25. Gene Transfer Technology in Marine Invertebrates

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Introduction

Invertebrates represent the most numerous animals in terms of species and individuals. They are ubiquitously distributed on the planet and their importance is extreme with regard to human activities: pollinisators and pests in agriculture; parasites and vectors of infectious diseases in medicine; shrimps, oysters and mussels in fishery and aquaculture.

Because of this economical and medical importance and by referring to the numerous applications of genetic transformation in plants and vertebrates, the development of gene transfer technologies for invertebrates appears as a prioritary topic that is still in its infancy.

For terrestrial invertebrates, gene transfer technologies have been developed and are routinely used only for *Drosophila* fruit flies (see Kaiser) and for a nematod, *Caenorhabditis elegans* (see D. Tierry-Mieg). Largely because of the availability and the reliability of these technologies, these species have acquired the status of models for basic research on molecular genetics and biology of development. For all the other terrestrial invertebrates groups, gene transfer technology is still speculative until the identification of efficient transformation systems (Handler and O'Brochta, 1991).

Concerning marine invertebrates, research on genetic transformation has been initiated in the eighties for sea urchins as models to study embryogenesis at the molecular level, a huge amount of informations being otherwise available about classical embryology of these primitive deuterostomes. A research strategy based on gene transfer technology has been developed as described below.

In the nineties, genetic transformation of molluscs and shrimps began to be explored. By referring to transgenic plants (Grumet, 1990 and Scholthof *et al.*, 1993) and vertebrates (see this book) and like for insects vector of diseases (Miller *et al.*, 1987 and Eggleston, 1991), genetic transformation has been actually considered as a promising strategy for producing pathogen-resistant animals, that could permit to control the infectious diseases that dramatically affect aquaculture productions all around the world (Mialhe *et al.*). Although significant progresses have been already achieved, as described below, shrimp and bivalve gene transfer technology is still considered as an uncertain project, specially because it is developed by a very limited number of scientists.

Gene Transfer Technology in Sea Urchins

The first studies related to sea urchin gene transfer technology were based on cytoplasmic microinjection of *Strongylocentrus purpuratus* unfertilized eggs that are then fertilized *in situ* by addition of a drop of sperm solution (Flytzanis *et al.*, 1985 and McMahon *et al.*, 1985).

Gametes are shed from sexually mature sea urchins after an intracoelomic injection of a 0.5 M KCl solution. The egg jelly coat is removed by treatment with acid sea water. The eggs are about 80 µm in diameter and their surface is negatively charged, that permits to immobilize them by electrostatic attraction to the surface of the injection dishes previously coated with a 1% protamine sulfate solution. Without the need of a holding micropipet, eggs are injected by using a continuous flowing micropipet of about 0.5–1.0 µm tip diameter. About

2 pL of DNA solution is injected into the cytoplasm, that is to say around 1.5% of the egg volume. Up to 300 eggs can be injected per hour with 90–95% undergoing normal fertilization and 40–50% completing normal embryogenesis to the pluteus stage, 3–4 days later. Occasionally, a batch of microinjected eggs completely fails to develop. Moreover, embryonic survival depends on the amount of DNA microinjected. In practice, it is recommended to use DNA solutions at concentrations below 32 μg/mL (that corresponds at a maximum injection of 12000 copies per 2 pL for 5 Kb DNA molcules). Microinjection of DNA solutions at concentrations equal to or greater than 320 μg/mL is completely toxic.

A protocol has been also developed to directly microinject the zygote nucleus, the main purpose of this procedure being to increase the number of embryonic cells containing DNA (Franks *et al.*, 1988). This microinjection procedure has been established for the Gulf Coast sea urchin *Lytechinus variegatus* that has almost transparent eggs whereas *S. pururatus* has opaque eggs. The collect and the treatment of gametes are similar for the two species. Microinjection into the unfertilized egg pronucleus is not possible because it moves inside the cytoplasm at the contact of the microneedle. After fertilization and fusion of pronuclei, the resulting zygote pronucleus is more stably anchored inside the newly assembled cytoskeleton, that permits to introduce the continuously flowing microneedle. DNA solution is thus delivered into the nucleus but also dispersed inside the cytoplasm during the needle penetration. For a same duration of operations, the number of eggs that can be microinjected in the zygote nucleus is almost the same than for microinjection inside the cytoplasm. The percentage of eggs that complete normal embryogenesis to the feeding pluteus stage is also lower, with an average of about 60–65%.

