATERSON 1989; TAKASU-ISHIKAWA *et al.* 1992; ATKINSON *et al.* 1993). Some of these sequences can be convincingly interpreted as the result of the repair mechanism (SAEDLER and NEVERS 1985; COEN *et al.* 1989).

Third, four germ line events were recovered that contained deleted or rearranged forms of the transposon. Two of these, named *Hog6* and *Hog2*, were recovered from homozygous females, and two (*G4* and *G9*) from males. *Hog6* (shown in Figure 3) is an internally deleted derivative of *Miw1* that is possibly a product of incomplete gap repair. Its structure was determined by a combination of PCR analysis and Southern hybridization (data not shown). *Hog2* (not shown in Figure 3) has a more complex structure, containing a partial duplication of the *w* sequences in the transposon. This structure can also be explained by an inaccurate gap repair event; in this case, each end has copied more than half the element and then the ends were joined out of alignment. The *G4* and *G9* events contain partial deletions of the right half of *Miw1* that are combined with a precise inversion of the transposon. The *G9* insertion contains a deletion of approximately half of the transposon, from the A of the TA duplication to bp 7227 of the *w* gene. The *G4* insertion is very similar. Such structures could result from two independent events: an inversion of the element, caused by homologous recombination of the inverted repeats, followed by an excision event and partial gap repair of the inverted element. However, inverted elements were not detectable by a sensitive PCR assay in A10.1 flies, either in the presence or in the absence of transposase (data not shown), suggesting that spontaneous or transposase-mediated homologous recombination between the inverted repeats does not happen frequently. Therefore,

