RESEARCH ARTICLE

Expression of microinjected foreign DNA in silkworm, *Bombyx mori*

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As a prelude to achieving transgenesis in *Bombyx mori*, conditions have been established for successful microinjection of cloned foreign genes into the silk worm eggs. A sharpened metallic needle is used to pierce the thick chorion layer of the eggshell, approaching through a droplet of DNA solution deposited on its surface. The microinjection is carried out within 2–2.5 h after oviposition and the injected eggs show 3–5% hatchability and 80–90% survival. Such larvae continuously expressed the microinjected cloned reporter gene, β-galactosidase, placed under the control of a constitutively expressed cytoplasmic actin A3 gene promoter from *B. mori*. The expression is seen in different tissues, viz. the fat body, tracheae and the silk glands, till the late larval instars. The microinjected DNA sequences are retained in the adult G_0 moths.

THE successful development of transgenesis methodologies has made the germ line transformation of metazoa a reality. This technique permits the modification of traits in the recipients through the introduction of foreign DNA sequences in their germ line and thus offers a great potential for the improvement of desired traits in animals and plants. Transgenesis techniques have been developed for a variety of animals ranging from insects such as Drosophila[1] and mosquitos[2] to mammalian species[3–5] and aquatic organisms or amphibians[6–9].

The microinjection of cloned DNA has also opened up new concepts in basic molecular biology for the analysis of gene expression *in vivo*, related to the study of tissue- and development-stage-specificity. In the absence of any well-documented methodologies for transgenesis in *Bombyx mori*[10], the *Drosophila* system has, in fact, been exploited as a model for *in vivo* regulation of the expression of silk fibroin and related genes. Microinjection of cloned foreign DNA sequences into *B. mori* eggs was first attempted by Nikolaev *et al.*[11] but they had examined only the survival of the injected embryos and not the expression of the injected genes. Subsequently, Tamura *et al.*[12] reported the microinjection into *B. mori* eggs of a cloned reporter gene *CAT* and its transient expression in the early stages of embryonic development. Most recently, Coulon-Bublex *et al.*[13] have demonstrated the expression of a microinjected β-galactosidase gene almost exclusively in the vitellophages of *B. mori* during the early embryonic stages. Here we report the introduction of the cloned foreign gene into the silk worm eggs and its expression in the tissues and organs of the larvae until the late stages of development.

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Experimental

Plasmid DNA

The plasmid DNA used in the present study was pA3-lacZ, containing the bacterial β-galactosidase gene (reporter) under the control of *B. mori* cytoplasmic actin A3 promoter element[13]. The actin coding sequences were almost completely replaced by the bacterial *lacZ* gene, which was fused at codon 54 on the 5' side and sequences starting from 129 bp downstream to the stop codon of the A3 gene on the 3' side. Since actin A3 is expressed constitutively in all tissues of *B. mori*[14], the activity due to the microinjected reporter gene was expected to be detected in all the tissues.

Microinjection

Newly emerged moths of *B. mori* (Pure Mysore, a multivoltine race) were allowed to mate for 4–6 h. The separated females were kept in a sterile chamber to lay eggs on Whatman No. 1 filter paper sheets. The paper strips containing 60–70 freshly laid eggs were treated with 2% formaldehyde and 70% alcohol and air-dried. The paper was fixed onto the inner side of the lid of a sterile petri dish using a double-sided tape.

The DNA to be microinjected was deposited on the egg surface using a fine-tipped glass capillary (a few nanolitres of the solution, 2–4 μg of DNA per μl of injection buffer containing 0.5 M KCl and 0.1 mM sodium phosphate, pH 6.8). A sharpened sterile metallic (stainless steel) needle was used to pierce the thick chorion layer of the *B. mori* eggs, approaching the surface through the deposited solution, the two processes being carried out almost simultaneously under a microscope. The injections were carried out near the micropyle. Care was taken to ensure that the glass capillaries do not get blocked with the vitellus emerging out from within the egg. The pressure of piercing the chorion should be well controlled to prevent the bursting of the whole egg.

