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Silke Jensen, L. Cavarec, O. Dhellin, T. Heidmann. Retrotransposition of a marked *Drosophila* line-like I element in cells in culture.. *Nucleic Acids Research*, 1994, 22 (8), pp.1484-1488. <10.1093/nar/22.8.1484>. <hal-01925042>

HAL Id: hal-01925042

<https://hal.science/hal-01925042v1>

Submitted on 4 Nov 2022

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Retrotransposition of a marked *Drosophila* line-like I element in cells in culture

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Received November 29, 1993; Revised and Accepted March 7, 1994

ABSTRACT

We have marked a *Drosophila* transposable element—the LINE-like I element—with an intron-containing indicator gene inserted in place of a large deletion in the I element second ORF encompassing the reverse transcriptase domain, and this marked element was placed downstream to a potent actin promoter. An expression vector for the I element ORFs was also constructed, under the same heterologous promoter. The indicator gene contains a *lacZ* reporter gene the expression of which is conditioned by retrotransposition of the marked element, thus allowing detection of transposition events by testing for either β -galactosidase expression or occurrence of spliced DNA molecules. The marked I element was introduced into *Drosophila melanogaster* cells in culture by transfection. Spliced DNA copies of the marked element and specifically stained β -galactosidase-expressing cells were detected only upon co-transfection with the I expression vector, thus indicating that an ORF2-deleted element can be complemented *in trans* for transposition. This simple assay for retrotransposition in *Drosophila* cells in culture provides a tool for the rapid analysis of the mechanism of I transposition in its *cis* and *trans* sequence requirements.

INTRODUCTION

LINEs (Long Interspersed Nucleotidic Elements) are highly reiterated mobile elements found in many eukaryotic species including plants, insects and mammals (reviewed in 1,2). LINEs are up to 7 kb-long elements which commonly possess two long open reading frames, one (ORF1) with a putative coding domain also found in the nucleic acid binding domain of the *gag* polypeptide of retroviruses, and the other (ORF2) displaying a highly conserved region with homology to retroviral reverse transcriptase (reviewed in 3). Reverse transcriptase activity was demonstrated for the *Drosophila melanogaster* Jockey (4), the human L1 (5) and the trypanosomatid *CRE1* (6) elements. In several instances, an internal pol II promoter has been characterized at the 5' end of full-length elements (7–9), but

many LINEs are heterogeneously truncated at their 5' end. LINEs possess an A-rich sequence at their 3' end usually, but not always, preceded by a polyadenylation signal.

These features have led to the suggestion that LINEs transpose through an RNA intermediate and its reverse transcription. This assumption was tested for a *Drosophila* LINE—the I element (reviewed in 10,11)—that was tagged with an intron-containing indicator gene (*neo^{RT}*), previously developed to detect RNA-mediated transposition in mammals by selective methods (12–15). *In vivo* transposition events of this marked element could be selected in transgenic *Drosophila*, and sequencing of the transposed copies disclosed precise splicing out of the intron thus unambiguously demonstrating 'retrotransposition' of this *Drosophila* LINE (16, see also 17).

Although several full-length LINEs have now been entirely sequenced and characterized—among which several elements from *Drosophila* and L1 elements from humans—still little is known on the refined molecular mechanisms of their retrotransposition. For instance, it has been questioned whether functional LINEs could *trans*-complement for retrotransposition defective (non-coding) LINE elements (10), and whether they could be involved in the mobility of the non-coding transposable SINE elements (reviewed in 18) as well as in processed pseudogene formation (15); similarly, ribonucleoprotein particles containing LINE transcripts were found associated with reverse transcriptase activity and LINE-encoded proteins (19–21), but it is still not known whether these structures are the functional intermediates for LINE retrotransposition.

