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Electroporation, an alternative to biolistics for transfection of *Bombyx mori* embryos and larval tissues

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Abstract

There are few powerful techniques available to transfect insect tissues. We previously used biolistics to transfect *Bombyx mori* embryos, and larval and pupal tissues (Thomas J-L et al. 2001. Journal of Insect Science 1/9, Kravariti L et al. 2001. Insect Biochemistry and Molecular Biology 31: 473-479). As the main limitation was the irregularity in results we explored electroporation as an alternative technique by adapting techniques used for chicken embryos to *B. mori* embryos. By injecting the DNA solution into the hemocoel of late embryos that were finishing organogenesis, we expressed marker genes in numerous tissues following electroporation. With some adaptation of the method this was also achieved for early embryos lacking a hemocoel. Some larval tissues were also transfected. During these technical studies we found that optimizing parameters such as electrical voltage, number of pulses and their frequency, and conductivity of the buffer was important. These results confirmed that electroporation is a reliable technique for transfecting *B. mori* tissues.

Abbreviation:
GFP Green Fluorescent Protein
CCD Charged Coupled Device

Introduction

Electroporation is well known today as a powerful transfection technique and is useful for the study of gene expression. Initially developed for the transfection of *in vitro* cultivated cells (Chang 1992; Neuman *et al.* 1982; Fromm *et al* 1985; Andreason *et al.* 1988; Shigikawa and Dower 1988), the technique was adapted to *ex-vivo*, *in-situ* and *in-vivo* DNA transfection of part of, or whole organisms (Titomirov *et al.* 1991; Weaver 1993; Dev and Hoffman 1994; Muramatsu *et al.* 1998; Itasaki *et al.* 1999).

In our silkworm transgenesis program we previously used biolistics to check the functionality of DNA constructs (Thomas *et al.,* 2001) or to study tissue-specific promoter regulation (Kravariti *et al.* 2001). Although some of the difficulties of applying biolistics to soft insect tissues were circumvented (Thomas *et al.* 2001), the irregularity of the results remained the main drawback. Moreover very early dechorionated embryos are excessively fragile as targets of the micro-projectiles. The work of Muramatsu *et al.* (1997a, 1998) compared the relative efficiency between three of the main transfection methods i.e. lipofection, biolistics, and electroporation. These studies were performed on early chicken embryos using a DNA construct in which the *lacZ* gene was under the control of the ubiquitous Rous sarcoma virus promoter. From their results it was concluded that electroporation was the best of the three tested methods. In fact, the early chicken embryo seems well adapted to such a technique because DNA can be confined in diverse organs such as the neural tube, the heart cavity or the cerebrum vesicle (Sakamoto *et al.* 1998; Louvi *et al.* 2000; Itasaki *et al.* 1999; Nakamura *et al.* 2000; Tabata *et al.* 2001). Furthermore, diverse organs have been electroporated, such as liver (Heller 1996), gonads (Muramatsu *et al.* 1997b; Sugihara *et al.* 2000; Yamasaki *et al.* 2000; Nakamura et al. 2002), muscle (Mir *et al.* 1999; Vica *et al.* 2000) or even tumors (Goto *et al.* 2000; Kishida *et al.* 2001). It is also interesting to notice that whole multicellular organisms can be successfully electroporated as in the case for the frog tadpole (Eide *et al.* 2000; Sasaqawa *et al.* 2002a,b), mouse and chicken embryos (Osumi and Inoue 2001; Yasugi *et al.* 2000) or explanted tissues (Fukuda *et al.* 2000; Harrison *et al.* 1998;] Miyasaka *et al.* 1999).

The main application field of electroporation is focused on vertebrate tissues and organisms but very little work has been done on insects. Only three papers describe insect electroporation: Kamdar *et al.* (1992) tried electroporation of *Drosophila melanogaster* embryos, Leopold *et al.* (1996), Devault et al. (1996), Hughes et al. (1997) of *Helicoverpa zea* embryos and finally Moto *et al.* (1999) of explanted *Bombyx mori* larval cerebrum. This work showed that electroporation is a powerful method and could be considered as an alternative to the biolistic technique to study somatic transient gene expression in *Bombyx mori* embryonic tissues. DNA can be easily confined within the hemocoel of a late *B. mori* embryo in which organogenesis is complete. Since the transfection of embryonic

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organs was the main concern, we tried to electroporate diverse *B. mori* organs considered as a pouch containing the target cells to be transfected. To validate the technique a densoviral DNA based construct, *pBRJZ* (Jourdan *et al.* 1990, Giraud *et al.* 1992) was used as a transfection vector that had been successfully used in our previous biolistic work (Thomas *et al.* 2001).

We demonstrate in this paper that electroporation can be successfully applied to transfect and express DNA in late and especially in early *B. mori* embryonic tissues, but also in larval tissues such as ovaries or epithelial cells of larval imaginal wing disc.

Materials and Methods

Silkworm strain

The Indian polyvoltine strain Nistari of *B. mori* was used as a source of embryonic larval tissues. This strain was obtained from a silkworm collection maintained at UNS/INRA (France). The silkworms were reared at 25° C and fed with mulberry leaves.

