ASSESSMENT OF MURINE EMBRYO DEVELOPMENT FOLLOWING ELECTROPORATION AND MICROINJECTION OF A GREEN FLUORESCENT PROTEIN DNA CONSTRUCT

by

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ABSTRACT

Transgenic techniques have rapidly evolved in recent years. However, the efficiency of these techniques to produce viable offspring is still disappointingly low. The purpose of this study was to assess in vitro development, transgene expression, and integration following pronuclear or cytoplasmic microinjection of condensed or linear green fluorescent protein DNA into murine embryos using electroporation. In experiment 1, the effect of embryo orientation (group or linear) within the electroporation chamber on development was evaluated using zygotes which received one pulse duration (10 ?sec), and one of two voltages (250 or 400 V). Zygotes that received 400 V had the lowest development score (Group, 2.06 ? 0.12; Linear, 1.97 ? 0.13), irrespective of orientation. Embryos that received 250 V had the highest development of the voltage treated groups (Group 3.42 ? 0.12; Linear 3.32 ? 0.12), irrespective of orientation, and development was lower than the control embryos (Control 4.28 ? 0.12; Mannitol control 4.36 ? 0.18). In experiment 2, the efficiency of utilization of the prepared enhanced green fluorescent protein (EGFP) construct as a visual marker of protein expression was

evaluated using pronuclear microinjection. Embryo development and fluorescence were evaluated following pronuclear injection of EGFP at a concentration of 3 μg/ml and compared to an uninjected control. Embryos injected with the EGFP had lower development scores (3.85 ± 0.15) than uninjected control embryos (5.72 ± 0.2). Of the embryos injected, 32.4% fluoresced due to expression of EGFP. Experiment 3 evaluated the effect of combining cytoplasmic injection of EGFP (425 μg/ml) with electroporation at 250 V on EGFP expression. The non-manipulated control embryos had significantly higher ($P < 0.01$) 4 d development scores (5.57 ± 0.11) than manipulated control embryos (4.6 ± 0.18), where the injection needle was inserted into the cytoplasm and no DNA was injected. Combining cytoplasmic DNA injection and electroporation caused a significant ($P < 0.01$) decrease in development scores, irrespective of DNA construct, when compared to embryos injected with a DNA construct alone. The mechanical effects of needle insertion combined with electroporation were not significantly different ($P > 0.05$) from embryos injected with DNA alone, irrespective of construct injected. Cytoplasmic injection of condensed DNA (0.38%), linear DNA (0.38%), and condensed DNA combined with electroporation (0.36%) resulted in one fluorescent embryo respectively. Cytoplasmic injection of linear DNA when combined with electroporation (3.57%) resulted in 13 fluorescent embryos. Pronuclear injection of the prepared EGFP construct results in lower development than control embryos. Electrical stimulation of zygotes reduces early embryo development. However, low amounts of electrical stimulation may allow for enhancement of gene integration in transgenic embryos.

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CHAPTER 1

INTRODUCTION

At Yale University Jon W. Gordan and his associates (1980) determined that a fertilized mouse embryo could incorporate foreign DNA. In the short time since this discovery, techniques for the production of transgenic animals have rapidly evolved. This evolution led to the remarkable development of animals such as Genie the sow, the first pig ever to produce a human blood protein in her milk (Velander et al., 1997). Although these advances are remarkable, the efficiency of the techniques used to produce transgenic animals such as Genie is still disappointingly low.

Pronuclear microinjection of DNA constructs continues to be the most efficient method of transgenic animal production; however, the procedure is limited in its applications and remains very costly. Microinjection requires a large number of fertilized embryos due to the high mortality rate. Pronuclear visualization is necessary and requires significant embryo manipulation, including centrifugation, in many domestic animal species. Skilled technicians are required, due to the highly technical nature of the procedure, and even in the hands of these highly skilled technicians, the efficiency of the procedure and in vitro culturing techniques remains unsatisfactory.