To answer these questions, the *Drosophila* I element is unique since a full-length element was previously cloned (22) that was demonstrated to be functional for autonomous transposition—after it was reintroduced into *Drosophila* as a transgene (23, see also 16). Until now, I element transposition has only been detected *in vivo* under very specific conditions characterized as I-R hybrid dysgenesis (24, reviewed in 10,11), and the aim of the present study was to develop a rapid assay, similar to that previously developed for mammalian transposable elements (25), to analyze *ex vivo*, in *Drosophila* cells in culture, the transposition of a marked I element. It further allowed us to demonstrate transposition of a deleted I element and its transcomplementation by an expression vector containing the I element ORFs.

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MATERIALS AND METHODS

DNA constructs

Construction of a lacZ^{RT} indicator gene for *Drosophila*. The pSP64-D-pA-A plasmid necessary for subsequent construction of lacZ^{RT} was constructed by insertion of the polyA-signal and splice-acceptor containing region of the described neo^{RT} gene (12) as a BamHI–KpnI–Klenow-treated fragment into the unique BamHI site of a D-containing pSP64 plasmid (26). The lacZ^{RT} gene was constructed upon insertion of the hsp70 promoter as a SalI–HindIII–Klenow-treated fragment from phspCAT (gift of P. Herbomel) into the unique BamHI-site of pSP64-D-pA-A, and subsequent insertion of the nlslacZ fragment derived from pGEMnlslacZ (27) as a SalI–BamHI–Klenow-treated fragment into the PstI site of the above plasmid.

Construction of the marked I element. The lacZ^{RT} gene was excised as a XhoI–Klenow-treated fragment and inserted into an XbaI- and Klenow-treated pI407 plasmid, thus eliminating a large part of ORF2 (positions 2579–5207 according to I element sequence in 28) of the complete I element in pI407 (22). The resulting marked I-lacZ^{RT} element was inserted into the EcoRV-restricted pAct5C-PPA expression vector (29) as a AseI-Klenow-treated fragment, thus giving the Act-I-lacZ^{RT} construct.

Construction of an expression vector for I element ORFs. Act[I] was constructed by inserting the ORF1-containing BanII–EcoRV–Klenow-treated fragment from pI407 into the pAct5C-PPA expression vector restricted by EcoRV, and subsequent

insertion of the ORF2-containing EcoRV–BstYI fragment from Ineo^{RT} (16) into the unique EcoRV site.

Cells, transfection and β -galactosidase staining

Drosophila melanogaster Kc cells (30) were grown at 25°C on Schneider medium (Gibco) supplemented with 10% fetal calf serum (Eurobio). 10⁷ cells per 60 mm dish were transfected by the calcium phosphate method with 10 μ g of Act-I-lacZ^{RT} and 10 μ g of either irrelevant carrier DNA or Act[I]. Seven days later the transfected cells were resuspended, part of them (1/40, i.e. about 2 \times 10⁶ cells) were replated into 35 mm diameter dishes and X-Gal stained less than one day after replating, and the remaining cells were used for DNA extraction; in experiments where DNA was not extracted the cells were X-Gal stained without replating. X-Gal staining was performed as described in (31) after a 2 h heat-shock at 37°C.

Nucleic acid purification and PCR amplification

Genomic cellular DNAs were prepared according to the salting out procedure in (32). For PCRs, the oligonucleotides used were: h1 = 5'-CTTCGTCTACGGAGCGACAATTC; h5 = 5'-CAA-CTGCAACTAC-TGAAATCTGCC; lz3 = 5'-CTTAGCAATT-CTGAAGGAAAGTCCTTGG; lz4 = 5'-GCG-GAATTCCGG-AAAACCTTTATCCATC; A = 5'-CACCGGTCGGTCCAGT-TGTTCTTG. Nested PCR amplifications were performed essentially as described in (14), in 50 μ l containing 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin (w/w), 0.2 mM of each dNTP, 1 μ M of each primer, 1.5U of Taq Polymerase (Amersham), and 10 μ g of DNA (equivalent to \approx 3 \times 10⁷ cells). After an