The microinjections were carried out within 2–2.5 h of egg laying. The injected eggs were maintained in a humid atmosphere at 26°C by putting upside down the petri plate lid with the eggs sticking to it over a dish containing a moist tissue or a cotton swab. After 6–8 h, the eggs were checked again under a stereomicroscope, and the damaged or uninjected ones were removed. The larvae were allowed to hatch out and were fed on freshly plucked tender mulberry leaves. They were subsequently reared under the normal rearing conditions at 26°C and 60% humidity.

Expression of microinjected DNA

The larvae at different stages of development were sacrified and the β-galactosidase activity due to the microinjected plasmid DNA was monitored histochemically[15]. The pH of the staining solution was maintained at 6.8–7.0, a condition under which the endogenous activity of the larval tissues, if any, was barely detectable. The individual tissues were separately processed for enzyme activity staining.

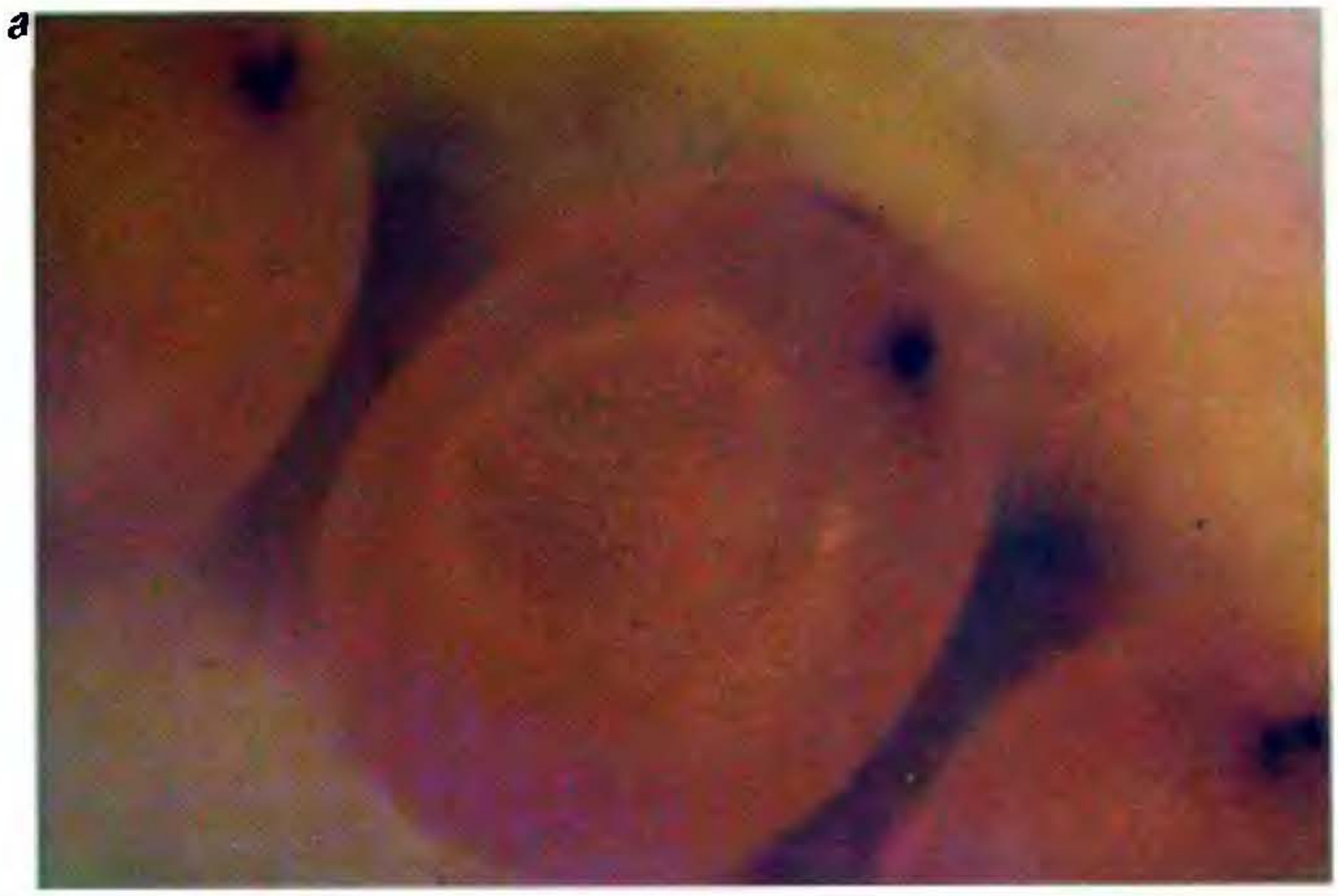
Some of the injected larvae (4 out of 10 surviving larvae, totally numbering 20, from the different batches) were allowed to spin the cocoons and the total DNA from the emerging adult moths (after clipping off the head, wings and limbs) was isolated. The presence of the microinjected DNA sequences in the isolated *B. mori* genomic DNA was examined by dot blot hybridization[16] using the nicktranslated plasmid pA3-lacZ DNA as the probe.

