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UCRL-ID-113636

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April 28, 1993

Lawrence
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Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under Contract W-7405-Eng-48.

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SPERM CELLS AS VECTORS IN THE PRODUCTION OF
TRANSGENIC ANIMALS

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18 December 1992

Prepared in partial fulfillment of the requirements of the Science and Engineering Research Semester under the direction of Rod Balhorn and Nicholas Hud, Research Mentors, in the Lawrence Livermore National Laboratory.

*This research was supported in part by an appointment to the U.S. Department of Energy Science and Engineering Research Semester (hereinafter called SERS) program administered by LLNL under Contract W-7405-Eng-48 with Lawrence Livermore National Laboratory.

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ABSTRACT

Transgenic animals are used in industry and in biomedical research in order to provide in vivo experimental model systems. Sperm cells have been reported used as vectors in the production of transgenic animals before, however no approach has of yet proven to be successful. Fertilizing eggs with genetically modified sperm would be advantageous in that sperm are readily accessible and stable, and eggs can be fertilized by modified sperm cells in vivo. Recent elucidations regarding the unique manner of DNA packaging in sperm chromatin by protamines has provided us with the insight for developing a method of introducing foreign DNA into sperm which is likely to succeed where others have failed.

We have developed a method for mimicking the in vivo system of sperm chromatin toroiu subunits in vitro, concentrating these toroids, and fluorescent visualization. Our present work concerns development of a method to successfully deliver DNA across the cell membranes and into the nucleus.

Introduction

Transgenic animals are used as biomedical research tools to provide *in vivo* model systems in the study of gene regulation, development, pathogenesis and disease treatment. There are also applications for their use in industry in terms of pharmaceuticals and genetically superior livestock production . The most commonly used method of microinjection is considered to be the most efficient in the production of transgenics (Brinster et al 1985). This laborious method involves several labor intensive, invasive steps that are traumatic to the embryo (Merlino, 1991).

Several laboratories are investigating the possibility of producing transgenic animals using sperm cells as the vector as this appears to be a much more attractive method. Sperm cells are far more readily accessible and stable than eggs, and eggs can be fertilized by modified sperm cells via noninvasive artificial insemination. However, groups working with the development of this hypothesis have not proven to have produced transgenic animals using transformation methods such as simple incubation (Brackett et al, 1971; Lavitrano et al, 1989). The results from these groups indicate that DNA introduced to the cells associates with the cell membranes instead of proceeding with the sperm nucleus into the egg (Atkinson, 1991). Other groups have tried to transform sperm cells using electroporation (Gagné et al, 1991), and liposomal delivery (Bachiller et al, 1991). Bachiller concluded that the foreign [naked] DNA was degraded in the egg cytoplasm by intense enzymatic activity.

It is proposed that the fundamental packing unit of sperm chromatin is a toroidal structure consisting of DNA highly condensed by protamine (Hud et al, 1992). When DNA is complexed with protamine, enzymatic processes are inhibited (Balhorn, 1990). If a synthesized toroid containing a gene of interest were to be transfected into a sperm cell, it should be indistinguishable from the native sperm chromatin and be protected from enzymatic degradation in the egg cytoplasm.

Materials and Methods

Labeling of fish protamine toroids

We purchased high purity salmon protamine (salmine grade X) from Sigma Chemical Co. (St. Louis, MO). Salmine was dissolved at 1 mg/ml in 100 mM NaCl 10 mM Tris pH 7. Fluorescein isothiocyanate (FITC, Sigma) was dissolved at 10 mg/ml in methanol (Johnson et al, 1982). 200 µg of FITC solution per milligram protein was added to the dissolved protamine (Fig. 1). This solution was incubated at room temperature under darkness conditions for three hours and dialyzed against 100 mM NaCl, 10 mM Tris pH 7 to remove excess FITC from the solution.

Synthesizing salmon toroids

Toroids were synthesized by combining calf thymus DNA (Worthington Biochemical Corporation, Freehold, NJ) with the labeled salmine.

Nonprecipitating concentrations were obtained by combining equal volumes of equal concentrations of DNA and protamine up to a final concentrations of 40 µg/ml in 100 mM NaCl, 10 mM Tris pH 7. All samples were imaged with a Zeiss Axioscop.

