ROLE OF GREEN FLORESCENT PROTEIN (GFP) GENE IN SOMATIC CELL CULTURES - A REVIEW
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ABSTRACT

Green fluorescent protein (GFP) is a single peptide of 238 amino acids, extracted from *Aequorea* jelly fish absorbs blue light and emits green light. Cloning of GFP came with breakthrough that expression of the gene in other organisms creates fluorescence and there is no jellyfish specific enzymes needed for the post-translational synthesis of the chromofore. The GFP is a noninvasive, effective marker for screening and allows the direct visualization of gene expression in living cells without the need for destructive techniques or the addition of cofactors. Prior to clone any livestock species for the purpose to fulfill the requirement of human need, the incorporation of transgene through transfection in the somatic cells is essential. Desired gene can be inserted in the somatic cells and actual transfected cells can be identified with the help of any reporter gene among them GFP is most popular due to its independence from any substrate or cofactor, and these cells can be separated through any suitable technique such as Fluorescent Activated Cell Sorting (FACS). Even very few no. of transfected somatic cells can lead to successful generation of Transgenic animal.

Improvements in technologies to produce transgenic farm animals are valuable, because the economic savings would benefit both biotechnology and basic research. The main barrier for transgenic animal production remains the identification of more efficient systems of transgene delivery and better mechanisms to optimize regulation of transgene expression levels. Transgenic techniques have rapidly evolved in recent years. However, the efficiency of these techniques to produce viable offspring is still disappointingly low. In vitro transfection of cultured cells combined with nuclear transfer is the most effective procedure to produce the transgenic livestock.

The Green Fluorescent Protein (GFP) can effectively be used to confirm the presence of functional transgene in cell culture. GFP was discovered by Shimomura *et al.*, (1962) is a single peptide of 238 amino acids, extracted from *Aequorea* jelly fish absorbs blue light and emits green light. GFP is the primary member of a revolutionary class of biologically compatible fluorescent protein probes that offer a wide applicability in cell, molecular and developmental biology. The acceptance of GFP as a widespread molecular probe is owing to its intense fluorescence, water solubility, photostability and its ability to be reconstituted in living cells. In just few years, the GFP has vaulted from obscurity to become one of the most widely studied and exploited proteins in the field of research. Its amazing ability to generate a highly visible, efficiently emitting internal fluorophore, is both intrinsically fascinating and tremendously valuable. GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms.

Cloning of GFP came with breakthrough that expression of the gene in other organisms creates fluorescence and there is no jellyfish specific enzymes needed for the post-translational synthesis of the chromofore. The GFP is a noninvasive, effective marker for screening and allows the direct visualization of gene expression in living cells without the need for destructive techniques or the addition of cofactors. GFP's independence from enzymatic substrates is likewise particularly promising in intact transgenic embryos and animals and for monitoring the effectiveness of gene transfer.
During the past decade, a variety of methods have been developed for introducing foreign genes into eukaryotic cells, including calcium phosphate precipitation, DEAE-dextran, protoplast fusion, electroporation, retrovirus and other viral vectors, and liposomes. Nowadays, it is possible to transfer DNA, RNA, oligonucleotides, proteins and small molecules into almost all types of cells by various gene transfer methods. Non-viral gene transfer agents offer several potential advantages over recombinant viruses. They are noninfectious, relatively non-immunogenic, have low acute toxicity, can accommodate a large DNA plasmid and may be produced simply on a large scale. They are limited by their lower gene transfer efficiency than viruses and transient gene expression. Electroporation and Lipofection are two powerful methods of transfection has several advantages to other DNA transfer methods including high reproducibility, relative ease of performance, and applications in a large number of cell types.

Promising new transfection method involving cationic lipids or liposomes is called Lipofection. In this method a cationic headgroups interact strongly with negatively charged phosphates on DNA, forming DNA-cationic lipids complexes, termed lipoplexes (Almofti et al., 2003). Net positively charged lipoplexes bind to negatively charged sialic acid residues on the cell surfaces and thus promote the passage of DNA through cell membrane.