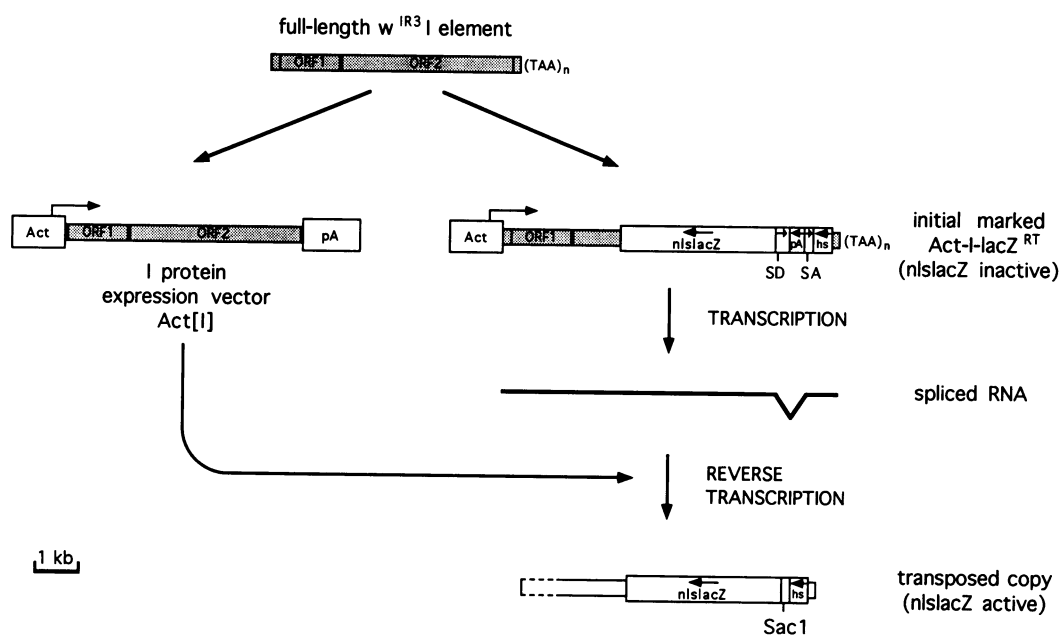


Figure 1. Structure of the marked I element and expression vector, and rationale of the assay. The structure of the full-length w^{IR3} I element with its two ORFs and 3' terminal TAA repeats, and the structures of the two derived constructs are schematized. The expression vector for the I element coding sequences (Act[I]) contains the two ORFs under control of the promoter (Act) and the polyadenylation signal (pA) of the actin-5C gene. The lacZ^{RT} indicator gene is inserted into the I element upon a large deletion in ORF2 encompassing the reverse transcriptase domain; the marked I element is placed downstream to the actin-5C promoter (Act) for high expression in cells in culture. The lacZ^{RT} indicator gene contains the β -galactosidase coding sequence fused to a nuclear location signal (nlslacZ), under control of the *Drosophila hsp70* promoter (hs); in the initial configuration of the indicator gene, nlslacZ is inactive because of a polyadenylation sequence (pA) inserted between the promoter and coding sequence (arrows in the indicator gene indicate the orientation of each genetic element); retrotransposition should result in a transposed copy in which the pA sequence—which is bracketed by splice donor and acceptor sites (SD, SA)—should have been removed by splicing of the RNA intermediate, thus resulting in an active nlslacZ gene; the SacI restriction site generated at the splice junction in a retrotransposed copy is indicated.

initial step at 94°C (5 min), a first round of 35 cycles of amplification was carried out with primers h1 and lz3 (30 s at 64°C, 1 min 30 s at 72°C, 10 s at 94°C). 2 μ l of the PCR 1 reaction were then amplified for 35 cycles in 50 μ l with a second pair of internal primers (h5, lz4), in the same conditions. When indicated, a third round of 35 cycles was performed on 2 μ l of PCR 2 in 50 μ l with primers A and lz4, under the same conditions.

