Germline Transformation of *Drosophila virilis* With the Transposable Element *mariner*

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Manuscript received October 9, 1995
Accepted for publication February 6, 1996

ABSTRACT

An important goal in molecular genetics has been to identify a transposable element that might serve as an efficient transformation vector in diverse species of insects. The transposable element *mariner* occurs naturally in a wide variety of insects. Although virtually all *mariner* elements are nonfunctional, the *Mos1* element isolated from *Drosophila mauritiana* is functional. *Mos1* was injected into the pole-cell region of embryos of *D. virilis*, which last shared a common ancestor with *D. mauritiana* 40 million years ago. *Mos1* PCR fragments were detected in several pools of DNA from progeny of injected animals, and backcross lines were established. Because G_0 lines were pooled, possibly only one transformation event was actually obtained, yielding a minimum frequency of 4%. *Mos1* segregated in a Mendelian fashion, demonstrating chromosomal integration. The copy number increased by spontaneous mobilization. *In situ* hybridization confirmed multiple polymorphic locations of *Mos1*. Integration results in a characteristic 2-bp TA duplication. One *Mos1* element integrated into a tandem array of 370-bp repeats. Some copies may have integrated into heterochromatin, as evidenced by their ability to support PCR amplification despite absence of a signal in Southern and *in situ* hybridizations.

ASHBURNER has highlighted the importance of transformation of nondrosophilids as a technical development essential for genetic programs of insect control (ASHBURNER 1995). First, germline transformation with transposable elements enables the genetic manipulation of organisms that are disease vectors or agricultural pests. Second, the potential ability of transposable elements to sweep through a population may enable the genetic manipulation of an entire natural population by propelling an engineered gene to virtual fixation. Although simple in principle, the practical implementation of transposable elements for population control must overcome numerous technical and ecological obstacles.

The *P* element functions as an efficient vector for transformation of exogenous DNA into *Drosophila melanogaster* (RUBIN and SPRADLING 1982), but it has been used successfully in only a few other species of Drosophila, such as the sibling species *D. simulans* (SCAVARDA and HARTL 1984), and has yielded highly unstable transformants in the more distantly related species *D. hawaiiensis* (BRENNAN *et al.* 1984). Use of the *P* element as a general transformation vector in insects was called into question by the observation that extensive attempts to transform *Anopheles gambiae* resulted in a single transformation event that occurred in a *P*-independent manner (MILLER *et al.* 1987). Subsequently, it was shown by excision assays in embryos that *P*-element mobility is phylogenetically limited (O'BROCHTA and HANDLER 1988). Possible reasons for failure are incompatibility of the *P*-element system with host-encoded functions required for transformation, failure of the promoter driving the reporter or *P*-helper genes, and integration of the transposon into regions of the genome that inhibit expression of the reporter gene. The failure of stable *P*-element transformation in species of Drosophila that are distant relatives of *D. melanogaster* and the desirability of transformation of nondrosophilid insects of agricultural and medical importance has motivated a search for other transposable element systems that might serve as general transformation vectors.

Among the candidates for use in biotechnology is the *mariner/Tc1* superfamily of transposable elements and, in particular, the *mariner*-like elements (MLEs) (ROBERTSON and LAMPE 1995a). MLEs have an exceptionally wide distribution in insect species (ROBERTSON 1993; ROBERTSON and MACLEOD 1993), resulting in part from horizontal transmission (LOHE *et al.* 1995a; ROBERTSON and LAMPE 1995b). MLEs are also found in planaria, hydra, and even in the human genome (A. LOHE and D. HARTL, unpublished results; H. ROBERTSON, personal communication). The vectors and molecular mechanisms of horizontal transmission are unknown. Of 404 insects examined for MLEs by ROBERTSON and MACLEOD (1993), 16.6% were positive in PCR amplifications with degenerate primers matching conserved regions of the elements. This value represents a minimum of the MLE distribution because only a small number of insects of each species were examined by PCR and because the primer pairs may not have detected all fami-

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lies of MLEs present in an insect. The wide species distribution of MLEs, which differs dramatically from the distribution of other transposable elements confined to one or a small cluster of species, suggests that *mariner* is an excellent candidate as a general transformation vector.

mariner is a small, intronless transposable element of 1286 bp that codes for a single polypeptide of 345 amino acids. *Mos1* is an autonomous *mariner* element isolated from *D. mauritiana* (MEDHORA *et al.* 1988) and has been shown to transform *D. melanogaster*, a sibling species in which *Mos1* is not normally present (GARZA *et al.* 1991). The *Mos1* system can also serve as a vector for delivering exogenous DNA fragments up to at least 12 kb (LIDHOLM *et al.* 1993), a size that is large enough to be useful in genetic engineering of insects. Transposase sources driven by heat shock or germline-specific promoters are also available as helpers for transformation (LOHE *et al.* 1995b).

We have examined *D. virilis* to explore the species boundaries of transformation by *mariner*. *D. virilis* is a member of the subgenus Drosophila, whereas *D. mauritiana* is a member of the subgenus Sophophora. The species shared a common ancestor ~40 million years ago (RUSSO *et al.* 1995). In contrast to many nondrosophilid insects, *D. virilis* is amenable to polytene chromosome cytology, and reasonably well developed molecular biology and genetics are available. Furthermore, *D. virilis* is easily cultured in the laboratory and, because the characteristics of early development are similar to those in *D. melanogaster*, the methodology and skills required for embryo manipulation, microinjection and rearing are similar to those already developed for *D. melanogaster*. *Mos1* plasmid DNA was injected into the pole-cell region of *D. virilis* embryos and the resulting G_1 progeny were assayed for the presence of *Mos1* by PCR. The results indicate that *Mos1* integrates readily into the germline of *D. virilis* and is functionally active in *D. virilis*. These experiments demonstrate the potential of *mariner* as a general transformation vector in insects.