Physical methods for this gene delivery have been developed more recently. They have become increasingly popular, although their application demands special equipment. The phenomenon of introducing DNA into cells by application of short electric field pulse is termed Electroporation or electropermeabilization. In essence, electroporation makes use of the fact that the cell membrane acts as an electrical capacitor which is unable (except through ion channels) to pass current. The applied high-voltage electric field results in transient elevation of the transmembrane voltage to approximately 1 V. Consequently, a dramatic membrane reorganization takes place - it breaks down and creates aqueous pathways or electropores. The DNA presumably diffuses into the cell through these pores which are believed to subsequently shrink and disappear. Although most electropores close rapidly, some may remain open for hours (Potter, 1996). Electroporation provides a valuable alternative to chemical and other physical methods that may be ineffective or toxic when transforming certain cell types.

TRANSGENIC CELL LINES

There are various types of cells which can be used to produce the transgenic lines. Fibroblasts cells have been used extensively in various species due to rapid growth, stable nature of these cells in culture. These cells can be harvested easily from decapitated and eviscerated fetuses at 40-60 days of gestation. Under standard culture conditions, the cells have population doublings between 17 and 24 hours and can have up to 60 population doublings prior to reaching senescence (Hill et al., 2000; Lanza et al, 2000). In nuclear transfer experiments fetal fibroblast from sheep, cattle, goat and pigs result in 0.5-25% of reconstructed embryos developing to live offsprings (Baguisi et al., 1999; Cibelli et al., 1998; Onishi et al., 2000).

Cumulus and mural granulosa cells have also been extensively used for nuclear transfer in cattle, mice, goats and pigs (Wells et al., 1997; Polejaeva et al., 2000). Wells et al., (1997) produced cloned calves by using quiescent mural granulosa cells as nuclear donors. Pre-implantation development of high quality blastocysts was 27.5% of reconstructed embryos cultured, out of these 10% of embryos produced calves. In mice, the nuclei of cumulus cells appear to be the best somatic cell for
supporting the normal embryonic development (Wakayama, et al 1998, 1999). The use of cumulus cells has certain technical advantages: they can be isolated from ovulated oocytes as a homogenous suspension and can easily be injected into recipient oocyte because of their small size. Further most cumulus cells are arrested in GO/G1 at the time of ovulation and their synchronization of cell cycle stage with chemical agent may not be necessary. However, one of the disadvantages of cumulus cell cloning is that only females can be obtained after nuclear transfer (Schnieke et al., 1997).

**GREEN FLORESCENT PROTEIN**

Wild-type green fluorescent protein (wtGFP) is a naturally occurring bioluminescence molecule emitted from photocytes in the umbrella of the northern Atlantic jellyfish, *Aequorea Victoria* (Gerdes and Kaether, 1996). Green fluorescent protein has become a valuable and non-invasive marker for labeling mammalian cells in culture, as well as, transgenic multicellular organisms (Hadjantonakis et al., 1998). These applications were made possible after the cloning of the complimentary DNA (cDNA) for GFP (Cubit et al., 1991). Further analysis of this cDNA revealed that GFP was a 238 amino acid protein with a 27 to 30 kDa molecular weight. Purified GFP absorbs blue light and emits green light. Maximum absorption of blue light occurs at 395 nm with a minor absorption peak occurring at 470 nm. The peak of green light emission occurs at 509 nm with a shoulder at 540 nm.

No exogenous substrates or cofactors are required for the activation of GFP. A bright green fluorescence occurs when GFP is expressed in eukaryotic or prokaryotic cells that have been illuminated with either blue or green ultraviolet light (Chalfie et al., 1994). The light absorbing chromophore that emits this fluorescence consists of a cyclic tripeptide, which will only produce fluorescence when embedded within the entire GFP protein (Breje et al., 1997; Li et al., 1997). Following oxidation this cyclic tripeptide is formed from the amino acids Ser65, Tyr66, and Gly67.

The development of several mutant forms of GFP has made it an important alternative to other reporter genes. Genes such as chloramphenical acetyltransferase and luciferase require multi-step treatments for proper detection of gene expression, including exogenous substrates and cofactors. Green fluorescent protein requires only UV light for activation (Gerdes and Kaether, 1996). However, UV light has been reported to be detrimental to the embryos. After exposing mouse embryos under a fluorescent microscope for several minutes, Mohr and Trounson (1980) reported the embryos failed to develop further, and were no longer viable. Eibs and Speilmann (1977) reported that prolonged exposure to UV light has a toxic effect on embryos, however morula stage embryos examined briefly under the fluorescent microscope showed no signs of toxicity due to this exposure. These embryos continued to develop normally, and were transferred successfully to recipient females while maintaining their vitality (Ikawa et al., 1995). The brief irradiation required for excitation did not affect embryo viability. These data suggest that if UV light is used appropriately in activation, the embryos will remain viable.

