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1. Introduction

The process of transformation, both natural and artificial, continues to play an important role in molecular biology. It is generally accepted that natural genetic competence, the ability of cells to bind to and to take up exogenous DNA, is widespread among bacteria and might be an important mechanism for the horizontal transfer of genes. In recent years, an exciting progress has been made in characterizing the pathways and signals that regulate the development of bacterial competence as an active process [1]. Observations that DNA, RNA, and oligonucleotides may also naturally associate with the surface of eukaryotic cells and become internalized were much less frequently reported in the past, and mechanisms of the development of natural eukaryotic competence have been only rarely studied and are poorly understood until now. Nevertheless, transfection of eukaryotic cells *via* an artificially induced competence (*e.g.*, by chemical or electrical treatment) is a widely used experimental procedure in laboratories of molecular and cellular biology.

Transgenic animal and plant technologies represent some of the most powerful tools in functional studies of various genes and serve for the preparation of organisms with new properties. Furthermore, because of many potential benefits, the research into gene transfer has become a very rapidly developing field with a special focus on *1*) human gene therapy protocols and *2*) production of biologically important macromolecules by transgenic animals. There are still many problems in preparing a 'perfect' transgenic organism, and today's transgenic technology is far from being satisfactory. One of the crucial steps is to find a simple way to efficiently introduce exogenous DNA into one-cell embryos. Furthermore, it is difficult to ensure integration of the DNA into the host DNA in all cells of the transgenic organism or only in cells of some 'target' tissues. Finally, the controlled expression of exogenous DNA in the whole organism or only in target tissues is the last essential step. DNA Microinjection into fertilized egg cells has been so far the most popular and widely used method for the production of transgenic animals. However, it requires costly and sophisticated equipment and considerable skills in micromanipulation, while the efficiency of the method is rather low. It does not exceed more than 4–5% in mice, the most successfully used organisms, and it is much lower with other species such as livestock or marine animals. Moreover, microinjection is always a non-physiological process, which may damage the cell. On the other hand, if the DNA uptake by spermatozoa developed into a well-controllable and defined process, these cells might become the most potent and, at the same time, the most natural tool for the production of transgenic animals. Compared to microinjection, a

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1. Introduction

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Transgenic animal and plant technologies represent some of the most powerful tools in functional studies of various genes and serve for the preparation of organisms with new properties. Furthermore, because of many potential benefits, the research into gene transfer has become a very rapidly developing field with a special focus on *1*) human gene therapy protocols and *2*) production of biologically important macromolecules by transgenic animals. There are still many problems in preparing a 'perfect' transgenic organism, and today's transgenic technology is far from being satisfactory. One of the crucial steps is to find a simple way to efficiently introduce exogenous DNA into one-cell embryos. Furthermore, it is difficult to ensure integration of the DNA into the host DNA in all cells of the transgenic organism or only in cells of some 'target' tissues. Finally, the controlled expression of exogenous DNA in the whole organism or only in target tissues is the last essential step. DNA Microinjection into fertilized egg cells has been so far the most popular and widely used method for the production of transgenic animals. However, it requires costly and sophisticated equipment and considerable skills in micromanipulation, while the efficiency of the method is rather low. It does not exceed more than 4–5% in mice, the most successfully used organisms, and it is much lower with other species such as livestock or marine animals. Moreover, microinjection is always a non-physiological process, which may damage the cell. On the other hand, if the DNA uptake by spermatozoa developed into a well-controllable and defined process, these cells might become the most potent and, at the same time, the most natural tool for the production of transgenic animals. Compared to microinjection, a

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further advantageous aspect of using spermatozoa as DNA vectors lies in the possibility of carrying out mass transgenesis, which could be of particular interest for, *e.g.*, agriculture and fisheries.

In the course of the last ten years, the idea of using sperm cells for the transfer of exogenous DNA into egg cells during fertilization and preparation of genetically transformed embryos and animals has been examined in several laboratories. Sometimes, sperm electroporation or sperm lipofection, and recently also sperm microinjection [2], were used to improve the rates of embryo transformation. All these reports (except for the sperm microinjection experiments) were summarized and critically evaluated by *Spadafora* [3]. In his review, it is shown that sperm-mediated gene transfer has been applied with variable degrees of success in various animals (abalone, silkworm, salmon, carp, *X. laevis*, rooster, mouse, rabbit, pig, cow). The technology appears to give the most reproducible results with invertebrates, fish, amphibians, and birds (*Table*). Sperm-mediated transgenesis of large mammalian species, such as pigs and cows, was also reported to be successfully performed, but due to a relatively low number of individuals, no statistically significant conclusion could be drawn. Despite these relatively encouraging data, many questions remain unanswered. The most important one is the great variability in the efficiency of transgenesis documented especially in mice, by far the most frequently and thoroughly examined species. We still do not understand why, in some experiments, completely negative results, *i.e.*, no transgenic animals, may be obtained. This indicates that the overall outcome of sperm-mediated gene transfer is influenced by factors, involved in the development of natural competence, and the identity of at least some of them remains still quite elusive (see also [3]). Further serious questions to be explained are the DNA-integration process, mosaicism, and modification of exogenous DNA in transgenic organisms. However, these problems are not limited to the sperm-mediated DNA transfer technology only, but represent a challenge to transgenesis in general.

