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Precise excision of the retrotransposon *gypsy* from the *forked* and *cut* loci in a genetically unstable *D.melanogaster* strain

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ABSTRACT

The genetically unstable Mutator Strain of *D.melanogaster* is characterised by a high frequency of spontaneous mutations and their reversions. Three *forked* mutants were obtained independently and several reversions arose spontaneously with frequency of $10^{-3}-10^{-4}$. The sites of integration and excision of the *gypsy* retrotransposon were analysed by Southern blot analysis and sequencing of PCR fragments. In all cases *gypsy* had inserted at the end of the third exon of the major transcript of the *forked* gene, causing the duplication of TCCA target sequence. All the reversions resulted from precise excision of the *gypsy*. A double mutant containing ct^6 and f^1, caused by *gypsy* insertions into untranslated regions of the corresponding genes, was constructed. Two spontaneous ct^6f^+ revertants as well as one ct^+f^1 revertant were obtained from this line. Sequence analysis of *gypsy* integration and excision sites revealed that in all cases *gypsy* excision was also precise. These experiments constitute the first demonstration of precise excision of LTR-containing elements from their host genomes.

INTRODUCTION

Retrotransposons are the retrovirus-like mobile genetic elements inhabiting eukaryotes of all kingdoms. *Gypsy* (mdg4) is one of the most thoroughly studied *D.melanogaster* retrotransposons. Its structure [1–3] and the main steps of its life cycle, based on reverse transcription [4], are well characterised. However, little is known about the mechanisms of its integration and excision from genomic DNA.

A previously described system of Mutator Strain (MS) [5,6] which has originated from Stable Strain (SS) is characterised by genetic instability caused by a high level of *gypsy* transposition. The latter is a result of combination of two factors: mutation in one of the genes regulating *gypsy* transposition which has preexisted in SS [7], and presence of active variant of this element [8], which was occasionally introduced into SS during several crossings with different laboratory strains. Mutator Strain and all its derivatives are characterised not only by a high frequency of spontaneous mutations but also by an elevated frequency of their reversions. Therefore, it seemed reasonable to use this system for the investigation of the excision process. The present paper describes the results of initial experiments which show that the *gypsy* excision from two loci in X chromosome is precise and occurs in the absence of homologous chromosome containing an empty target site, eliminating the possibility that these excisions are merely gene conversion events.

MATERIALS AND METHODS

D.melanogaster strains

D.melanogaster strains used in this work were the following: SS [5]; MSf^{14K} [9]; MSn^1; MSn^2 [7]; *sc ec cv* ct^6v g^2 f^1/FM3,*y B*; *B*/C(1)RM,*y w f*/Y [10]. Eight spontaneous reversions: four in case of MSf^{14K} (MSf^+ 1, 2, 3, 4) and four in case of MSn^1 (MSn$^1f^+$ 1, 2, 3, 4) were obtained spontaneously among male offspring of MSf^{14K} and MSn^1 males individually crossed with *B*/C(1)RM,*y w f*/Y females. Large scale experiments on screening for reversions were performed for MSf^{14K} and MSn^1 but not MSn^2. Double mutant MSct^6f^1 was constructed as follows: FM3,*y B*/*sc ec cv* ct^6v g^2f^1 female flies were crossed with SS male flies and the progeny was screened for recombinant males containing ct^6f^1 in the SS genetic background. These males were crossed with FM3 females to maintain the resulting MS-like stock MSct^6f^1 exhibiting all the features, characteristic for MS. Screening for reversions was performed among the offspring of MSct^6f^1 males crossed with *B*/C(1)RM,*y w f*/Y females. Two spontaneous *forked* (MSct^6f^+ 1, 2) and one *cut* (MSct^+f^1) revertants were obtained. Revertant stocks were maintained either in the *B*/C(1)RM,*y w f*/Y background or as homozygotes.

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Preparation and treatment of DNA

DNA extraction, restriction enzyme treatment, DNA labelling, and Southern blot experiments were performed according to Sambrook *et al.* [11].

