44. *Single P-element Insertional Mutagenesis in Drosophila melanogaster*

By Motojiro YOSHIHARA, Etsuko TAKASU-ISHIKAWA, Yutaka SAKAI,
Yoshiki HOTTA, and Hitoshi OKAMOTO

Department of Physics, Faculty of Science,
University of Tokyo, Hongo, Tokyo 113

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Introduction. P-M hybrid dysgenesis in *Drosophila melanogaster* has been used for transposon-tagging mutagenesis.¹⁾ Once a gene of interest is mutated by a P-element insertion, the gene can be cloned with ease by using P-element DNA as a probe. The original method, however, has some inherent difficulties for producing a stable single-site mutation. When P strain males with many P-elements in the genome are crossed to M-cytype females, multiple P-elements are mobilized simultaneously. Furthermore, the transposed P-elements tend to undergo further transpositions. Recently, molecular basis of the P-M hybrid dysgenesis has been extensively worked out. The transposon, P-element, consists of a transposase gene surrounded by two terminal repeats.¹⁾ While the transposase is *trans*-acting, the terminal repeats are necessary for its own transposition.

We have developed a new insertional mutagenesis system to overcome the difficulties discussed above. In this system, a single copy of P-element transposes, and the transposed element can be stabilized by removing the source of transposase. Our system is very useful for transposon tagging,²⁾ since the mutated loci can be easily cloned by a plasmid rescue method.³⁾ Cooley *et al.*⁴⁾ have recently developed a similar system. The advantages of our system compared to theirs are the high frequency of transposition and easy removal of the transposase gene which is readily confirmed by a change in eye color of the offspring bearing the P-element insertion.

Materials and methods. Fly strains, w, C(1)DX,y w f/FM6,y^{31d} sc⁸ dm B (hereafter designated as C(1)DX), SM1,al² Cy cn² sp²/Pm (hereafter designated as Cy/Pm) were from our stock collection. All these strains have M cytype after repetitive backcrosses to the Canton-S strain for many generations. All the experiments were carried out at 25°C without heat shock.

The plasmid p π 25.7wc⁵⁾ was digested completely with HindIII and partially with SalI. 1.9 Kb HindIII-SalI fragment containing 3' two thirds of the transposase gene was recovered. The plasmid pCN4123⁶⁾ was digested completely with BamHI and partially with HindIII. 2.3 Kb BamHI-HindIII fragment containing hsp70 promoter and 5' one third of the transposase gene was recovered. Both fragments were ligated with pW8⁷⁾ digested with XhoI-BamHI to create pP[hsP, hsW⁺] (Fig. 1(a)).

The second plasmid, pUCHsneo (Fig. 1(b)), used in our transformation experiments is a gift from Drs. H. Steller and V. Pirrotta,⁸⁾ which contains pUC8 sequence for plasmid rescue method and neo^R gene under the control of hsp70 gene promoter within the P terminal repeats.

Germ-line transformations were carried out by the standard method as de-

Fig. 1. (a) Structure of the pP[hsP, hsW⁺]. The vector has a transposase gene under the control of the hsp70 gene promoters to enhance its expression.¹³⁾ It has also a white gene as a marker for selecting and maintaining the transformant lines. Major restriction sites are indicated as S:SalI, P:PstI, H:HindIII, K:KpnI, E:EcoRI, B:BamHI. (b) The pUCHsneo vector from Steller and Pirrotta.⁸⁾ The vector has a pUC8 sequence for plasmid rescue and a neo^R gene as a selective marker under the control of a hsp70 gene promoter. These are incorporated to make easier the gene cloning to follow the mutagenesis. The restriction sites of XhoI and EcoRI are indicated as X and E, respectively.

scribed by Rubin and Spradling.⁹⁾ A strain w (white eye) was used as a recipient. Embryos were injected with 100 μ g/ml of p π 25.7wc and 500 μ g/ml of pP[hsP, hsW⁺] or pUCHsneo. To select transformed progeny with pUCHsneo, flies were reared on medium containing 1 mg/ml G418.