While new techniques are emerging every day, none have been proven to be as effective as pronuclear injection. The cost of all of this inefficiency is considerable, and that cost is passed onto the consumers in need of the pharmaceutical products produced by the transgenic animals. A scientist at the US Department of Agriculture (USDA) has estimated the cost of a founder animal with a functional transgene at $25,000 for a pig,

$60,000 for a sheep, and $300,000 to 500,000 for a cow (Wall, 1997). These numbers clearly demonstrate the need for a more efficient method of transgenic animal production.

The use of electroporation to facilitate gene delivery is one avenue worthy of investigation. Electroporation has several advantages over other DNA transfer methods including high reproducibility, relative ease of use, and application in a large number of cell types. This temporary disruption of the cell membrane, due to the application of electrical stimulation, creates transient aqueous pores through which charged molecules, such as DNA, may travel.

Evaluating the coupling of electroporation with DNA injection continues the search for alternatives to traditional transgenic animal production techniques.

The objectives of these experiments were to 1) evaluate the effects of embryo orientation within the electroporation chamber on embryo survivability and to establish a voltage to be used for additional experimentation; 2) to validate a prepared DNA construct as a visual marker for protein expression using a known method of transgenic animal production, pronuclear injection; and 3) to evaluate the effects of coupling cytoplasmic microinjection with electroporation on green fluorescent protein expression in murine embryos.

CHAPTER 2

LITERATURE REVIEW

The following is a selected review of research that provides a background in the areas of transgenic technologies. This section will explain DNA condensation, electroporation and DNA transfer, cytoplasmic injection of DNA, and the utilization of the green fluorescent protein (GFP) construct as a marker for protein expression.

Transgenic Techniques

The most efficient and widely used method for the production of transgenic animals, animals that carry other species' genes, is pronuclear DNA injection. The pronuclear stage occurs after fertilization, but before the fusing of the male and female pronuclei and subsequent cellular cleavages. Pronuclear injection involves the injection of the gene of interest along with a promoter sequence into the male or female pronucleus of a fertilized ova. In the murine zygote, the male pronucleus, which contains half of the genetic information for the embryo, is larger than the female pronucleus (Watson et al., 1983). For this reason, the male pronucleus is most often used for microinjection of foreign DNA into the murine zygote.

After microinjection the foreign DNA is usually found in both somatic and germ-line cells, therefore further integration should occur early in development as a Mendelian trait. These genes are usually found in tandem head to tail arrangements (Watson et al., 1992). This transgene integration varies from mouse to mouse, with different chromosomes carrying the foreign DNA. The number of copies of the foreign gene

found within the transgenic animal can vary greatly as well, from only a few copies to more than a hundred, even in groups of animals where the zygotes have been treated identically (Watson et al., 1983). The mechanism behind foreign DNA integration into the host genome is unknown; however, the ends of DNA molecules may be involved. Folger et al. (1982) reported that linear DNA molecules are integrated more efficiently than circular molecules.

Following microinjection the embryo is either directly implanted into the oviduct of a recipient mother, or is allowed to develop in long term culture medium to the blastocyst stage. The blastocyst may then be transplanted into the uterus of a recipient, where implantation can occur. Even though pronuclear injection is currently the most efficient method in mammalian transgenesis, the number of successes, when compared to the number of injected embryos, is unsatisfactory. In the murine embryo, only about 10 to 30% of the embryos microinjected survive the manipulation. The number of surviving embryos that integrate the foreign DNA into their genome can vary significantly as well, from only a few percent to as high as 40% (Watson et al., 1983). Wilkie et al.(1986) reported that after microinjection about 12% of the zygotes survive to birth, and only 30% of those are transgenic. Brinster and associates (1985) had a similar result, that a well-trained technician working with superovulated murine embryos will have a birth rate ranging from 15 to 25%. The number of transgenic animals born can range from 15 to 30% (Hogan et al., 1986). In the bovine, the efficiency for production of a transgenic animal is only 0.2% (Bondioli et al., 1988 ; Krimpenfort et al., 1991). Brem et al. (1990) reported a slightly higher efficiency (0.5%) in the porcine species. The overall efficiency of pronuclear injection is about 10% (Watson et al., 1983).