Polymerase chain reaction

PCR was performed according to standard techniques [11]. As primers, oligonucleotides complementary to *forked* (Fig.1a), *cut* (Fig.3c) and *gypsy* (Fig.3b) sequences were used in appropriate combinations. All the oligonucleotides were designed to direct the synthesis of PCR fragments of suitable for the analysis size in the range 0.4−0.6 kb. As a template genomic DNA isolated from one fly of corresponding stock was used. After 30 cycles of PCR, material was analysed in a 1.2% agarose gel. PCR-derived fragments were isolated using low melting agarose and purified using a Geneclean kit (BIO 101, USA).

DNA sequencing

Sequence analysis of the *cut* locus fragment, containing a *gypsy* insertion was performed according to Maxam and Gilbert [12] and Sanger [13]. The latter technique was used for the analysis of *gypsy* sites of integration and excision in both *cut* and *forked* genes.

RESULTS

Analysis of the *gypsy* integration and excision site in three independent *f* mutants and derived spontaneous revertants

The first three MS-like stocks, MSf^{14K}, MSn^1 and MSn^2, containing the *forked* mutation were obtained independently. MSf^{14K} was obtained as spontaneous mutation in the MS genetic background [9]. *Forked* mutations and genetic instability were revealed in MSn^1 and MSn^2 [7] which were obtained as transformed derivatives of SS after microinjection of plasmid DNA, containing a transposition-competent *gypsy* [8], inserted into Casper vector. The *gypsy* insertion in MSf^{14K} was mapped by Hoover *et al.* [14]. Southern blot analysis (data not shown) of these three *forked* mutants revealed no difference between them in the location of *gypsy* insertion. Therefore, we have used the sequence data on *forked* gene and all the *gypsy* insertions (including MSf^{14K} and f^1 alleles), kindly provided by V.Corces [14] and designed PCR primers, located at position 16940−16921 (primer 1) and 16325−16344 (primer 2) according to Hoover *et al.* (Fig. 1a). PCR was performed using primers 1 plus 7, and 2 plus 8 (primers 7 and 8 are complementary to internal sequences near XhoI site of the *gypsy* LTR; see Fig. 3b).

PCR-derived fragments were the same size for all the mutants and coincided with the expected for MSf^{14K} 0.62 kb long (in case of primers 1 plus 7) and 0.48 kb long (in case of primers 2 plus 8). The fragments were isolated and sequenced using the same primers. As a result, the exact site of *gypsy* integration into *forked* locus in all three mutants was determined. In all cases *gypsy* was inserted into the same sequence, position 16517 according to Hoover *et al.* [14], causing the duplication of TCCA target DNA sequence (Fig. 1b).

The *f* mutants were maintained either as homozygotes or over attached-X chromosomes (in this case only males contained *f* mutation). Males were monitored for the appearance of reversions. The analysis of the reversion rate is presented in Table 1.