Sperm collection and preparation

Rabbit semen was obtained from healthy, mature, New Zealand white bucks by use of an artificial vagina and a teaser female (Hartman et al, 1974). Sperm were washed twice in a 305 mOsm/kg defined media as previously described by Brackett et al (1974). For visualizing sperm cells, cells were mounted with propidium iodide (PI) in AntiFade (Johnson et al, 1981).

Electroporation

Washed sperm cells were subjected to a variety of parameters using the Cell-Porator electroporation system (Gibco-BRL). The capacitance ranged from 10 to 1980 μ F. The resultant electrical fields applied ranged from 125 to 1062.5 V/cm ($d=0.4$ cm). Fields were applied one, two and three times to suspensions containing sperm, sperm with labeled salmine, and sperm with toroids. Parallel controls were prepared using no electroporation. All electroporations were accomplished on ice as described (Potter et al, 1992) in pre-cooled disposable micro-electroporation chambers (Gibco-BRL). The effect on motility was observed before and after electroporation (Gagné et al, 1991). Post-electroporation washes were performed with Brackett's defined media.

Labeling bull toroids

Bull protamine was isolated as previously described (Balhorn et al., 1977). We reduced the protamine at 1 mg/ml in 100 mM NaCl, 10 mM Tris pH 7 with 10 mM dithiothreitol (DTT) overnight under a nitrogen atmosphere. FITC was dissolved at 10 mg/ml in methanol. 200 μ g of FITC solution per milligram protein was added to the dissolved protamine. This solution was incubated at room temperature under darkness conditions for three hours and dialyzed

against 100 mM NaCl, 10 mM Tris pH 7 to remove excess FITC from the solution. It was then centrifuged to remove any insoluble precipitated FITC.

Synthesizing salt-stable, concentrated bull toroid solution

27 ml of 20 µg/ml calf thymus DNA and 27 ml of 20 µg/ml bull protamine were combined in 100 mM NaCl 10 mM Tris pH 6. 54 ml of 100 mM NaCl 50 mM Tris pH 10 was added and the solution allowed to incubate at room temperature five to seven minutes. 108 ml of 100 mM NaCl 100 mM Bis Tris (Sigma) was added then to the solution. NaCl was added to a final concentration of 2M. To each of six thin-wall polyallomer ultracentrifuge tubes was distributed thirty-five ml of the above solution. The tubes were centrifuged for 20 minutes at 25,000 rpm (Beckman L8-50M ultracentrifuge with SW-28 rotor). The upper 30 ml from each tube was removed and the lower aliquots vigorously mixed and pooled into one tube. This was centrifuged at 25,000 rpm for 40 minutes. The upper 25 ml was removed and the lower aliquot vigorously mixed to remove any toroids from the sides of the tube.

Some concentrated samples were lyophilized and resuspended in water.

Results

Fluorescent visualization of toroids

When salmine was labeled with FITC, complexed with calf thymus DNA at a final concentration of 40 µg/ml or lower, and placed on a slide under excitation by wavelengths in the blue range, discrete point sources of yellow green light are observed with a constant green background. Control slides

with labeled protamine alone exhibit no points of light but instead a constant green background. DNA and salmine complexed to final concentrations greater than 40 $\mu\text{g}/\text{ml}$ exhibited points of light and large aggregates.

Toroids synthesized from bull protamine and calf thymus DNA (Fig. 3) appeared identical to those synthesized from salmine with the exception that aggregates appeared as the final solution reached concentrations greater than 10 $\mu\text{g}/\text{ml}$ (Fig. 2). However, we observed that toroids subjected to the pH swing for salt stability exhibited a density on a slide many times that of the initial solution. The constant green background was absent (Fig. 4). The calculated theoretical concentration of toroids concentrated in this method is 108 $\mu\text{g}/\text{ml}$ assuming complete recovery. Like the other toroid solutions described, this aggregates over a period of hours as well.

The lyophilized, salt-stable toroids resuspended well in water; we observed fields of point sources more dense than when initially concentrated on slides of them, corresponding to the amount of water the lyophilized product was resuspended in.

Sperm collection and preparation

The sperm cells retained a high percentage of motility after being allowed to sit on a desk top over a period of 48 hours after being washed as described above. Cells visualized under blue excitation after mounting in PI-AntiFade appear are seen as red sperm heads with barely visible tails.