DNA blot analysis was carried out by the standard method as described by Southern.¹⁰⁾ The probe was ³²P-labeled by hexanucleotide priming.¹¹⁾

In situ hybridization to salivary gland chromosome was performed using biotin label as described by Engels *et al.*¹²⁾ pUC18 was used as a probe to detect presence of the P-element derived from pUCHsneo.

Results. We constructed the plasmid pP[hsP, hsW⁺] (Fig. 1(a)) as described in Materials and methods.

We have injected embryos of the w strain with pP[hsP, hsW⁺] and obtained three transformant strains with a single P-element inserted on autosomes. They were named AP1, AP2 and AP3, respectively, and maintained them for further analyses by crossing the males to virgin C(1)DX females with M cytotype. The P-element insertions in AP1 and AP2 are on the second chromosome, while in AP3 is on the third chromosome. Eye color of the transformants varied from pale orange to deep red, depending on the position of the integrations. The insertions of pP[hsP, hsW⁺] in AP2 and AP3 are stable for generations. Changes in eye color of the transformant flies were rarely observed.

Embryos of the w strain were also transformed with pUCHsneo, and three X-linked transformant strains were obtained. The location of the insertions were determined by *in situ* hybridization (see Table I). We named them as Xneo1, Xneo2, and Xneo3, respectively.

Fig. 2 shows the mating scheme performed for mutagenesis. Male Xneo flies were mated with virgin AP females in mass to introduce a pUCHsneo and a transposase gene into the germ-line of F1 individuals. Each F1 male with red eye color was selected and individually mated to w females. The F1 male necessarily had the non-autonomous element on its X-chromosome inherited from father, since its mother had Y-chromosome and C(1)DX (attached-X) chromosome. F2 flies were raised on 1 mg/ml G418 medium without heat shock to see if males survived. Each F2 male survivor is expected to carry a transposed non-

Table I. Transposition of pUChsneo induced by P[hsP, hsW⁺]

	Xneo1(1A)	Xneo2(6C-F)	Xneo3(3C)
AP1	0/115(6)	3/1237(5)	2/ 238(6)
AP2	6/102(6)	11/ 956(5)	442/ 956(41)
AP3	1/154(6)	0/ 740(6)	396/2105(136)
C(1)DX	0/138(5)	3/1147(6)	2/ 377(9)

Values for males/females resistant to neomycin are shown for each combination. Numbers in the parentheses after strain names indicate the cytological localization of the original inserts. Numbers in the parentheses after the ratios indicate the number of single male crosses.

Fig. 2. Mutagenesis with Xneo strains and AP strains. Male survivors of F2 have a transposed pUChsneo on autosome.

autonomous P-element on its autosome.

The number of males and females that survived on G418 medium was counted (Table I). The ratio of males/females is a measure of the frequency of transposition from X to autosomes. The combinations of AP2 or AP3 and Xneo3 gave high frequency transposition (about 20 to 45%). Thus we chose these combinations in the following experiments.

XhoI digest of the genomic DNA of F2's offspring was compared with one another by DNA blot hybridization with pUC18, which has the same sequence with pUC8 without polylinker, as a probe. The experiments confirmed that the non-autonomous P-element had really transposed in F2 males. The results are shown in Fig. 3. Since a single XhoI recognition site exists in the non-autonomous P-element from pUChsneo, the number of genomic fragments thus detected by the probe corresponds to the number of integration sites. In the transposed lines, fragments containing the non-autonomous P-element were detected at positions different from that in the original Xneo3 strain (Fig. 3, lane 8). To examine whether a single F1 male could produce multiple inserts of the non-autonomous P-element at different positions in the genome of germ line cells, genomes of seven white⁻ neo^R males (F2) derived from a single F1 male were compared by DNA blot analyses. In the case of F1 between Xneo3 and AP3, the same single fragment was detected in all the F2 strains (Fig. 3(b), lanes 1-7). This suggests that the transpositional event occurred once in the combination of Xneo3 and AP3. In the case of F1 progeny between Xneo3 and AP2, on the other hand, F2 derived from a single F1 male has different hybridizing fragment patterns from individual to individual (Fig 3(a), lanes 1-7). This revealed that multiple insertions of the non-autonomous element must have taken place in the combination of Xneo3 and AP2.

