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Extrachromosomal transmission of microinjected DNA to the progeny of silkworm *Bombyx mori*

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Abstract

Vector DNA (pBmFRT) microinjected into the silkworm eggs (preblastoderm stage) persisted in different conformational forms throughout the period of embryonic development. Southern blot analysis confirmed the persistence of DNA as extrachromosomal copies. Slot blot analysis showed the inheritance of the injected DNA to the subsequent progenies; however the copy number of the injected vector declined in the progenies.

Introduction

Genetic transformation of *Drosophila* was achieved using transposable element (P-element) based vectors (Rubin & Spradling 1982). Since a P-element based vector system is not amenable for efficient transformation of non-drosophilid insects (O'Brochta & Atkinson 1996), there is a need for species specific transformation vectors. The transposable elements such as *Mariner, Minos, Hermes, hobo* and *piggyBac* have been identified in insects and their use as transformation vectors has been reported (Marshall 1998). Creating transgenic insects like honey bee (*Apis mellifera*) and silkworm (*Bombyx mori*) will have many applications (Crampton & Eggleston 1994). These transgenic insects may act as bioreactors for the high level production of useful foreign proteins.

For genetic transformation of silkworm, a number of vectors have been tested with limited success (Nikolaev *et al*. 1993, Nagaraju *et al*. 1996). Repetitive element based vector has not been attempted in genetic transformation of silkworm *Bombyx mori*. *Bm1* sequence (mid-repetitive sequence) is present in 2.3×10^4 copies per haploid genome in *Bombyx mori* (Adams *et al*. 1986). In the present study, we have injected a vector containing Bm1 and FRT sequence (derived from the 2 μm plasmid of *Saccharomyces cerevisiae*). The assumption was that the *Bm1* sequence would facilitate the integration of the injected DNA with the silkworm genome by homologous recombination. This paper reports the fate of pBmFRT DNA injected into the silkworm eggs.

Materials and methods

Bombyx mori (Pure Mysore) eggs collected 3 h and 8 h after egg laying (a.e.l) [preblatoderm stage eggs] were microinjected with 500 μg/ml of pBmFRT vector DNA (Figure 1; gift from Dr S. Mahalingam, USA). The vector dissolved in TE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA) was microinjected following the method of Shamila & Mathavan (1996). The control eggs were injected with TE buffer. After microinjection, eggs were kept at 10 °C for a few minutes and incubated at 27 ± 1 °C for hatching.

Genomic DNA was extracted from (i) microinjected eggs during the entire period of embryonic development, (ii) larval tissues from all instars (from Vth instar onwards both somatic and reproductive tissues), (iii) pupae, (iv) moths, (v) their progenies and (vi) corresponding controls.

For Southern blot analysis, the DNA samples (digested or undigested) were electrophoresed on 0.9%

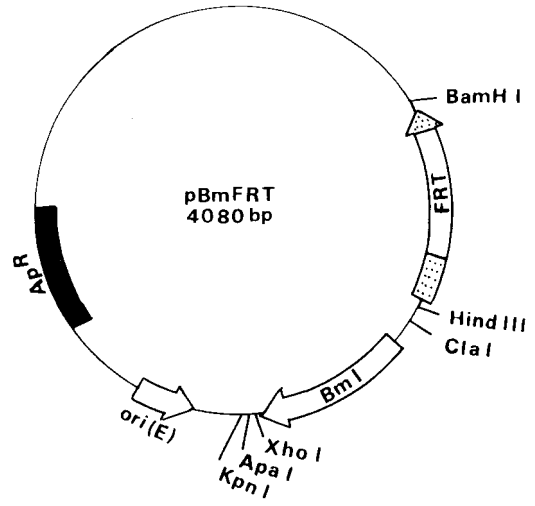

Fig. 1. Restriction map of the plasmid DNA (pBmFRT).