More recently, transfection of *Strongylocentrotus purpuratus* sperm has been investigated as a mass transfection procedure permitting to vehicle DNA directly into the egg nucleus (Arezzo, 1989). Based on previous observation related to permeability of sperm membrane, experiments have been performed to transfect sperm (2 μL) by incubation for 1 hour in a solution of supercoiled or linearized plasmids (50 μg) diluted in sterile artificial sea water (30 μL). The transfected sperm is then mixed with eggs for *in situ* fertilization. The use of the reporter gene CAT placed under the control of heterologous promoters led to transient expression in embryos. Consequently, it was assumed that sea urchin sperm can be loaded with DNA that is then vehicled to the egg nucleus where it can express.

It has been established that the fate of of transfected DNA depends essentially on its physical form (Flytzanis *et al.*, 1985; McMahon *et al.*, 1985 and Franks *et al.*, 1988). DNA microinjected as supercoils persists in the embryos, mainly in supercoiled or relaxed circular form, but without DNA amplification. Linearized plasmids are transformed into high-molecular-weight DNA after injection into the egg. Random ligation of heads and tails are observed resulting in concatenate structures. The concatenation of linear molecules is independant of the presence of staggered ends since it has been shown that "blunt-ended" as well as "sticky-ended" DNA molecules are end-to-end ligated after injection. Moreover, the terminal restriction sites are reformed, indicating little if any modification of the ends by intracellular nucleases. Based on experimental data, it may be assumed that the ligations occur soon after injection of linearized DNA, high molecular-weight DNA being found as soon as 60 min after injection. During early embryogenesis there is an amplification of the concatenated DNA, this amplification occuring independently of the presence of sea urchin sequences. Replicated DNA is present in more than 50% of 5-week larvae. Two to three months after metamorphosis, the frequency of positive animals ranged from 4 to 16% when the injection was made in the egg cytoplasm and about 36% when the injection was made in the zygote nucleus. Plasmid DNA is chiefly present as extrachromosomal concatenated structures. Integrated plasmid DNA concatenates have been found and homologies have been observed near the breakpoint between the end plasmid sequences and repeated sequences of the adjoining sea urchin DNA; The latter consist of short repetitive AT-rich sequences. Integration may thus result from homologous recombination (Flytzanis *et al.*, 1985). Integrated sequences have been also found in the sperm of adult sea urchin issued from microinjected eggs.

Expression of reporter genes have been reported following microinjection into unfertilized egg cytoplasm or zygote nucleus and following sperm transfection. On the basis of transient expression analyses, a lot of studies have been performed on sea urchin promoters and genes that show the present routine character of gene transfer for sea urchin.

Gene Transfer Technology in Bivalve Molluscs and Shrimps

Molluscs

Gametes of bivalve molluscs are easily obtained by injection of 0.5 M KCl into the adductor muscle and fertilization is simply achieved by mixing gametes.

By using Lucifer Yellow as a reporter molecule, microinjection has been developed for 1-cell and early embryo stages, but unfertilized ovocytes revealed too sensitive to survive. Microholder and microneedles made with a two-stroke puller are necessary. Embryo microinjections are performed with the aid of micromanipulators mounted on an inverted microscope. In practice, it is possible to microinject up to 100 embryos per hour with survival rates ranging between 10% and 55% (Cadoret, 1992a).

Electroporation has been applied on early embryos to induce polyploïdy (Cadoret, 1992b) and will have to be evaluated as a possible mass transfection technique.

Aqueous biolistics, initially developed to transfect mosquito eggs, has been recently applied to mass transfection of ovocytes, uni- and pluricellular embryos of mussel and oyster (Cadoret *et al.*, submitted). The determination and the optimization of biolistic parameters have been made on the basis of luciferase reporter gene transient expression.

In parallel to these investigations on transfection that are directly oriented to the obtention of transformed animals, lipofection of heart cells in primary culture has been developed and been proved suitable to analyse the functionality of heterologous promoters (Boulo *et al.*, submitted).

Analyses about the fate of transfected DNA were first aimed at determining the functionality of heterologous promoters on the basis of transient expression of luciferase reporter gene. It has been shown that the hsp 70 (heat shock protein) promoter of Drosophila and the early promoter of CMV (cytomegalovirus) are efficient both in embryos and in heart cells. In the latter, the promoter of SV (simian virus) is also efficient (Cadoret *et al.*, submitted and Boulo *et al.*, submitted).

Shrimps

In vitro fertilization has been announced for the shrimp *Penaeus atzecus* (Clark *et al.*, 1973) but this result revealed to be practically non reproducible. Consequently, transfection procedures have been only explored on naturally fertilized eggs and early embryos.