Above results were confirmed by an independent experiment using another enzyme, EcoRI (data not shown). The number and pattern of hybridizing fragments were the same as in Fig. 3. We also found some transpositional events to autosomes by *in situ* hybridization in several other cases (data not shown).

The results indicate that the combination of Xneo3 and AP3 is suitable for

Fig. 3. DNA blot hybridization of genomes containing transposed non-autonomous element in the case of Xneo3-AP2(a) and Xneo3-AP3(b). DNA from the 50 sons from the cross between white⁻ neo^R F2 male and normal(Canton-S) virgin females was digested with XhoI, separated on a 0.6% agarose gel, and blotted. The blot was probed with ³²P-labeled pUC18 DNA. Under the condition, a single band is expected from each integrated pUChsneo. The size of that fragment depends on the distance from the integrated site to the nearest XhoI site in the flanking *Drosophila* DNA. Lanes 1–7: F2 progeny derived from a single F1 male. Lane 8: males of Xneo3. Lanes 9–15: F2 progeny derived from different F1 males. Several non-autonomous elements are contained in each lane in (a), while a single element is detected in each lane in (b). These results indicate that transposition in the germ-line occurred once in (b), but, many times in (a). Molecular weights are shown at the left side.

producing a single P-element insert. Consequently, attempts were made to isolate insertional mutant strains, using this combination.

Each independently mutated F2 fly with white eyes was individually mated to Cy/Pm virgin females, and F3 flies were reared on G418 medium. Survived Cy F3 was back-crossed to Canton-S and the offspring was reared on G418 food to examine whether insertion was on the 2nd chromosome. Second-chromosomal mutants in F3 progeny derived from the same F2 male were further mated among themselves to establish homozygous lines.

Of the 53 strains thus tested, 24 were found to be linked to the second chromosome. Among them, 2 were found to carry recessive lethal mutations.

Discussion. Our results indicate that the ratio of transposition depends on the combination of the two strains to be mated. In the combinations other than Xneo3-AP2 or Xneo3-AP3, the ratios were low (Table I), probably because level of transposase expression is low in AP1, or Xneo3 strain might have the integrated non-autonomous P element at a suitable site for excision.

Robertson *et al.*¹³⁾ also devised a similar system for mutagenesis, and proposed that the deletion of the 2–3 intron is required for a high frequency of transposition. However, our experiments demonstrated that the transposition frequency can be high by using the transposase gene with all the introns. The higher frequency of transposition of our system may be due to the use of hsp70 promoter, or to the selection of suitable combinations of the non-autonomous P-element and transposase gene integrated into proper positions.

The frequency of transposition was very high in the combination of Xneo3-AP2, and many insertions took place during the development of the F1 male. This combination may be useful for efficient screening of new alleles of an already identified locus. Once an allelic mutant is obtained, other additional insertions,

if any, can be removed by chromosomal segregations and recombinations.

For tagging previously unknown loci, a mutation induced by a single P element insertion is preferred, so that the combination of Xneo3-AP3 should be chosen. The transposition frequency of the non-autonomous P-element is sufficiently high and a single copy is inserted in most cases (Table I and Fig. 3(b)). Cooley *et al.*⁴⁾ developed a similar mutagenesis system with a relatively lower transposition frequency. They reported that 40% of the F1 males containing both a non-autonomous element and a transposase gene yielded useful insertions within their progeny. In our system, most, if not all, of the F1 males produced transposition with a single autosomal insertion. Once the non-autonomous P-element is inserted to mutate a locus, the DNA surrounding the element can be easily cloned by the plasmid rescue method. This is possible because, when the genomic DNA of mutant flies is digested with appropriate enzymes and self-ligated, only the fragment with the pUC8 sequence can replicate in *E. coli* and the flanking gene responsible for the mutation can be cloned.

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