Electroporation

The percentage of motile sperm subjected to a 10 μ F, 1062.5 V/cm field was equal to that of non-electroporated controls. However, approximately 50% of the cells appeared contorted and approximately 10% appeared to have undergone the acrosome reaction after receiving three pulses of the same voltage at 800 μ F capacitance.

Approximately 50% of cells subjected to a 1180 mF, 875 V/cm field in a suspension of Brackett's media containing toroids were observed to have fluorescent greenish-yellow point sources associated with their cell membranes. Approximately 0.1% of cells that had not been electroporated but incubated with toroids exhibited this fluorescence. 100% of cells incubated or electroporated in suspension with only labeled salmine exhibited bright yellow-green fluorescence over their entire membranes. In order to determine whether the point sources were outside or underneath the membrane, the cells were serially washed. After three washings, approximately 5% of those cells previously exhibiting fluorescence retained this property to some degree.

The density of fluorescent point sources on slides made from aliquots from the chambers after the post-pulse incubation was observed to be less than the predicted density.

Discussion

Other groups have attempted and failed to produce transgenic animals using sperm cells as vectors. The successful genetic alteration of spermatozoa intended to be used in the generation of transgenic animals can only be

accomplished by packaging foreign DNA in such a way that it mimics the *in vivo* system. In sperm chromatin, the genetic material is packaged into high density units. These toroids can be synthesized *in vitro* and fluorescently labeled.

The method for toroid concentration was arrived at by studying the molecular structure of protamine (Fig. 5). Toroids can be concentrated through the pH swing procedure which operates on the principle that cysteines can be manipulated by altering the pH; $pK_a \text{ cysteine} = 8.3$ (Fig. 6). The observed insoluble aggregates are the result of cysteines making disulfide bridges between neighboring toroids made from mammalian protamine in solution. The interior of a toroid benefits from these disulfide bridges in that they give the structure stability, in fact they are stable in 2M NaCl. As we complex DNA and reduced protamine at a pH less than 7.3, toroids still form because of the electrostatic attraction between the phosphodiester backbone of DNA and the arginines of protamine. However, the cysteines are reduced and do not disulfide bond. As we change the pH of the solution to 9.3, cysteine loses its proton and is available for disulfide bonding. The bridges form between neighboring cysteines in the toroid's interior, but as the solution is kept very dilute, there is very little chance of one toroid bumping into another and bonding. When the pH is changed to neutral, the outer cysteines are protonated and therefore prevented from disulfide bonding (Balhorn et al, 1992). This procedure works as we have seen after concentration.

FITC-labeled toroids and PI-labeled sperm are readily distinguishable under blue excitation as point sources of yellow green light and red sperm heads,

respectively. We have observed that when sperm are electroporated with varying parameters of voltage and capacitance that toroids will appear to associate with the cells. However, 95% or more toroids are removed when cells are washed, indicating that toroids are only transiently adhered to the outer membrane.

Also, toroids are very dense structures which we propose sink to the bottom of the chamber instead of diffusing through pores created by electroporation. Therefore, electroporation would not be the ideal tool for delivering packaged DNA through the membranes to the sperm nucleus. We are presently looking into a liposomal delivery system.

Acknowledgments

Thanks so much to Rod Balhorn, Nick Hud, Joe Mazrimas, Shelley Corzett, Joe Lee, and Jeff Ferguson for their as of yet undying patience and a wonderful introduction to scientific research.

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•fluorescein isothiocyanate couples to the amino terminus of proteins

• $\lambda_{\text{excitation}} = 480\text{nm}$

• $\lambda_{\text{emission}} = 520\text{nm}$

FIGURE 1: FITC + protamine reaction

FIGURE 2: aggregate - 15 ug/ml

FIGURE 3: 10 ug/ml toroids

FIGURE 4: 100 ug/ml toroids

FIGURE 6

Concentrating Toroids by Manipulating Cysteine Sulfhydryls

- $pK_{a\text{cysteine}}=8.3$
- at pH 8.3, there is a 1:1 equilibrium between these two forms:

• Favored 10:1 at pH 7.3 • Cannot form disulfide bonds	• Favored 10:1 at pH 9.3 • Ready to form disulfide bonds

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