**LIPOFECTION**

The ability to introduce nucleic acids into cells has enabled the advancement of our knowledge of genetic regulation and protein function within eukaryotic cells, tissues and organisms. The successful pioneering studies of Graessmann and Graessmann (1976) with DEAE dextran and calcium phosphate-mediated transfection techniques, paved the way for future experiments necessitating DNA transfer into cultured eukaryotic cells.

The next advancement in liposomal vehicles was the development of synthetic
cationic lipids. Liposome-mediated delivery offers advantages such as relatively high efficiency of gene transfer, ability to transfect certain cell types that are intransigent to calcium phosphate or DEAE-dextran, successful delivery of DNA of all sizes from oligonucleotides to yeast artificial chromosomes (Felgner, et al., 1987).

Cells transfected by liposome techniques can be used for transient and for longer term experiments that rely upon integration of the DNA into the chromosome or episomal maintenance. Unlike the DEAE-dextran or calcium phosphate chemical methods, liposome-mediated nucleic acid delivery can be used for in vivo transfer of DNA and RNA to animals and humans (Capechhi, 1980). A lipid with overall net positive charge at physiological pH is the most common synthetic lipid component of liposomes developed for gene delivery. Often the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE). The cationic portion of the lipid molecule associates with the negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex. For cultured cells, an overall net positive charge of the liposome/nucleic acid complex generally results in higher transfer efficiencies, presumably because this allows closer association of the complex with the negatively charged cell membrane. Following endocytosis, the complexes appear in the endosomes, and later in the nucleus. It is unclear how the nucleic acids are released from the endosomes and traverse the nuclear membrane. DOPE is considered a “fusogenic” lipid (Lanza et al., 2000) and it is thought that its role may be to release these complexes from the endosomes, as well as to facilitate fusion of the outer cell membrane with the liposome/nucleic acid complexes.

Bondioli et al., (2001) established Fibroblast cell cultures from a slaughterhouse-derived male bovine fetus at approximately 50 days of gestation, and transfected with the CEEGFP plasmid containing the enhanced, humanized version of the GFP-reporter gene using Lipofectamine (Gibco BRL) as nuclear donor cells in oocyte reconstructions producing cloned calves. Skin biopsies were performed on two cloned calves at 48 h after birth to establish fibroblast cell cultures and analyzed by fluorescence-activated cell sorting (FACS) to determine the percentage of cells expressing GFP. The FACS analysis of fibroblast cultures revealed approximately 50% GFP-positive cells.

Koo et al., (2000) cultured Bovine pulmonary aortic endothelial cells (BPAECs) and transfected pEGFP-N1 (CLONTECH) through lipofection reagent Fugene 6 and expression was studied with the help of flowcytometry. To optimize transfection conditions, they measured the effects of increasing amounts of plasmid DNA while maintaining a constant volume of Fugene 6. Maximum efficiency was found to occur between 5 and 10 μg of plasmid per 100 mm dish; 60–70% of cells were typically GFP(+). Using more than 10 μg DNA per dish lowered the transfection efficiency. They tested various DNA : Fugene 6 (μg : ml) ratios using 5, 10, or 20 μg of plasmid DNA. The efficiency of transfection increased with increasing DNA:Fugene 6 ratios for both the 5 and 10 μg DNA per samples. At 20 μg DNA, the percentage of GFP(+) cells was slightly reduced at all DNA:Fugene 6 ratios tested. They established the optimal transfection conditions using 5-10μg DNA and 60 μL of Fugene 6 in 100 mm plates.

Bhuiyan et al., (2004) constructed an expression plasmid by inserting a green fluorescent protein gene and human ProU gene
into a pcDNA3 plasmid and transfected into bovine ear fibroblasts cell culture by lipid mediated method using Fugene 6. GFP expressing cells were selected under ultraviolet (UV) light using a Standard Fluorescent Isothiocyanate (FITC) filter set and used as donor nuclei. After Nuclear Transfer by these transfected cells 47.8 % blastocysts expressed GFP under FITC filter with some mosaic expression.