Preparation of embryos and description of electroporation experiments

Eggs newly laid on a sheet of paper were placed in an incubator at 25° C and 80% R.H. for 6 days and the occurrence of stemmata pigmentation (the first pigmented structures) was controlled. The eggs were used after the appearance of stemmata pigmentation until cephalic black pigmentation appeared. A constant supply of eggs was maintained by keeping them at 5° C for no longer than one week. This temperature stops development without disturbing the normal resumption. Eggs were collected by incubation for 5 minutes in tap water bath (approximately 20° C) and dried on paper towels before being glued onto Petri dishes with cyano-acrylate glue ensuring that they were laid flat. Eggs were disinfected with a 4% formaldehyde solution in PBS 10 mM, pH 7.4, for 10 min, rinsed with distilled water and finally dried with absolute ethanol. Eggs were dissected in Grace's medium containing antibiotics (Sigma www.sigmaaldrich.com, cat. # A-5955). Just before being electroporated 6 day old embryos were put on a wet GF/C Whatman glass filter (diameter 25 mm, Figure 1D) and injected with the DNA solution (0.5 µg/µl, 0.5% eosin) using a sharpened glass capillary (tip diameter: approximately 30 µm). Injection of DNA was carried out two ways; the first location was just behind the head through the translucent soft integument, the second, directly through the front of the head. Embryos were placed between the electrodes (Figure 1E) connected to the BTX ECM 830 electroporator and electroporated (conditions specified in the Results). The embryos were then placed in Grace's medium containing antibiotics for 2 days at 25° C for subsequent development. It was possible to cultivate embryos in wells of 16 mm or 35 mm in diameter, or in standard 1.5 ml micro-tubes filled with one ml of culture medium. We used a variant method with the early embryos (3 and 4 day old embryos) for which manipulation with forceps is impossible. After dechorionation using forceps, embryos were manipulated using a pipette and always kept floating in liquid. To be electroporated early embryos were put in agarose cast wells previously placed between the electrode set (Figure 1A and B). After the electroporation was completed, the piece of agarose was put in a 35 mm diameter culture well containing Grace's medium (Figure 1C).

Preparation of ovaries and imaginal wing disks for electroporation

Ovaries and imaginal wing disks were dissected from 5 day old fith instar larvae killed with diethyl ether. Just before dissection, the integument of the larvae was carefully disinfected using bleach water, rinsed with deionised water and dried with 90% ethanol. Ovaries were excised through two incisions done on the back of the eighth abdominal segment, whereas imaginal wing disks were excised from incision done just above the second pair of forelegs. The DNA solution was microinjected into the ovaries and into the peripodial pouch of the imaginal disk. These organs were then treated as the 6 day-old embryos.

Description of the electrodes set

Several kinds of BTX electrode sets are usable with the ECM 830 electroporator including a BTX microslide electrode set having a gap of 3.2 mm and 4 millimeters high. A custom set of platinum wire electrodes (diameter: ~ 0.3 mm) was necessary because of the small size of embryos. They were glued on a microscope slide to create a leak proof slot (Figure 1E). This customised electrode set allowed the embryos to be handled more easily. Another set of customised electrodes was made to allow the easy handling of the very fragile 3-4 day old embryos. This electrode set was made of two parallel aluminium bars with a gap of 8 mm and 6 mm high. The bars were attached with epoxide glue on a microscope slide to create a leak proof slot (figure 1A), . The width of the gap was designed to fit easily inside a piece of agarose consisting of several cast wells (Figure 1A) into which the early embryos were placed (figure 1B) and submitted to electric pulses after the DNA solution was added. The whole block of agarose was then transferred in the insect Grace's culture medium (Figure 1C).

DNA vectors and their preparation

Four vectors were used that had ubiquitous promoters.

1) *pBRJZ*,The more widely used, especially as a positive control, was a densoviral vector with the *LacZ* marker gene under the control of the *P9* viral promoter (Jourdan *et al.* 1990, Giraud *et al.* 1992). A variant was used having the *GFP* coding sequence (a gift of Hervé Bossin) in place of the *LacZ* coding sequence,.

2) The *pA3ΔSBmZ* vector with the cytoplasmic *Actine-3 Bombyx mori* promoter controlling the *LacZ* coding sequence (Mangé *et al.*, 1997).

3) The *pIE1BmZ* vector with the *LacZ* coding sequence under the control of the *IE1* (Immediate Early 1) promoter of the *Bombyx mori* baculovirus (Vulsteke *et al.*, 1993).

Two vectors were electroporated carrying tissue specific promoters:

1) The *pB3xP3EGFP* vector (Horn and Wimmer, 2000) that express *GFP* in embryonic stemmata and nervous tissues (Thomas *et al.*, 2002).

2) The *(-1451)p25LacZ* in which the *LacZ* gene coding sequence is under the control of the *fibrohexamerin/P25* promoter (Horard *et al.*, 1997).

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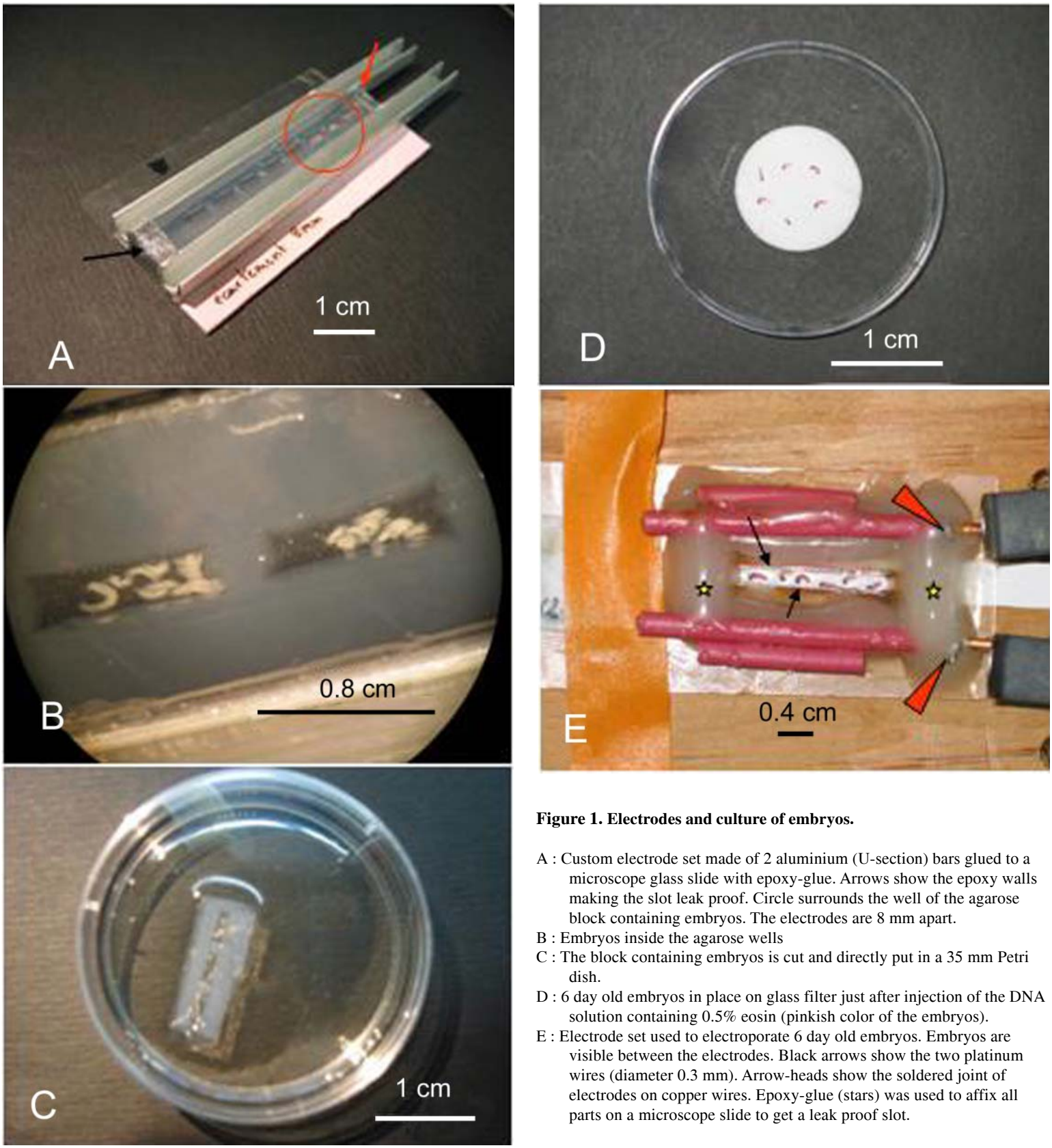