Results and discussion

The technique for injecting into the eggs has to be evolved depending upon the surface through which the injected needle has to go through. For mammalian eggs/embryos a sharpened glass capillary is used as a microinjection needle. In case of eggs with soft chorion outer layers too, glass capillaries are useful for microinjecton. The egg shell of *B. mori* being comprised of a thick chorion layer (Figure 1) presents the problem of penetration with glass capillaries because it tends to break during the injection process. Hence, we used a sharpened stainless-steel needle to create a pore on the egg shell surface by gently piercing through a microdroplet of the DNA solution to be injected, which was simultaneously placed at the site of injection. A schematic representation of this procedure is shown in Figure 2. Injections were carried out by a single needle prick near the micropyle but away from it (Figure 1 *a*, *b*). Once the micropyle is damaged, the survival chances of the embryos are totally lost. In the case of *Drosophila* dechorionation of the embryos without disturbing the internal structure has been possible, permitting the microinjection of DNA through such a surface with glass capillaries. Similar attempts to dechorionate partially or fully the *B. mori* eggs, either by using bleach solutions or sticky tapes, always resulted in the nonviability of *B. mori* eggs in our hands. Tamura *et al.*[12] had used a tungsten needle and air-pressure system for injecting into the silkworm embryos.

Since the turgid pressure inside the *B. mori* eggs is high, the vitellus tends to leak out as soon as the surface is pierced. However, a small quantity of the DNA gets injected by the time the metallic needle is removed, since the piercing is achieved through this solution. The pressure of injection should be gentle enough to avoid too much loss of embryonic fluids, which will otherwise interfere in the viability of the embryos.

Coating the surface of the injected eggs with a microdrop of mineral oil also reduces the leakage of