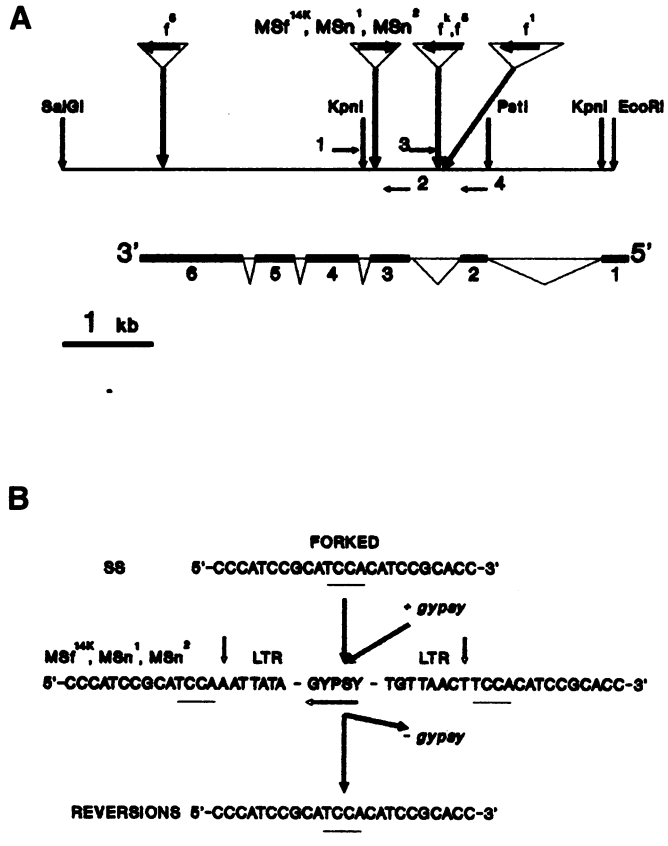

Figure 1. (A) The restriction map of the *forked* locus with known *gypsy* insertions. All of them, except MSn^1 and MSn^2 were localised by Hoover *et al.* [14]. The structure of 2.5 kb *forked* transcript is presented according to Hoover *et al.* Solid boxes with numbers indicate exons, triangles indicate introns. Arrows in *gypsy* insertions show the direction of *gypsy* transcription. Arrows with numbers indicate primers used in PCR. (B) Nucleotide sequence of the same fragment of the *forked* locus in SS, MSf^{14K}, MSn^1, MSn^2 and their revertants. The sequence of the target site DNA is underlined. The empty vertical arrows indicate the first and the last nucleotides of *gypsy*. The empty horizontal arrow shows the direction of *gypsy* transcription.

DNA isolated from males of each revertant stock was used as a template in PCR. Primers 1 and 2 directed the synthesis of a DNA fragment 0.6 kb long, indicating the absence of any significant difference between the structure of the *forked* gene in revertants and wild type flies. Sequence analysis of this region (Fig. 1b) revealed precise excision of *gypsy* in all revertants.

The analysis of *gypsy* integration site in the *forked* locus of a double mutant and its reversions

The above mentioned experiments suggested to us the existence of a special mechanism providing precise excision which is activated in MS. However, these experiments could not provide any information about the frequency of imprecise excision, since the *gypsy* in these mutants was inserted into the protein coding region and imprecise excisions were unlikely to be detected in this case. Therefore, we constructed an MS-like strain, containing *gypsy* insertions in untranslated regions of both *forked* and *cut* loci.

MSct^6f^1 was obtained by introduction of ct^6 and f^1 mutations from laboratory FM3, *sc ec cv* ct^6v g^2f^1 strain [10] into the SS

Table 1. The frequency of reversions of *forked*

Strain	Direction of mutation	Number of chromosomes analysed	Number of clusters	Cluster size*	Frequency of reversions
MSf^{4K}	$f \rightarrow f^+$	5603	4	4/40; 3/26; 1/43; 3/41	7×10^{-4}
MSn^1	$f \rightarrow f^+$	5214	4	3/83; 4/74; 4/84; 1/23	7.6×10^{-4}

*The first figure in cluster size shows the number of f^+ individuals, the second figure indicates the total number of male offspring derived from an individual *f* male

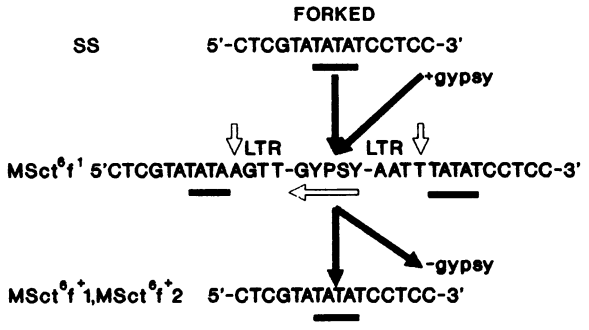

Figure 2. Nucleotide sequence of the same fragment of the *forked* locus in SS, MSct^6f^1 and derived revertants MSct^6f^+ 1, MSct^6f^+ 2. The sequence of the target site DNA is underlined. The empty vertical arrows indicate the first and the last nucleotides of *gypsy*. The empty horizontal arrow shows the direction of *gypsy* transcription.