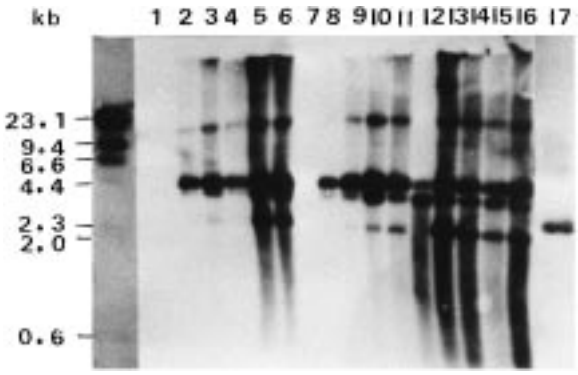

Fig. 2. Southern blot analysis of the genomic DNA (undigested) extracted from eggs injected with pBmFRT: lanes 1 and 7: DNA extracted from mock injected eggs (negative control); lanes 2–6: DNA from 1st, 2nd, 6th, 8th day developing eggs and freshly emerged larvae (3 h a.e.l); lanes 8–16: DNA from 1st–8th day developing eggs and freshly emerged larvae (8 h a.e.l); lane 17: pBSK+/*Cla* I digested (positive control).

agarose gel and transferred onto a nylon membrane by passive transfer method (Sambrook *et al.* 1989). The DNA in the gels were depurinated (0.2 N HCl for 10 min), denatured (0.5 M NaOH, 1.5 M NaCl for 10 min, twice) and neutralized (0.5 M Tris/HCl pH 8.0; 1.5 M NaCl for 15 min, twice) prior to transfer. The DNA were fixed to the membranes by exposing it to UV light for 3 min.

For slot blot analysis, genomic DNA (15 μg) was transferred onto a nylon membrane using slot blot apparatus. This was then placed on Whatman 1 filter paper pre-wetted with 0.4 M NaOH for 30 min to denature the DNA.

Standard protocols were followed for pre-hybridization and hybridization. The plasmid Bluescript SK+ was used for the probe which formed the backbone of pBmFRT. The entire pBmFRT plasmid was not used as probe because the vector contained the *Bm1* sequence (repetitive sequences in *Bombyx mori* genome). The probe DNA was radiolabeled with [α^{32}P] dCTP following random priming method (Boehringer-Mannheim, Germany). All the other techniques were followed as described by Sambrook *et al.* (1989).

For reverse probing, genomic DNA extracted from the Ist instar larvae that emerged from microinjected eggs was digested with *Hin*dIII and used as a radiolabeled probe. The genomic DNA extracted from microinjected batch, uninjected control and plasmid DNA were hybridized against this probe.

For plasmid rescue experiment, an aliquot of the genomic DNA extracted from the larvae that emerged from the injected batch was transformed in *E. coli* HB101. The plasmid DNA was rescued from transformed colonies and characterised

Results

A total of 2978 eggs (3 h and 8 h a.e.l) were injected with pBmFRT DNA. Figure 2 represents the Southern analysis of genomic DNA (undigested) extracted from microinjected eggs during embryonic stages and also from freshly emerged larvae. The injected DNA persisted in different conformational forms such as supercoiled, nicked, open dimers and multimers of high molecular weight forms.

The genomic DNA samples tested in the above experiment were digested with *Bcl* I (*Bcl* I has no site in the injected vector DNA) and subjected to Southern analysis (Figures 3A, B). The pattern of hybridization was almost similar to that of hybridization in Figure 2. Analysis of the hybridization data revealed the injected DNA persisted as unintegrated copies in the developing embryos and also in the freshly emerged larvae. The intensity and pattern of hybridization in lane 7 of Figure 3A (DNA from 5th day old egg) is different from the pattern observed in other samples. Reasons for the difference in the pattern of hybridization is not known. However, such types of hybridizations have been reported in other insect transgenic studies also (*Locust migratoria* – Mathi *et al.* 1991; *Bombyx mori* – Nagaraju *et al.* 1996).