Microinjection of embryos has been successfully developed for different *Penaeus* species. The efficiency of the procedure has been controlled by checking the introduction of Lucifer Yellow and the subsequent survival. A chief limitation of this technique is linked to the rapid division of blastomers and consequently the small number of embryos that can be injected at the 1-cell stage (Gendreau, 1992).

Classical biolistics, that is referred to as "dry biolistics", has been tested on decapsulated artemia cysts and shown not to be very efficient (Gendreau *et al.*, 1995). The modified biolistic procedure, named "aqueous biolistics" because DNA-coated particles are kept in suspension to be shooted (Mialhe and Miller, 1994), has been investigated and optimized with embryos of *P. stylirostris*. This technique is efficient to transfect thousands of early embryos (from 32 blastomer stage to blastula), survival reaching 60% (Rousseau *et al.*, in prep., Rousseau, 1995).

By using aqueous biolistics to transfect embryos and by lipofecting ovarian cell in primary cultures, it has been showed on the basis of luciferase transient expression that the promoters for the Drosophila hsp 70 and human CMV early genes are efficient in shrimps (Rousseau, 1995).

Prospects

Gene transfer technology is presently studied in a number of marine invertebrates with very different prospects according to the group.

For sea urchins, investigations concern essentially embryogenesis and molecular biology of development. Because of this specific fundamental orientation, current gene transfer technology appears sufficiently controlled. As a matter of fact, DNA concatenation, extrachromosomal amplification and persistence are compatible with experimentations that are based on transient expression of constructs designed to analyse developmentally regulated promoters and genes. A limitation is related to the mosaic distribution of transfected DNA. However this limitation can be reduced by performing microinjection into the zygote nucleus rather than the egg cytoplasm and mass transfection of sperm could lead to non mosaic distribution. Evidence of DNA integration, that seems to result from homologous recombination between small AT-rich sequences shared by the plasmid and the chromosome of DNA, is a very promising way to make easily transgenic animals. As a matter of fact, it would be possible to flank transfected construct with repetitive sequences present in the host chromosome, such as telomeres (Edman, 1992). A chief advantage of gene transfer technology, based on homologous recombination, consists in the easiness to identify repetitive sequences compared to the identification of specific and/or functional transformation system, such as transposon or integrative virus.

Concerning gene transfer technology for bivalves and shrimps, the main objective is to produce transgenic strains that resist to pathogen through the expression of immune genes, viral genes or viral antisense sequences. It is consequently primordial to control integration of transfected DNA in order

to get stable and inheritable resistance. Experiments to evaluate potential transformation systems are complicated by the generation times (about 9 months) and by the lack of cell lines although one has been recently established from shrimp lymphoid organs through transformation (Tapay *et al.*, 1995). In the short term, experiments will be advantageously performed with heterologous transformation systems that are available and have already proved efficient in other animal systems. Particularly promising systems correspond to pseudotyped retroviral vectors (Burns *et al.*, 1993) that have been successfully used to transform fishes (Lin *et al.*, 1994) and rare possibly efficient in molluscs (Cheng, pers. comm.). A suitable way to use these transformation vectors could be to infect sperm in order to limit mosaic effect. Other potential heterologous transformation vectors have to be analyzed, some derived from insect densoviruses that are able to replicate or to integrate and others derived from transposons. In parallel, investigations are necessary to identify and to characterize transposons, viruses, repetitive sequences, promotors and immune genes of molluscs and shrimps. Taking into account the extent of these researches on genetic transformation of molluscs and shrimps, international networks are currently organized to create the necessary interactions between scientists from these different topics (Mialhe *et al.*, 1995). The organization of these networks is supported by cooperation agencies because control of diseases is the priority for world shrimp aquaculture.

References

Arezzo, F. (1989) Sea urchin sperm as a vector of foreign genetic information. *Cell Biology International Reports* **13,** 391–403

Boulo, V., Cadoret, J.P., Le Marrec, F., Dorange, G., Mialhe, E. (submitted) Transient expression of luciferase reporter gene after lipofection in oyster (*Crassostrea gigas*) primary cell cultures

Burns, J.C., Friedmann, T., Driever, W., Burrascano, M. (1993) Vesicular stomatitis virus G glycoprotein pseudo typed retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037

Cadoret, J.P. (1992a) Mise au point de méthodes de manipulation embryonnaire de mollusques bivalves. Application en génétique et pathologie infectieuse. Thèse *EPHE*, pp. 1–100 (IFREMER libraries)