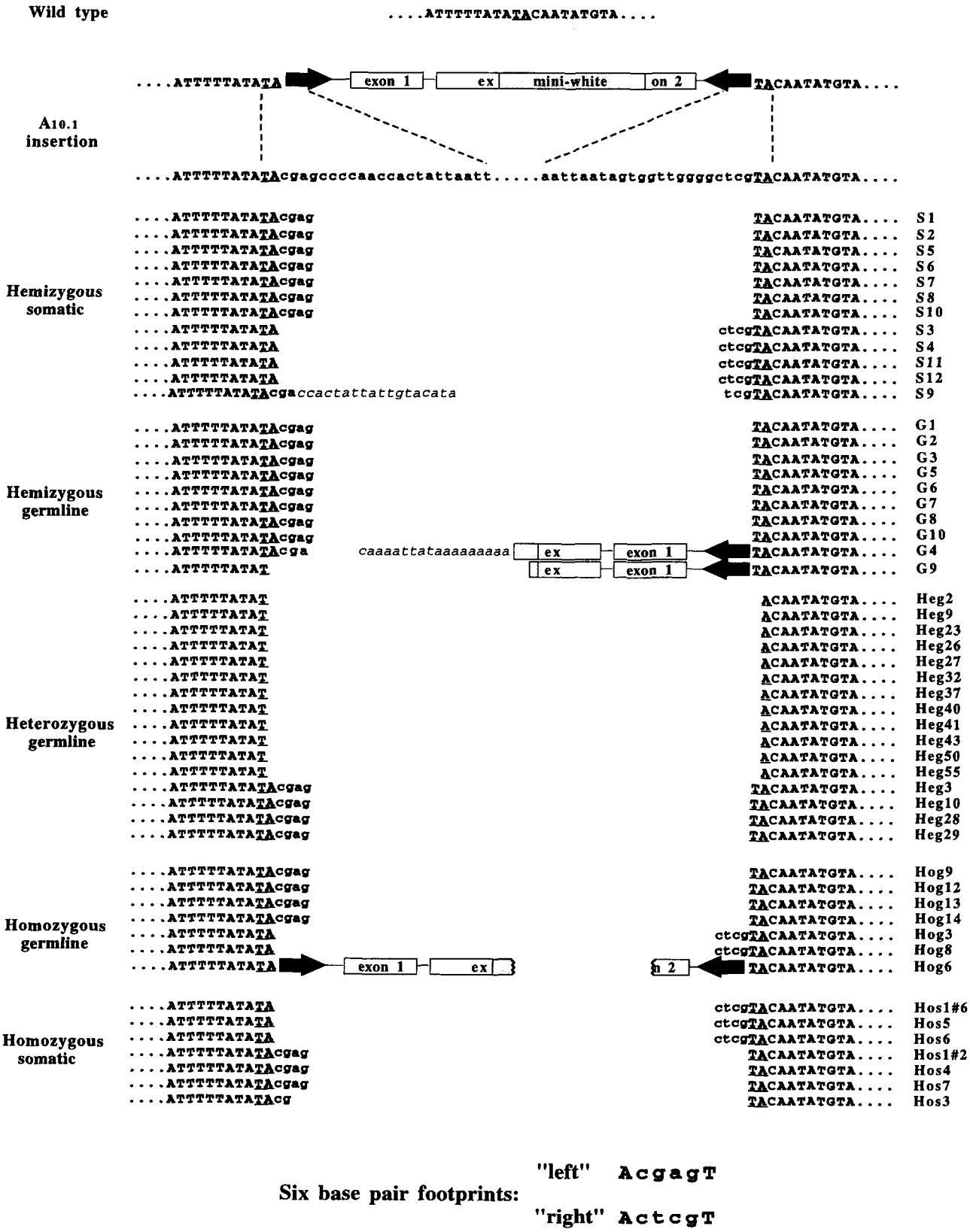

FIGURE 3.—Summary of the sequence analysis of donor sites after *Miw1* excision. PCR amplified fragments obtained as shown in Figure 2 were cloned and sequenced. From top to bottom: the wild-type sequence, the A10.1 insertion and the structure of the donor sites are shown. Uppercase letters, *D. melanogaster* genomic flanking regions; lowercase letters, *Minos* sequences; TA, duplicated target site; italic lowercase letters, sequences of unknown origin. The two symmetrical most commonly recovered footprints are shown at the bottom.