Figure 1. Electrodes and culture of embryos.

A : Custom electrode set made of 2 aluminium (U-section) bars glued to a microscope glass slide with epoxy-glue. Arrows show the epoxy walls making the slot leak proof. Circle surrounds the well of the agarose block containing embryos. The electrodes are 8 mm apart.
B : Embryos inside the agarose wells
C : The block containing embryos is cut and directly put in a 35 mm Petri dish.
D : 6 day old embryos in place on glass filter just after injection of the DNA solution containing 0.5% eosin (pinkish color of the embryos).
E : Electrode set used to electroporate 6 day old embryos. Embryos are visible between the electrodes. Black arrows show the two platinum wires (diameter 0.3 mm). Arrow-heads show the soldered joint of electrodes on copper wires. Epoxy-glue (stars) was used to affix all parts on a microscope slide to get a leak proof slot.

The DNA was prepared from *Escherichia coli* using Qiagen (http://www.qiagen.com) appropriate kits and resuspended in demineralised water. The DNA solutions were used at a concentration of 0.5 µg/µl in PBS 10 mM, pH 7.4.

Xgal staining

All samples were treated in the same manner. They were fixed with 4% formaldehyde solution in 10 mM PBS (phosphate buffered saline), pH 7.4 (Sigma cat. N°1000-3) for 10 min and

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washed with PBS for 5 min. They were subsequently incubated in Xgal solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-Beta-D-galactopyranoside; 15 mM potassium ferricyanate; 15mM potassium ferricyanate; 2 mM MgCl 2 in 10 mM PBS, pH 7.4) for 15 hours (overnight) at 37° C.

EGFP expression detection

The *EGFP* expression was detected using a Leica GFP-II filter set mounted on a Leica MZFL-III binocular microscope.

Results

Determination of electroporation parameters

Electroporation of *Bombyx mori* embryos using the BioRad Gene Pulser II (www.bio-rad.com) was attempted using the electrical conditions of Moto *et al.* (1999): five 50 ms square pulses of 250 volts/cm at a frequency of 0.5 hertz. Unfortunately, X-gal staining of the *LacZ* gene expression was not obtained. By recording the shape of electrical pulses of current using an oscilloscope the expected square pulses were not seen but rather biphasic pulses showing exponential decay. The ECM 830 device from BTX (San Diego, CA, USA) was then tried as this device is often cited in the field of tissues electroporation. The same parameters were used but the frequency was changed to 1 hertz instead 0.5 hertz. Eight variations were tried around these parameters to find the optimum parameters (Table 1). The best results were obtained with the P2 and P7 pulse programs (Table 2) followed by the P1, P8, P3, P4, P5 and P6 programs in decreasing order of efficiency. The 1 hertz pulse frequency was maintained constant. Polarisation of the X-gal spot distribution was noted using the P2 electroporation program (Figure 2A), The positive spots were spread mainly on the embryonic side facing the anode. Running the P2 program twice but with an inversion of the polarity during the second run of pulses significantly attenuated the polarised spreading of the spots but with no dramatic increase of the number of spots (Table 3). This program was named P'2 and was adopted for use.

In toto embryonic expression of several kinds of DNA constructs

The benefit of transfection lies in the ability to study diverse DNA constructs each one carrying different combinations of promoters and marker genes. For that purpose, four more DNA constructs were tested that can be distributed into two groups, one carrying the *GFP* marker gene and the second the *LacZ* gene. For the *GFP* marker gene the *P9* promoter of the *Junonia coenia* densovirus (*pJGFP* derived from *pBRJZ*, Jourdan *et al.* 1990), and the *3xP3* recombinant promoter (*pB3xp3EGFPaf* vector, Horn and Wimmer 2000) were used. For the *LacZ* gene the *IE1* (Immediate Early 1) promoter of the *Bombyx mori* baculovirus (*pIE1BmZ,* Vulsteke *et al. 1993*), the *(-1451)P25* silk promoter (*(-1451)p25LacZ*, Horard *et al.* 1997), the *P9* densoviral promoter (*pJGFP*) and the *A3ΔS* promoter (Mangé *et al.* 1997) (*pA3ΔSZ*) were used. Among these DNA constructs only, *pIE1BmZ*, *pA3"SZ* and *pJGFP* yielded expression of the marker gene (Table 4, Figure 2B, C and F). The number of X-gal spots was of the same order for the three ubiquitous promoters tested (i.e. *A3ΔS, P9, IEBm*) controlling the *LacZ* coding sequence, but for the *GFP* marker, fluorescence was only obtained for the *pJGFP* vector and apparently

