Excision of one of two defective P elements as the cause of alternate mutational events (sn+ and sne) of the singed bristle allele snw in Drosophila melanogaster

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ABSTRACT

The X-linked singed locus is concerned with the bristle phenotype and female sterility, and is known as a hot spot of P element insertion. A moderate allele of singed, singed-weak (snw) (Engels, 1979; 1984) is inserted with P elements. It is used as an index of P element activity, since it mutates at a high frequency to either a more extreme allele, singed-extreme (sne), or to a phenotype that is equivalent to the wild type (sn+) when an autonomous P element exists. We show here that snw is inserted with two defective P elements in reverse orientation, and the two alternate mutational events (sn+ and sne) are caused by the excision of one or the other of the P elements present in the singed gene. It is interesting that sn+ and sne are inserted with a single P element in the same position, but show very different phenotypes. The insertional sites of P elements in the singed locus possibly contain an unidentified repetitive sequence, which is repeated dozens of times per haploid genome of the wild-type strain Canton-S.

1. INTRODUCTION

The P element is one of the best characterized transposable elements in Drosophila, and responsible for the phenomenon of P–M hybrid dysgenesis. Hybrid dysgenesis, which induces various symptoms such as high rates of mutation, chromosomal rearrangement, gonadal sterility and male recombination, is caused by transposition of P elements (for reviews, see Engels, 1983; Bregliano and Kidwell, 1983; O'Hare, 1985). The P element transposition occurs when males of a P strain, carrying many active P elements, are mated with females of a M strain with no active P element.

Many transposable elements often affect the gene expression of their insertion sites (e.g., McGinnis, Shermoen and Beckendorf, 1983). Insertion mutations by transposable elements tend to be unstable, and have been well studied in the white locus (Green, 1967; Rubin, 1983). Insertion mutations by P elements have been found by many researchers, and P elements are now used as a mutagen and

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The X-linked singed locus is associated not only with the bristle phenotype but with female sterility, and also is a hot spot for the insertion sites of P elements (Green, 1977; Engels, 1979). A weak phenotypic allele of the singed bristle locus, singed-weak (sn\(^w\)) was identified by Engels (1979; 1984). It mutates at a high frequency to \(sn^e\), a more extreme singed bristle phenotype, and to \(sn^+\), equivalent to the wild bristle phenotype when a complete P element is present (\(sn^w\) hypermutability). Thus \(sn^w\) hypermutability is often used as an index of the P element activity (e.g., Spradling and Rubin, 1982; Laski, Rio and Rubin, 1986; Nitasaka, Mukai and Yamazaki, 1987). The secondary mutation rate of \(sn^w\) is about 40 to 60 percent, but that of other P insertional mutations is relatively low (approximately \(10^{-8}\); Rubin, Kidwell and Bingham, 1982) in a strong dysgenic state. Defective P element(s) in the \(sn^w\) locus are involved in \(sn^w\) hypermutability (Spradling and Rubin, 1982). The precise molecular characteristics of this phenomenon still remain to be clarified. Moreover, the molecular cloning of the singed gene has not been completed.

In the present study, we found that two defective P elements were inserted in the \(sn^w\) locus, and that two alternate mutational events (\(sn^+\) and \(sn^e\)) are caused by the excision of one or the other of two defective P elements inserted in reversed directions in the singed gene. The flanking sequence of the insertional site of P elements in \(sn^w\) was also found to contain an unidentified repetitive sequence repeated more than 20 times at various sites in a haploid genome of the wild-type strain Canton-S. Though this repetitive sequence was not examined in detail it appears unrelated to \(sn^w\) hypermutability, since none of the restriction maps of \(sn^w\), \(sn^+\) and \(sn^e\) differed with each other.

2. MATERIALS AND METHODS

Most of the experimental methods used in this study are described by Maniatis, Fitsch and Sambrook (1982).

Drosophila strains

A description of the genetic symbols and balancer chromosomes is given by Lindsley and Grell (1968).

The two strains \(y sn^w f^+ Y; bw; st(sn^w) (M)\) and \(C(1)DX, y w f\) are described in Nitasaka, Mukai and Yamazaki (1987). \(l (2) Icarus-neo/CyO, DTS_{519}\) is described by Steller and Pirrotta (1986). This strain has a modified P element (Icarus-neo) under the control of hsp70 promoter on the second chromosome, and is resistant to antibiotics G418. This strain was kindly provided by V. Pirrotta.