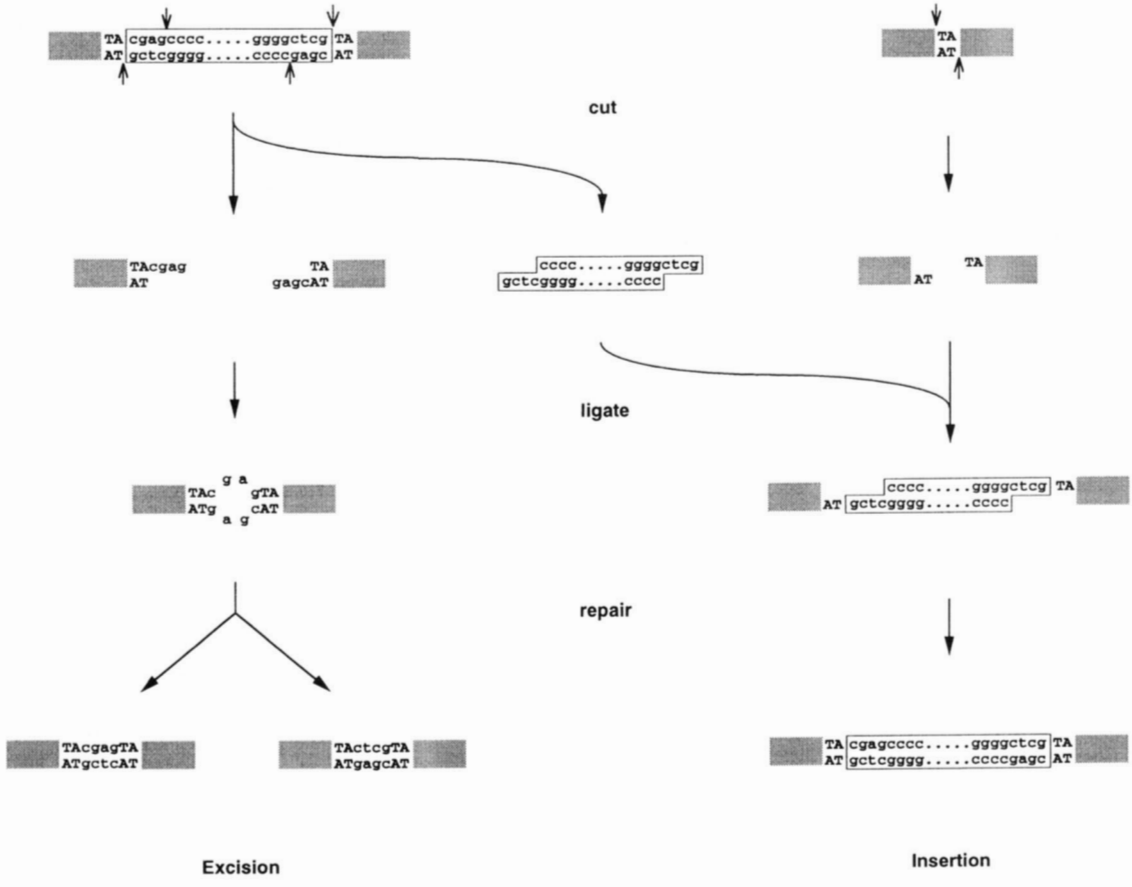

FIGURE 4.—Mechanism for generating the 6-bp footprints at *Minos* donor sites after excision and for regenerating the ends of *Minos* after integration. During excision (left), staggered cuts introduced by the transposase (open arrows) are followed by joining of the chromosomal ends and formation of a heteroduplex that contains a 2-bp mismatch. Repair or replication of the heteroduplex produces the two alternative 6-bp footprints. At integration (right), staggered cuts are introduced at the TA donor site (open arrows) and the excised transposon intermediate (shown here in linear form) is inserted, generating six-nucleotide gaps at each end. The gaps are filled by DNA polymerase activity, reproducing the transposon ends, and generating the TA taget site duplication. Transposon sequences are shown in lower case. Stippled bars represent *D. melanogaster* genomic regions flanking the TA target site.

it is more likely that the inverted and partially deleted structures were generated during the excision repair process itself. A possible mechanism may be incomplete gene conversion that initiated against the opposite inverted repeat, perhaps facilitated by pairing between the inverted repeats in the chromatid used as template.

The 6-bp footprints result from direct ligation and heteroduplex formation: Footprints similar to the ones left upon *Minos* excision have been found at donor sites of other transposons of the *Tc1-mariner* superfamily. Loss of the related transposons *Tc1* and *Tc3* in somatic cells of *C. elegans* leaves, in most of the cases, footprints consisting of 2 bp from either end of the transposon, flanked by the TA duplication (RUAN and EMMONS 1987; EIDE and ANDERSON 1988; VAN LUENEN *et al.* 1994). Similarly, the most common footprint left after *mariner* excision in *D. mauritiana* consists of 3 bp from either the 5′ or the 3′ end of the transposon, flanked by the TA duplication (BRYAN *et al.* 1990). Staggered cuts within the transposon ends have been proposed to explain the origin of these footprints (EIDE and ANDERSON 1988; BRYAN *et al.* 1990; VAN LUENEN *et al.* 1994). Moreover, the structure of linear extrachromosomal *Tc3* molecules is compatible with a double-strand cut staggered by 2 bp at the transposon ends (VAN LUENEN *et al.* 1994). The two nucleotide "tails" left at each end of the donor site after *Tc3* or *Tc1* excision could give rise to the observed footprints by one of two alternative mechanisms: ligation followed by mismatch repair (EIDE and ANDERSON 1988) or exonucleolytic attack at one of the two ends, followed by ligation and repair of the two-nucleotide gap (VAN LUENEN *et al.* 1994).

Figure 4 shows a model for the production of the 6-bp footprints after *Minos* excision and the generation of the TA target site duplication upon insertion. The model is based on a "ligation-repair" mechanism originally proposed for *Tc1* (EIDE and ANDERSON 1988). According to this model, a 4-bp staggered cut is introduced at each end, leaving four nucleotides of the inverted repeats at each end of the chromatid break and the complementary single-stranded tails at the ends of the transposon. Ligation of the chromatid ends to

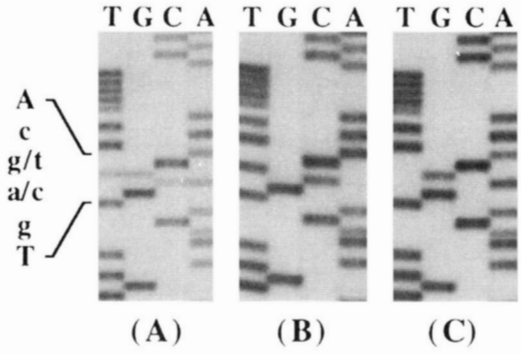

FIGURE 5.—Two-base pair mismatch at a directly cloned repaired donor site. The sequence around the donor site is shown. The sequence in A was obtained from DNA that was isolated from bacteria grown from a primary colony containing a cloned donor site. In B and C the sequences from two secondary clones are shown, that were derived from the primary colony and represent the two different footprints, AcgagT and ActcgT.