Table 1. Electroporation programs

ID of the programs	Number of pulses	Duration of the pulses in ms	Voltage of the pulses in V/cm
P1	5	10	250
P2	5	50	250
P3	5	10	500
P4	5	50	500
P5	5	100	125
P6	5	100	250
P7	10	50	250
P8	5	50	125

Frequency of the pulses : 1 hertz

Table 2. Optimization of electroporation parameters

Electroporation program	Assay number	Number of electroported embryos	Number of positive embryos	Percentage of positive embryos	Number of positive spots	Mean number of positive spots per embryo
P1	1	6	6	100	130	21.6
(5, 10, 250)*	2	6	6	100	114	19
P2	1	6	6	100	470	**78.3**
(5, 50, 250)	2	6	6	100	296	**49.3**
P3	1	6	4	66.6	97	16.16
(5, 100, 250)	2	6	5	83.3	171	28.5
	3	6	4	66.6	72	12
P4	1	6	6	100	34	5.6
(10, 50, 250)	2	6	5	83.3	65	13
P5	1	6	3	50	7	1.16
(5, 100, 125)	2	8	0	0	0	0
P6	1	5	0	0	0	0
(5, 50, 125)						
P7	1	6	6	100	469	**78.1**
(5, 10, 500)						
P8	1	6	1	16.6	4	0.66
(5, 50, 500)	2	4	3	75	87	21.75
	3	6	6	100	164	27.3
	4	6	5	83	53	8.83

* : first number : number of pulses, second number : duration of one pulse, third number : voltage of the pulses. The frequency of the pulses was fixed at 1 hertz.

For each assay the electrode set used was A13122001 except for the assays marked with the star which was done using the BTX microslide electrode set model number 453.

DNA : pBRJZ (0.5µg/µl)

Number in bold and underlined are the markedely highest values.

Table 3. Comparison between single polarity electroporation with successive inversion of polarity (pooled results of seven P2 assays and nine P'2 assays in four independent experiments)

Electroporation program	Number of electroporated embryos	Number of positive embryos	Mean percentage of positive embryos	Number of spots	Mean number of spots per embryo
P2	36	32	88	1165	36.4
P'2	**44**	**42**	**95**	**1786**	**45.5**

Electrode set: A13122001, DNA: pBRJZ

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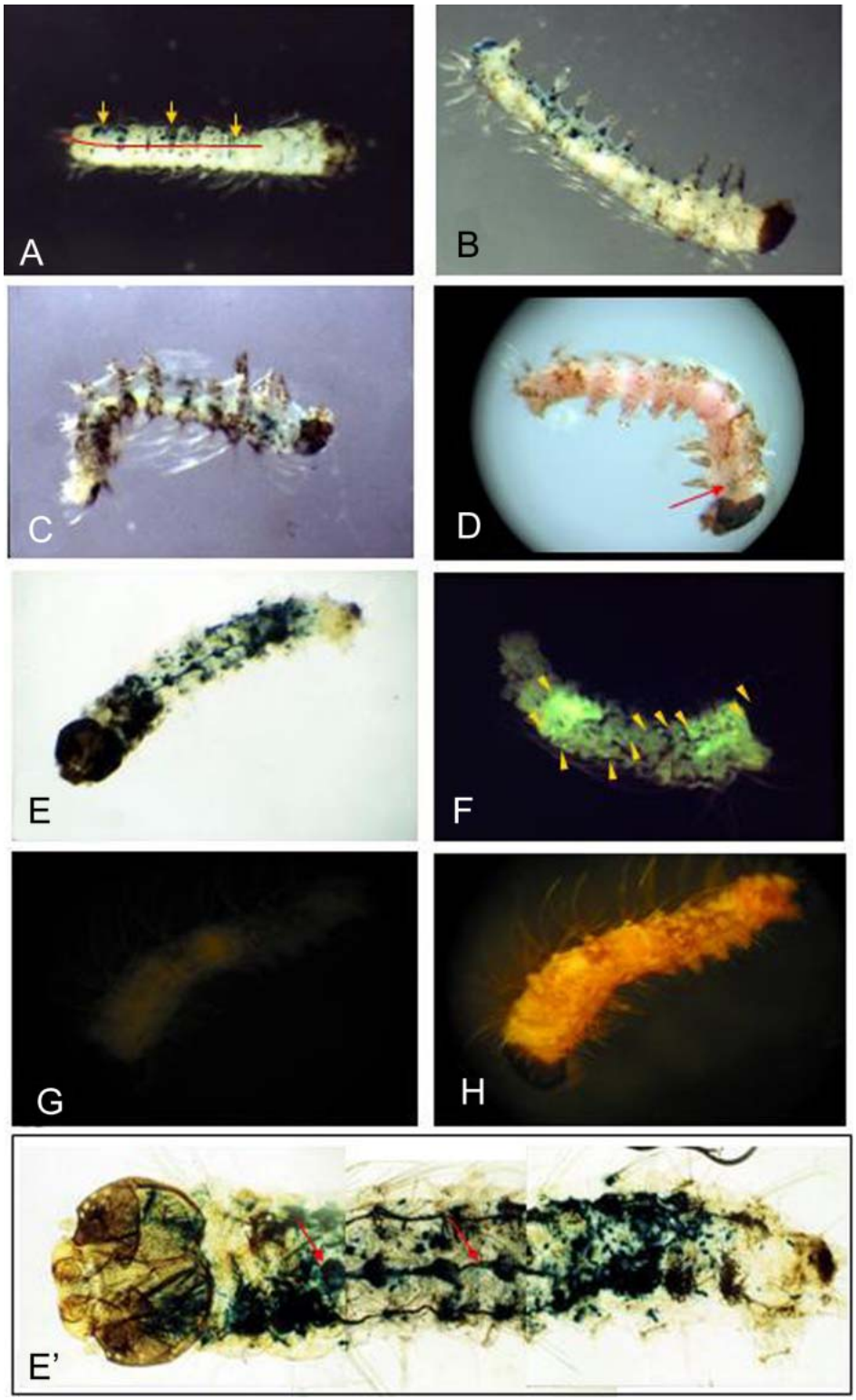

Figure 2. 6 day old electroporated embryos.