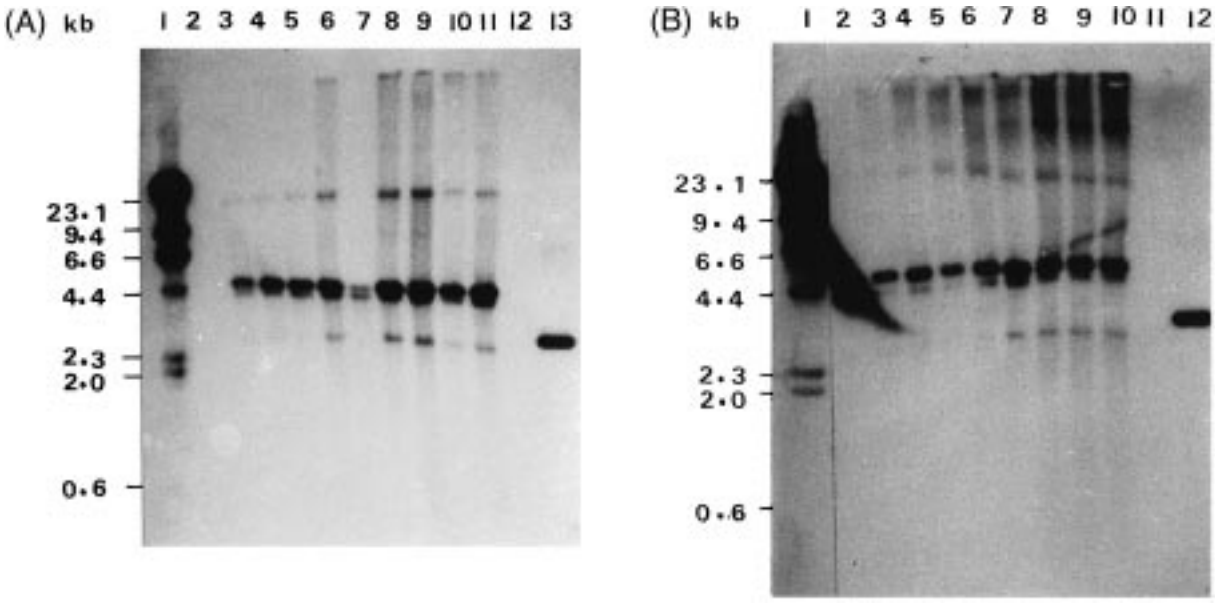

Fig. 3. Southern blot analysis of the genomic DNA extracted from microinjected/mock injected (control) eggs (3 and 8 h a.e.l). DNA was digested with *Bcl* I enzyme. (A) lane 1: λ/*Hin*d III; lanes 2 and 12: negative control; lanes 3–11: DNA from 1st–8th day developing eggs and freshly emerged larvae (3 h a.e.l); lane 13: pBSK$^+$/*Hin*d III (positive control). (B) lane 1:λ/*Hin* dIII; lanes 2–10: DNA from 1st–8th day developing eggs and freshly emerged larvae (8 h a.e.l); lane 11: negative control; lane 12: pBSK+/*Hin*d III (positive control).

DNA extracted from injected batch was digested with *Bam*H I (single site in the injected vector DNA) and hybridized with pSK$^+$ probe; the hybridization signals at 4.08 kb indicate the persistence of the injected plasmid without modification. However, the hybridization above the plasmid size suggests a partial digestion of the plasmid (Figure 4). There is no evidence to indicate the genomic integration of the injected DNA.

Since it has been confirmed that the vector DNA persisted in the injected batch as extrachromosomal copies, reverse probing technique was adapted to re-establish the persistence. Reverse probing technique was carried out using genomic DNA extracted from injected larvae as probe (Figure 5). The DNA was digested with *Hin*d III before preparing probe. A hybridization at 4.08 kb in *Hin*d III digested samples from injected batch (3 h and 8 h) confirms the persistence of the injected plasmid. Since the genomic DNA was labeled and used as probe, it hybridized at high molecular weight form with the homologous sequence present in the undigested genomic DNA (lanes 4 and 5 in Figure 5). In the same figure, a band in between 4.4 and 6.6 kb is observed (lane 4). The DNA loaded in this lane was undigested and it contained circular and relaxed forms of injected DNA. The band corresponds to a relaxed form of the injected DNA. A faint signal