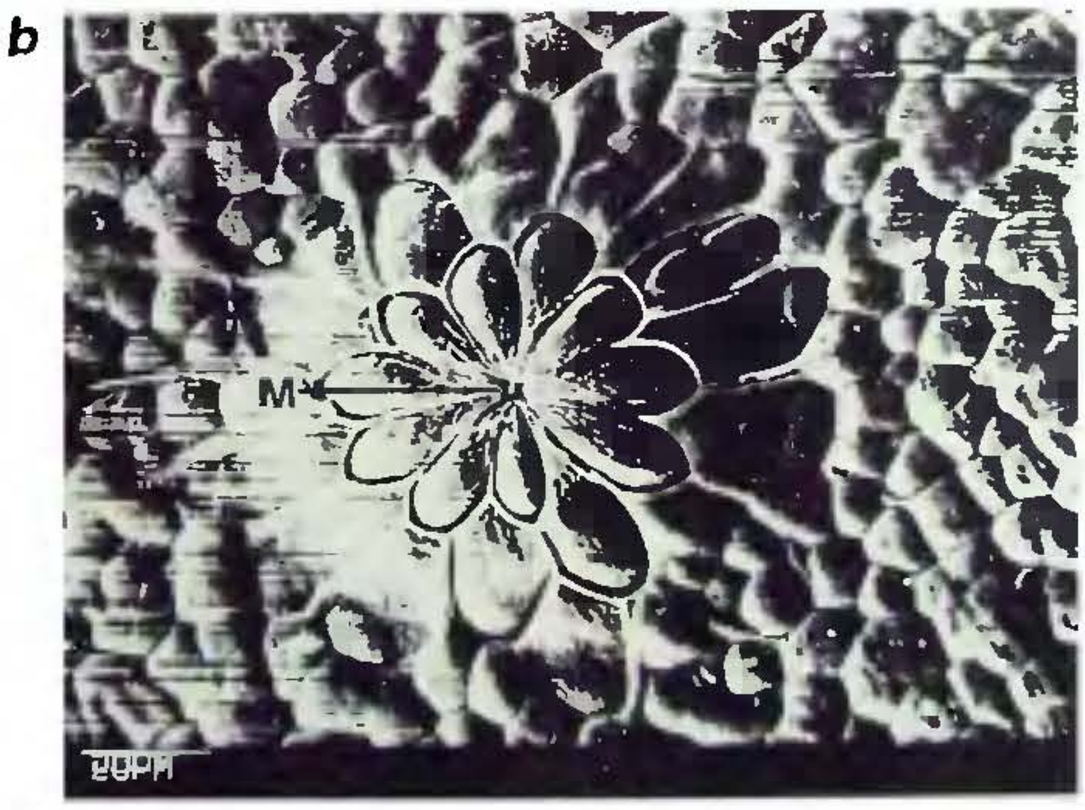


Figure 1. *Microinjection of B mori* eggs *a*, *B mori* eggs, 24 h after injection seen under a stereomicroscope (× 350) The piercing spot on the egg shell during microinjection can be seen *b* Scanning electron micrograph of *B mori* eggs (× 1000) showing the thick chorion surface and micropyle region (M)

vitellus and prevents entry of air into the eggs. Since the embryonic development of *B. mori* takes about 10 days for completion, care should be taken to avoid dehydration of the injected embryos as well as bacterial/fungal contamination during incubation. Usually, a moist tissue paper placed in the development chamber (inside the petri dish in which the eggs are being incubated) ensures this. By smearing the eggs and the inside of the petri plates with gentamycin (300 U/ml), the contaminations could be avoided.

Recombinant plasmid DNA was introduced into the *B mori* eggs within 2-2.5 h of oviposition, i.e. at around the time pronuclear fusion takes place. Injection within 2-6 h of laying has been generally found to be most suitable[13]. The results on the effect of microinjection on the hatchability and survival of the eggs are presented in Table 1.

In general, the hatchability of microinjected embryos of *B mori* was low and ranged from 3 to 4.5%. However, the survival of the hatched larvae was high, in

Table 1. Survival of the microinjected *B. mori* larvae

No. of eggs injected	No. of eggs damaged destroyed during injection	Percentage hatchability	Percentage survival	Larvae +ive for β-galactosidase activity
512	42	4.5	89	9 (10)*
396	29	3	92	7 (7)
210	18	3.8	86	4 (5)
518	36	3.1	79	7 (9)
450	31	3.2	84	4 (4)
280	15	3.7	82	4 (4)

*The numbers in parentheses indicate the number of larvae screened for β-galactosidase expression

The *B. mori* eggs were microinjected within 2–2.5 h of oviposition, and incubated at 26°C in a moist, sterile atmosphere till hatching. The larvae were fed on mulberry leaves till the fifth instar, and screened for expression of β-galactosidase activity at different larval stages. A few larvae (altogether 20, from different batches of microinjection) were allowed to pupate and emerge as adult moths.