each other would produce a heteroduplex with a 2-bp mismatch which, by repair or replication, would give rise to the observed footprints. Re-insertion of the "free" transposon into a new chromosomal site by an analogous ligation-repair mechanism would require a 2-bp staggered cut at the TA donor site followed by ligation of the transposon. The resulting 6-bp gaps would then be repaired, generating, in this way, the TA target duplication and the complete transposon.

A direct test of one prediction of this model was performed by cloning A10.1 donor sites after excision of the *Miw1* transposon and looking for the presence of the 2-bp mismatch. To clone the donor sites, DNA was purified from A10.1;M56 males after a heat shock and, using appropriate restriction sites, a plasmid minilibrary was constructed that was expected to contain the "empty" donor sites. A prediction of the ligation-repair model is that DNA molecules containing the 2-bp mismatch should be present among the cloned fragments. A primary colony from a plasmid that contained such a fragment should, therefore, comprise two subpopulations of plasmids: one with the footprint ACGAGT and the other with the footprint ACTCGT.

Two positive clones were isolated and sequenced, from a low-density screen of ~30,000 colonies. One of these clones contained the predicted footprints. Figure 5 shows the sequence obtained from the primary colony of this clone, along with representative sequences from two secondary colonies, that were obtained from the primary colony by streaking. Each of the secondary colonies contained one of the two alternative footprints (ACGAGT or ACTCGT), while the banding pattern of the primary colony clearly shows the sequence AC(G/T)(A/C)GT. Assuming that the bacterial colony examined started from a cell that was transformed with a single plasmid molecule, this result clearly shows that this plasmid molecule contained the footprint with the mismatch predicted by the ligation-repair model.

The other clone contained a 3-bp footprint (ACT), consisting of the duplicated target TA plus a C, and is similar to the 4-bp footprint (*Hos3*) previously isolated by PCR amplification. This kind of footprint, which is shorter and is observed at much lower frequencies compared with the most common 6-bp footprints, might be generated by a repair mechanism involving exonuclease action, as proposed previously by VAN LUENEN *et al.* (1994).

Extrachromosomal *Minos* copies are circular: There is evidence from other Class II transposable elements that mobilization is correlated with the existence of free forms of the transposon. It is assumed that some of these forms are intermediates in the excision-transposition process. To investigate the fate of an excised *Minos* transposon, DNA from A10.1; M56 flies that were subjected to a 1-hr heat shock followed by 20-min recovery, was size fractionated and free copies of the *Miw1* transposon were detected in the fractions by Southern analysis using a *Minos* probe. As shown in Figure 6A, in addition to the strong hybridization signal of high molecular weight that corresponds to the integrated *Minos* sequences, a band can also be detected in one of the fractions, where DNA of lower molecular weight migrates. This band, which migrated at the 8-kb region, was also detectable in nonheat shocked flies and in flies that were allowed to recover for longer periods (1 and 2 hr) after the heat shock, but was undetectable in A10.1 flies not carrying a helper chromosome (data not shown).

The electrophoretic mobility of the extrachromosomal copies on agarose gels is not compatible with a linear form of ~6 kb, suggesting that they may correspond to relaxed circles. The structure of these extrachromosomal forms was determined by restriction analysis of the sucrose fractions enriched in these forms. As shown in Figure 6C, digestion of a linear form of the transposon with *Sal*I would yield one fragment of 3.2 kb, detectable with the left-arm (M) *Minos* probe used, while a circular form should yield only one M-hybridizing band of ~5 kb. The results of hybridization with the M probe (Figure 6B) clearly show the 5-kb band diagnostic for circles; they cannot rule out the presence of linear forms, however, because of the existence of a band at 3.3 kb, which is present in both chromosomal and extrachromosomal DNA, derived from the left arm of the A10.1 insertion (LOUKERIS *et al.* 1995a). Hybridization with the W probe (Figure 6B) confirmed the presence of the 5-kb diagnostic band and also showed that circular molecules are the only extrachromosomal forms detectable. Linear forms should yield three fragments of 3.2, 1.8, and 0.9 kb with this probe (Figure 6C). Because the 3.2- and 0.9-kb fragments comigrate with endogenous fragments, a 1.8-kb fragment would be diagnostic for linear forms.