Keefer et al., (2001) isolated Fetal fibroblasts from Day 27 to Day 30 fetuses from a dwarf breed of goat. The CE-eGFP plasmid was delivered into the cells using ipofectamine (Gibco) according to the manufacturer's instructions. Presence of the eGFP transgene was confirmed by polymerase chain reaction, Southern blotting, and fluorescent in situ hybridization analyses. A total of 27 embryos derived from transgenic cells and 70 embryos derived from nontransgenic cells were transferred into 13 recipients. Five recipients (38%) were confirmed pregnant at Day 35 by ultrasound. Of these, four recipients delivered five male kids (7.1% of embryos transferred) derived from the nontransfected line. One recipient delivered a female kid derived from an eGFP line (7.7% of embryos transferred for that cell line) showing that both in-vitro transfected and nontransfected fetal fibroblasts can be used successfully as donor cells.

Elnitski and Hardison (1999) had shown the lipofection for MEL (Mouse Erythroleukemia) cells as a reliable, effective and reproducible method. The Lipofection reagent Tfx50 (Promega) was used to transiently transflect 1 x 10^5 cells. The reagent and DNA remained in the cell culture for 48 hr. The fraction of cells expressing "enhanced" green fluorescent protein was determined by flow cytometry. The optimal amount of reagent for use with MEL cells was determined by titrating increasing amounts of DNA at each ratio. Transfection conditions were determined to be optimal at a 2:1 lipid to DNA ratio. 2.0 μg DNA most frequently seen to be optimal MEL cells. The level of expression consistently declined above 4.0 μg of DNA, presumably due to cell death at higher concentrations of the lipid reagent.

Naito et al., (2000) isolated primordial germ cells from the blood of early chicken embryos and transfected the cells by lipofection using SuperFect transfection reagent (Qiagen). One μg DNA (lacZ gene) was diluted with 29 μL of serum free medium, mixed with 4μL SuperFect solution, and incubated for 60 min. at room temperature. Then mixture was added in the collected donor Primordial Germ cells and incubated for 24 hrs. at 38°C. The transfected cells were washed with culture medium and dispersed in 100 μL of fresh culture medium. Of the 52 embryos manipulated, 43 embryos survived at day 5.5 of incubation. Expression of the gene in the gonads of chimaeric embryos was observed in 34 (65.4 %) embryos.

Lascombe et al., (1996) cultured the guinea pig endometrial stromal cells and transfected the 10^3 to 10^5 cells by taking 2.5 μg a-galactosidase in the serum free Chemically Defined Medium (CDM) with an amount ranging from 2.5 to 30 μg of lipofectin (Life Technologies). The DNA solution and Lipofectin solution were slowly mixed in a total volume of 2 mL/well and incubated at 25°C for 10 min. Cells were incubated with the liposome-DNA complexes for 12 hrs. a-gal activity was spectrophotometrically measured. The activity observed with ratios of 6 and 7 were the highest: there were significantly 3.2 fold and 3.1 fold increases, respectively, compared with the activity for a ratio of 3.

Ayoob et al., (2000) cultured the Potorous tridactylis (rat kangaroo) kidney cell line (PtK2 cell lines) in PtK2 medium used for lipofection with Liposomal transfection reagent: Lipofectin™ (Life Technologies Inc.). 10 μL of
Lipofectin diluted in 200 μL of Opti-MEM and 1 to 2 mg of EGFP DNA was diluted in the 200 μL of Opti-MEM then both were mixed and incubated for 15 min. Then 600 μL of Opti-MEM was added in the DNA-Lipofection mixture and added in the cells and incubated for 3 hrs. then DNA-Lipofection mixture was removed from the cells 2 ml of complete media was added in the cells. Cells were observed for GFP expression with epifluorescence optics and filters optimized for the species of GFP used and yielded transfection rates with GFP-alpha-actinin of 40-60 % for PtK2 cells.

Arat et al., (2002) took Tissue biopsy from the ear of a 13-yr-old cow for adult skin fibroblast cell lines. A Day 70 fetus cloned from the same donor cow was surgically removed from the recipient’s uterus and cells were obtained from the lung of the fetus Adult and fetal cells were transfected with a plasmid-containing EGFP gene under control of the cytomegalovirus promoter and neomycin resistant gene, which allows selection using geneticin under control of an SV40 promoter (pEGFP-N1, Clontech) using a polyamine transfection reagent (GeneJammer, Stratagene). Briefly, 2 - 3 X 10^5 cells at passage 4 were seeded in a 35-mm culture plate 1 day before transfection. Cells were transfected with 2 μg of linearized pEGFP-N1. After transfection the cells were exposed to 600 μg/ml of geneticin (G418, Sigma) for 20 days. After 20 days, 300 μg/ml of geneticin was used during the selection process to obtain stable expression, and single colonies were isolated in the presence of G418 and expanded. Transfected cell colonies were picked up by using cloning discs and placed in 24-well plates. Cells in each well were examined for EGFP expression under fluorescent light using a standard fluorescein isothiocyanate (FITC) filter set and marked as positive and negative cell lines. 49 adult and 35 fetal cell colonies were obtained. Green fluorescence expression was observed in 35 out of 49 (71.4%) adult clones and in 30 out of 35 (85.7%) fetal clones.

