Mol. Cells, Vol. 10, No. 1, pp. 61–64

Molecules and Cells

Isolation of True *Rbp9* Null Alleles by Imprecise P Element Excisions

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(Received on October 2, 1999)

The P element has been widely used as a mutagen because of its convenience in locating the site of mutagenesis. However, P element-induced mutations often result in varied mutant phenotypes, making it difficult to identify the null phenotype. Previously, three *Rbp9* alleles were isolated using P element mutagenesis. Although the coding regions of *Rbp9* were disrupted by P elements in all three cases, they showed different degrees of defects. In order to characterize the null phenotype of *Rbp9*, *Rbp9* alleles with chromosomal deletions were created by inducing imprecise excisions of the P elements. All *Rbp9* alleles generated from imprecise excisions showed the same mutant phenotype: female flies were sterile and cystocyte differentiation was blocked. This result reveals that the primary function of *Rbp9* resides in the regulation of cystocyte differentiation. In addition, this result shows that a P element does not always completely inactivate gene activity, even when it is incorporated into the coding region.

Keywords: *Drosophila*; Imprecise Excision; Null Allele; P Element; *Rbp9*.

Introduction

To generate mutations in the *Drosophila* genome, transposons are often used as mutagens. The P element has been widely used (Engels, 1983; Tsubota and Schedl, 1986) because it provides easy tools for locating the site of the mutation and recovering the neighboring DNA. Previously, we used P element (Torok, 1993) mutagenesis to create mutations in the coding region of *Rbp9* (Kim-Ha *et al*., 1999). *Rbp9* encodes a putative RNA binding protein (Kim and Baker, 1993). Its nearest homologs are *Drosophila elav* (Robinow and White, 1991) and the human Hu gene family (Levin *et al*., 1993; Ma *et al*., 1996; Szabo *et al*., 1991), which are involved in neurogenesis. We previously reported that three *Rbp9* alleles contained P elements inserted into the coding regions of the *Rbp9* genome (Kim-Ha *et al*., 1999). Phenotypic analysis revealed that the P element mutations in *Rbp9* affected female sterility, not neurogenesis. Moreover, this female sterility phenotype varied among different alleles, even though the P element had been inserted into the coding region in all three cases. As no RBP9 protein was detected in these mutants, the phenotypic variation could result from variation in some residual RBP9 protein, not detectable by western blotting. This residual activity could potentially occur if the P element were spliced out of a small fraction of the transcripts. If this were the case, the deletion of some genomic regions of *Rbp9* by imprecise excision of the P element would result in the most severe mutant phenotype. To test this, I induced imprecise P element excisions in two *Rbp9* alleles. I chose the $Rbp9^{P[2775]}$ and $Rbp9^{P[2398]}$ strains because they showed different degrees of female sterility phenotypes. In both cases, fly strains from precise excision restored wild-type female fertility, while strains from imprecise excision showed 100% female sterility. These results suggest that some functional transcripts can be made despite the incorporation of P elements into the coding regions.

Materials and Methods

Fly stocks The *Rbp9* alleles used in this study are described in Kim-Ha *et al*. (1999). The *w*/*w*; *Sp*/*CyO* strain was created by crosses between w^{1118} and WR13S stocks (Lindsley and Zimm, 1992).

Southern hybridization Genomic DNA was isolated from 30 adult flies each of homozygous $Rbp9^{P[2775]}$ or excision lines $Rbp9^{\Delta 1}$ through $Rbp9^{\Delta 3}$, and $Rbp9^{\Delta 5}$. Half of this quantity of each DNA was digested with *Hin*dIII and blotted on a nitrocellulose membrane as described in Lee *et al*. (1998). The blotted

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nitrocellulose membrane was hybridized with a ^{32}P-labeled probe prepared from the genomic DNA fragment encompassing the entire *Rbp9* locus (the region from 3.4 kb upstream of the first transcriptional initiation site to nucleotide position 11347). Equivalent blots were probed with smaller *Hin*dIII-digested genomic DNA fragments corresponding to nucleotide positions 1854 to 3815, 3815 to 6250, and 6250 to 9823.