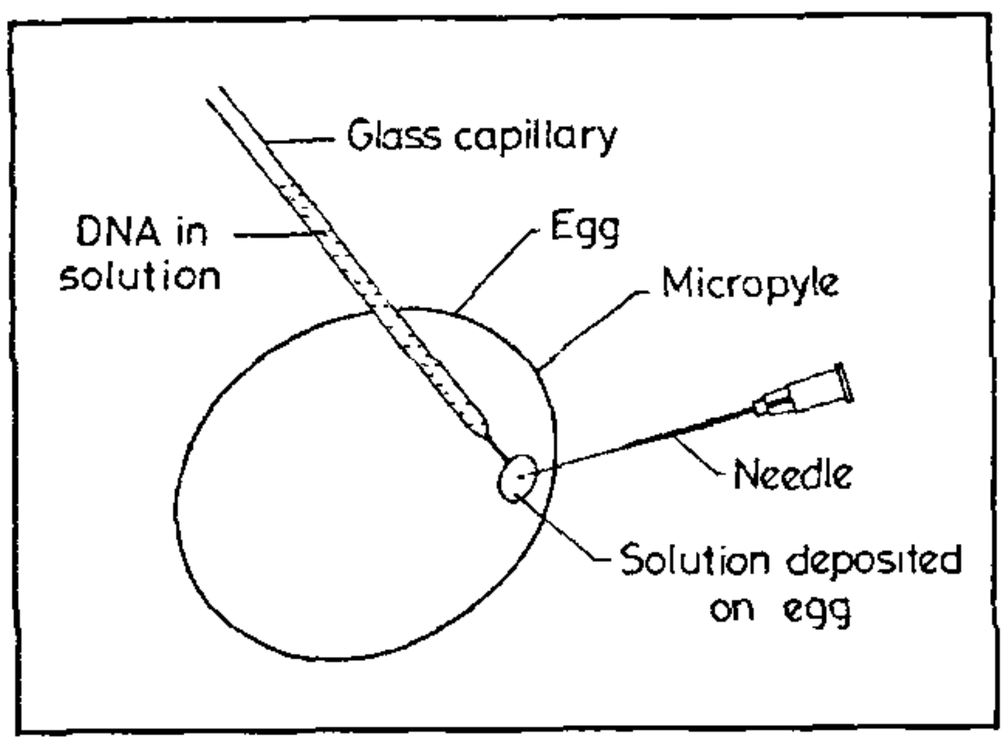


Figure 2. Schematic representation of the microinjection process. The eggs were viewed through a stereomicroscope and the DNA solution to be injected was placed on the egg as a microdroplet using a fine glass capillary. Simultaneously, a sharpened stainless steel needle was used to pierce gently the egg shell surface through the deposited solution. The withdrawal of the needle permits the entry of a small amount of the DNA solution into the eggs.