RESULTS

Rationale of the assay

The rationale of the assay (see Figure 1) relies upon the use of a *Drosophila*-adapted version of the lacZ^{RT} indicator gene, previously developed to detect retrotransposition events in mammalian cells by an *in situ* assay (26). This indicator gene contains the β -galactosidase-encoding gene (*lacZ*), and is engineered in such a way that in its initial configuration *lacZ* is silenced by a polyadenylation sequence (pA) inserted between the *lacZ* coding sequence and a promoter for this gene (*hs*, see below). The polyadenylation sequence is bracketed by a donor and an acceptor splice site, so that it should be removed by splicing from the transcript of the transposon marked with the indicator gene. Accordingly, in the retrotransposed copy generated by reverse transcription of the RNA intermediate, *lacZ*

should be active, thus allowing direct detection of transposition events by an *in situ* assay for β -galactosidase activity; the *lacZ* gene used is fused to a nuclear location signal (nls), resulting in an unambiguous nuclear staining. Precise splicing out of the intron in the transposed copies can further be detected by PCR amplification of the intron-containing domain of the indicator gene, which should result in a PCR fragment of reduced length with a *SacI* restriction site generated at the splice junction (12) (see Figure 2). Finally, since the thymidine kinase promoter in the original lacZ^{RT} gene is poorly expressed in *Drosophila* cells (33), it was replaced by the *Drosophila hsp70* promoter (*hs*, heat-shock-inducible). The *Drosophila lacZ^{RT} gene was then introduced into a cloned I element that was previously demonstrated to be functional for transposition (*w*^{IR3}) (23). This element contains two ORFs, and lacZ^{RT} was inserted in place of a large deletion in ORF2 encompassing the reverse transcriptase domain (see Figure 1). To ensure high expression of this marked element in cells in culture, in a non-dysgenic genetic context, it was inserted downstream to the strong *Drosophila actin-5C* promoter (previously used for expression vectors in 29), resulting in the Act-I-lacZ^{RT} construct (see Figure 1).*

Since the marked I element is defective (at least for the reverse transcriptase function) we constructed an expression vector to tentatively complement *in trans* the marked element for