P2 electroporation program in A and P'2 electroporation programs from B to F.
A : *pBRJZ*: Arrows show the main distribution of the staining. The red line displays the longitudinal axis of the embryonic body.
B : *pIE1BmLacZ*
C : *pA3ΔSLacZ*
D : Xgal Control: *pBRJZ* injected embryo and Xgal stained without electroporation. Arrow shows the low Xgal staining background in the sub-oesophagian corpus.
E et E': *pBRJZ*
F : pJGFP
G and H: GFP control: pJGFP injected embryo without electroporation, G : GFP filter, H : GFP filter in episcopic ordinary light.
A-H (x25), E' (x50) embryos.
E': magnification (photomontage) of the view E. Arrows show the ventral ganglionic chain.

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Table 4. Assays of different promoters and expression markers

Experiments	Assays	ADN	Number of electroporated embryos	Number of positive embryos	Percentage of positive embryos	Number of spots	Mean number of spots per electroporated embryo
1	1	*pBRJZ*	4	1	25	30	7.5
	2		5	4	80	129	25.8
	1	*pIE1BmZ*	5	4	80	47	9.4
	2		5	5	100	33	6.6
	3		5	5	100	146	29.2
	1	*pA3 ΔSZ*	5	4	80	24	4.8
	2		5	4	80	31	6.2
	3		6	6	100	94	15.6
2	1	*pJGFP*	5	5	100	12	2.4
	2		5	5	100	8	1.6
	3		15	5	30	17	1.13

Electroporation program : P'2
The DNA solution were at 0.5µg/µl
Electrode set: A13122001

at a relatively lower number. Probably the low-level yellow-green fluorescent background hindered the view of the weakest fluorescent spots and as a consequence the number of cells expressing the GFP was underestimated.

The tissue specific promoter *(-1451)P25* gave doubtful positive results. The low level or absence of viewable expression of these three tissue specific promoters could be associated with the differential sensitivity of the two markers, *GFP* versus *LacZ*, the strength of the promoters, or the DNA accessibility to the target tissues. *LacZ* gene expression, revealed by X-gal staining, seems to be more sensitive than the *GFP* marker. The location of X-gal staining seemed to be mainly internal but sometimes it appeared that the staining was on the surface, possibly because of expression in the integument (Figure 2E'). When we tried to electroporate late embryos bathed in DNA solution we never observed X-gal staining. The same result was obtained if the embryos were injected with DNA without electroporation and Xgal stained. Only a very low level background could be seen sometimes in the sub-oesophagian corpus (Figure 1, arrow). From histological sections of the positive embryos it appeared that essentially internal tissues were transfected but also occasionally integument (Figure 3H) confirming the *in toto* view. Head muscle and unidentified head tissues, as well as gut and unidentified tissues of the body often expressed *lacZ* marker (Figure 3E and G). Unfortunately, we did not find positive cells in the silkgland of embryos treated for histological sectioning, although *pBRJZ* is known to be expressed in embryonic silkgland (Thomas *et al.* 2001). Nervous tissue was transfected as can be seen on the ventral ganglionic chain of the whole embryo (Figure 2E', see arrow), but positive cerebrum was not seen as was the case using biolistics (Thomas *et al.* 2001). We did not observe any expression using the *pB3xP3EGFP* vector although it is known to be expressed in the central and peripheric nervous tissues (Thomas *et al.* 2002).

Electroporation of 3 and 4 day old embryos

As there might be some advantages of electroporation over biolistics for transfecting tissues of early embryos, we electroporated 3 and 4 day old embryos. At this stage of development such early embryos are very fragile but easy to handle using a pipette. Embryos were dechorionated under Grace's medium and put in PBS in an agarose cast well (Figure 1B). The agarose block containing the embryos was placed between the electrode (Figure 1A) and after electroporation, returned to Grace's medium in a culture Petri dish

Table 5. Electroporation of 3 and 4 day old embryos

Experiments	Age of embryos	Assays	Number of electroporated embryos	Number of positive embryos	Number of spots	Mean number of spots	Percentage of positive embryos	Electrodes
1	4d	1	19	11	133	7	57.8	BTX
		2	8	3	11	1.37	37.5	
2	3d	1	10	2	2	0.2	20	Aluminium
		2	10	0	0	0	0	
		3	15	0	0	0	0	
		4	10	2	4	0.4	20	

Electroporation program : P'2
ADN: *pBRJZ* (0.5 µg/µl)
Electrodes: aluminium, customised aluminium electrode set having 8 mm gap width
BTX, microslide electrode set model 453 having 3.2 mm gap width

(Figure 1C). For this purpose special electrodes were made of two 6 mm high aluminium parallelepipedic rods with a gap of 8 mm. The gap allowed one to manipulate the agarose block containing several embryos with forceps (Figure 1A). The density of the DNA solution was increased by adding glycerol at 1% final concentration to ensure that as embryos fell on the floor of the agarose well the DNA solution surrounded the embryos. Moreover to control the filling of the well, we added eosine to the DNA solution at a final concentration of 0.1%. As it can be seen in Table 5 and Figure 4A, transfection of 3 and 4 day-old embryos was achieved. The number of spots is rather low compared to the number obtained in late dechorionated embryos but they are distributed all along the embryonic body. These results were obtained using the P'2 program and the conditions would probably be optimised by an enlarged study of systematic variations of electrical parameters and DNA concentration. No background was visible on Xgal stained control embryos (Figure 4A').