A common problem in transgenic animals produced through pronuclear microinjection is mosaicism. Mosaic animals occur when the transgene is not integrated into every cell of the animal. Wilkie et al. (1986) reported that about 30% of the mice produced through pronuclear microinjection were mosaic in the germline. This can occur if DNA replication occurs prior to integration of the foreign DNA, and results in transgenesis within only a fraction of the embryonic cells. To prevent mosaicism, the transgene must be integrated at the one-cell stage.

Other methods are also used in the production of transgenic animals including, retroviral infection, the use of embryonic stem cells, and the use of spermatozoa as vectors. Viral vectors are highly efficient for introducing foreign DNA into cells, as would be expected since their job is to spread infection. While lytic viruses kill their host cell in the process of replication, retroviruses are RNA viruses that usually have little effect on the viability of their host cell (Watson et al., 1992). The viral enzyme *reverse transcriptase* is the reason for this different action within the cell. When retroviruses infect the host cell their RNA sequence is converted to DNA form, due to the action of *reverse transcriptase.* This new viral DNA is then efficiently integrated into the host genome, where with each cell division it will be replicated along with the original DNA of the host cell.

The retrovirus is now considered a provirus as it has been permanently integrated into the host cell, where it will continuously produce viral RNA. All of this newly produced RNA acts as genomic RNA for the production of new viruses, and as mRNA for the production of viral proteins. These new viruses are made within the cytoplasm of the host cell, and will ultimately bud off of the cytoplasm of the host cell to infect other

cells. These steps all occur within the host cell and have little or no effect on the cell's stability. When these viruses are used as vectors, the foreign DNA becomes a permanent part of the host cell, along with the virus itself. Retroviral infection is limited in that the animals that are produced are mosaic. Another limitation with the use of retroviral infection is the size of the transgene that can be integrated. In retroviral infection of blastomeres the transgene can only be about 7 kb in length, limiting it to packaging only small foreign DNA constructs (Rulicke, 1996).

Another method of transgenic animal production involves the use of embryonic stem (ES) cells, however this is a very time consuming process. Murine ES cells were first isolated about ten years ago. Embryonic stem cells are cultured in the presence of leukemia-inhibiting factor (LIF) to maintain the cells in an undifferentiated state. The ES cell genome can be disrupted, new genes inserted, or existing genes modified. Once the cells are modified, they are introduced to developing embryos to produce a chimera. If the ES cells integrate into the germ line of these chimeras they are outcrossed. This outcrossing produces offspring heterozygous for the particular genomic modification. These animals are then mated to produce progeny that are homozygous for the modification of interest (Wigley et al., 1994). Even with the short generation interval of the mouse, it can take 6 to 12 mo before germline transmission is evident (Nottle et al., 1997). The resulting transgenic animals also have a high level of mosaicism (Anderson et al., 1992).

Transgenesis has also been achieved through the use of live spermatozoa as vectors for the introduction of recombinant DNA into the oocyte in vitro. This technique has been used in the mouse (Lavitrano et al., 1989), cattle (Schellander et al., 1995), and

in pigs (Lavitrano et al., 1997). The sperm is incubated with foreign DNA prior to in vitro fertilization or artificial insemination. However, the repeatability of the technique is in question (Perry et al., 1999). Perry et al. (1999) examined the use of intracytoplasmic sperm injection (ICSI) to inject membrane-disrupted sperm heads and exogenous DNA encoding green fluorescent protein (GFP) or B-galactosidase reporters into mouse oocytes. This technique resulted in 64 to 94% transgene expressing embryos. However, only about 20 % of the offspring produced from embryos transferred with the GFP series expressed the integrated transgene.

DNA Condensation

Deoxyribonucleic acid is made up of small basic units called nucleotides, or mononucleotides. Each mononucleotide is made up of a nitrogenous base, a pentose sugar, and a phosphate group all covalently bound together. Two types of nitrogenous bases exist: purines and pyrimidines. The two purine bases are adenine (A) and guanine (G). The three types of pyrimidine bases are cytosine (C), thymine (T), and uracil (U). While both DNA and RNA consist of bases A, C, and G, only DNA contains T (Klug and Cummings, 1991).