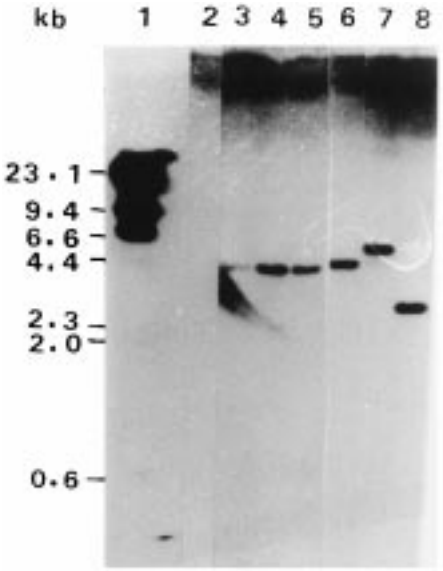

Fig. 4. Southern blot analysis of DNA extracted from microinjected/mock injected (control) eggs (3 and 8 h a.e.l). The randomly selected samples were linearized with *Bam*H I: lane 1: λ/*Hin*d III; lane 2: negative control; lanes 3–5: DNA from 3rd, 6th and 7th day developing eggs (3 h a.e.l); lane 6: DNA from freshly emerged larvae (8 h a.e.l); lane 7: DNA from 1st day eggs undigested (8 h a.e.l); lane 8: pBSK+/*Bam*H I (positive control).

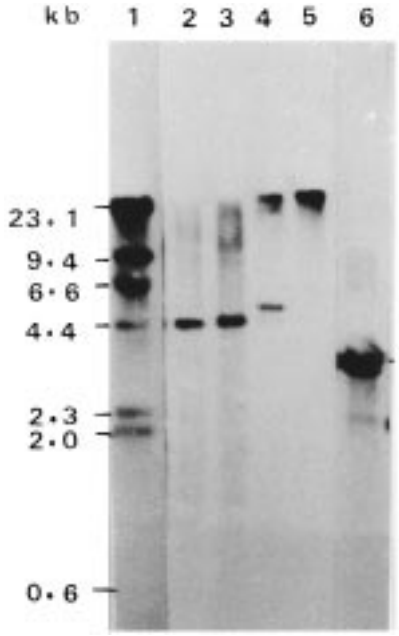

Fig. 5. Reverse probing: the genomic DNA extracted from the freshly emerged larvae eclosed from eggs injected with pBmFRT was used as probe. lane 1: λ/*Hin*d III; lane 2: DNA from 8th day eggs digested with *Hin*d III (3 h a.e.l); lane 3: DNA from 8th day eggs digested with *Hin*d III (8 h a.e.l); lane 4: undigested DNA from 1st day developing eggs (8 h a.e.l); lane 5: negative control; lane 6: pBSK+/*Hin*d III (positive control).

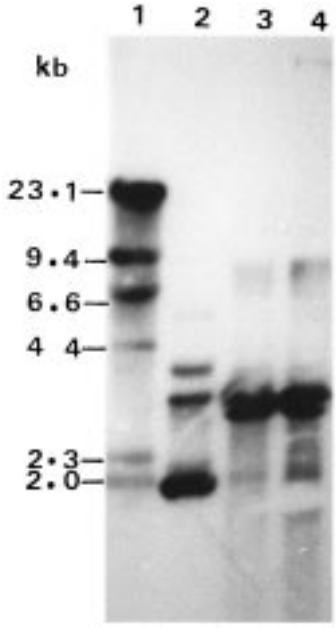

Fig. 6. Southern hybridization of rescued plasmid obtained from pBmFRT injected batch (3 and 8 h a.e.l). lane 1: λ/*Hin*d III; lane 2: rescued plasmid (undigested); lanes 3 and 4: rescued plasmid digested with *Hin*d III. pBSK+ was used as probe.

is also observed in the lane at (4.08 kb) representing another form of the persisting DNA.

The plasmid DNA was rescued from the genomic DNA extracted from freshly emerged larvae of the injected batch. The restriction pattern of the rescued plasmid and injected plasmid was almost identical (Figure 6). This result confirms the persistence of the injected DNA without modifications.