All flies were grown on a standard cornmeal, molasses and agar medium at 25°C. As required, the standard medium was supplemented with antibiotics.
G418 (Geneticin, SIGMA) at a final concentration of 500 µg/ml.

**Construction of sn\(^+\) and sn\(^-\) strains**

Several females of the sn\(^w\) (M) strain were mated with a single male fly of l (2) /icarus-neo/CyO, DTS513. After two days, the culture was subjected to heat-treatment for 1 hr. at 37°C to cause expression of the transposase activity. G1 males with sn\(^w\) bristle and wild wing phenotypes were crossed individually to attached-X females (C(1)DX, y w f). The G2 sn\(^+\) and sn\(^-\) males were crossed individually to attached-X females, and this step was repeated to eliminate Icarus-neo transposons for two generations. By this process, a number of sn\(^+\) and sn\(^-\) strains were obtained. The mating scheme is shown in Fig. 1.

![Mating scheme for construction of sn\(^+\) and sn\(^-\) strains](image)

Fig. 1. Mating scheme for construction of sn\(^+\) and sn\(^-\) strains.

**DNA preparation**

Fly DNA was extracted by the method of Steller and Pirrotta (1986). It should be noted that only male flies were used for DNA preparation of sn\(^+\) and sn\(^-\) strains.

Probe plasmids were constructed as follows; the transformation-helper plasmid, p\(n25.7\) wc (Karess and Rubin, 1984) was completely digested with BamHI and was partially digested with SalI. A 4 kb fragment was gel-purified, and was ligated into pUC9 which was then digested with BamHI and SalI. The helper plasmid thus obtained was designated as pWC, which was partially digested with HindIII, and the 3 kb fragment containing most of a P element was gel-purified. This fragment was ligated into the HindIII-digested Bluescript SK- (STRATAGENE), and the resulting plasmid was designated as pPORF0123A. It contained most of a P element, and was free from any genomic sequence of Drosophila. Single stranded plasmid DNA of pPORF0123A can be rescued when bacteria harboring these plasmids are infected with helper phage. This is because Bluescript SK- has a intergenic region of the M13 phage and its restriction map is shown in Fig. 2A. A plasmid containing an I factor (pI407: Bucheston et al., 1984) was kindly provided by D. J. Finnegan.
Probe DNA was prepared using the multiprime DNA labeling kit (Amersham) following the procedure specified. The single stranded plasmid DNA rescued by helper phage (R408) was used as the substrate of the labeling reaction.

**Cloning of singed gene**

We constructed genomic DNA libraries using \( \lambda \)EMBL3 by the method of Frischauf et al. (1983), but without the phosphatase treatment. The genomic DNA of all strains used was partially digested with BamHI, and was used for construction of libraries. These were first screened on a lawn of bacteria, P2392 (a P2 lysogen of LE392, STRATAGENE). The second screening was conducted on the bacterial lawn of LE392 (\( recBC^+ \), \( sbcB^+ \) ; see Maniatis, Fitsch and Sambrook, 1982). To rescue the \( sn^w \) clone, the bacterial host, DG30 (\( recBC^- \), \( sbcB^- \), \( tyrB^- \), \( aspC^- \) ; see text) obtained from the National Institute of Genetics was used. It should be pointed out that this strain was propagated in a standard medium supplemented with tyrosine and phenylalanine at a final concentration of 50\( \mu \)g/ml each, since it has a mutation in \( tyrB \) and \( aspC \) each.

**In situ hybridization to salivary gland chromosomes**

Salivary gland chromosome squashes were prepared as described by Engels et al. (1986). Hybridization with sulfonated DNA probes was carried out using DNA CHEMIPROBE (Orgenics Ltd., and Takara Shuzo).

### 3. RESULTS

**Construction of \( sn^+ \) and \( sn^e \) strains**

The mating scheme is summarized in Fig. 1. All the resulting strains turned out to be sensitive to antibiotics G418, and their singed phenotypes were stable for several generations, indicating none of them to have the \( Icarus-neo \) transposon. We used only the male flies of \( sn^+\)-2 and \( sn^e\)-3 for DNA preparation.