Hyun et al., (2003) prepared a foetal fibroblast cells from pig fetuses on day 30 of gestation. The plasmid PEGFP-N1 (Clontech) that has been optimized for brighter fluorescence and higher expression in mammalian cells was transfected in confluent fetal fibroblasts dishes by adding 1 μL (1 μg) of pEGFP-N1 and 3 μL of FuGENE-6 (Roche Diagnostics) were diluted with 97 μL of serum-free DMEM and incubated for 15min. at room temperature. After this 101 μL of mixture was added into 2 ml of cell culture medium. The cells were cultured for 2-3 days. In order to detect the EGFP gene in cloned piglets, genomic DNA was extracted and PCR amplification was performed. Expression frequencies of GFP gene during development were 100%, 78 %, 72 %, 71 %, and 70 % in fused, two cell, four to eight cells, morulae, and blastocysts, respectively.

Diaz et al., (2003) established a primary culture of fetal fibroblasts from a porcine fetus of approximately 60 days old and transfected with pCXN-EGFP using Lipo TAXI Mammalian Transfection Kit (Strategene). 300 μL of LipoTAXI reagent and 2.7 ml of DMEM/F12 were mixed and settled 30 min. at room temperature before dilution of with 5 ml of DMEM/F12 containing 30 μg vector. Subconfluent fetal fibroblasts were incubated with this preparation for 5 hr. in a 100 mm culture dish followed by the addition of 1 ml of FCS and cultured until confluent. EGFP expression in the gene transfected fibroblasts was determined by using an epifluorescence microscope with a standard FITC filter (Eclipse TE, Nikon). Transfection rate of EGFP gene after 2 day culture of fibroblasts was very low (1.7 ± 0.5% ± SD of 5 replicates) in the preliminary experiment. However after the successive selection culture, all of the fibroblasts, which were stained with Hoechst 33342,
emitted green fluorescence under the epifluorescence microscope.

**ELECTROPORATION**

Electroporation involves subjecting a cell and DNA suspension to an electrical impulse, which is believed to induce local areas of reversible membrane breakdown. This breakdown creates pores through which DNA enters the cell. Electroporation has several advantages to other DNA transfer methods including high reproducibility, relative ease of performance, and applications in a large number of cell types. Electroporation occurs in a chamber between two charged electrodes. Upon discharge the electrical impulse is transmitted through the cell and DNA suspension (Catalado et al., 1998). In order for electroporation to be efficient, several critical physical parameters must be identified and met. Critical physical parameters include field strength and pulse duration (Anderson and Evans, 1989). Field strength (kV/cm) depends on the voltage that is applied to the cells, and the distance separating the electrodes of the electroporation chamber (Catalado et al., 1998). Pulse duration, or the duration of exponential decay, depends on the amount of energy stored in the capacitors of the electroporation apparatus. This energy is measured in microfarads (μF). Pulse duration is also dependent on the resistance created by the suspension medium in use.

The setting where approximately 20 to 50% of the cells remain viable after electroporation is considered sufficient for DNA transfer in most cells (Chu et al., 1987; Andreason and Evans, 1988). Temperature plays an important role in this viability, and is believed to affect the efficiency of electroporation. In most cells electroporation takes place at room temperature, and the cells are then incubated on ice. Andreason and Evans (1989) reported that this incubation period extends the period of time the cell membrane pores remain open, and improves cell viability. The effect of this incubation on DNA transfer varies among cell types. Variation is also found in the electroporation protocol for each individual cell type, for this reason, conditions must often be empirical for each experimental situation. Biological parameters that affect the efficiency of electroporation include the ionic strength of the cell suspension medium, as well as, the size and type of the cell being transfected (Catalado et al., 1998). The optimal medium for transmission of the electrical impulse will have low resistance and high conductivity. The efficiency of electroporation, for some cell types, can be adversely affected by divalent cations found within the media. These divalent cations can act to stabilize the cell membrane, and thus cause resistance to the disruption of the membrane. Careful consideration is needed when selecting a medium with the proper properties for the specific cell type in use. Cell diameter also contributes to the efficiency of electroporation. Cells with a smaller diameter, as compared with large diameter cells, can survive electroporation with larger voltage and capacitance (Chu et al., 1987, Potter, 1996). Therefore, cell diameter plays an important role in selecting voltage and capacitance parameters. A larger cell requires a lower voltage to maintain subsequent viability.