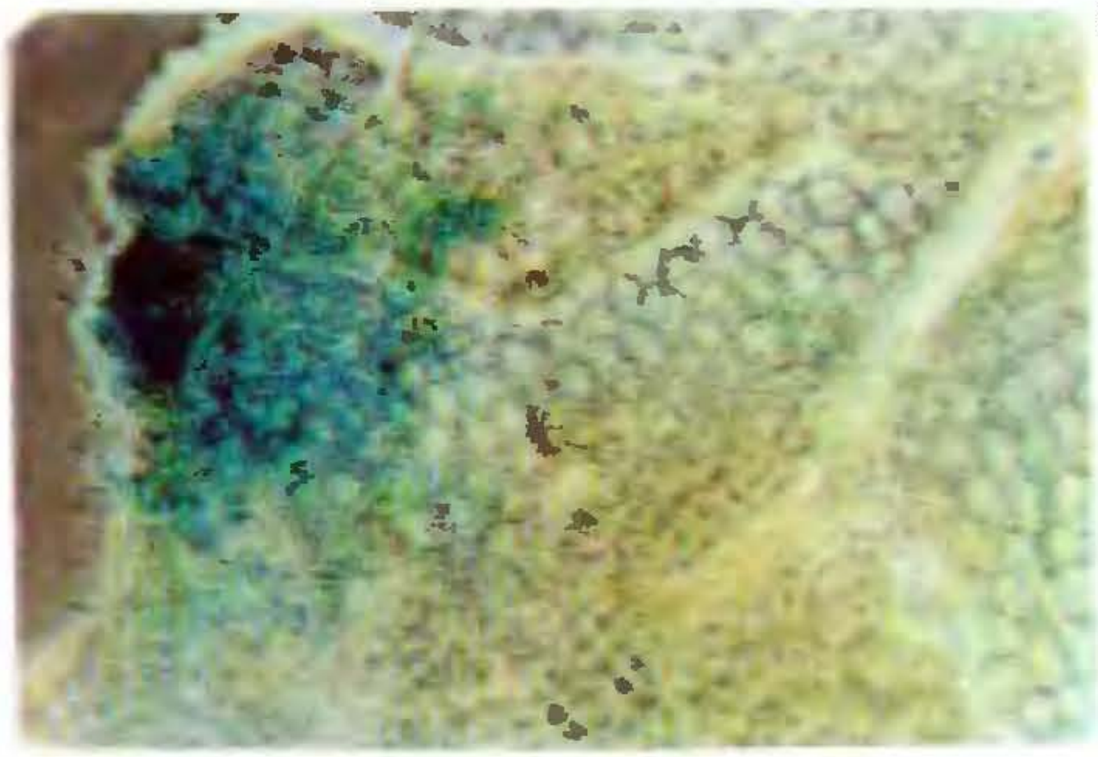
the range 80–90%. The failure rate due to external physical damages during injection itself was always lower than 10%. Nevertheless, a large percentage of the microinjected eggs did not hatch out, sans external damage. Amongst the larvae which hatched out, 80–100% showed high levels of β-galactosidase activity. The observed activity was evidently due to the presence of the microinjected DNA because the endogenous level of the enzyme activity under the assay conditions employed was barely detectable. The β-galactosidase activity in the isolated fat bodies, trachea, or the silk glands from the control and from the microinjected *B. mori* larvae is presented in Figure 3 *a–f*. The high levels of β-galactosidase activity in these tissues from the injected larvae are clearly evident. In tracheae and fat bodies, the blue colour due to the enzyme activity staining was spread from the head to anal regions on both sides of the intestines. There was detecable staining for β-galactosidase also in the silk glands of the microinjected larvae. This could be anticipated because the actin A3 gene, whose promoter has been exploited in the present studies, is known to be expressed in this tissue[17]. However, the appearance of the enzyme activity was restricted to the lower parts of the anterior silk gland close to its junction with the middle silk gland (Figure 3 *d, e*). Under identical conditions, the tissues from the uninjected control larvae did not show detectable levels of this enzyme activity (Figure 3 *c, d, f*). For controls, at least 10 times the number of larvae was always examined.

The transient expression of the β-galactosidase activity due to the microinjected DNA was seen all through the larval development. When the total genomic DNA from the adult moths emerging from such microinjections were analysed by dot blot hybridization, they were found to harbour the injected sequences (data not shown). The presence of microinjected plasmid DNA sequences in up to 7–10% cases of the G_0 moths of *B. mori* has been reported previously[11]. Partial integration of foreign DNA in the absence of any homologous or recombinogenic sequences in the mosquito *Anopheles gambiae* has also been reported[2]. The expression of neomycin resistance in *A. gambiae* due to the microinjected plasmid containing *hsp-G418* and the *P* element of *Drosophila* was found to be independent of the *P* element sequences. The features responsible for determining how the foreign DNA sequences are retained in the plasmid form for such long times of larval development, or how they are integrated into the genome in the absence of any recombinogenic or homologous sequences, are not clear at present. If the microinjected DNA sequences are integrated into the genome of *B. mori*, the resulting transgenesis would