Electroporation of larval organs : ovaries and imaginal wing disk

In order to evaluate if this technique could be applied to samples other than embryos, we tried electroporation on ovaries and imaginal wing disks (Table 6). The interesting cells to be transfected in these organs are surrounded by a closed cellular sheet that could be considered as a pouch into which DNA solution could be injected. DNA solutions were injected into the ovaries and into the imaginal wing disks through their envelope and submitted to electroporation using the platinum electrode set. Although it was difficult to obtain expression in imaginal wing disks (Figure 4B and C), it was relatively easy to obtain numerous X-gal staining on ovaries (Figure 4D and E). The transfected tissues in ovaries were not the follicular cells nor the oocytes but the interstitial connective tissue surrounding the ovarioles. In fact, the expression of the *lacZ*

Table 6. Electroporation of larval ovaries and imaginal wing disk

Experiments	Assays	Organs	Number of electroporated organs	Number of positive organs	Number of spots	Mean number of spots per organs	Percentage of positive samples	Electroporation programs
1	1	wing disk	16	2	9	4.5	12.5	P2
2	1	ovaries	5	0	0	0	0	P2
	2		5	3	nd	nd	60	P2(15)

ADN: *pBRJZ* (0.5 µg/µl)
Electrode set: A13122001
P2(15) means that the DNA was left 15 mn in contact with the tissues before applying P2 electroporation program

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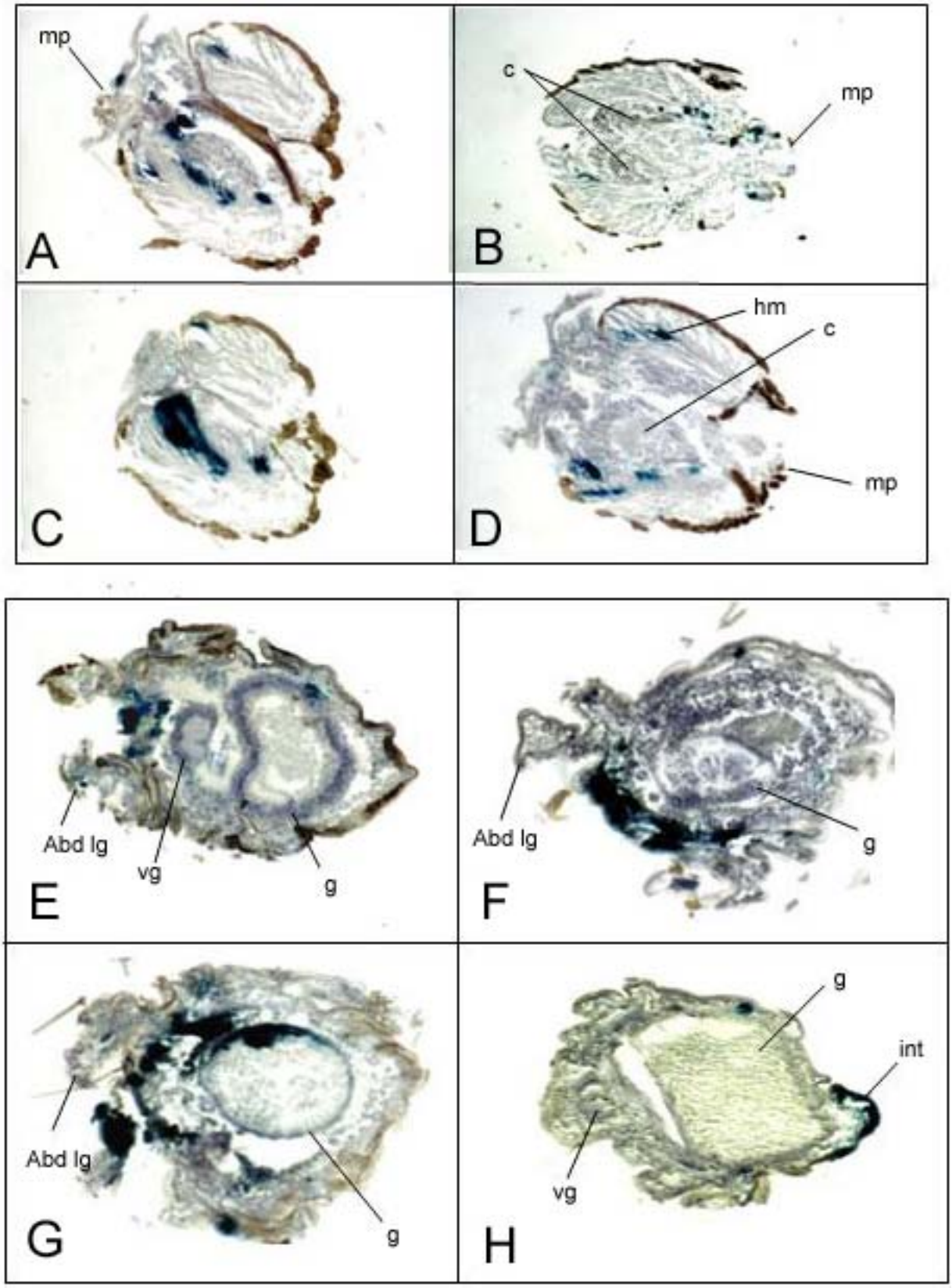

Figure 3. **Histological sections of embryos electroporated with *pBRJZ***.

Magnification x240.
A to D: head
E to H: abdomen
c: cerebrum, **hm**: head muscles, **abd lg**: abdominal leg, **vg**: ventral ganglion, **g**: gut, **int**: integument, **mp**: mouth part.