DISCUSSION

We have shown that a defective *Minos* transposon (*Miw1*) that is inserted into the *D. melanogaster* genome

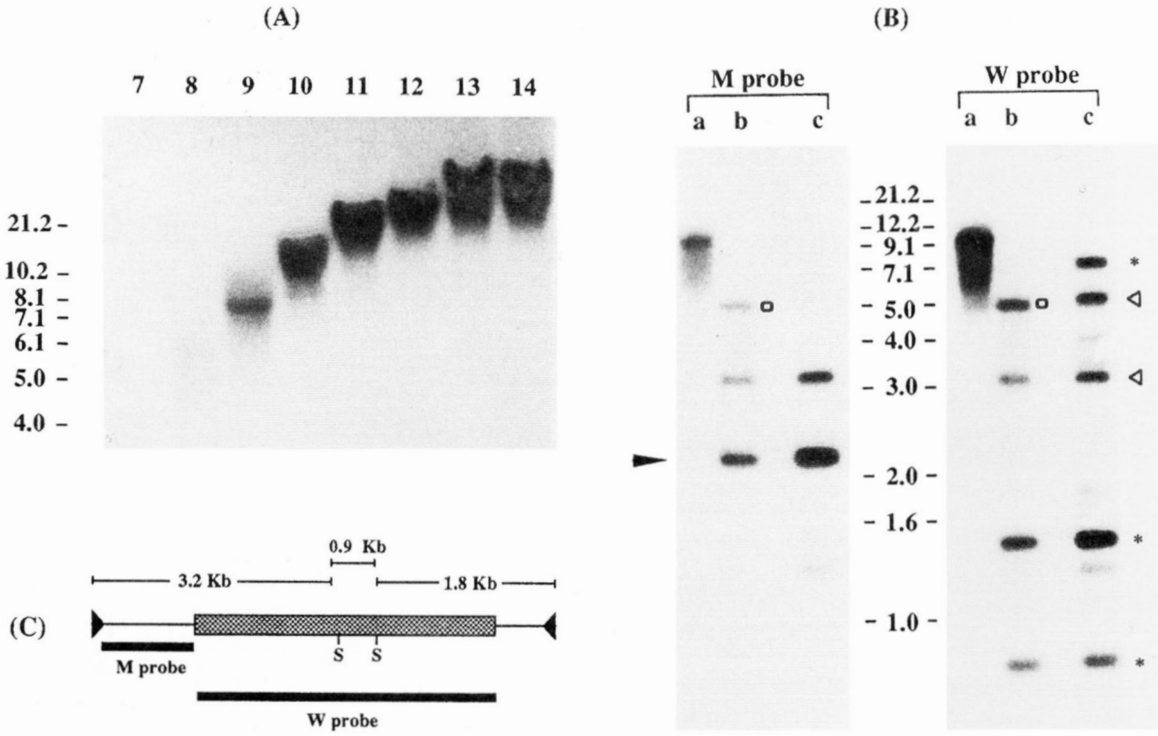

FIGURE 6. Detection and analysis of extrachromosomal forms in A10.1; M56 flies. Flies were heat shocked for 1 hr at 37° then left to recover at 25° for 20 min. (A) Detection of free forms: About 350 μg of genomic DNA were size fractionated by sucrose gradient centrifugation and the collected fractions, after precipitation, were subjected to electrophoresis on a 0.7% agarose gel and analyzed according to Southern with a Minos probe. Lane numbers correspond to fractions, from top to bottom of the gradient. (B) Restriction analysis of the free forms. DNA corresponding to fraction 9 was digested with *Sal*I and blotted after electrophoresis. The blot was first hybridized with a *Minos* probe (M), dehybridized, and reprobed with a *w* probe (W). The lanes are: a, uncut fraction 9 DNA; b, fraction 9 DNA digested with *Sal*I; c, total DNA digested with *Sal*I. The dark arrowhead indicates a 2.2-kb band from the *Hsp70-Minos* fusion; asterisks indicate the endogenous *w* gene fragments and open arrowheads indicate fragments contaning the ends of the A10.1 insertion (LOUKERIS *et al.* 1995a); boxes indicate the diagnostic 5-kb fragment. (C) Map of the *Miw1* transposon showing the *Sal*I fragments and the probes used in Southern analysis.