Interaction between DNA of retroviral origin, *Rous* sarcoma virus DNA, and *Xenopus laevis* oocytes and one-cell embryos has been studied in our laboratory in the last few years. DNA was originally introduced into the cells by microinjection, and its fate and effect on *X. laevis* development was examined [4]. Among the vertebrate models, embryos of the amphibian *X. laevis* offer several distinct advantages for such studies. The embryos are large, they develop quickly, and they are easily accessible throughout the development (see also [2]). Retroviruses developed one of the most efficient natural mechanisms for the integration of foreign DNA into the genome of their hosts. Another advantage of using a retroviral construct in gene-transfer experiments is that host-DNA rearrangements do not usually occur, which is of great practical significance. Furthermore, the presence of strong regulatory elements in the retroviral LTR sequences is considered to efficiently suppress the influence of the integration site on the expression of introduced foreign genes.

Later, we found out that the DNA-microinjection technique we used in the frog system could be efficiently substituted by sperm-based technology described by *Spadafora* and his colleagues for mice [5], *i.e.*, by sperm cells preincubated with the retroviral DNA [6][7]. This paper summarizes results of our sperm-mediated transgenic experiments with *X. laevis*.

Table. *Sperm-Mediated Transfer of DNA in Various Species* (according to [3], adapted)

Class	Number of offspring analyzed	% transgenic offspring
Mollusks (1 species)	20	65
Insects (1 species)	300	n.d.
Fish (5 species)	761[a])	2.6–85[a])
Amphibians (1 species)	650	20–30
Birds (1 species)	273	23–67
Mammals (4 species including mice)	3292[a])	2.4–92[a])
Mice	2055	7.4–92

[a]) In some reports, only the best obtained results from a variety of tested experimental conditions were included; n.d., not determined.

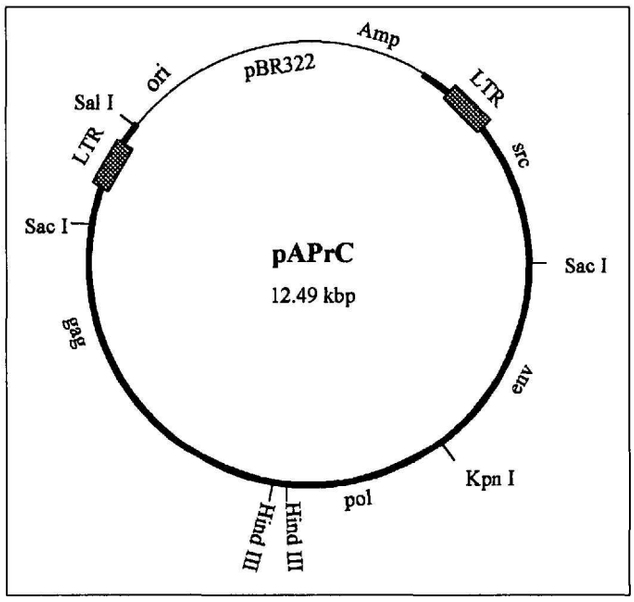

Figure. *Schematic representation of pAPrC* (according to [8]). A thicker line depicts the proviral part of the plasmid. LTR, long terminal repeat; Amp, ampicillin resistance; Hind III, Kpn I, Sac I, Sal I, restriction sites. The picture is not drawn to scale.

2. Results and Discussion

The sperm-mediated transfer of retroviral DNA into *X. laevis* eggs was carried out with a recombined plasmid pAPrC (12.5 kbp) carrying a pBR322-cloned proviral form of the *Rous* sarcoma virus (RSV) genome (*Fig.*, [8]). The proviral RSV genome consisted of four retroviral genes *gag, env, pol* and *src*, and two long terminal repeat (LTR) regulatory regions involving strong promoter and enhancer elements. The v-*src* gene is a well-established oncogene with transforming activity in birds, which are RSV-permissive organisms. The main aim of the study was to find out whether the *src* gene, besides its transforming activity, might have a function in the development, and to set up a suitable model system in which the effect of the *src* gene in early embryogenesis could be studied. Naturally RSV-nonpermissive frog organisms appeared to be particularly suitable for this purpose. Frog sperm cells prepared from testes of adult males were found to spontaneously bind