Immunoblot analysis Immunoblot analysis was performed as described by Park (1999).

Nuclear staining of ovaries Ovaries were dissected in 150 mM NaCl and fixed with 4% paraformaldehyde. After washing with 150 mM NaCl, they were incubated with 1μM Syto16 in 150 mM NaCl for 20 min. Images were analyzed by confocal microscopy (Bio-Rad).

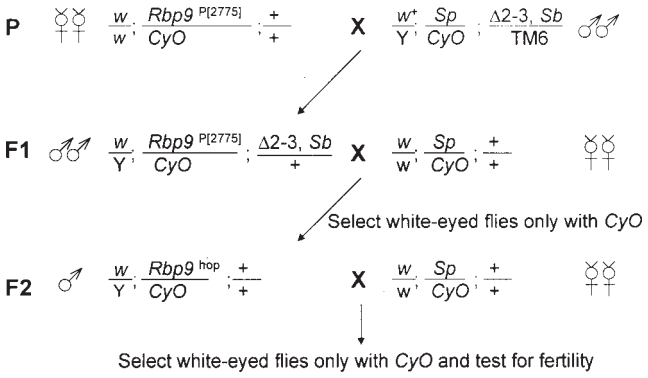

Fig. 1. Genetic scheme used to induce P element excision. The crosses are described in the text. The strains used in these crosses were described by Kim-Ha *et al*. (1999).

Results and Discussion

Mobilization of P element to induce imprecise excision The genetic scheme used to remobilize the P element from the *Rbp9*$^{P[2775]}$ strain is shown in Fig. 1. Five bottles were set up, each containing 20 males carrying transposase activity on their third chromosome (Δ2-3, *Sb*; Robertson *et al*., 1988) and 20 *Rbp9*$^{P[2775]}$ heterozygous P element females (P). Flies were transferred to new bottles every day. When the total number of transferred bottles reached 20, the adult flies were discarded. Males that carried both the *Rbp9*$^{P[2775]}$ chromosome and the transposase-containing chromosome were recovered from this cross. Thirty to 40 males of this genotype were crossed to 20 females carrying both the *Sp* marker and the *CyO* balancer (F1). Thirty-five bottles were set up for F1 cross. Among the F1 progenies that had the *CyO* genotype, but not the *Sb* marker, 4% of the flies had the white-eyed phenotype. Fifty-six males with white-eyes and the *CyO* balancers, but not the *Sb* marker, were selected to isolate the chromosomes that lost the P element (*Rbp9*^{hop}, Fig. 1). Individual males with this genotype were crossed to three females carrying both the *Sp* marker and the *CyO* balancer (F2). Among the F2 progeny, white-eyed males and females with the *CyO* balancer were selected to create stock lines. Homozygotes of these lines, which carried the chromosomes with either precise or imprecise excision of the P element, were used in further analysis.

Remobilization of the P element from the *Rbp9*$^{P[2398]}$ strain was also performed, following the same genetic scheme used for the *Rbp9*$^{P[2775]}$ strain. Forty bottles were set up for F1 cross and 34 P element excised lines were obtained. The frequency of the flies carrying P element excised chromosomes among the F1 progeny was 2%. The lower frequency seem to be due to the presence of two P elements in the *Rbp9*$^{P[2398]}$ strain, whereas there is only one in the *Rbp9*$^{P[2775]}$ strain.