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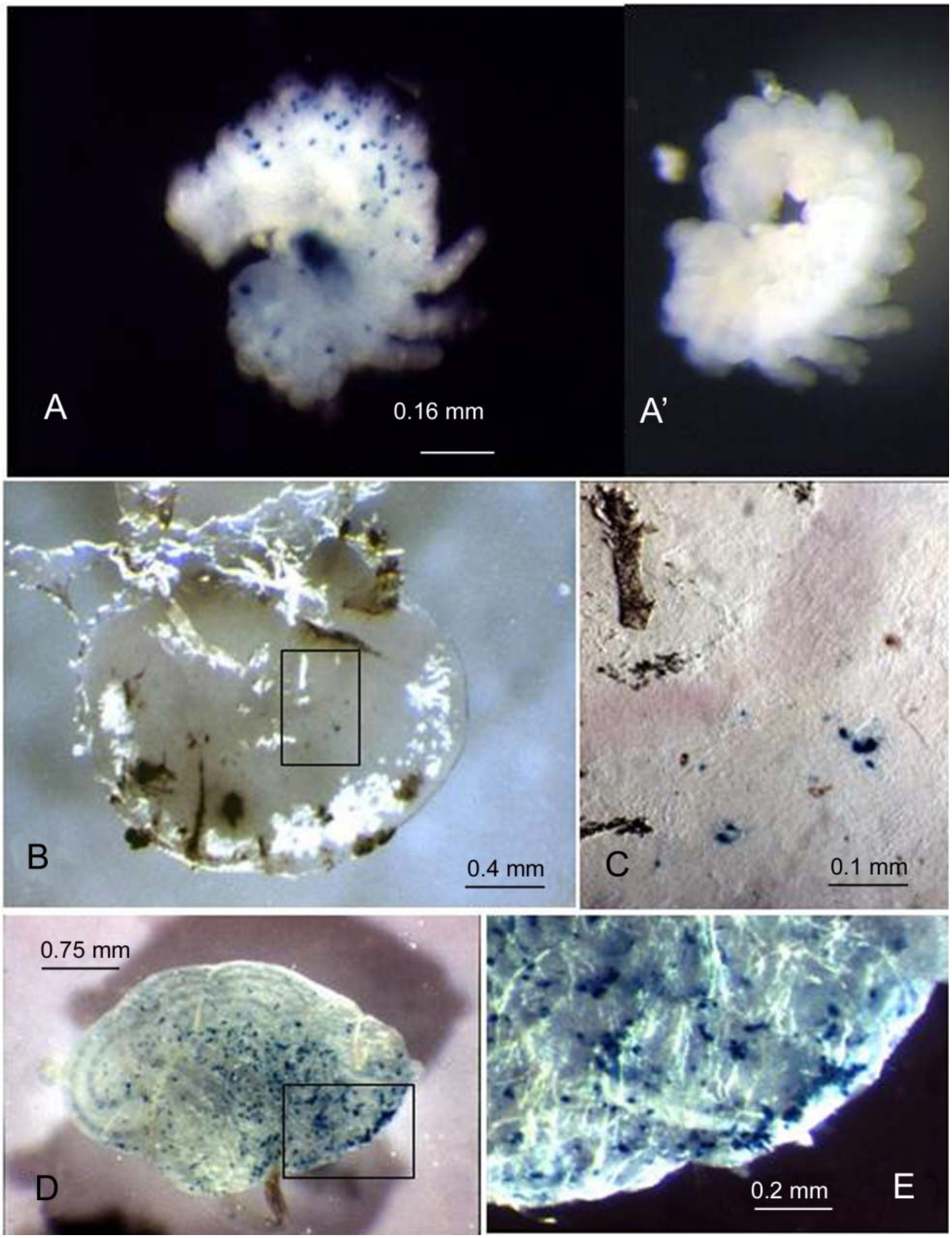

Figure 4. Electroporation of early embryos, fifth day larval imaginal wing disk and ovary.

P'2 electroporation program was used for all samples.

A and A': two embryos at E3 developmental stage (about 4 days of incubation at 25° C for Nistari strain). A : Embryo electroporated with pBRJZ vector and A' control embryo electroporated without DNA. Embryos were stained using Xgal.

B and C : imaginal wing disk of fifth instar larvae (C: detail of enclosed area in B)

D and E : ovary of fifth instar larvae (E: detail of enclosed are in D)

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gene was obtained after the DNA was in contact with tissues for 15 minutes before submitting ovaries to the electrical pulses. In the imaginal wing the epithelial cells of the wing buds were transfected. Despite the presence of a connective tissue in the ovaries the DNA solution spread easily on all parts of the gonad. This was not the case for the imaginal wing disks. Although it was easy to prick through the peripodial sheet, once the glass needle tip penetrated the cellular sheet, it was difficult to spread the DNA solution around the wing buds. Therefore the amount of the injected DNA solution was low which probably explains the low level of *LacZ* gene expression in wing discs compared to the ovaries.

Discussion

These experiments show that the electroporation technique is an efficient technique to transfect *B. mori* embryos and larval tissues. Previous work showed the possibility to transfect insect embryos by using this technique (Kamdar *et al.* 1992, Leopold *et al.* 1996, Devault et al. 1996, Hughes et al. 1997). Kamdar *et al.* (1992) obtained transfected gene expression in *D. melanogaster* embryonic tissues whereas the others proved the transfection of embryonic tissues by detecting the vector sequences in embryonic and larval tissues with the aim of germinal transgenesis. To our knowledge the results presented here are the first to obtain expression of a foreign gene in Lepidopteran embryonic tissues using electroporation. This technique can be helpful and complementary to biolistics knowing that it is very difficult if impossible to get DNA expression in *B. mori* embryos by microinjection of chorionated eggs.

Thomas *et al.* (2001) and Kravariti *et al.* (2001) previously used the biolistic method to test the functionality of DNA constructs using somatic transfection before engaging in the tedious time-consuming work of germinal transgenesis. As biolistics allows one to quickly record data that can be then more accurately completed by germinal transgenesis experiments, this technology becoming today a routine technique for *B. mori* (Tamura *et al.* 2000, Thomas *et al.* 2002, Uhlirova *et al.* 2002, Tomita *et al.* 2003, Yamada *et al.* 2002). However, biolistics had some drawbacks for our purposes, even if partially corrected (Thomas *et al.* 2001). In particular, the irregular results obtained with biolistics remained a problem. Moreover, using this technique we were unable to transfect early dechorionated embryos which were too fragile to be handled outside a liquid medium.