is stable in the absence of *Minos* transposase, but is mobilized at relatively high frequencies in the presence of transposase that is encoded by a modified element inserted in a different position of the genome. (LOUKERIS *et al.* 1995a). The stability of the *Miw1* transposon in the absence of *Minos* transposase suggests that there is no appreciable interaction between *Minos* ends and transposases that may be produced by other active transposable elements of the *Tc1* family that are present in the *D. melanogaster* genome, such as *Bari*-1 and the *S* element (CAIZZI *et al.* 1993; MERRIMAN *et al.* 1995).

We have also shown that chromosomes carrying a transposase source can induce transposition of a defective *Minos* element to new locations in the genome, at relatively high frequencies. This suggests that *Minos* transposons can be of use for gene tagging and enhancer trapping in *D. melanogaster*.

Analysis of the structures remaining after excision events can provide important insights about the mechanism of transposase action. Both the sequence data of donor sites after transposon mobilization and the higher frequencies of transposon loss in flies heterozygotes for the *Miw1* insertion support the involvement of a double-strand break gap repair mechanism for rejoining the chromatid after *Minos* excision, as proposed for the *P* element by ENGELS *et al.* (1990).

Of 33 germ line and 19 somatic events that were sequenced after amplification, 12 events represented precise transposon loss, while the rest contained additional sequences at the donor site. As mentioned above, precise events were recovered only from the germ line of heterozygous mothers, in accordance with the gap repair model. Four of the nonprecise germ line events, two recovered from homozygous females and two from males, contained deletions or rearrangements of the transposon which are also compatible with the gap repair model. Defective *P* and *hobo* elements with structures similar to *Hog6* are quite common and heterogeneous in *D. melanogaster* (BLACKMAN and GELBART 1989; ENGELS 1989), but they have not been detected in naturally occurring *Minos* elements in *D. hydei* (FRANZ *et al.* 1994) and generally they are rarely found in members of the *Tc1* family (LEVITT and EMMONS 1989). The source of this difference between *Tc1*-like and other elements is not understood; however, our results clearly show that deletion derivatives of a *Tc1*-like element can be generated, most likely by incomplete gene conversion, as hypothesized for the *P* element.

Most of the nonprecise events (35 of 41) contain a six-base footprint at the position that was occupied by the transposon. This footprint consists of the four terminal nucleotides of either end of the transposon flanked by the TA target site duplication. The gap repair-gene conversion model cannot give a satisfactory explanation for this structure. Similar footprints, consisting of the target site duplication flanking small stretches of nucleotides from the ends of the inverted repeats, have been found at donor sites after excision of other elements of the *Tc1-mariner* superfamily (RUAN and EMMONS 1987; EIDE and ANDERSON 1988; BRYAN *et al.* 1990; VAN LUENEN *et al.* 1994; COATES *et al.* 1995). Two alternative mechanisms for their generation have been proposed, both involving double-strand staggered cuts within the inverted repeats, that leave protruding ends at the donor site. One of the models postulates limited exonucleolytic attack of one of the ends followed by ligation and filling the gap (VAN LUENEN *et al.* 1994). According to the other, the symmetrically cut ends are ligated, resulting in a short heteroduplex structure, which gives rise to the two alternative footprints by mismatch repair or after replication (EIDE and ANDERSON 1988).

By directly cloning a repaired donor site, we have shown that, as predicted by the second model, chromatids containing the heteroduplex are present in flies in which *Minos* is transposing. This finding strongly suggests that the *Minos* transposase introduces staggered cuts at the transposon ends, leaving 4-bp-long protruding ends at the donor site; ligation of these ends generates a heteroduplex at the excision breakpoint. Mismatch repair or replication of the heteroduplex generates the 6-bp footprints found after *Minos* excision, as shown in Figure 4. It is likely that a similar mechanism may be responsible for the generation of excision footprints left by the transposons *Tc1* and *Tc3* in *C. elegans* and *mariner* in *D. mauritiana*. Heteroduplex structures similar to those reported here have recently been detected after transposase-induced excisions of a mariner element from plasmids (COATES *et al.*, 1995). It should be noted that the presence of 3′, rather than 5′, protruding ends suggests a simple mechanism for regeneration of the full sequence of the transposon after re-insertion and for generation of the TA target site duplication (Figure 4), as has been previously suggested for *Tc3* by VAN LUENEN *et al.* (1994).