**Cloning of singed gene**

It was found that the sizes of the BamHI digested fragments containing P elements of the \( sn^w \) (M) strain were less than the cloning capacity of the bacteriophage \( \lambda \) vector by genomic Southern blotting (15–20 kb, data not shown). Thus BamHI was used to construct a library of random genomic DNA fragments from the \( sn^w \) (M) strain in the bacteriophage \( \lambda \) vector EMBL3 (Frischauf et al., 1983; Kaiser and Murray, 1985). It was expected that we could isolate phages which had a single BamHI fragment containing P element(s) in the singed locus from this library.

The library was first screened using pPORF0123A containing most of a P element (Fig. 2A) as a probe on a bacterial lawn of \( recBC^+ \) host (P2392 and LE392), and 6 positively hybridizing phages were obtained. HindIII cuts a
P element excision at singed locus

complete P element at the 5' tip (position 39), and SalI cuts a complete P element 
at position 2410 (O'Hare and Rubin, 1983). Therefore, we expected that the 
defective P elements retained the HindIII and/or SalI sites. One of them (λ w1) 
were characterized by restriction mapping and Southern blotting with HindIII, 
and SalI. The P-homologous fragments for double digestion of HindIII and SalI 
were 4 kb in size. It has been reported that when the DNA of the snw (M) strain 
is digested with HindIII and SalI and is probed with a P element, a number of 
P-homologous fragments are produced, but only fragments with less than 1 kb are 
associated with snw hypermutability (Spradling and Rubin, 1982). The AvaII 
cuts a complete P element at four different sites, including those within the left 
and right terminal repeats. It is thus possible to determine the size of a given P 
element following its digestion with AvaII. The results of Southern blotting 
following its digestion using AvaII of λ w1 indicated that it contained a very small 
single P element (about 0.5 kb). In contrast, the snw allele is thought to be 
inserted with larger P elements (Spradling and Rubin, 1982). These results 
suggest that these clones do not contain any portion of the singed locus. Other 
positively hybridizing phages were characterized in the same manner and were 
found to contain very small (about 0.5 kb) P elements, and size of P-homologous 
fragments as determined by HindIII and SalI was found not to be consistent with 
the result of genomic DNA blotting of the snw strain. We therefore concluded 
that we could not isolate the singed locus by this approach.

Much difficulty has been encountered in the cloning of a region having a long 
parindromic structure using a λ phage vector (e.g., Leach and Stahl, 1983; 
Wyman, Wolfe and Botstein, 1985). This difficulty can be circumvented by using 
a recBC− host. We thought this was the case; thus a host having recBC and 
sbcB mutations (DG30) was used for screening of bacteriophage λ libraries. 
Unfortunately, since phage P2 for the Spi selection was not capable of lysogenizing 
recBC− hosts (Kaiser and Murray, 1985), we could not use the Spi selection to 
eliminate non-recombinant phages in the first screening of the libraries. But the 
arms of the λ vector (EMBL3) were prepared by a double digestion of BamHI and 
EcoRI; thus, the non-recombinant background was kept low.

Using the recBC− host, we obtained 14 P-homologous clones of the snw strain. 
Five of these phages were selected at random for further analysis. Three of the 
five phages did not correspond to the singed gene by plaque hybridization with a 
P-flanking probe of a previously isolated non-singed clone (λ w1, data not shown), 
and the other two were analyzed as described above. One phage clone (λ w102) 
was found to carry two copies of P elements, and was considered to correspond to 
the singed gene, since its fragment size as determined by Southern blotting 
analysis with HindIII, SalI and AvaII was exactly the same as that found by 
Southern blotting of genomic DNA from the snw (M) strain (Spradling and Rubin, 
1982). Another phage clone (λw105) appeared to have undergone self- 
rearrangement during the cloning procedure. Preliminary results from the
analysis of remaining positively hybridizing clones indicated some had the same maps to \( \lambda \) w102, while others underwent self rearrangement during the cloning.