Bombyx mori larvae emerged from the microinjected eggs (random samples from 3 and 8 h a.e.l. injected batch) were reared in order to asses the persistence of the injected sequences in the larval stages. Moths emerged from the injected batch (G_0) were either self crossed or crossed with uninjected controls. From the injected batch F_1 and F_2 progenies were reared; DNA was extracted from the progenies and subjected to slot blot analysis to trace the persistence of transgene in the progeny.

About 200 samples from G_0 were subjected to slot blot analysis; of this 20% showed positive response (6% from reproductive tissues and 14% from somatic tissues). Similarly, about 155 samples were analysed from F_1 generation; of this 17% showed positive signals (4% from reproductive tissues and 13% from somatic tissues). In F_2 generation, 15% of the samples showed positive response (3% and 12% positive from reproductive and somatic tissues, respectively). The intensity of response was reduced in F_2 batch compared to the G_0 (Figure 7).

Southern blot analysis of the DNA from these samples (F_1 and F_2) did not give discrete hybridization. Faint hybridization was observed at 4.08 kb corresponding to the plasmid (data not shown). The faint response may be due to low copy of the plasmid DNA persisting in the progenies.

Discussion

Southern blot analysis showed that the pBmFRT DNA injected into the eggs mostly persisted as extrachromosomal copies and these copies were transferred to their progenies. This pattern of extrachromosomal persistence (without replication) was reported in *Drosophila* (Shen & Sofer 1991); *Locust migratoria* (Mathi *et al*. 1991) and *Bombyx mori* (Nikolaev *et al*. 1993). Nagaraju *et al*. (1996) observed intense degradation of the DNA (pBR322) injected into silkworm eggs during early part of development. The DNA was subjected