Transposase-catalyzed staggered cuts within the transposon ends have been shown directly for another member of the *Tc1* family, the *C. elegans* transposon *Tc3*, and have been proposed to be a general property of the *Tc1* family of transposases (VAN LUENEN *et al.* 1994). Similar staggered cuts that leave ~16 nucleotides from the ends of the transposon at the donor site, have recently been proposed to explain footprints left after *P*-element excision (STAVELEY *et al.* 1995). It seems, however, that production of staggered cuts within the inverted terminal repeats is not a general property of eukaryotic Class II transposases. A different mechanism might be employed by transposases of the *hAT* superfamily where more complex structures, containing inverted duplications of the target site rather than nucleotides from the transposon ends, are found at the donor sites after excision (COEN *et al.* 1989; ATKINSON *et al.* 1993).

In summary, chromatid breaks generated at the donor sites after *Minos* excision can be repaired by two alternative mechanisms. The first (gap repair) involves exonuclease action at the broken ends, followed by gap repair against homologous sequences; the second (ligation repair), consists in direct ligation of four-nucleotide-long 3′ protruding ends, resulting in the formation of a heteroduplex, which is "corrected" by replication or by mismatch repair. The net result of gap repair is restoration either of the original target sequence (generating precise loss events) or of the transposon, depending on the chromosomal configuration. The net result of ligation repair is, in most of the cases, formation of the characteristic 6-bp footprints. The 3:1 ratio observed between precise loss events and events with footprints in the progeny of heterozygous A10.1 females suggests that breaks induced by *Minos* excision in *D. melanogaster* are repaired at least threefold more frequently by the gap repair mechanism.

Extrachromosomal circular copies of the *Minos-white* transposon can be detected with or without a heat shock but are absent from flies that are not carrying a *Minos* transposase gene. The detection of extrachromosomal copies in the absence of heat-shock induction probably results from basal levels of transcription from the *Hsp70* promoter (FRANZ *et al.* 1994). This "leaky" transcription is sufficient to promote somatic and germ line mobilization of the *Minos-white* transposon (LOUKERIS *et al.* 1995a).

Whether the extrachromosomal circular copies of *Minos* represent transposition intermediates or byproducts is unclear. Circles are the only extrachromosomal form that can be detected, either at 25° or shortly after a heat shock. It is possible that inactive circular forms might accumulate from the basal expression of the *Hsp70* promoter. Moreover, low numbers of highly reactive linear intermediates could easily escape detection. Use of a more tightly controlled system for expression of *Minos* transposase may help address this question.

The presence of extrachromosomal circular copies has been shown for other elements of the *Tc1* family (ROSE and SNUTCH 1984; RUAN and EMMONS 1984; RADICE and EMMONS 1993; VAN LUENEN *et al.* 1993), for transposon-like elements in protozoa, such as *TBE1*, *Tec1* and *Tec2* (JARACZEWSKI and JAHN 1993; WILLIAMS *et al.* 1993) and for bacterial transposons of the *IS3* family, such as *IS911* and *IS1* (POLARD *et al.* 1992; TURLAN and CHANDLER 1995), all encoding D35E-type transposases. Transposable elements unrelated to this

superfamily, such as the maize transposon *Mu* and the prokaryotic transposon *Tn916*, also generate circular forms (SUNDARESAN and FREELING 1987; CAPARON and SCOTT 1989). For most of the above mentioned transposons, circular forms are not thought to be transposition intermediates, and, up to now, only the covalently closed circular form of the conjugative transposon *Tn916* has been shown to act as an intermediate in transposition (CAPARON and SCOTT 1989). However, the widespread occurence of extrachromosomal circles suggests that they may have a functional role in transposition, rather than being inactive byproducts.

Direct cloning of extrachromosomal circles and/or transposition intermediates may help in answering this question. Until cell-free transposition assays become generally applicable, molecular analysis of transposon mobilization *in vivo* with simple genetic systems, like the one now available for *Minos* in *D. melanogaster*, may provide important insights into mechanisms of transposition of eukaryotic transposable elements.

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