MATERIALS AND METHODS

Injection of *D. virilis*: The *Mos1* element is not normally present in *D. virilis*, although repeated invasions of new hosts by horizontal transmission of transposable elements in the *mariner-Tc1* superfamily have been identified in many insect species. A *D. virilis* strain carrying the w^- mutation was used for injection of *Mos1* because the w^- mutation serves as a convenient strain marker and is also undoubtedly highly detrimental to any potential escapees.

D. virilis carrying a w^- mutation was grown at 25° on standard Drosophila media. To improve viability of embryos, the strain was outcrossed to a wild-type strain and the w^- mutation was reisolated. Conditions for manipulation of embryos and injection were the same as used for *D. melanogaster*. The concentration of *Mos1* plasmid DNA (MEDHORA *et al.* 1988) used for injection was 500 μg/ml.

DNA and PCR: G_1 progeny from a single bottle were collected in pools of ~20. A rapid method of DNA preparation for PCR (GLOOR and ENGELS 1991) was modified by increasing the volume of squishing solution to 150 μl. Conditions for PCR screening of *D. virilis* for *Mos1* transformation were essentially as described in LOHE *et al.* (1995a) except that 3 μl of DNA from pooled flies were used in a 20 μl reaction, the number of cycles was 30, and the reannealing temperature was 58°. Southern hybridizations were carried out as described in SAMBROOK *et al.* (1989).

The primers to the *Mos1* sequence used in the initial screen for transformants were as follows: forward primer (628F) 5′-GTTACTGGAGATGAAAAATGG-3′ and reverse primer (791R) 5′-AGCTCATAGTAAATGACACCG-3′. Primers used to demonstrate full-length and deleted *Mos1* elements in line 17 (Figure 1) were 524F (5′-GATGGGTGCCACATGAGTTGA-3′) and 1035R (5′-GAATAGGTGGTAATCGGATGG-3′). Primers for PCR synthesis of a single copy probe for the *alcohol dehydrogenase (Adh)* gene were provided by D. NURMINSKY (personal communication).

For inverse PCR (OCHMAN *et al.* 1993), 5 μg of DNA from lines 17 or 69 were digested to completion with *Nde*II, *Msp*I or *Dde*I. The enzymes were heat inactivated at 65° for 15 min and the sample was ethanol precipitated. One-fifth of the sample was ligated with T4 ligase in a final volume of 100 μl (12° for 16 hr) and the DNA was ethanol precipitated. DNA was dissolved in 20 μl of water, and 10 μl was used in a PCR reaction with 58° as the annealing temperature for *Mos1* primers. To obtain an inverse PCR product of the 5′ end of *Mos1* insertions, *Mos1* primers used were 628F and 53R (5′-CGTTTGCGAGACATCTATATG-3′), and for the 3′ end, the primers were 1174F (5′-AAATGTGTAGCTAGCGACGGC-3′) and 1035R. In some cases, when one end of a *Mos1* insertion had been cloned, the other end was obtained by inverse PCR using primers to the genomic sequence and DNA from wild-type *D. virilis* as template. For inverse PCR of the 3′ end of the 69S insertion, the primers were 69SII (5′-CAACCTTTCCTTATGATTTTT-3′) and 69SR (5′-ATTTTCGGGTCCACATCTTTA-3′). For inverse PCR of the 5′ end of the 69L insertion, the primers were 69LF (5′-TGACCACAGAGCACACCCAAC-3′) and 69LII (5′-GATGATGACGGCACGACTCCC-3′). PCR products representing the genomic region before insertion of *Mos1* were used as hybridization probes. The primers used to amplify 163 bp of genomic DNA surrounding the 69S insertion were 69SII and 69SIII (5′-GATGTAACCCCTTGCCATTTT-3′). For a 210-bp genomic region surrounding the 69L insertion the primers were 69LF and 69LR (5′-TTTTATCACAGCAGTGATAGC-3′), and to amplify the 206 bp of genomic DNA surrounding the deleted element in line 17, the primers were 17F (5′-AAGTAACTTGAGCGCTTTATG-3′) and 17R (5′-ACATAAGTTCAGCGCTCAGCT-3′). The annealing temperature of the primers was determined empirically in 2° increments. PCR products were cloned using a TA cloning kit (Invitrogen) and sequenced as described (LOHE *et al.* 1995a).

RESULTS

Rationale for transformation strategy: The failure of germline transformation by the *P* element in species of Drosophila that are distantly related to *D. melanogaster*, or *P*-element transformation of nondrosophilid insects, is widely acknowledged and accepted. The *a priori* expectation for the transformation efficiency for other transposable elements, such as the active *mariner* element *Mos1*, is that these elements too might fail or, at best, might integrate into the germline inefficiently. An additional source of uncertainty is also contributed by

the use of any heterologous selectable marker, such as the *white* gene from *D. melanogaster,* because the marker must function at least minimally in a distantly related species to be detected. Furthermore, the efficiency of transformation in *D. melanogaster* of *Mos1* constructs that carry the *white* gene is ~20-fold lower than the transformation efficiency by the intact *Mos1* element (LOHE 1995b). Although not fully understood in molecular terms, the inefficiency of transformation by *Mos1* constructs that carry exogenous DNA may result from insertion of the DNA into a *Sac*I site in a region of *Mos1* that is necessary for efficient integration into the chromosome (LOHE and HARTL 1996).