Cadoret, J.P. (1992b) Electric field-induced polyploidy in mollusc embryos. *Aquaculture* **106,** 127–139

Cadoret, J.P., Boulo, V., Gendreau, S., Mialhe, E. (submitted) Transient expression of luciferase reporter gene in bivalve embryos using particle bombardment

Clark, W.H., Talbot, P., Neal, R.A., Mock, C.R., Salser, B.R. (1973) *In vitro* fertilization with non-motile spermatozoa of the brown shrimp *Penaeus aztecus*. Marine Biology **22,** 353–354

Edman, J.C. (1992) Isolation of telomerelike sequences from *Cryptococcus neoformans* and their use in high-efficiency transformation. *Molecular and Cellular Biology* **12,** 2777–2783

Eggleston, P. (1991) The control of insect-borne diseases through recombinant DNA technology. *Heredity* **66,** 161–172

Flytzanis, C.N., McMahon, A.P., Hough-Evans B.R., Katula, K.S., Britten, R.J., Davidson, E.H. (1985) Persistence and integration of cloned DNA in postembryonic sea urchins. *Developmental Biology* **108,** 431–442

Franks, R.R., Hough-Evans B.R., Britten, R.J., Davidson, E.H. (1988) Direct introduction of cloned DNA into the sea urchin zygote nucleus, and fate of injected DNA. *Development* **102,** 287–299

Gendreau, S. (1992) Recherches sur la transformation génétique des crustacés. *Thèse Université de Bretagne occidentale*, pp. 1–116 (IFREMER libraries)

Gendreau, S., Lardans, V., Cadoret, J.P., Mialhe, E. (1995) Transient expression of luciferase reporter gene after biolistic introduction into Artemia franciscana (Crustacea) embryos. *Aquaculture* **133,** 199–205

Grumet, R. (1990) Genetically engineered plant virus resistance. *HortScience* **25,** 508–512

Handler, A.M., O'Brochta, D.A. (1991) Prospects for gene transformation in insects. *Annual Review of Entomology* **36,** 159–183

Lin, S., Gaiano, N., Culp, P., Burns, J.C., Friedman, T., Yee, Y.K., Hopkins, N. (1994) Integration and germ line transmission of a pseudotyped retroviral vector in Zebrafish. *Science* **265,** 666–669

McMahon, A.P., Flytzanis, C.N., Hough-Evans B.R., Katula, K.S., Britten, R.J., Davidson, E.H. (1985) Introduction of cloned DNA into sea urchin egg cytoplasm: replication and persistence during embryogenesis. *Developmental Biology* **108,** 420–430

Mialhe, E., Bachère, E., Boulo, V., Cadoret, J.P. (1995) Strategy for research and international cooperation in marine invertebrate pathology, immunology and genetics. *Aquaculture* **132,** 33–41

Mialhe, E., Bachére, E., Boulo, V., Cadoret, J.P., Saraiva, E., Carrera, L., Calderon, J., Colwell, R. Bio-technology-based control of disease in marine invertebrates: development of molecular probe diagnostics and disease-resistant transgenic shrimp andmolluscs. *Molecular marine Biology and biotechnology*

Mialhe, E., Miller, L.H. (1994) Development of biolistic techniques for transfection of mosquito embryos (*Anopheles gambiae*). *Biotechniques* **16,** 924–931

Miller, L.H., Sakai, R.K., Romans, P., Gwadz R.W., Kantoff, P., Cran, H.G. (1987) Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. *Science* **237,** 779–781

Rousseau, C. (1995) Développement de technologies de transformation génétique d'embryons et de cellules de crevettes pénéides. Thèse EPHE, pp. 1–76. (IFREMER libraries)

Rousseau, C., Cedeno, V., Robalino, J., Cadoret, J.P., Boulo, V., Mialhe, E. (in prep.) Transient expression of luciferase reporter gene in embryos of White Shrimp *Penaeus stylirostris* and analysis of two promoters using aqueous biolistic

Scholthof, K.B.G., Scholthof, H.B., Jackson, A.O. (1993) Control of plant virus diseases by pathogen-derived resistance in transgenic plants. *Plant Physiology* **102,** 7–12

Tapay, L.M., Lu, Y., Brock, J.A., Nadala, E.C. Jr, Loh, P.C. (1995) Transformation of primary cultures of shrimp (*Penaeus stylirostris*) lymphoid (Oka) organ with simian virus 40 (T) antigen. *Proc. Soc. Exp. Biol. Med.* **209,** 73–77