A link formed between two of these mononucleotides consists of a phosphate group linked to two sugars. When mononucleotides are linked together in this way, they form a long chain called a polynucleotide. These long single strands of DNA bind to corresponding nitrogenous bases through hydrogen bonds; these bonds will only form between bases A and T, and G and C. This specific binding forms double strands of

DNA (Klug and Cummings, 1991). The ability of a base to form hydrogen bonds governs the specificity of this binding. Bases G and C can form three hydrogen bond pairs, and A and T can form only two hydrogen bond pairs (Watson and Crick, 1953). The complementarity of their binding capabilities creates a natural binding affinity. These two helical chains are coiled around a central axis, with each complete turn of the helix containing 10.4 base pairs. The three-dimensional double helical structure of DNA is due to the negatively charged phosphodiester backbone. This phosphodiester bond results from the ester linkage of phosphoric acid to the two hydroxyl groups on the two sugars (Klug and Cummings, 1991).

The DNA molecule is highly stable. While one hydrogen bond alone provides little stability, many hydrogen bonds together in a long chain provide a great degree of structural stability. The specific arrangement of sugars and bases along the central axis of the DNA structure adds to the existing hydrogen bond stability. This specific arrangement allows for water interactions, which provide chemical stabilization to the helix. The hydrophobic nitrogenous bases are sheltered from the water on the interior of the central axis, and the hydrophilic sugar-phosphate backbone is on the exterior of the axis (Klug and Cummings, 1991).

The DNA of nearly all organisms is supercoiled, creating a complex tertiary structure. This folding and coiling is theorized to be required so that the DNA and the associated proteins can fit into the cell nucleus (Klug and Cummings, 1991). This supercoiling results from the interaction of the negatively charged phosphodiester backbone and the positively charged proteins and histones present in chromatin

molecules. Chromatin molecules are tightly condensed complexes of DNA, RNA, histones, and non-histone proteins, which make up chromosomes.

Histones play a crucial role in the condensation process because they are rich in lysine and arginine (Tobias and Olson, 1993). These basic amino acids bond electrostatically to the negatively charged phosphate group of nucleotides, neutralizing the phosphodiester backbone of the DNA at the polar lysine and arginine regions (Lente et al., 1975). When examined through X-ray diffraction intact chromatin structures produced regularly spaced diffraction rings, however, removal of histones from the chromatin structure alters the diffraction pattern. This is clearly a case of structure explaining function. Ada and Olins (1974) reported that regularly occurring linear arrays of spherical particles, like beads on a string, occur along the axis of a chromatin strand. These particles are now referred to as nucleosomes or V-bodies. A nucleosome is a complex where each of 4 histone molecules is present in duplicate, with two turns of a DNA molecule wrapping around this complex. These histone groupings are essential for condensation of chromatin molecules (Klug and Cummings, 1991). Salt concentration and DNA osmotic pressure also affect DNA condensation in vivo (Lente et al., 1975; Vologodskii and Cozzarelli, 1995).

In vitro DNA condensation occurs in low salt solutions when cations containing +3 valency or more cause compaction of condensed linear or covalently closed-circular DNA (Trubetskoy et al., 1999). Many different substances are used to induce in vitro DNA condensation in aqueous solutions including: multivalent cations, cationic polypeptides, divalent metal cations, and ethanol.

Cobalt (III) hexammine and spermidine^{3+} are examples of multivalent cations, also called cationic ligands, that cause DNA condensation. These ligands bind to the extended DNA double helices causing highly organized condensation and compaction. The cationic ligand binds to negatively charged DNA, DNA condensation occurs after a critical ligand concentration is reached (Matulis et al., 2000). The ligand concentration needed to cause DNA condensation appears to vary depending on the multivalent cation used. With Cobalt(III) hexammine, condensation occurred when about 67% of the DNA phosphate backbone was neutralized, however, spermidine had to neutralize 87% before condensation would occur.

Monovalent cations do not make good condensing agents because they require a very high salt concentration for neutralization of the phosphodiester backbone, thus their neutralizing capabilities are not as strong as 2+, 3+, and 4+ cations (Bloomfield, 1991). High salt concentrations interrupt DNA base pairing causing a destabilization of DNA secondary structure.