As a first step in evaluating the potential of *mariner* to serve as a general transformation vector in insects and to circumvent some of the potential pitfalls in the use of exogenous genetic markers, it was decided to attempt germline transformation in *D. virilis* with the intact *Mos1* element and to screen for potential G_1 transformants by the PCR technique. To implement this strategy successfully, several considerations had to be taken into account. First, sufficient numbers of G_2 larvae must be visible in cultures before the G_1 parents are sacrificed for PCR, so that a large number of G_2 adults can be screened subsequently for *Mos1* sequences from those cultures in which a parent (or parents) was positive in the PCR assay. Second, PCR screening of G_1 flies must be completed while the G_2 progeny are still viable and fertile, or else the number of cultures becomes unmanageable. The pooling strategy can be used to detect a rate of transformation as small as 1/500, which it sometimes is for *P*-element transformation in *D. melanogaster.* Control experiments with *Mos1* demonstrated that, under our experimental conditions, a positive PCR result was obtained when one *D. melanogaster* containing a single copy of *Mos1* was mixed together with 29 *D. virilis* (1:30 ratio), but the result was not consistent for a ratio as great as 1:100. Therefore, DNA for PCR analysis was prepared from ~30 G_1 adults. Despite the saving in time by screening groups of 30 adults in each PCR reaction, the number of pools to be screened remains excessive in a large-scale injection experiment, given the time constraint to complete G_1 screening before G_2 adults age or are lost. (For example, assuming that all G_0 individuals are fertile, as few as 30 G_0 adults could produce up to $15 \times 500 = 7500$ G_1 progeny from male parents and $15 \times 100 = 1500$ progeny from female parents; to screen 9000 G_1 individuals in groups of 30 would require 300 DNA preparations and PCR reactions).

In view of the expectation of a low transformation efficiency of *D. virilis* by *Mos1,* we took the unusual step of pooling G_0 adults to obtain G_1 progeny for screening by PCR. Although pooling G_0 adults has the obvious drawback that the number of independent transformation events could not be determined, and therefore only a minimum transformation efficiency could be calculated, the chance for detection of an infrequent event is increased significantly at the same time that the number of DNA preparations and PCR reactions is decreased by about an order of magnitude.

Transformation of *D. virilis* by *Mos1*: From ~250 embryos of *D. virilis* that were injected with *Mos1* DNA, 115 larvae hatched and 27 G_0 adults were recovered. Adults were pooled as they emerged and transferred to fresh media every 2–3 days until egg laying ceased. This yielded 40 bottles with varying numbers of G_1 progeny. Pools of 20–30 G_1 flies from the bottles were used to initiate separate cultures, and the parents were sacrificed for PCR when G_2 larvae were visible.

DNA preparations were assayed for *mariner* sequences by PCR using a pair of primers internal to *Mos1.* DNA from the strain of *D. virilis* used for injection gave no PCR products with these primers or with any of the available primers to *Mos1.* However, DNA from 15 of 66 pools of G_1 flies showed a faint band of the correct size (163 bp). The presence of *mariner* sequences in the DNA preparations was verified by repeating the PCR with three other pairs of primers. Ten samples gave the predicted size of fragment for each pair of primers, four other samples gave the correct size for three primer pairs but were negative for the fourth pair, and one sample was positive only for the primer pair used in the initial screen. Lines that failed to show a PCR product for all of the four primer pairs were not investigated further.

Mendelian segregation of *Mos1* in transformed lines: Lines were initiated by backcrossing single G_2 adults from cultures with putative transformants, and G_2 parents were sacrificed for PCR when progeny were visible in the vial. Ten lines were retained, each derived from a G_2 parent that was positive by PCR. G_3 progeny from three of these crosses, used to initiate lines 14, 17 and 69, were also assayed for *mariner* sequences by PCR. The *Mos1* element segregated in a 1:1 Mendelian ratio in the three lines, as would be expected if one copy of the *Mos1* element had integrated into the chromosome (Figure 1 shows the results for line 17). An attempt to amplify by PCR the region spanning the junction of *Mos1* and flanking genomic sequences in the *Mos1* plasmid failed, which would be expected if the *Mos1* element had integrated by transposition, leaving plasmid and flanking DNA behind. Since the injected G_0 parents were pooled to generate the G_1 progeny, the number of independent transformation events cannot be calculated. For example, there could be as few as one transformation event that is present in the 10 lines that were retained, because the 10 G_2 parents may have derived from a single G_1 parent (or from G_1 transformants that arose from a single premeiotic transformation event in a G_0 individual). Therefore, a minimum estimate for the transformation efficiency of *Mos1* in *D. virilis* is 4% (1/27).

Spontaneous deletion of an element: PCR screening

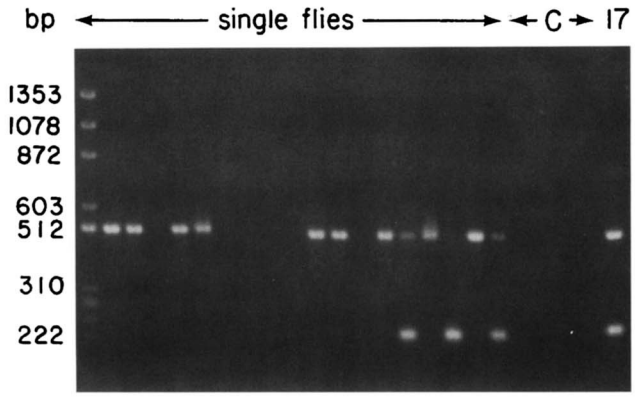

FIGURE 1.—Independent assortment of full-length and deleted *Mos1* elements. In lanes labeled single flies, 19 progeny from a backcross of the founder of line 17 were subjected to PCR amplification. There are two size classes of *Mos1* elements and there is independent assortment of the classes. The 512-bp band corresponds to the expected full-length product and the smaller fragment of 222 bp contains a 290-bp deletion. A molecular weight marker of ϕX174 DNA digested with *Pal*I (bp) was also loaded into the first lane. Lanes under C are controls in which water was substituted for DNA. The lane labeled 17 contains DNA from a pool of 20 flies in line 17.

of one G_2 individual (the founder of line 17) with *Mos1* primers gave two bands, one of the expected length and another ~300 bp smaller, suggesting a deletion had occurred in a *Mos1* element. Further characterization by PCR and sequencing confirmed the presence of a 290-bp deletion from nucleotides 683–972 inclusive. The progeny obtained by backcrossing the G_2 individual containing the deleted and full-length PCR products were also examined by PCR (Figure 1). Progeny could be grouped into four classes based on the size of the PCR product: those with a full-length product only, with deletion product only, with both products, or with neither product. This result indicates that the G_2 individual contained at least two copies of *Mos1* at different chromosome locations. The presence of two elements can be explained either by a single transformation event followed by self-mobilization of *Mos1* to a new chromosome location (either preceding or followed by an internal deletion in an element) or by two independent transformation events in the parents of the G_2 individual, one yielding a full-length element and a second yielding a deleted element.