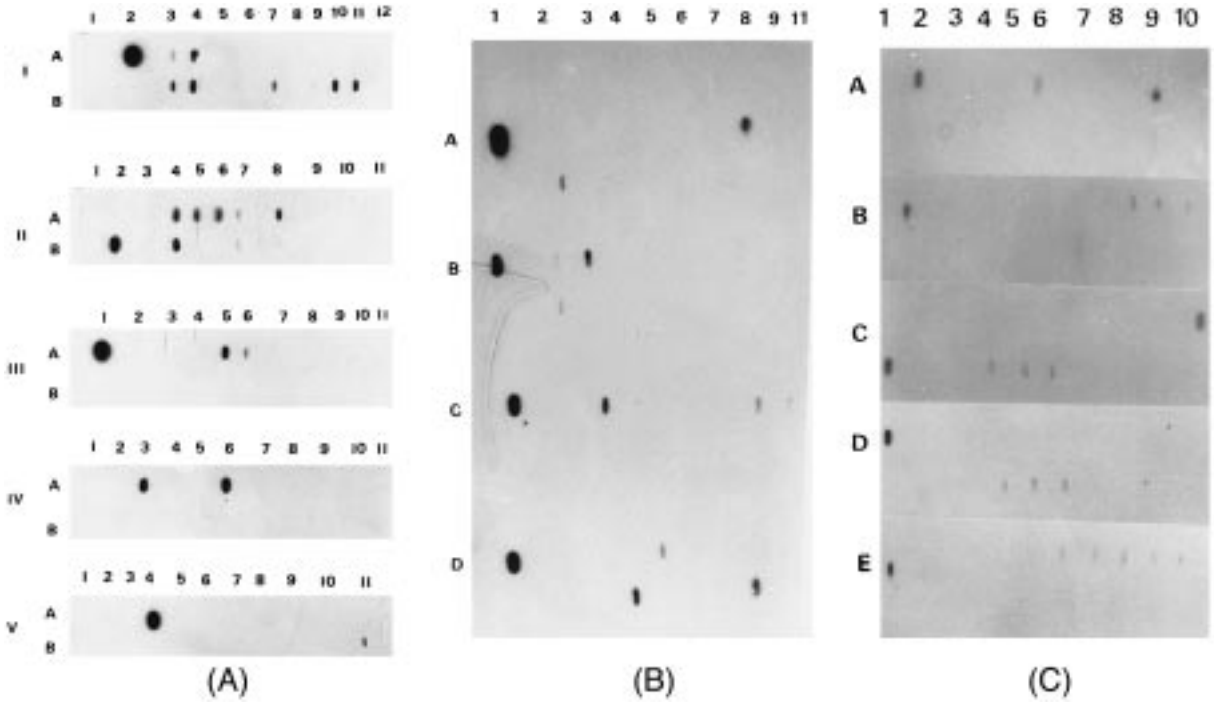

Fig. 7. Slot blot analysis of the genomic DNA extracted from G_0, F_1 and F_2 individuals that descended from the eggs injected with pBmFRT. Each slot was loaded with 15 μg of genomic DNA. (A) Positive controls (1 μg/slot) – Slots: A2, B2, A1, A3, and A4 in blots I–V. Negative controls (3 μg/slot): slot B1 in blots I–V. Positive signals. The following slots represent reproductive tissues (I – B9, B11; II – A4, A6, A7 and A8; III – A6; V – B11). The remaining positive slots represent the DNA from somatic tissues of larvae, pupae and moths of G_0. (B) Slots A1, B1, C1, D1: upper slots positive control (3 μg/slot) and lower slot negative control (3 μg/slot); positive signals in slot B2, C3, C8, D4 and D5 represent reproductive tissues and the remaining positive signals in the blots A, B, C and D represent the DNA from somatic tissue of larvae, pupae and moths of F_1. (C) Slots A2, B2, C10, D1, E1 upper slot: positive control (1 μg); lower slot: negative control (3 μg/slot); positive signals in slot B10, C5, C10, D6, E7 and E8 blots represent reproductive tissues and the remaining positive signals in the blots A, B, C, D and E represent the DNA from somatic tissue of larvae, pupae and moths of F_2.

to endonuclease activity soon after injection and the pool of unaffected DNA remained stable throughout embryogenesis. The above authors have detected the presence of exogenous DNA in the freshly hatched larvae and reported progressive elimination of injected DNA before the larvae attained IInd instar. In the present work, the injected DNA persisted even during late larval stages and also in the progeny. However, based upon the intensity of hybridization signals, it is inferred that the concentration of the injected DNA has been reduced progressively in the larvae and progeny. Recently, Jeyaprakash *et al*. (1998) reported that the injected DNA (via maternal injection) persisted as extrachromosomal copies in predatory mites (*Metaseiulus occidentalis)* and transmitted up to 30 generations; afterwards the extrachromosomal plasmid DNA was completely lost.

The episomal persistence of injected sequence may be due to the following reasons: (i) The embryogenesis of *Bombyx mori* has an unequal syncytial division, i.e., all the nuclei are not simultaneously resting at the periphery prior to cellularization; it can reduce the contact of injected sequence with host chromosome and result in considerable loss of injected DNA in yolk (Mathi *et al*. 1991). (ii) As reported by Steller & Pirrotta (1985) in *Drosophila*, the formation of pseudonuclei can minimise the probability of injected DNA to interact with chromosomal DNA. (iii) As reported by Etkin *et al.* (1984) intranuclearly localized extrachromosomal DNA may persist in the developing embryo and larvae while those molecules in the cytoplasm might have been degraded.

In the present study, the *Bm1* element, a mid-repetitive sequence in the *Bombyx mori* genome was used with the assumption that it would undergo integration through homologous recombination. The pBmFRT persisted as extrachromosomal copies in the injected batch; however, *Bm1* sequence in the vector failed to mediate integration of pBmFRT by homologous recombination. It is known that yeast recom-

binase FLP acts on a specific target FRT sequences to catalyze intramolecular and intermolecular recombination in insects like *D. melanogaster* (Golic *et al*. 1997) and *Aedes aegypti* (Morris *et al*. 1991). In the current study, we used FRT in the vector; if the FLP recombinase has been applied in *trans* it would have helped in homologous recombination as reported in other insects.

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