The sites of the insertion of the P element in the singed locus of \( sn^+ \) and \( sn^e \) strains were also cloned by the above procedure with a whole P probe (pPORF123A). Six and eight P-homologous phage clones were obtained for \( sn^+ \) and \( sn^e \) strains, respectively. Two of the six for \( sn^+ \) and two of the eight for \( sn^e \) phages strongly hybridized to the P-franking probe of the non-singed phage (\( \lambda w1 \), data not shown). It is thus evident that these phages do not contain the singed locus. They were mapped with restriction enzyme and were found to have the same maps as the non-singed phage (\( \lambda w1 \)) isolated from \( sn^w \) library. This locus in which a small P element is inserted was thus concluded to have no association with \( sn^w \) hypermutability. Two of the remaining four for \( sn^+ \) (\( \lambda + 101 \) and \( \lambda + 105 \)) and two of the remaining six for \( sn^e \) (\( \lambda e102 \) and \( \lambda e107 \)) phages were randomly selected, and were mapped using HindIII, SalI and AvaII. Each two phages of \( sn^+ \) and \( sn^e \) had the same maps, respectively. It was found that both \( sn^+ \) and \( sn^e \) phages had a single P element in the same position of the singed gene of \( sn^w \), though the direction of one insertion was the reverse of the other. The size of P element in \( sn^w \) was somewhat larger than \( sn^+ \), and both possess HindIII and SalI sites. To confirm the location of putative singed clones, an in situ hybridization was carried out. Phages were hybridized to the singed locus (7D;

![Fig. 2. Restriction maps.](image)

(A) The restriction map of pPORF0123A used as a probe. This plasmid contains most of P-sequence, but the AvaII site, originally present in the right terminal repeat, was eliminated through plasmid construction. The right terminus of this map also contains a portion of pUC9 poly-linker (SalI-PstI-HindIII). (B) Restriction maps of singed clones from \( sn^w \), \( sn^+ \) and \( sn^e \) libraries. The maps of \( sn^w \), \( sn^+ \) and \( sn^e \) correspond to the \( \lambda \) phage clone, \( \lambda w102 \), \( \lambda + 101 \) and \( \lambda e102 \), respectively. The size of both sides of \( sn^+ \) and \( sn^e \) has been shortened. The bold region between S and H of \( sn^e \) includes unidentified repetitive sequence.
Lindsley and Grell, 1968) region on the X chromosome (see below), and thus those cloned from sn\textsuperscript{w}, sn\textsuperscript{+} and sn\textsuperscript{e} libraries were concluded to certainly possess the \textit{singed} locus. Their restriction maps are shown in Fig. 2B. A comparison of the maps of sn\textsuperscript{w}, sn\textsuperscript{+} and sn\textsuperscript{e} clearly indicate that sn\textsuperscript{w} mutates to sn\textsuperscript{e} or sn\textsuperscript{+} following excision of P elements.

A wild-type library (Canton-S; Maniatis et al., 1978) was also screened so as to isolate the \textit{singed} locus using the 5' flanking-probe of a P element from sn\textsuperscript{e} allele (1.3 kb \textit{SalI-HindIII} fragment, indicated by bold line in the Fig. 2B map). Approximately 60,000 phages of the Canton-S library were screened, and, unexpectedly, as many as 106 positively hybridizing phages were obtained (data not shown). This sequence of flanking P elements in the \textit{singed} locus contains a repetitive sequence \textit{(e.g., a transposable element)}. To estimate its copy number, the same filters were rehybridized with a plasmid containing the I factor (pI407; Bucheston et al., 1984) and 65 positively hybridizing phages were obtained. The Canton-S strain is an I strain, with 10 to 15 copies of I factor on its chromosomal arms and several copies in its chromocenter. This unidentified repetitive sequence may thus possibly disperse more than 20 sites in the Canton-S genome. Its insertional position and structure have yet to be determined. It is not yet known whether the unidentified repetitive sequence exists in the \textit{singed} locus of the wild-type genome.