Integration of *Mos1* results in a 2-bp TA duplication: Chromosome integration of *mariner* results in a characteristic 2-bp duplication of the TA target site adjacent to each of the inverted repeat termini (JACOBSEN *et al.* 1986). To determine whether *Mos1* integration into the *D. virilis* chromosome generated the expected 2-bp duplication, the 5′ and 3′ ends of *Mos1* were cloned using inverse PCR (OCHMAN *et al.* 1993). Two lines of transformants were used as a source of DNA: line 17, which contained the deleted element, and line 69. Inverse PCR of the 3′ end of *Mos1* in line 69 gave two strong bands and several faint bands. Nucleotide sequencing of clones corresponding to each of the strong bands showed that the 3′ genomic sequences flanking *Mos1* were different (Figure 2), indicating that line 69 contains at least two different *Mos1* insertions. The 5′ flanking DNA sequences were obtained by repeating inverse PCR using DNA from line 69 or with DNA from wild-type *D. virilis* and primers synthesized from the 3′ genomic sequences. In both insertions in line 69, the 5′ and 3′ sequences of *Mos1* were flanked by a 2-bp TA duplication. Similarly, both ends of the deleted *Mos1* element in line 17 contained a TA dinucleotide, and the genomic DNA sequence flanking the DNA in line 17 was different from either of the sequences flanking *Mos1* in line 69. Hence, line 17 and line 69 contain at least three independent sites of integration of *Mos1* into *D. virilis* DNA. The nucleotide sequences at the sites of integration have two features in common: first, the sequences surrounding the insertion sites are highly A + T rich (69–75%); and, second, the sequences at the insertion site are TTTTA, where the TA dinucleotide is duplicated at both ends of the element.

Accumulation of the *Mos1* element: Unexpectedly, initial attempts at Southern hybridizations of *Mos1* to DNA from the 10 transformant lines proved unsuccessful, despite repeated hybridization attempts and long autoradiographic exposures. However, the presence of *mariner* sequences in each of the DNA samples was verified by PCR. When the same filters were reprobed with a fragment of the *Adh* gene, a single copy band was visible in an overnight exposure. In the absence of genetic drift, the signal strength of a *Mos1* element was expected to be one-fourth as strong as a single copy sequence in the genome because each line was initiated by backcrossing a G_2 individual that was positive by PCR. Because of repeated failures to obtain a signal in Southern hybridizations with 2 μg of rapid "miniprep" DNA made from pools of 20–30 flies, highly purified, CsCl-banded DNA was prepared from pools of 100–200 flies and the amount of DNA in the Southern blots was increased to 5 μg per sample. Nevertheless, no signal was obtained in any of the lines with a *Mos1* probe, despite long exposures of autoradiographs and digestions with various combinations of restriction endonucleases.

We also attempted to exclude the possibility that the *Mos1* element had integrated into a highly underreplicated region of the genome (such as heterochromatin) by preparation of DNA from heads of adults from lines 14, 17, 67 and 69. DNA from heads does not show significant underrepresentation of satellite DNAs due to underreplication of heterochromatin in polytene cells, which contribute disproportionately to DNA from adults. No signal was observed with ~1 μg of DNA (50 heads) per lane from each of the four lines, although a control with *D. melanogaster* DNA containing a euchromatic *Mos1* insertion was positive. However, interpretation of this experiment is difficult, in part because of the lack of a suitable positive control, such as a single-

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69S:  .....CCGATTTTCAAATTTTATACCATTTTGGACTC.....(370-bp satellite)

69L:  .....CCAATTGTTCTCTTTTAAAGACATTCTGCAAA.....(unique sequence)

17:   .....AGGCATTATCCGTTTTAATTACATTAAAAGTT.....(highly repeated)
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FIGURE 2.—Genomic sequences flanking sites of three *Mos1* insertions. Two of the insertions are present in line 69 DNA, and the other insertion, corresponding to the *Mos1* element with an internal deletion, is present in line 17. The site of insertion at the TA dinucleotide is shown in bold.

copy insertion into heterochromatin of *D. virilis*. Furthermore, the proportion of adults bearing a *Mos1* insertion two to three generations following establishment of a line is uncertain, and, as stated previously, the signal strength of *Mos1* is expected to be about one-quarter as strong as a genomic single-copy sequence.

Approximately 8 months after the transformants were first isolated (eight generations), Southern hybridizations with the *Mos1* probe were repeated, and, this time, a faint band was visible in line 69 in an overnight exposure. By increasing the time of exposure to 120 hr, signals of differing intensity and size were visible in many, although not all, lines (Figure 3). In four of the samples, there was significant hybridization to uncut DNA migrating at limiting mobility near the top of the gel. Since the *D. virilis* DNA had been digested to completion with *Hin*dIII and *Bam*HI, and these enzymes do not cut within *Mos1*, each band in the hybridization represents a different *Mos1* element. Because some lines contain seven to eight elements, the *Mos1* copy number appears to increase with time. The hybridization control using *Adh* in Figure 3 also demonstrates that lanes that are negative with the *Mos1* probe after a 120-hr exposure contain approximately the same amount of DNA as the other lanes.