Figure 3. *In vivo* expression of microinjected foreign gene in different tissues of *B. mori*. The eggs microinjected with pA3-lacZ plasmid were allowed to hatch out and develop up to the moth stage. The larvae at different stages were sacrified and the tissues were stained *in situ* for β-galactosidase activity. The picture shows the pattern seen in 5th instar larvae. For controls, at least 10 times the number of larvae were examined. *a, b*, Tracheae and fat bodies from microinjected larvae (× 400). *c*, Tracheae and fat bodies from control larvae (× 400). *d*, Silk gland from microinjected (left) and control (right) larvae (× 100). *e, f*, A close up view (× 400) of the region of anterior silk gland at its junction near the middle silkgland, where the β-galactosidase activity is clearly seen in the injected (*e*) but not in the control uninjected (*f*) larvae. ▶

a

b



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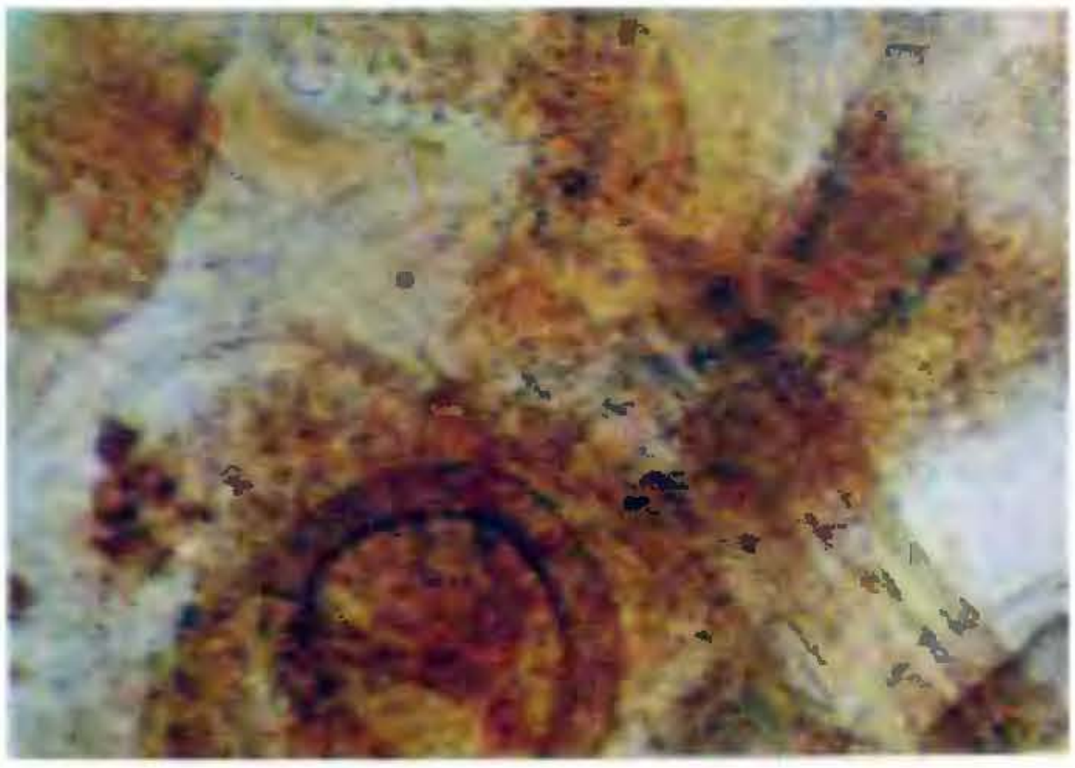

d

e

f

permit the transfer of desired traits into the germ line of silk worm If a judicious choice of genes for introduction into the germ line is made, this technique could revolutionize sericulture. However, at present, the availability of sequences that would readily permit germ line integration in *B mori* is not documented. Nevertheless, in recent times, the retention of microinjected plasmid DNA sequences and their expression in tissues and muscles for several weeks after injection in mammalian species has been reported[18]. The latter observation suggests the possibility of application of this technique for gene therapeutics or direct DNA-mediated protective vaccination against viral infections in humans.

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