Li et al., (2002) assessed and compared the efficiency of two methods of transfection, Effectene (Qiagen) and electroporation. Plasmid DNA (pEGFP-N1, Clontech) was transfected into fetal fibroblasts, cumulus cells, and adult ear skin cells. For electroporation, cells(5 × 10^6 cells/ml) were mixed in perrim buffer + 15 μg pEGFP-N1, and subjected to two pulses of 0.38 kV and 400 μF delivered by Gene Pulser (BioRad). Assesment of EGFP transfected cells by green fluorescence was carried out under an inverted epifluorescence microscope equipped with a filter for FITC. The efficiency of gene
transfection into foetal fibroblast by effectene reagent (14.2 ± 1.7%) was significantly (P < 0.05) higher than that by electroporation (5.1 ± 1.0%). Among the three types of cells, the efficiency of gene transfection by Effectene and electroporation of Foetal fibroblast was significantly higher than those of cumulus cells and Ear skin fibroblast cells (9.4 ± 1.5 and 3.3 ± 0.8; 8.8 ± 0.07 and 2.1 ± 0.4%, respectively).

Chen et al., (2002) attempted to develop efficient strategies for the generation of human bile salt-stimulated lipase (BSSL) transgenic cows. In this a construct was made from pBSSL III by inserting EF-n-GFP and was electroporated in the Bovine primary foetal fibroblasts by suing 5μg DNA in a 2 mm cuvette containing 2.0 X 10^6 cells for 1 msec at 275 volts for three pulses. Cells were sorted by repeating flow cytometry for stable expression of GFP and used for NT. 165 out of 331 cell cytoplast couplets were fused out of which 99 (60 %) embryos cleaved, 68 (41.2%) were developed in the GFP Positive blastocysts.

Kalina et al., (2003) isolated Spermatogonial cells from testes of mature avian males (White Leghorn). Firstly they prepared a lipocomplex by adding Lipofectin® Reagent (GIBCO BRL®) with pEGFP-C1 DNA (Clontech) in 3 : 1 ratio. 100 μl of the medium (M 199, Sigma) containing a correct cell concentration (10^5 to 10^6/ml) of cells were put into the electroporator cuvette (Bio Rad Pulser, Cuvette, 0.1-cm gap) and the solution of Lipofectin-plasmid (ratio 3 : 1) was added. The parameters to optimize the use of the RF Gene Pulser module were voltage, burst duration, percent modulation, radio frequency, number of bursts and burst interval. The following electroporation parameters were tested in the experiments: group 1 (10 V, 50 msec), group 2 (20 V, 40 msec), group 3 (200 V, 30 msec), group 4 (400 V, 5 msec), group 5 (400 V, 10 msec), group 6 (400 V, 20 msec). Other parameters were constant: percentage of modulation 100%, RF frequency 40 kHz, number of burst 1. Cell cultures were checked under a fluore-scent microscope (Olympus IX50, U-RFL-T) with th89ge excitation wavelength of 488 nm and emission wavelength of 507 nm, magnification 400×, and gene expression was analysed. The X-test was used for a statistical comparison of the percentage of GFP positive spermatogonial cells with different electroporation condition. The high-est number of positive cells (35.8%) was achieved by the following electroporation parameters: total volts 400 V, percent modulation 100%, RF frequency 40 kHz, 20 msec burst duration, number of bursts 1. These results were statistically significant in comparison with other groups of transfected cells where different important parameters were used: 10 V, 50 msec (2.1%), 20 V, 40 msec (3.9%), 200 V, 30 msec (4.5%), 400 V, 5 msec (5.3%). In the experiment, stable transfection of spermatogonial cells was achieved, and the expression of GFP was observed in with in one month during the cell proliferation.