pAPrC DNA during incubation at room temperature in a simple medium consisting of 60 mM NaCl, 1 mM KCl, and 15 mM Tris-HCl at pH 7.4. The binding was essentially completed after about 30 min. Efficiency of the plasmid DNA binding to *X. laevis* sperm cells determined by filtration and hybridization techniques was found to be in the range of 50–160 molecules of DNA bound in a DNase-resistant form per one sperm cell, if spermatozoa were exposed to the DNA at a concentration of 1 μg DNA/10^7 sperm cells. The binding was strongly inhibited by heparin. The fertilization capacity of the sperm cells was not affected by treatment with pAPrC [7][9]. *In vitro* artificial insemination of batches of approximately 150 freshly collected frog eggs was carried out in parallel either with control, untreated, or pAPrC-treated spermatozoa. Fertilization with the latter spermatozoa reproducibly induced developmental defects in about 20–30% of all 5-day larvae (*Table*, [7]). Head and eye defects, edemas, axial deformations, impaired yolk utilization, and a decreased size were the most typical ones. Strongly aberrant embryos died. Less aberrant embryos sometimes spontaneously recovered and lived longer, but usually died before metamorphosis. Exceptionally, originally defective and later recovered embryos passed the metamorphosis, but with a large delay. Thus, in a very few cases, phenotypically normal transgenic frogs (except for a smaller size) were obtained. Fertilization with sperm cells treated with plasmid pBR322, which does not contain any retroviral DNA sequences, never induced defective morphogenesis, and phenotypic appearance of embryos did not differ from that of control embryos [7]. Immunohistochemical analysis of tissue sections from aberrant frog embryos with a specific anti-Src monoclonal antibody, supplemented with Western blot analysis of tissue extracts, revealed that the defective morphogenesis is accompanied with a high level of v-Src kinase (coded by the v-*src* gene) expressed mainly in myoblasts, neuronal tube, and epidermis. PCR/*Southern* hybridization experiments with appropriate probes proved the presence of characteristic v-*src* and RSV-LTR sequences in DNA isolated from aberrant embryos. Probing of Northern-blotted RNA isolated from defective embryos revealed the production of RSV-LTR *src* mRNA in their tissues. Transcription of *gag*, *env*, or *pol* sequences was not detected [7].

All these positive findings, which were obtained simultaneously at four experimental levels (phenotype, genotype, RNA, protein), fully agree with the view that frog sperm cells of *X. laevis* can serve as a natural vector for the transfer of retroviral DNA into eggs and for the production of transgenic frogs. Defective morphogenesis following the fertilization with the pAPrC-treated spermatozoa perhaps provides the most compelling evidence for their proviral DNA binding and transfer potential. The DNA was expressed into RNA (RSV-LTR-*src* mRNA) and protein (v-Src kinase), and an increased expression of the latter apparently interfered with the developmental program of frog organisms [7].

Several second generations of transgenic frog embryos were obtained by mating a transgenic male frog of the first (sperm-mediated) generation with a normal female frog [10]. Fifteen to twenty percent of frog embryos of the second generation (named B_1) displayed a spectrum of morphogenetic defects similar to but a little milder than those observed in the first generation of transgenic frogs prepared by direct sperm-DNA transfer. PCR and *Southern* analyses with a pAPrC probe revealed that proviral RSV DNA sequences were detectable in about 50% of all B_1 offspring, which is consistent with the *Mendelian* hereditary pattern. They were found in all defective and defective-repaired tadpoles and in about 30% of the phenotypically normal ones. Immunohistochemical examination of tissue sections of RSV DNA-positive, phenotypically normal and aberrant embryos demonstrated close correlation between the severity of defects and intensity of immunostaining for Src kinase. High level of Src kinase expression was accompanied with strong defects in frog morphogenesis. Low level of expression of the kinase did not seem to disrupt the embryogenesis. The results were consistent with the view that the defective frog development correlates with the expression of the *src* gene above a certain threshold. Low Src concentration in tissues (even though still higher than that in normal, control embryos) does not seem to be phenotypically relevant [10]. Detailed screening by *Southern* blot analysis with appropriate probes for RSV genes and structures transferred into the second, B_1 generation of transgenic frog embryos by sperm of the transgenic male frog, accompanied with the same DNA analysis of individual tissues of the male frog, provided an insight into RSV DNA structure and distribution in transgenic frogs and frog embryos, and into the process of transmission of these structures to the next generation [10]. This analysis proved the integration of RSV DNA structures into the frog DNA and revealed the absence of the v-*src* gene (but not LTR) in DNA of B_1 embryos and in sperm DNA of the parental male frog. The presence of RSV-LTR in embryonic DNA correlated with the occurrence of developmental defects and an elevated expression of endogenous c-*src* mRNA and c-Src protein. This led to the hypothesis that the high increase in the Src concentration observed in tissues of aberrant B_1 embryos was induced by the integrated LTR portion of RSV DNA that, apparently due to its strong enhancer element, insertionally activated expression of one or both *X. laevis* endogenous c-*src* genes [10].

In summary, our experiments indicate that sperm-mediated DNA-transfer technology is applicable also to the frog system. The retroviral DNA, introduced into *X. laevis* eggs by sperm cells integrated into the frog DNA, was expressed and transmissible to the progeny. On the other hand, a germ-line and somatic mosaicism was detected, and the integrated RSV DNA was modified by deletion. Systematic examination of all individual steps of the sperm-mediated transgenesis, and the sperm-DNA interaction in particular, is now required to develop the technology into a fully controllable process.

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