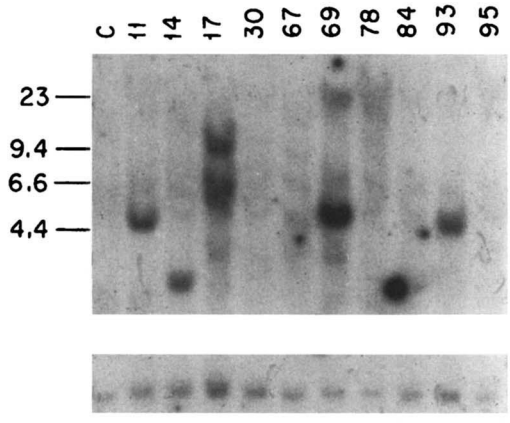

FIGURE 3.—Multiple copies of *Mos1* are present in different lines of transformants of *D. virilis*. Southern hybridization to DNA from 10 different lines (numbered 11–95) with a *Mos1* probe (top). Control DNA from the line used for transformation is in lane C. DNA samples were obtained from pools of ~100 flies and 5 μg of DNA were digested with *Hin*dIII plus *Bam*HI. The exposure was for 120 hr with a DuPont Quanta III intensifying screen. After probing with *Mos1*, the filter was stripped and reprobed with a PCR product from the *Adh* gene (bottom). Molecular weight markers in kb are shown at left.

The increase in copy number of *Mos1* with time, despite the absence of deliberate selection, was also evident in PCR experiments. One year after the initial *Mos1* transformation event, individuals from four lines were examined for *mariner* sequences by PCR. Almost 90% of the individuals in each of two lines contained *mariner* sequences, line 69 (n = 32) and line 84 (n = 15), supporting the Southern results of multiple copies of *Mos1*. However, the number of *Mos1* elements did not increase to high levels in all of the lines. In line 95, only 33% of the individuals were positive for *mariner* sequences (n = 15) and in line 14, 20% were positive (n = 15). It is interesting to compare these results with the Southern hybridization in Figure 3, which was carried out at roughly the same time as the PCR experiments. No clear bands were visible in DNA from line 84 in the Southern blot after a 120-hr exposure, despite the presence of at least one copy of *Mos1* in ~90% of the individuals, as determined by PCR. Although only 20% of the individuals in line 14 were positive for *Mos1* by PCR, a band of moderate intensity was visible (note, however, that this band was not visible after an overnight exposure that was sufficient to easily detect other single-copy sequences in the *D. virilis* genome). Apparently, the degree of overreplication of *Mos1* varies significantly in DNA from adults of lines 14 and 84 and depends on the genomic locations of *Mos1*. These differences illustrate that the strength of the signal in Southern hybridizations is a function of the genomic location of *Mos1* (such as euchromatic *vs.* heterochromatic) and the proportion of individuals in a population that carry the element, as well as copy number of the element within an individual.

***Mos1* insertions into highly repeated DNA sequences:** As described above, genomic DNA flanking three *Mos1* insertions was isolated by inverse PCR. To investigate this DNA further, primers were made to genomic DNA some distance from the insertion site on both sides, and a PCR probe for each insertion site was synthesized from total *D. virilis* DNA for use in Southern hybridization. A 163-bp probe from the 69S PCR product (69S refers to the shorter of two inverse PCR products from the *Mos1* 3′ end in line 69 DNA) hybridized strongly to a single band of 370 bp when genomic DNA was digested with any of six different enzymes, and a ladder of bands at multiples of 370 bp was visible in many of the lanes. A ladder of 11 monomers was visible in a *Sca*I digest, indicating tandem repetition of the 370-bp

repeat across >4 kb. Three other enzymes with a 4-bp recognition sequence digested much of the genomic DNA into small molecular weight fragments, as shown by ethidium bromide staining, but hybridization was principally to uncut DNA remaining near the top of the gel. Only two strong bands of hybridization were present in the *Hin*fI digest, and the sum of their sizes was ~370 bp. These patterns of hybridization are characteristic of complex satellite DNA sequences that have zero, one, or a small number of restriction enzyme sites in a short sequence repeated in tandem. The nucleotide sequences of the 5′ and 3′ inverse PCR products were compared and found to belong to the same 370-bp repeated sequence family. In this case, a *Mos1* element has integrated into a classical satellite DNA sequence, presumably in the heterochromatic region of the genome.

A search of the nucleic acid database with the 370-bp repeated sequence (Genbank accession number U37102) revealed an 80-bp homology to part of a dispersed, moderately repetitive element in *D. virilis* (ZELETSOVA *et al.* 1986). The homology is 90% over an 83-bp region that was identified as the terminal direct repeats of the element, but there was little homology of the 370-bp repeat to other regions of the element. The insertion site of *Mos1* is in a region of the 370-bp repeat distinct from this homology.

Southern hybridization with a PCR probe from genomic DNA flanking the deleted *Mos1* element in line 17 labeled a smear of DNA for all enzymes tested, showing that the *Mos1* element in this line is also located in highly repeated DNA sequences. In contrast to the locations of 69S and 17 insertions in highly repeated DNA sequences, the 69L probe hybridized to a single copy sequence.

Bacteriophage P1 clones of insertion sites: A bacteriophage P1 library of *D. virilis* DNA (LOZOVSKAYA *et al.* 1993) was screened for clones that corresponded to the region of the genome targeted by the *Mos1* insertion events, using probes from the genomic PCR product. The screening yielded a large number of clones for the 17 deletion and 69S insertions of *Mos1,* confirming their locations in highly repeated DNA sequences. Primers flanking the *Mos1* insertion in line 17 produced a 206-bp PCR product with total *D. virilis* DNA as a template. When the P1 clones that hybridized to the 206-bp fragment were analyzed by PCR with the same primer pair, a product of the correct size was obtained from P1 clone 85-94, suggesting that it contains a representative fragment of genomic DNA at the *Mos1* insertion site in line 17. None of the 12 P1 clones examined with primers that flanked the 69S insertion gave a band of the expected 163-bp size, despite strong hybridization of the same clones in the P1 library screen. However, one clone from inverse PCR of the 69S insertion contained three 370-bp repeats in tandem. The sequences of two of the repeats differed by ~10%, including a site at the 3′ nucleotide of one primer used for PCR. It is therefore possible that sequence divergence in the region corresponding to a primer could explain the absence of a PCR product from the P1 clones. Using genomic DNA as template, a PCR product of the expected length was visible after only 15 cycles, indicating a high copy number of 370-bp repeats.