Thomas (2003) used the electroporation technique for the transfection in the Bombyx mori embryos. He used pB3xP3EGFP vector that express GFP in embryonic stemmata and nervous tissues. He used different electroporation program, through a device able to deliver square pulses of current, BTX- ECM- 830 Electroporator, (Tables 1 and 2) The EGFP expression was detected using a Leica GFP II filter set mounted on a Leica MZFL-III binocular microscope.

Kato et al., (1999) collected the fertilized one cell embryos from the oviducts of superovulated BDF1 females that were mated to BDF1 males. The purified DNA fragment (EGFP gene) was microinjected into the pronuclei of zygotes. These were cultured in a microdrop of M16 medium in a humidified atmosphere of 5% CO2 and 95% air at 37°C until they developed into morulae or
TABLE 1: Electroporation program

<table>
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<th>ID of the program</th>
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<th>Duration of the pulses in msec</th>
<th>Voltage of the pulses</th>
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Thomos, 2003

TABLE 2: Optimization of Electroporation parameters

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<th>Mean no. of positive spots per embryo</th>
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Naito et al., (2000) collected chicken blastoderm of freshly laid and unincubated eggs at stage X and were transfected by lipofection and electroporation. Lipofection was carried out by using a SuperFect transfection reagent (Qiagen) 9 µg DNA was diluted with 18 µL serum free medium and mixed with 36 µL SuperFect (Qiagen) solution, and incubated for 60 min at room temperature. 2 µL of of The SuperFect-DNA solution, filled in micropipette was inserted into the blastoderm and the tip of the micropipette was placed in the central part of the blastoderm and between the yolk membrane and blasoderm layer. For electroporation DNA was diluted with medium at a concentration of 0.25 µg/µL and injected one µL in each blasotderm by the same way as used in lipofection treatment. Electroporation was carried out using an CUY-21 electroporator with 4 mm apart in parallel on both sides of the blastoderm. Electric square pulses were applied 5 times at 5-50V for the loading periods of 50 msec. per pulse with one second intervals. Expression of GFP gene was detected under a fluorescent microscope (MZFL III, Leica), with the filters 480 nm excitation and 510 nm emission wavelength. Blastoderms were transfected by lipofection, 70% of the blastoderms at day 1 of incubation expressed the GFP gene and percentage of GFP positive blastoderms decreased gradually with the incubation period proceeds reached at 40% at day 3 while Electroporated blastoderms expressed the GFP gene was less than 30 % at day 1 of incubation when 5 V were applied, whereas it was 70-80 % when 10-25 V electric pulses were applied.

Paulssen et al., (1990) grew Rat Pituitary tumour cells (GH2C, and GH3) as monolayer cultures. Cell suspension of
concentration 10^6 cells/ml of PBS mixed with the DNA were prepared and 0.5 ml was transferred to presterilized disposable cuvettes (interelectrode distance 0.4 cm). Electroporation was carried out using the Bio-Rad Gene Pulser by selecting 60 msec pulse and 960 μF capacitance at voltage settings in the range of 200 to 800V. The electroporated cells were divided into three equal portions, and each were diluted with an appropriate volume of culture medium and plated in 9 cm Petri dishes resulting in cell viability ranged from 98 % at 200 V to 6 % after 600 V. As effective electroporation seems to occur when cell viability is about 20-50%, cell viability about 50%, resulting in optimal transfection for both types of rat pituitary cells, was obtained at 300V for GH2C1 cells and at 240 V for GH3 cells. Result showed the same time course of expression with peak activities at 24 hrs. Highest expression was observed by using 50 μg DNA for GH2C1 cells and with 25 μg DNA for GH3 cells and decreased significantly with higher DNA concentrations.

Lin et al., (2002) studied a total of 126 male Sprague-Dawley rats weighing 300-350 g, received direct intrathecal injection of 100 μg pE-GFP plasmid (Clontech). The intrathecal electrode was contacted with a negative electrode clamp through the sheath. A positive electrode was placed on the tail. A current of 200 V, pulsing duration at 50 ms; nonpulsing duration at 950 ms; and the number of shocks at 5 was applied by the pulse generator (BTX). The spinal cords were retrieved and analyzed with fluorescence microscopy, reverse transcription polymerase chain reaction (RT-PCR), and Western blotting. At day 1, 3 or 7 following electroporation a clear GFP expression in spinal cord tissue was detected. The most prominent transfection occurred in the meningeal cells and superficial layer of the spinal cord. The expression of GFP protein was peaked between 3 and 7 days after electroporation and significantly decreased at 14 days.