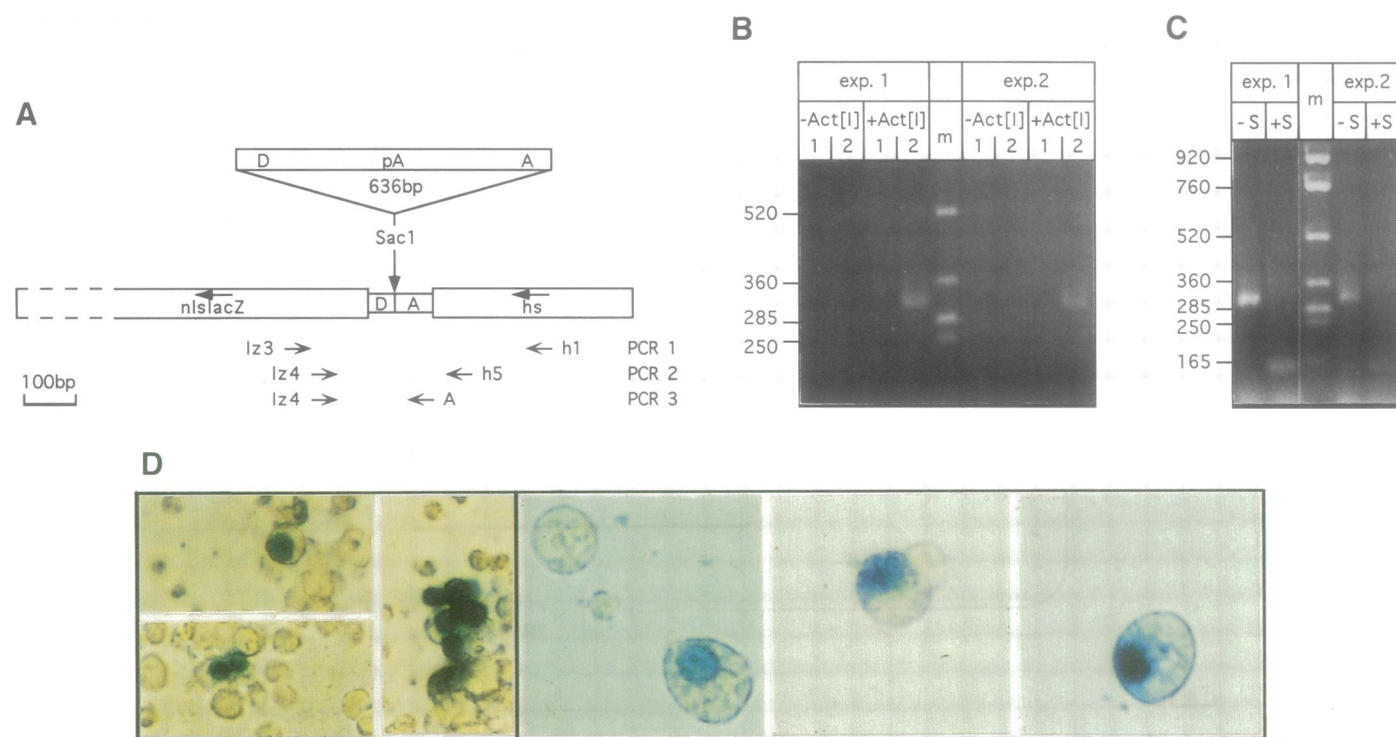


Figure 2. PCR amplification of the lacZ^{RT} intron-containing domain and β -galactosidase-expression in transfected Kc cells. (A) PCR strategy: the structure of the intron-containing domain of the indicator gene in a transposed copy is indicated; PCRs were performed with couples of nested primers flanking the intron, namely (h1, lz3), (h5, lz4) and (A, lz4) for PCR 1, PCR 2 and PCR 3 respectively; for retrotransposed copies, the length of the h5-lz4 fragment in PCR 2 should be 324 bp, and yield two fragments of 164 bp and 160 bp after *SacI* restriction. (B) Analysis of PCR products after amplification of genomic DNA from Kc cells co-transfected with the marked I element, Act-I-lacZ^{RT}, and either an irrelevant carrier DNA (-Act[I]), or the Act[I] expression vector (+Act[I]) (PCR 1 in lanes 1, PCR 2 in lanes 2). The results of two independent transfection experiments are presented. PCR fragments were analysed by electrophoresis of 10 μ l of the PCR reactions in 1.5% agarose gels and UV ethidium bromide staining; m: size marker, in bp. (C) The PCR-amplified fragments in (B) were restricted (+S) or not (-S) with *SacI* to demonstrate precise splicing out of the intron during the retrotransposition process. (D) X-Gal stained β -galactosidase-expressing cells transfected with Act-I-lacZ^{RT} and the Act[I] expression vector: (left) large fields of blue-staining cells (single, couple and cluster) as observed seven days after transfection (40-fold magnification); (right) higher magnification (70-fold) demonstrating nuclear location of the X-Gal staining.

transposition. The two ORFs of the I element were therefore introduced into the pAct5C-PPA expression vector (29), between the promoter and polyadenylation signal of the actin-5C gene (Act[I], see Figure 1). In this construct, the I element promoter (9) and almost all the 3' untranslated domain—among which the TAA repeats possibly involved in the initiation of reverse transcription—are deleted.