Chromosome locations of *Mos1* insertions: The locations of *Mos1* insertions in polytene chromosomes were mapped by *in situ* hybridization using *Mos1* as a probe. Initially, no signal was observed in experiments using larvae from several different lines, even though both parents of the larvae were positive for *mariner* sequences by PCR. *In situ* hybridizations using *Mos1* as a probe were repeated ~1 year after the original transformation event. In contrast to the lack of hybridization signal in the initial experiments, hybridization was observed to multiple euchromatic sites in some lines, although not in others. In one cell from a larva in line 17, four sites of hybridization were observed, including one site within or close to the chromocenter (Figure 4, a–d). Moreover, many of the sites were polymorphic, suggesting that *Mos1* is mobile in these lines.

To localize the site of the deleted *Mos1* element in line 17, the P1 clone 85-94, which includes the target site of integration of the deleted element, was used as a probe for *in situ* hybridization. Strong hybridization was observed to a single major site in euchromatin (Figure 4e) and faintly to other bands. The single site of hybridization is the presumed location of the deleted *Mos1* element because the signal extends over two bands, typical of a P1 clone with an insertion of 75–100 kb. Surprisingly, when larger amounts of labeled P1 DNA were applied to the slide, >200 sites of hybridization were observed (Figure 4f). The multiple sites of hybridization probably result from one or more classes of transposable elements present in the P1 clone. These results are in accord with the Southern hybridization, which showed that the sequences immediately flanking the *Mos1* element in line 17 are highly repeated in the genome. In this case, the repeated sequences appear to correspond to a transposable element that is present in high copy number in euchromatin.

Although no P1 clones corresponding to the exact 69S insertion site of *Mos1* were isolated, clone 54-66 hybridized strongly to a PCR probe to the 370-bp satellite in the P1 library screen. In polytene chromosomes, the P1 clone labeled the tips of the five major chromosome arms and a band close to one of the tips (Figure 4g). The labeling pattern was unusual in some telomeres and appeared as a speckled region, suggesting a location at the extreme terminus. The telomeres of Drosophila chromosomes are considered to be heterochromatic (PARDUE 1994), and the telomeres of *D. melanogaster* contain tandem copies of repeats, including the HeT-A (BIESSMANN *et al.* 1994; DANILEVSKAYA *et al.* 1994) and TART transposable elements (LEVIS *et al.* 1993).