Atkins et al., (2000) electroporated the avian embryos. Plasmid (eGFP-N1, Clontech) was diluted to 250 ng/μl in chick Ringer solution. Fertile chicken eggs (stage 10 -13) were windowed and the vitelline layer over the target area was carefully reflected with a fine tungsten needle. The double-barreled electrode was backfilled with DNA solution and a small pool of DNA solution was injected. They optimized the electroporation for 1 sec. train number (1, 3, 6, 3+3 of alternating polarity), pulse frequency (10, 100, 500, 1000 pulses/sec.) and Stimulator voltage (40, 50, 60, 70, 80) were varied accordingly. Train length was always one second. The egg was resealed with adhesive tape and returned to the incubator for 48 hours. Whole embryos were removed into phosphate buffered saline (PBS) and the amnion was taken off. GFP expressing cells were visualized using either an epifluorescent compound microscope (Zeiss). Early GFP expression was visible less than two hours post-electroporation but did not reach maximum intensity until 24-48 hours. Expression continued until incubation was halted (>8 days). Six alternating trains (3+3) were significantly more efficient than a single train (P<0.01). Five hundred pulses per second (pps) produced the optimal number of transfected cells (36.7%). Higher and lower frequencies resulted in approximately half the number of transfected cells. The highest mean numbers of GFP-expressing cells were observed at 60-70 V. Stimulator voltages of 40 V and 80 V produced significantly lower efficiencies (P<0.05).

Li et al., (2002) obtained Suspension human acute T leukemia Jurkat cells and cultured in complete media. Cells were then resuspended in pulsing buffer at 1 to 8 x 10^7 cells/mL together with pEGFP-N1 plasmid (Clontech) DNA at a concentration ranging...
The cell/molecule mixture (volume 1.5 to 50 mL) was then pumped into the disposable flow chamber by peristaltic pump and electroporated during flowing. The flow rate and pulsing sequence were synchronized such that each cell received 4 square pulses, 400 μs each. The processed cells were collected and incubated at 37°C for 30 min before plating in complete medium. Optimization, electrical fields were set at 1 kV to 2.3 kV/cm, depending upon the cell line. To evaluate eGFP transfection, cells were analyzed 40-48 hr post processing, examined the ability of flow electroporation to transflect mammalian cells for gene expression. A plasmid carrying the gene coding for enhanced green fluorescence protein (eGFP), driven by the CMV promoter, was used.

Chopra and Sharma (2005) observed that electroporation was more efficient than lipofection for skin fibroblast cells as well as cumulus cells of Bhadawari buffaloes when observed under fluorescent microscope. Expression was maximum at 48 hrs. post transfection incubation in both methods and both cells. In electroporation expression was maximum at 25 msec. exposure in both types of cells which was up to 9% in skin fibroblast.
PLATE III: LIPOFECTION IN CUMULUS CELLS

PLATE IV: LIPOFECTION IN SKIN FIBROBLAST CELLS

cells and up to 8% in cumulus cells. Transfection by Lipofecton method for cumulus cell has given the maximum expression by using 0.8μg DNA with SuperFect in ratio of 1:6, whereas for skin fibroblasts cells expression was maximum at 0.6μg DNA when added with the SuperFect in the ratio of 1:4 (Plates 1-4).

Many studies and advances have been achieved in the field of gene transfer, but the main obstacle, poor efficiency, still remains. Despite widespread use of the chemical and physical gene transfer methods and heaps of literature filled with successful experiments, one still faces a problem of optimization for the cell type being studied. The optimal conditions for each particular transfection method should be determined experimentally.

CONCLUSION

The production of somatic cell clones derived from different tissue types of cultured...
cells opens new horizons for transgenic technologies. Indeed, results obtained from various species, including bovine, ovine, goats, and pigs, have indicated that, when transfected donor cells are properly selected, a high proportion of offspring derived by nuclear transfer are transgenic. In addition to improved nuclear transfer efficiencies, more accurate methods for screening both genetically modified donor cells and reconstructed embryos before transfer to surrogate females are needed. GFP selection of donor cells has been used to produce transgenic offspring by nuclear transfer in mice, pigs, goats and cattle. Furthermore, GFP gene can be used to confirm the presence of a functional transgene in embryos derived from transgenic animals, which would greatly enhance the efficiency of propagating transgenic livestock.

REFERENCES