\textit{In situ hybridization}

To confirm the location of putative \textit{singed} clones, \textit{in situ} hybridization was carried out. Several salivary gland chromosomes of the sn\textsuperscript{w} (M) strain were hybridized with the whole P probe (pPORF0123A). The results in Fig. 3A

![Fig. 3. Chromosomal locations of the phages cloned. Chromosomes of the sn\textsuperscript{w}(M) strain were hybridized with all probes. Arrows indicate the hybridizing signals for each probe. Only the half tip of each X chromosomes is shown since all the probes used were only hybridized to the 7D region on the X chromosome. (A) P element probe (pPORF0123A). (B) non-\textit{singed} probe (\lambda w1). (C) \textit{singed} probe (\lambda e102).]
indicate the snw (M) strain to possess P elements only in two very closely linked loci on the X chromosome corresponding to the singed locus (7D). Chromosomal squashes of the same strain were hybridized with the whole phage DNA containing putative singed locus (λ e102), since the flanking region of P elements in the singed locus contained an unidentified repetitive sequence (see above). This probe hybridized to the 7D region on the X chromosome (Fig. 3B) and these putative singed clones certainly contain the singed locus (see above).

A phage containing another P-insertional site (λ w1) was also hybridized to snw chromosomes for determination of its location. Fig. 3C shows that λ w1 also hybridized to the 7D region on the X chromosome. These results are in agreement with the result using the whole P probe.

4. DISCUSSION

In the dysgenic state, snw mutates at a high frequency to sn+ and sn* (Engels, 1979; 1984). That the mutation rate of the snw allele is much higher than those of other insertional mutations of P elements in the dysgenic state is a matter that should be investigated. It was thought that the mechanism of P element transposition might be understood through clarification of the snw hypermutability.

From the present study, it is evident that two alternate mutational events result from a simple excision of one or the other of the P elements inserted in reversed direction in the singed gene. The mutated two alleles (sn+ and sn*) have P elements of a similar size in the same position, but their directions of transcription are opposite. We cannot exclude the possibility of other mechanisms for snw hypermutability, such as specific recombination between the two defective P elements catalyzed by a domain of P-transposase (e.g., resolution of the two cointegrated transposons catalyzed by resolvase activity in bacterial transposon Tn3, Heffron, 1983). The basis of this possibility is that the P-transposase is thought to have some domains such as DNA binding, endonuclease, etc., and the P elements inserted into the snw may be in a state similar to that referred as "cointegrated" in bacterial transposons.

The flanking sequence of P elements in the singed locus was also found to contain an unidentified repetitive sequence that is present at more than 20 different sites per haploid genome of the Canton-S strain. It does not correspond to P, I and Hobo elements at least according to hybridization experiments. Though neither the structure nor degree of insertion of this repetitive sequence was determined, it does not appear responsible for the snw hypermutability, since the restriction maps of the sequence flanking P elements of snw, sn+ and sn* alleles were all the same.

Because cloning of the singed locus has not been completed and we have not analyzed the transcripts of the singed locus, we cannot know the direction and location of the transcribed region. Therefore we are not able to clarify the effects
of P elements to the singed gene in sn\(^w\), sn\(^+\) and sn\(^e\) alleles. However, the levels of singed transcripts for sn\(^w\), sn\(^+\) and sn\(^e\) may be intermediate, normal and low, respectively, based on the observation of bristle phenotypes. Though all P elements of sn\(^w\), sn\(^+\) and sn\(^e\) were inserted in the same position of the singed gene, their bristle phenotypes differed greatly from each other in this respect, possibly due to the reversed directions of transcription of the P elements in the singed gene. At least they do not appear to be inserted in the structural gene of singed, since the sn\(^+\) allele has a bristle phenotype equivalent to the wild-type, though it contains a single P element in the singed locus.

The singed gene is associated not only with bristle phenotype but also with female sterility (Lindsley and Grell, 1968). Some alleles of the singed gene such as sn\(^{x2}\) show female sterility.

We know that the sn\(^w\) (M) strain is completely fertile, but the sn\(^w\) (P) strain cannot be maintained as a homozygous stock. Our preliminary results showed that a female fly homozygous for sn\(^w\) having a chromosome derived from a Q strain with no transposase activity (WY113: Nitasaka, Mukai and Yamazaki, 1987), turns out to be sterile. Thus a product of P elements other than transposase (e.g., putative repressor of P elements) may affect P elements in the singed gene. We considered that such a product would inhibit the transcription of this gene. From the above reasoning, we think that we can approach the mechanism of transposition and regulation of P elements by analyzing the structure and transcript of the singed gene.

Note added in proof: Since acceptance of this manuscript, the article by Roiha et al. (1988) has appeared in Genetics 119: 75–83, with results generally comparable to those of this study.

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