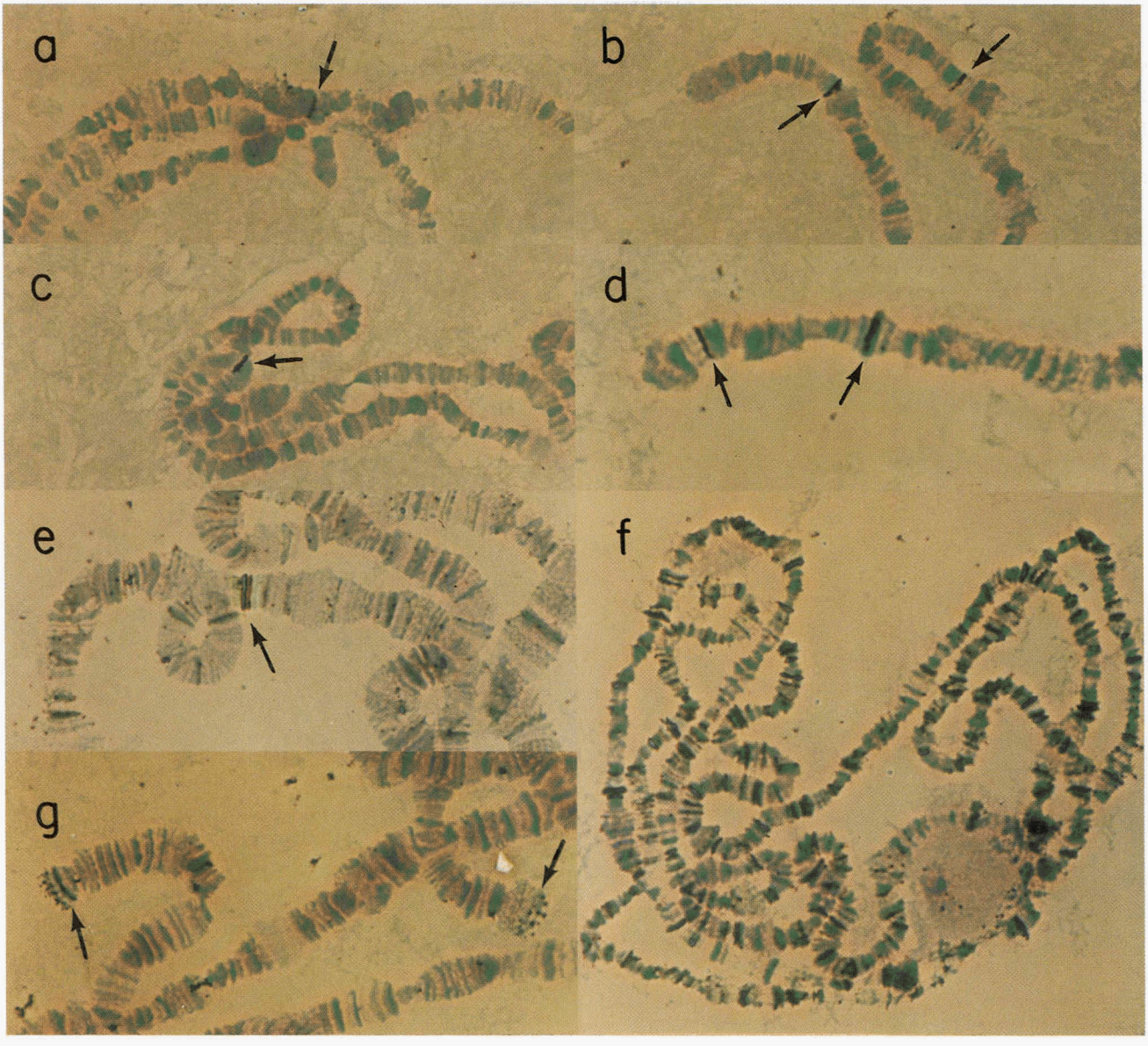

FIGURE 4.—*In situ* hybridization to polytene chromosomes of *D. virilis*. (a–d) Hybridization of a biotinylated *Mos1* probe to polytene chromosomes from line 17, showing the locations of *Mos1* copies (arrow). a–c are from the same cell and show four different locations of *Mos1*. (d) Polymorphism in *Mos1* locations. This chromosome contains two sites of *Mos1*, one site near the tip of the chromosome being the same as in b. (e) Hybridization of P1 clone 85-94 to a euchromatic site. This clone contains the site of insertion of the deleted *Mos1* element in line 17. Note the labeling over two bands that is characteristic of P1 hybridizations and the faint labeling of several other bands. (f) Hybridization of P1 clone 85-94, but more DNA was applied to the slide compared with e. There are >200 sites of hybridization in the euchromatin. (g) Hybridization of P1 clone 54-66 to the telomeres of two chromosomes.

Location of a *Mos1* insertion at the terminus of a chromosome would be difficult to detect if telomeric DNA sequences are underreplicated. It is noteworthy that although the 370-bp repeats share some homology with a transposable element in *D. virilis*, they are not homologous to transposable elements at the telomeres of *D. melanogaster*. Telomeres in *D. melanogaster* are unusual because they lack telomerase repeats (PARDUE 1994). Hence, the location of 370-bp repeats at or near the telomeres of *D. virilis* may indicate that the sequence of at least some repetitive elements near the telomeres is not conserved in different species of Drosophila.

Underrepresentation of *Mos1* DNA: The *in situ* hybridization and DNA sequencing results described above suggest two explanations for the variability in Southern hybridizations with a *Mos1* probe. First, at least the 69S element and one of several elements in line 17 are located in heterochromatin. Since DNA in heterochromatin is highly underrepresented in DNA preparations from adults, a heterochromatic insertion of *Mos1* could explain the absence of a signal in Southern blots and *in situ* hybridizations. Subsequent mobilization of *Mos1* to euchromatin would result in detection by both hybridization methods. Each of 10 lines be-

haved similarly in showing a lack of signal in the initial Southern and *in situ* hybridizations. If the original transformation events occurred in heterochromatin, there could have been as few as one heterochromatic insertion that was replicated because the lines do not necessarily represent independent events. Second, to prepare large amounts of purified DNA for extensive Southern hybridizations, DNA was made from pools of 100–200 flies approximately eight generations after injection of the embryos. Mobilization of *Mos1* from its presumptive initial heterochromatic location in G_1 flies to new chromosome locations would lead to additional bands in Southern hybridizations, but the intensity of hybridization would depend on the proportion of flies carrying a given *Mos1* insertion. For example, a jump of *Mos1* to a different euchromatic location in generations six or seven would be present in only a small number of adults in a pool of 100–200 flies at generation eight.

DISCUSSION

The apparent ease of transformation of *D. melanogaster* by the *P* element, as well as by other transposable elements such as *hobo* and *Minos*, has reinforced the impression that transformation of species of insects that are distantly related to *D. melanogaster* should be a simple matter. However, transformation of other insects by transposable elements, even by an element lacking exogenous DNA, may not be trivial. There has been only one reported case of transformation of a distant relative of *D. melanogaster* by the *P* element (BRENNAN *et al.* 1984), and the genetic instability of the transformed lines was markedly different from that expected from *P*-element behavior in *D. melanogaster*. It is entirely possible that the laboratory strains of *D. melanogaster* used routinely in transformation are not generally representative of either naturally occurring *D. melanogaster* or of other Drosophila species. *D. melanogaster* has been extensively inbred since Sturtevant's time and, in most laboratories, strains of *D. melanogaster* used for transformation are carefully chosen, following personal communication from other researchers that the strain works well. As molecular and evolutionary studies extend to species of Drosophila other than *D. melanogaster*, it will be important to enhance their utility by opening up the possibility of reciprocal transformation experiments.

One frequently contemplated strategy for germline transformation of a target species entails the use of a vector developed from a transposable element isolated from the target species itself. The rationale for this approach is that the presence of the element in the target species is an indication that the species supplies whatever host factors may be necessary to support transposition. There are other reasons for thinking that this strategy might not be optimal, however. Some transposable elements, such as the *P* element, promote their own regulation either through the production of a repressing "cytotype" in the oocyte, through the production of defective transposase proteins from deleted or mutated elements, or through titration of functional transposase by the ends of numerous copies of the element present in the host genome (reviewed in LOZOVSKAYA *et al.* 1995). Based on the properties of certain missense mutations in the *Mos1* transposase, we have suggested that *mariner* might be regulated in part through dominant-negative effects of transposase mutations (LOHE and HARTL 1996). Inducible host genes that mediate repression or silencing may also contribute to the regulation of transposition. Whatever the particular mechanism, if an endogenous transposable element is subject to repression, then it may be difficult to develop a transformation system based on that element. In as much as the mechanisms of genomic regulation of MLEs are not fully understood, it seemed wise in the initial experiments to use a host genome in which *mariner* elements closely related to *Mos1* are not present.

This reasoning helped motivate the choice of *D. virilis* for examining the transformation potential of *Mos1* outside the *melanogaster* species subgroup to which *D. mauritiana* belongs. The species *D. mauritiana* and *D. virilis* are in different subgenera of the genus Drosophila and shared a common ancestor ~40 million years ago (RUSSO *et al.* 1995). We have demonstrated that *Mos1* can transform *D. virilis* and estimate that the transformation efficiency is $\geq 4\%$. The true value may be higher because the G_0 adults were pooled and a transformation event may well have occurred in more than one G_0 individual contributing to a positive DNA pool. Given that injection into the posterior region of an embryo often leads to sterility, it is also unlikely that all G_0 adults were fertile. In retrospect, it would have been more informative to backcross G_0 adults individually to better estimate the efficiency of transformation; however, the observed value of $\geq 4\%$ was much higher than expected.