Female sterility in the P element excision lines P element mutations in *Rbp9* coding regions caused female-specific sterility (Kim-Ha *et al*., 1999). Therefore, precise P element excision from them should rescue the sterility phenotype. Individuals homozygous for the P element excisions were tested for fertility. Among these, complete reversion of the mutant phenotype occurred in only five of the 56 excision lines generated from *Rbp9*$^{P[2775]}$, and only seven of the 34 strains derived from *Rbp9*$^{P[2398]}$. However, the sterility phenotype of the infertile excision strains was more severe than that of the *Rbp9*$^{P[2775]}$ and *Rbp9*$^{P[2398]}$ strains; in fact, no adult flies eclosed. It was previously reported that the number of progeny eclosed from *Rbp9*$^{P[2775]}$ and *Rbp9*$^{P[2398]}$ homozygous parents were 3% and 23% of the wild type, respectively. The complete sterility phenotype seems to result from imprecise excisions, causing a deletion of genetic information in the *Rbp9* coding region or neighboring regions. The high frequency of this mutant phenotype indicates that most of the excision events were imprecise.

Southern and Western analysis of P element excision lines Analysis of the female-sterile P element excision lines revealed that, for an as yet unknown reason, imprecise excision occurred more frequently at the *Rbp9* locus than at most other loci. To characterize the chromosomal alterations caused by the imprecise excisions of the P element from the original *Rbp9* mutants, four excision lines that did not rescue the sterility phenotype (*Rbp9*^{Δ1} to *Rbp9*^{Δ3}, and *Rbp9*^{Δ5}) were examined; all four originated from the *Rbp9*$^{P[2775]}$ strain. Genomic DNAs from *Rbp9*$^{P[2775]}$ and from the four excision lines were digested with *Hin*dIII and analyzed by Southern hybridization using a probe prepared from genomic DNA encompassing the entire *Rbp9* locus (Fig. 2). Wild-type and *Rbp9*$^{P[2775]}$ DNAs were first compared for *Hin*dIII digestion pattern (Fig. 2B, lanes 1 and 2). Surprisingly, the *Hin*dIII digestion pattern was already altered in the *Rbp9*$^{P[2775]}$ strain, not only for the DNA fragment that

contained the P element, but also for other regions of *Rbp9*$^{P[2775]}$ chromosome. To determine the identity of each fragment, the same blot was hybridized with probes prepared from the small *Hin*dIII-digested genomic DNA fragments corresponding to the nucleotide positions 1854 to 3815, 3815 to 6250, and 6250 to 9823 (data not shown). This analysis revealed that the alteration occurred in the 1.96 and 2.43 kb DNA fragments where the P element resided originally in parental l(2)K12901 and *Rbp9*$^{P[1374]}$ strains, respectively (Fig. 2A). As has been previously described (Kim-Ha *et al*., 1999), the P element was mobilized from l(2)K12901 to the *Rbp9*$^{P[1374]}$ strain, and then to the *Rbp9*$^{P[2775]}$ strain. Therefore, the alteration in the 1.96 and 2.43 kb DNA fragments suggests that imprecise excisions occurred in both P element mobilization events.

None of the four P element excision lines restored the 3.57-kb DNA fragment that would indicate precise excision of the P element. On the contrary, large deletions were detected in *Rbp9*$^{\Delta1}$, *Rbp9*$^{\Delta2}$ and *Rbp9*$^{\Delta3}$ strains. Only in *Rbp9*$^{\Delta5}$ was there no detectable deletion. This analysis revealed that imprecise excision of the P element removed various amounts of chromosomal DNA, from a small undetectable deletion, to two to more kilobases (Fig. 2B). To further confirm the nature of the excisions, we tested the production of *Rbp9* protein in homozygous flies from four imprecise (*Rbp9*$^{\Delta1}$ to *Rbp9*$^{\Delta3}$, and *Rbp9*$^{\Delta5}$) and two precise (*Rbp9*$^{\Delta56}$ and *Rbp9*$^{\Delta64}$) excision lines. As expected, *Rbp9* protein was produced in the precise excision lines, while none was detected in the imprecise excision lines (Fig. 3A). Two imprecise (*Rbp9*$^{\Delta105}$ to *Rbp9*$^{\Delta106}$) and one precise (*Rbp9*$^{\Delta123}$) excision lines from the *Rbp9*$^{P[2398]}$ strain were also tested, and they showed similar results (Fig. 3B).