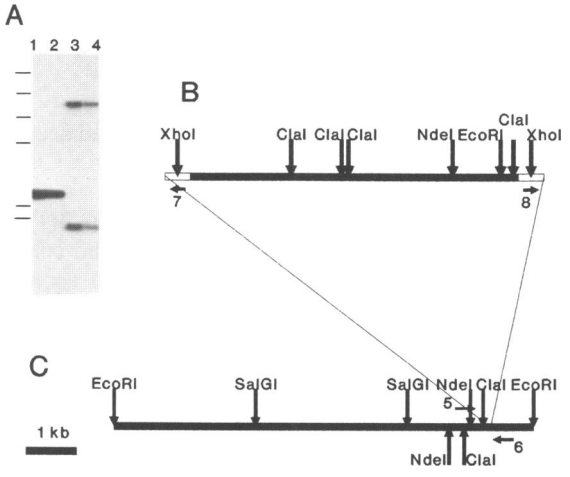

Figure 3. (**A**) Southern blot analysis of DNA isolated from SS (1), MSct^+f^1 (2), MSct^6f^+ (3) and MSct^6f^1 (4). As molecular weight marker, a HindIII digest of lambda DNA (23.1 kb, 9.4 kb, 6.7 kb, 4.4 kb, 2.3 kb, and 2.0 kb) was used. (**B**) Restriction map of *gypsy*. Empty boxes indicate LTRs. Orientation of *gypsy* transcription is from the left to the right. (**C**) Restriction map of the region of the *cut* locus, containing the *gypsy* insertion in MSct^6f^1. Arrows with numbers indicate primers used in PCR.

genome. As it is known, many mutations induced by *gypsy* insertions into untranslated regions of various genes can be suppressed by mutations in other genes regulating *gypsy* expression [15, 16]. The *gypsy* insertion into untranslated regions of *cut* and *forked* genes in MSct^6f^1 was confirmed by suppression of these mutations by *su(Hw)* and *su(f)* [16] (data not shown). The location of *gypsy* insertion in f^1 allele was determined by Hover *et al.* [14]. Therefore, it was possible to analyse the *gypsy* integration site in *forked* using the same approach as for MSf^{4K} and MSn^1. PCR was performed using primers 3 plus 8, and 4 plus 7 (Fig. 1a and Fig. 3b). Primers 3 and 4 were complementary to sequences located at position 15872–15853 (3) and 15486–15508 (4) according to Hoover *et al.* [14].

The analysis of PCR products in a 1.2% agarose gel allowed us to identify and isolate DNA fragments 0.49 kb long (using primers 3 plus 8) and 0.39 kb long (using primers 4 plus 7). The size of revealed fragments was that, which was expected according to sequence data of Hoover *et al.* [14]. Sequence analysis of these PCR fragments demonstrated that in MSct^6f^1 flies the *gypsy* element was inserted into the *forked* gene at the position 15676, causing the duplication of TATA target DNA sequence. The latter coincides with usual for *gypsy* TAYA (where Y is a pyrymidine) target site DNA [1, 2, 17].

MSct^6f^1 males were crossed with attached-X females and only male offspring, carrying the father's X chromosome and mother's Y chromosome, were scored for the reversions either of *ct* or *f*. Since the analysed X chromosomes had never met their homologues, no gene conversion could interfere with the results of our experiments. Moreover, the maternal attached X chromosomes were marked, one with *B* and the other with *y w f*. Therefore, the rare case of accidental detachment of X chromosomes would be monitored by the appearance of males with *B* or *y* mutations. Finally, the ct^6 mutation in MSct^6f^+ and f^1 mutation in MSct^+f^1 revertants could be considered as additional controls to the usual *w* genetic marker of all the strains. In total 10553 X chromosomes were analysed and two revertants were detected (a frequency of 2×10^{-4}).

The revertants MSct^6f^+ 1 and MSct^6f^+ 2 were analysed using above mentioned approach. PCR was performed using primers 3 plus 4. The expected PCR fragments should be 386 bp if the *gypsy* was excised completely, and 868 bp if the single *gypsy* LTR was left behind. PCR-derived fragments were 0.39 kb long, they were sequenced and like the case of other *f* reversions, *gypsy* excision was shown to be precise, restoring the original sequence (Fig. 2).