Our strategy in using PCR to detect transformants, rather than employing a selectable marker, may have been important in the success of the experiment. Screening by PCR is both sensitive and rapid and does not rely on expression of a reporter gene in a foreign host. In particular, PCR will also detect insertions into regions of the genome that suppress expression of a reporter gene or that are underrepresented in adult DNA, such as insertions into heterochromatin. It will now be possible to study *mariner* transformation of *D. virilis* with confidence to determine the frequency of integration of *Mos1*, the frequency of integration of a *Mos1* vector carrying a marker gene, the ability of the marker gene to be expressed, the rate of mobilization of a *Mos1* vector carrying exogenous DNA in the presence of transposase, and its stability in the absence of transposase. Our results also suggest that in transformation experiments with insects that are distantly related to *D. melanogaster* it may

be helpful to carry out PCR screening in conjunction with selection for a reporter gene.

Chromosomal integration of *Mos1* in *D. virilis* is accompanied by duplication of the TA target site and transfer of DNA sequences bounded only by the inverted repeats, as expected if the integration is a genuine transposition event mediated by *mariner* transposase. The integrated *Mos1* element is active, and a spontaneous 290-bp deletion was recovered in the G_2 generation of line 17. The position and size of the deletion in line 17 are similar to other internal deletions induced by transposase on a *mariner* target element in *D. melanogaster* (A. LOHE, C. TIMMONS and D. HARTL, unpublished results). PCR amplifications of DNA from five of the 15 pools of flies from the G_1 generation were positive with some primer pairs but negative with others, which provides additional evidence for *Mos1* deletions occurring soon after the initial transformation event. In the absence of selection, it was expected that the proportion of individuals carrying the *Mos1* element in a line might decrease with time and that the element might ultimately be lost by random genetic drift. However, as would be expected from an active transposable element, the number of *Mos1* copies increased with the number of generations.

Initial Southern and *in situ* hybridizations using *Mos1* as a probe were negative for each of 10 lines, despite the lines being positive for *mariner* sequences by PCR amplification. In later generations, however, a positive signal was obtained in some of the lines by Southern and *in situ* hybridizations. We propose that the initial *Mos1* insertions occurred in heterochromatin, which is underreplicated in DNA from adults used in Southern hybridizations and underrepresented in the chromocenter of polytene chromosomes. Indeed, there is evidence for at least two *Mos1* insertions into heterochromatin because the 69S element is flanked by a 370-bp tandemly repeated sequence, and another element in line 17 is located in the chromocenter of polytene chromosomes (Figure 4a). It is not known whether either insertion represents a primary transformation event. However, 10 out of 10 lines failed to hybridize to a *Mos1* probe in the initial Southern blots. Insertion of *Mos1* exclusively into heterochromatin of *D. virilis* in G_0 individuals seems unlikely, because the element is capable of inserting into euchromatin (Figure 4, b–d). Since different lines do not necessarily represent independent insertion events, the result is more consistent with a heterochromatic insertion of *Mos1* in the germline of one or a small number of G_0 individuals. Therefore, each of the G_2 adults used to initiate the 10 lines may have contained one or more heterochromatic insertions in common, and these were subsequently mobilized spontaneously to different euchromatic and heterochromatic sites in later generations.

Insertion of *Mos1* into heterochromatin may also explain why a signal was not visible by Southern hybridization in some lines after eight generations (Figure 3). Although heterochromatic insertion of *Mos1* is likely to result in poor expression of the transposase gene because of the suppressive effects of heterochromatin on neighboring euchromatic sequences (reviewed in LOHE and HILLIKER 1995), heterochromatic suppression of genes that are normally present in euchromatin is not necessarily absolute. An example is the variegated phenotype of euchromatic genes when subjected to classical heterochromatin-induced position effect. Hence, an active transposable element need not necessarily be "trapped" in heterochromatin, even if the initial insertion is into heterochromatin.

Insertions of the *P* element into heterochromatin of *D. melanogaster* are rare but can be detected in a genetic background with two *Y* chromosomes that ameliorate the suppressing effects of heterochromatin (ZHANG and SPRADLING 1994). About 50% of the genome of *D. virilis* is heterochromatic (GALL 1973) as compared with ~33% in the *D. melanogaster* genome. This suggests that transpositions into heterochromatin in *D. virilis* may be more frequent than in *D. melanogaster,* a relation that may apply to other insects with a large proportion of their genome represented by heterochromatin. The ability of *Mos1* to increase its copy number rapidly when integrated into the genome is a property that could be used advantageously to drive a gene into a natural population, a prerequisite of some proposals for genetic engineering of insects of economic or agricultural importance. Increase in copy number of a suitably engineered *mariner* transposon in this manner, following its release into the wild, would be appropriate only if the source of transposase could be tightly regulated.

The evolutionary separation between *D. melanogaster* and *D. virilis* has made *D. virilis* useful as a reference species for the identification of conserved features of genes. For example, *P*-element transformation of the *bride of sevenless, hunchback, exuperantia* and *nanos* genes from *D. virilis* rescues the corresponding mutations in *D. melanogaster* (HART *et al.* 1993; LUK *et al.* 1994; LUKOWITZ *et al.* 1994; CURTIS *et al.* 1995). The complementation demonstrates good conservation of both protein function and the regulatory sequences controlling expression, despite considerable sequence divergence in some parts of the genes. On the other hand, the *D. virilis* orthologue of *oskar* rescues body patterning in *D. melanogaster* mutants, but pole-cell formation is impaired and a dominant maternal-effect lethality is induced in a wild-type background (WEBSTER *et al.* 1994). To further investigate this and other unusual phenotypes, it will be important to carry out the reciprocal transformation experiments for which the *mariner* transformation system as well as *hobo* are available (LOZOVSKAYA *et al.* 1996). The successful transformation of *D. virilis* by *Mos1* suggests that *Mos1* should be considered for use in germline transformation of nondrosophilids.

This work was supported by U.S. Public Health Service Grant GM-33741.

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Communicating editor: R. S. HAWLEY