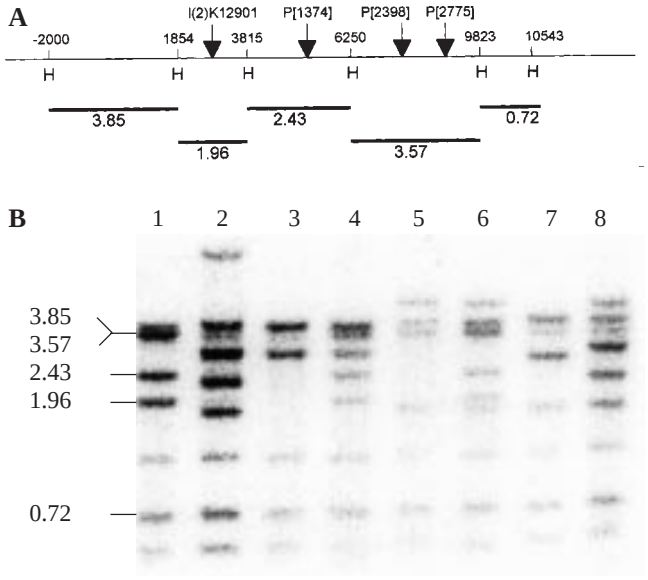

Fig. 2. Southern analysis of P element excision lines. **A.** Location and size of each *Hin*dIII fragment are indicated. Sizes are shown in kilobases. The numbers above the horizontal bar indicate the *Hin*dIII restriction enzyme recognition sites. Numbers correspond to the nucleotide positions described by Kim and Baker (1993). The location of each P element in l(2)K12901, *Rbp9*$^{P[1374]}$, *Rbp9*$^{P[2775]}$, and *Rbp9*$^{P[2398]}$ are indicated by arrows. **B.** *Hin*dIII-digested genomic DNAs were hybridized with the ^{32}P-labeled probe prepared from the genomic DNA fragment encompassing the entire *Rbp9* locus. DNAs isolated from w^{1118}, homozygous *Rbp9*$^{P[2775]}$, and homozygous *Rbp9*$^{\Delta1}$, *Rbp9*$^{\Delta2}$, *Rbp9*$^{\Delta3}$, and *Rbp9*$^{\Delta5}$ were loaded in lanes 1, 2, 3, 5, 7 and 8, respectively. DNAs isolated from heterozygous *Rbp9*$^{\Delta1}$/*CyO* and *Rbp9*$^{\Delta2}$/*CyO* strains were loaded in lanes 4 and 6, respectively.

Defective ovary development in imprecise excision lines The complete sterility and absence of *Rbp9* protein in the imprecise excision lines suggested that the variability in the degree of sterility in the previously reported P element insertion lines (*Rbp9*$^{P[2398]}$, *Rbp9*$^{P[2775]}$ and *Rbp9*$^{P[2690]}$) was due to some residual activity of *Rbp9*. As imprecise P element excision lines would no longer produce any intact *Rbp9* message, analysis of these lines should reveal the true *Rbp9* null phenotype. Ovaries from homozygous imprecise P element excision lines were examined and all ovaries showed virtually the same set of defects. In all mutant ovaries examined, the egg chambers never developed into one oocyte and 15 nurse cells, indicating that female germ cell development was blocked in the transition from cystocyte proliferation to differentiation. This ovarian phenotype is more severe than that of their parental strains (Figs. 4B and 4D). On the other hand, ovaries from precise P element excision lines showed wild type phenotype (Figs. 4A and 4C).