The analysis of *gypsy* integration and excision sites in the *cut* locus of MSct^6f^1 and its revertant

The first step of the analysis of *cut* in MSct^6f^1 was more detailed restriction mapping and sequence analysis of the *cut* region adjacent to the *gypsy* insertion in ct^6 [18, 19]. The only

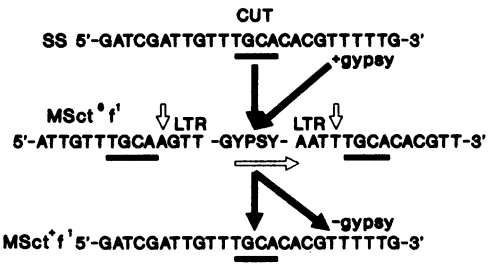

Figure 4. Nucleotide sequence of the same fragment of the *cut* locus in SS, MS$ct^{6}f^{1}$ and MS$ct^{+}f^{1}$. The sequence of the target site DNA is underlined. The empty vertical arrows indicate the first and the last nucleotides of *gypsy*. The empty horizontal arrow shows the direction of *gypsy* transcription.

sequence data available at that time was 40 bp adjacent to the *gypsy* insertion in ct^{MR2} mutation [20]. There was a ClaI site in this sequence, and this site was used for initial sequencing of *cut* [12]. For the sequence analysis the plasmid p8.3(1), kindly provided by N.Tchurikov, was used. This plasmid contains an EcoRI fragment 8.3 kb long from −7.2 to +1.1 according to Jack [18]. The restriction map of this region is presented in Fig. 3c. Sequence analysis was performed initially from the first ClaI site (denoted in Fig. 3c by the arrow below the line) in both directions, and later using synthetic primers according to Sanger [13]. The second ClaI site (denoted in Fig. 3c by the arrow above the line) was identified only by sequence analysis (for some unknown reason this ClaI site was never cut during ClaI digestion). In total, a 900 bp fragment, covering the region from the first NdeI (denoted in Fig. 3c by the arrow below the line) to the second ClaI site, was sequenced.

The results of Southern blot analysis of MS$ct^{6}f^{1}$, two revertants (one f^{+} and one ct^{+}) and SS as a control are presented in Fig. 3a. DNA isolated from flies of corresponding stocks was digested with SalGI and EcoRI. The 2.5 kb SalGI–EcoRI fragment from plasmid p8.3(1) was used as a probe for hybridisation. It can be seen that in SS (Fig. 3a, lane 1) only a 2.5 kb fragment is detected, while in MS$ct^{6}f^{1}$ (Fig. 3a, lane 4) and MS$ct^{6}f^{+}$ (Fig. 3a, lane 3) there is no such fragment. Instead, there are two fragments: 8.2 kb long (containing 6.7 kb of *gypsy* and 1.5 kb of *cut* sequences adjacent to *gypsy* insertion) and 1.8 kb (containing 0.8 kb of *gypsy* and 1.0 kb of the *cut* gene). The localisation of *gypsy* insertion and its orientation was confirmed by Southern blot analysis using SalGI-NdeI-EcoRI digestion (data not shown).

To localise *gypsy* insertion at the nucleotide level PCR was performed, using MS$ct^{6}f^{1}$ DNA as a template and primers 5 (complementary to 20 bp located 70 bp downstream from the second NdeI site denoted in Fig. 3c by the arrow above the line) plus 7 (Fig. 3b); and 6 (complementary to 20 bp situated 370 bp downstream the second NdeI site) plus 8 (Fig. 3b, c). PCR fragments 0.38 kb long in the first and 0.43 kb long in the second case were of expected size. They were isolated and used for sequence analysis using the same primers. The results of sequence analysis are presented in Fig. 4. The *gypsy* insertion was localised in exactly the same position as ct^{MR2} [20] and a TGCA duplication of target site DNA was identified.