A comparative study showed that electroporation may be more efficient than biolistics or lipofection (Muramatsu *et al.* 1997a). One reason for the success of this technique especially for the chicken embryos may be due to the containment of the DNA solution in the neural tube or natural embryonic vesicles. Moreover, positive results were obtained even with living swimming frog embryos immersed in the DNA solution (Eide *et al.* 2000). The hemocoel of late *B. mori* embryos could also be very convenient to confine a DNA solution close to the target organs such as silk glands. Several other larval organs such as gonads or imaginal wing disks can be considered as a pouch allowing the containment of the DNA solutions. It may also be possible to transfect early *B. mori* embryos in a liquid medium.

Electroporation can be performed using three main modes comprising capacitive discharge, radio-frequency pulses and square wave pulses (Chang 1992). It has been shown that square pulses of current could have better properties for the transfection of tissues and allow a better control of the membrane permeation and the resealing of the created pores (Sukharev *et al.* 1992). In almost all recent publications concerning electroporation of embryos, especially chicken embryos, square pulses of current were applied. Recently conclusions of Sukharev *et al.* (1992) on the benefit of the use of square wave pulses were confirmed by Satkauskas *et al.* (2002) and Golzio *et al.* (2002). Satkauskas *et al.* (2002) showed that the combination of two square pulses of current, the first one of very short duration but of high voltage, and the second of long time but low voltage is particularly beneficial for tissue DNA electrotransfer. Golzio *et al.* (2002) clearly visualised the effect of the variation of the electrical parameters on the efficiency of DNA electrotransfer into the cells. Given these results, the choice of a device able to deliver square pulses of current was important, and the one chosen was the BTX ECM-830 electroporator which gave positive results using the Moto *et al.* (1999) parameters.

Expression of the marker gene in late *B. mori* embryos was achieved only when the DNA solutions were injected into the embryonic hemocoel. Control DNA injected in the hemocoel without electroporation never gave gene marker expression. Electroporation applied to late embryos bathed in the DNA solution gave negative results for all trials performed, probably because secretion of a thin cuticle had begun. This was not the case for early embryos for which positive results were obtained under the same conditions. In fact at this stage of development embryos have no hemocoel cavity that could be injected and bathing them in the DNA solution is the sole approach that can be used to transfect them. In the conditions studied, these results were easily and regularly obtained, but only with DNA constructs carrying strong ubiquitous promoters such as the *P9* densoviral promoter (Royer *et al.* 2001; Kravariti *et al.* 2001; Thomas *et al.* 2001), the *IE1Bm* promoter (Immediate Early baculoviral promoter, Vulsteke *et al.* 1993), or the recombinant *B. mori Actine-3* promoter (Mangé *et al.* 1997).

Unfortunately uncertain results were obtained with *fibrohexamerin/P25* promoter (Horard *et al.* 1997) and none were obtained with *3xP3* promoter (Horn and Wimmer 2000), whereas they were expressed in bombarded silk gland (Horard *et al.* 1997) and embryos following egg injection (Thomas et 2002) respectively. It may be that the target organs for such tissue-specific promoters represent a relatively punctual and topologically restricted target to be reached by the DNA constructs. However it is possible that the parameters could be optimized to improve the quality or the frequency of the transfection with such tissue specific promoters. Nevertheless, in regards to the arguments cited above, the frequency of expression with such tissue specific promoters would probably remain rather low. Another possibility could be to explore the efficiency of restricted area microelectroporation (Sasagawa *et al.* 2002a; Momose *et al* 1999). The combination of the *P9* densoviral promoter with *EGFP* marker gave a lower number of bright spots than were obtained using the LacZ marker. This difference could be due to the weak fluorescent background of the embryos using GFP filter set, or to a greater sensitivity of the detection method of the *lacZ* gene marker as shown by Nakamura et al. (2002) in mouse embryonic gonads. In fact for the *LacZ* marker, apart from the sub-

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oesophagian corpus, there is no blue background and the X-gal substrate accumulates as a blue precipitate during several hours, whereas the fluorescent signal of the GFP is seen instantaneously from some accumulated GFP molecules submitted to a synthesis-degradation turnover. In this situation, the accumulation of the detection signal could be lower. Perhaps it could be better to use the *Luciferase* gene as a vital marker of expression, and a CCD camera accumulating the photons of the luminescent signal (Muramatsu *et al.* 1996; Honigman *et al.* 2001; Wang *et al.* 2002).

We previously tried to transfect early embryos using different lipopolyamine/*pBRJZ* vector DNA complexes, but very poor results were obtained. Electroporation offered the occasion to test the transfection of early embryos that can be handled only in a liquid medium. For this purpose it was necessary to use an agarose well containing embryos bathed by the DNA solution. We took care to prepare agarose in phosphate buffered saline to allow a good conduction of the electrical field. Under these conditions it was easy to express the *pBRJZ* vector even if the frequency of positive early embryos was lower than for the late embryos. The P'2 electroporation program gave better results on 4 day old embryos than on 3 day old embryos. Perhaps a different set of parameters need to be developed as several differentiated tissues appear step by step during embryonic development.

Electroporation was also used on larval tissues. For this purpose keeping the principle of the DNA solution containment, we focused the assays on organs that could be viewed as a pouch containing the target cells. These assays were successful on ovaries and imaginal wing disks, but not on testis (data not shown). We also tried electroporation on silk gland and ovarioles as an alternative to the biolisitics because damage might be reduced. In the case of biolistics applied to the silk gland, damage resulting in immune melanization and necrotic process of the gland lead in the most dramatic situation to the death of the larvae. Furthermore, applied under good conditions electroporation is less traumatic than the biolistics microcarrier perforations that damaged the ovarioles resulted in the partial disruption of the oocyte string. We performed electroporation on fifth instar larval silk gland or pupal ovarioles bathing them in DNA solution. Unfortunately positive Xgal staining was not obtained.

In conclusion, we show here that it is possible to successfully electroporate living *B. mori* late embryos, as well as early embryos or even some explanted larval organs. Optimizing electrical parameters, DNA concentration and buffer electrical conductivity could really be fruitful. For the first time we present *in situ* transient foreign gene expression in Lepidopteran embryos using this technique. Our results, and those of Kamdar *et al.* (1992) and Moto *et al.* (1999), confirm the relevance of the electroporation technique to transfecting and efficiently expressing foreign DNA constructs in insect tissues.

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