Mobility of the marked I element

The marked I element, Act-I-lacZ^{RT}, was introduced into *Drosophila melanogaster* Kc cells by transfection with either an irrelevant carrier DNA or the Act[I] expression vector. Kc cells are derived from a 'reactive' strain, and therefore do not contain functional I elements (30). For each experiment about 10⁷ cells per dish were transfected, and seven days later DNA was extracted for one fraction of the cells, whereas another fraction was tested for β -galactosidase activity, by an *in situ* X-Gal staining (see Materials and Methods).

To test for retrotransposition of the marked I element, fragments in the intronic domain of the indicator gene were tentatively amplified as described in (14) by PCR amplification of genomic DNA from the transfected cells, using nested couples of oligonucleotide primers (see scheme in Figure 2A). PCR amplification of retrotransposed copies should yield fragments of reduced size, in which the 636 bp-intron has been removed. Actually, as illustrated in Figure 2B for two independent transfection experiments, the expected 324 bp-fragment can be detected specifically under conditions where the Act[I] expression vector was co-transfected with the marked I element. This fragment could be easily detected (upon ethidium bromide staining) after the second PCR amplification (PCR 2), whereas for co-transfections without the I expression vector (i.e. with an irrelevant carrier DNA) no fragment could be detected (Figure 2B), even after a third PCR (primers in Figure 2A) which further increases PCR sensitivity at least ten-fold (data not shown). As previously noted (12), splicing out of the intron in the indicator gene generates a *SacI* restriction site at the splice junction. As illustrated in Figure 2C, *SacI* restriction of the amplified 324 bp-fragments yielded the expected 164 bp and 160 bp fragments, thus unambiguously demonstrating retrotransposition of the marked I element.

Finally, an *in situ* X-Gal assay for β -galactosidase activity in the transfected cells disclosed 'positive' cells—sometimes grouped in clusters (2–8 cells)—with a specific nuclear location of the staining (see Figure 2D). These were only detected in the co-transfection experiments with the Act[I] expression vector, at a frequency in the 10⁻⁵ to 10⁻⁴ range. These values are consistent with the numbers of transposed copies in the same cell populations, as estimated from the PCR experiments above, using serial dilutions of control plasmids for a quantitation of PCR sensitivity (data not shown) (see 34).

CONCLUSION

We have marked the *Drosophila melanogaster* LINE-like I transposon with a *lacZ*-based intron-containing indicator gene. This gene was inserted in place of a large deletion in the second ORF of the retrotransposon encompassing the reverse transcriptase domain. Retrotransposition of this defective marked element could be demonstrated in cells in culture that do not contain endogenous functional I elements, upon co-transfection of the marked element with an expression vector for I element ORFs.

In the presence of this expression vector—and only under these conditions—(i) PCR amplification of the intronic domain of the indicator gene disclosed splicing out of the intron and (ii) β -galactosidase-expressing cells with specific nuclear X-Gal staining were detected. These results demonstrate that an I element defective for its reverse transcriptase coding domain can be complemented *in trans* for retrotransposition. They further indicate that I element retrotransposition is not restricted to the *in vivo* situation encountered under the very specific conditions of hybrid dysgenesis (reviewed in 10,11), but can also take place *ex vivo*, in cell lines in culture. This transfection assay for retrotransposition of the I element in *Drosophila* cells in culture should allow analysis of the transposition mechanism in its *cis* and *trans* sequence requirements.

ACKNOWLEDGEMENTS

We thank Dr A. Bucheton for the gift of the pI407 plasmid, Dr G. Echalier for the Kc cells, Drs J.-F. Nicolas and C. Bonnerot for the pGEM-nlslacZ plasmid, and Dr C. Laviolle for critical reading of the manuscript. M.-P. Gassama is gratefully acknowledged for active participation in plasmid constructions. This work was financed by the Centre National de la Recherche scientifique (Unité associée 147), the Institut National de la Santé et de la Recherche Médicale (Unité 140), the Association pour la Recherche sur le Cancer (contract 6552 to T.H., grant to S.J.) and the Ligue Nationale contre le Cancer (grant to L.C.).

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