A single *cut* reversion was seen in the MS$ct^{6}f^{1}$ stock. MS$ct^{+}f^{1}$ was found as a spontaneous premeiotic reversion (cluster 2/74) among 8178 analysed males of MS$ct^{6}f^{1}$. Therefore, the frequency of reversion was 1.22×10^{-4}. Southern blot analysis (Fig. 3a) revealed no difference between MS$ct^{+}f^{1}$ and SS (Fig. 3a, lanes 2 and 1 respectively). PCR was performed using primers 5 plus 6. The expected size of PCR fragment was 303 bp in case of complete *gypsy* excision and 785 bp long if a single LTR was left behind. PCR-derived fragment was 0.3 kb long. It was isolated and sequenced. The results of these experiments are presented in Fig. 4. The *cut* reversion was caused by *gypsy* excision and it was precise, like all the other cases reported here.

DISCUSSION

The present paper is devoted to the analysis of *gypsy* integration and excision sites in mutations and derived reversions arising in the system of genetically unstable Mutator Strain in *D.melanogaster* [5–8, 21]. This is the first direct demonstration of precise excision of a retrotransposon and its frequency in this particular system is high ($10^{-3}-10^{-4}$). Although it was reported before [20] that one of *cut* revertants in another genetically unstable system (ct^{MR2}) retained no detectable *gypsy* sequences, no sequence data were provided to confirm that there had been a precise excision. Most reversions of *gypsy*-induced mutations described before, resulted from either the integration of other mobile elements into the *gypsy* regulatory region [15, 20, 22, 23], or presumably from recombination events when a single LTR was left behind in the target site [20]. 'Solo' LTR left behind in the reversion of w^{a} was also demonstrated for *copia* [24]. Therefore, it was supposed that retrotransposons can be excised only due to recombination between LTRs and precise excision, resulting from recombination between 4–5 bp direct repeats flanking an element must be extremely rare and practically undetectable.

The initial expeiriments described here, were performed using three independent *forked* mutants. Surprisingly, all three *gypsy* insertions in these mutants occurred in the same TCCA sequence. It is interesting that this sequence differs from all previously known targets PyrPurPyrPur [1, 3, 27, 20].

Since *gypsy* was integrated into the end of the third exon of the main 2.5 kb *forked* transcript [14], it was expected that all reversions could be associated with precise excision. The latter was confirmed by sequence analysis of *gypsy* integration site in revertants. The most significant result of these experiments was the high frequency of precise excision which seemed very unlikely to be due to recombination events.

MS$ct^{6}f^{1}$ was constructed in attempt to detect not only precise, but imprecise excision too, since ct^{6} and f^{1} alleles contain *gypsy* insertions into untranslated regions of corresponding genes. Experiments with this strain also allowed us to exclude the interference of gene conversion as an explanation for the precise nature of all observed excision events. It is interesting that three spontaneous reversion events in an MS$ct^{6}f^{1}$ background (one ct^{+} and two f^{+}) also resulted from precise *gypsy* excision. We cannot exclude the possibility that imprecise excision and recombinative excision (leaving behind solo LTR) may also take place, since only three reversions were obtained and analysed at the molecular level. Screening for reversions on a larger scale is necessary to find other cases of *gypsy* excision. Still, we propose that the high frequency (10^{-4}) of precise *gypsy* excision in MS$ct^{6}f^{1}$ revertants is the strongest evidence for existence of novel mechanisms providing this process.

Precise excision of retrotransposons at the molecular level has never been reported previously and, of course, nothing is known

about excision mechanism. Excision can be regarded as an event reverse to integration. The latter is provided by element's integrase as it was demonstrated for retroviruses [25] and for yeast retrotransposon *Ty1* [26]. Therefore, we suggest that one of the candidates for a major role in precise *gypsy* excision is its integrase. Additionally, since high frequency of reversion is characteristic for all analysed MS-like strains, we assume that activation of *gypsy* precise excision in this system is associated with *flamenco* mutation [Kim, unpublished data] which is responsible for the mutator behaviour of all these strains. Analysis of the possible roles of *gypsy* products in its precise excision will require a more sensitive assay for the process.

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