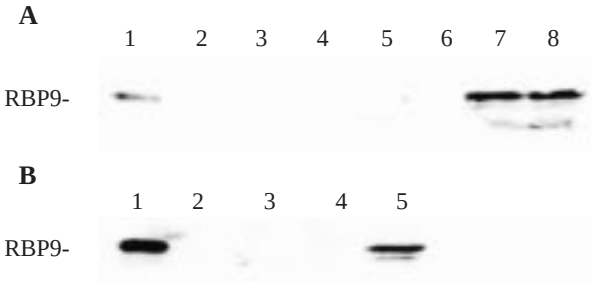

Fig. 3. Immunoblot analysis of P element excision lines. Crude fly extracts (4 μg) were analyzed by western blotting with anti-RBP9 Ab. **A.** Wild-type and *Rbp9*$^{P[2775]}$, and imprecise and precise excision lines were tested for the RBP9 expression. Extracts from wild-type, *Rbp9*$^{P[2775]}$, and imprecise excision lines *Rbp9*$^{\Delta1}$, *Rbp9*$^{\Delta2}$, *Rbp9*$^{\Delta3}$, and *Rbp9*$^{\Delta5}$ were loaded in lanes 1 to 6, respectively. Extracts from precise excision lines *Rbp9*$^{\Delta56}$ and *Rbp9*$^{\Delta64}$ were loaded in lane 7 and lane 8, respectively. **B.** *Rbp9*$^{P[2398]}$ derived excision lines were tested. Lanes 1 to 5 corresponds to wild-type, *Rbp9*$^{P[2398]}$, and imprecise excision lines *Rbp9*$^{\Delta105}$, *Rbp9*$^{\Delta106}$ and precise excision line *Rbp9*$^{\Delta123}$.

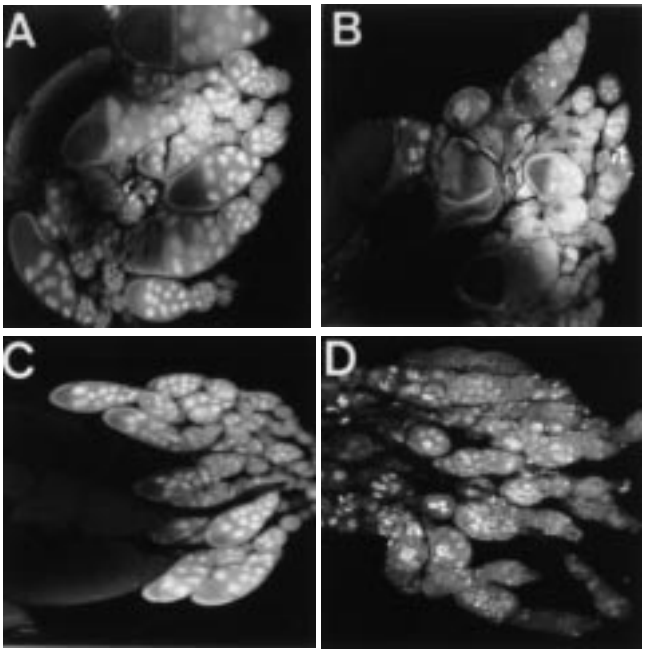

Fig. 4. Egg chamber formation in precise and imprecise excision lines. The anterior end of the ovaries is oriented to the right on each panel. **A.** Wild-type ovaries. Each egg chamber consists of one oocyte and fifteen nurse cells surrounded by follicle cells. **B–D.** Ovaries from $Rbp9^{P[2398]}$, $Rbp9^{\Delta 123}$ (precise excision line), and $Rbp9^{\Delta 105}$ (imprecise excision line).

In this study, we demonstrated the null phenotype of *Rbp9*: complete female sterility due to a block in the transition from cystocyte proliferation to differentiation during oogenesis. Furthermore, this and previous results indicate that P elements in the coding regions of *Rbp9* did not completely abolish *Rbp9* activity. Analysis of P element excised lines further revealed that imprecise excision occurs more frequently than precise excision at the *Rbp9* locus.

Acknowledgments This work was supported by grants from SBRI (B-98-023) and the Korea Science and Engineering Foundation, Republic of Korea (98-0403-04-01-5).

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