$MnCl_2$ and $MgCl_2$ are divalent cations that can induce DNA condensation in water at elevated temperatures (Bloomfield,1991). When alcohol is added to the water these divalent cations interact more readily with the DNA, lowering the dielectric constant. While $MnCl_2$ can condense both linear and supercoiled DNA, greater condensation occurs in supercoiled DNA (Ma and Bloomfield, 1994).

Polylysine, a cationic polypeptide, has also been used as a successful condensing agent. A DNA construct containing a complementary DNA (cDNA) encoding for human protein C was successfully condensed with polylysine and cytoplasmically injected into

pronuclear stage murine embryos, resulting in a 12.8% integration efficiency (Page et al., 1995a).

Once the DNA is condensed through the use of one of the above condensing agents, the DNA becomes either rod-shaped or toroidal in morphology, and these individually condensed particles can aggregate and assemble together in toroidal or rod-shaped aggregates. Rod-shaped DNA contains occasional sharp kinks or bends, while toroidal DNA is continuously wrapped. More energy is required to produce the sharp kinks of the rod-shaped DNA particles, and therefore they are less common than the toroidal-shaped particles (Bloomfield, 1991).

Toroidal condensates have inner radii of about 150 Å and outer radii of about 500 Å, consistent for DNA from 400 to 40,000 base pairs in length, and all condensates have a similar packing density. DNA molecules that are shorter in length are unable to condense to a distinct rod or toroidal morphology in low salt solutions, possibly due to a lack of free energy availability (Bloomfield, 1991).

DNA condensation is an important phenomenon, offering insight into DNA packaging in viruses and in cell nuclei, acting as a model for chromosome folding, and presenting an opportunity for the construction of artificial gene delivery vectors (Trubetskoy et al., 1999; Matulis et al., 2000).

Electroporation and DNA Transfer

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 (Zimmerman and Vienken, 1982). 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 though the cell and DNA suspension (Catalado et al., 1998).

In order for electroporation to be efficient, several critical parameters must be identified and met. Critical physical parameters include field strength and pulse duration (Anderson and Evanson, 1988). 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 (uF). 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 (Neumann et al., 1982). 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, 1988). Therefore, cell diameter plays an important role in selecting voltage and capacitance parameters. A larger cell requires a lower voltage to maintain subsequent viability.

Cytoplasmic Injection

Cytoplasmic injection involves inserting a glass injection pipet through the zona pellucida and into the embryonic cytosol where injection of the gene or construct of interest occurs. During cytoplasmic injection care must be taken to avoid injection of the pronuclei. When compared to pronuclear injection, cytoplasmic injection has

significantly lower efficiency. Brinster and associates (1985) conducted a comparison study between pronuclear and cytoplasmic injection. Pronuclear injection of linear DNA molecules resulted in an integration efficiency of 25 to 30%. Cytoplasmic injection of either linear or supercoiled DNA resulted in only 2 fetuses testing positive for the microinjected DNA out of 224 fetuses examined (0.89%).

The previously mentioned study by Page and associates (1995a) had 12.4% successful integration of the cytoplasmic injected polylysine condensed DNA (1:1 L:P ratio, 50 ug ml^{-1}). This success rate was still 41% less efficient, when looking at the number of animals testing positive for the DNA, than pronuclear injection of the same DNA. Pronuclear injection of linear DNA (1.5 ug ml^{-1}) resulted in 21.7% transgene efficiency, while cytoplasmic injection of linear DNA was completely ineffective. Pronuclear injection is the most commonly used and most efficient technique for the production of transgenic animals, however several advantages exist for the pursuit of more efficient cytoplasmic injection techniques.

Cytoplasmic injection can occur before the pronuclei are visible and may result in greater transgene integration efficiency, since injection could be performed during the zygote phase (Page et al., 1995a). Cytoplasmic injection would also make transgenic animal production easier in species such as pigs (Wall et al., 1985), cows (Biery et al., 1988), and sheep (Hammer et al., 1985), where the embryos must be centrifuged to visualize the pronuclei due to the cytoplasmic lipid layer. The use of cytoplasmic injection would decrease the amount of embryo manipulation required in these species, possibly resulting in higher embryo survivability.

Green Fluorescent 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* (Gereder, 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 1992 cloning of the complimentary DNA (cDNA) for GFP (Cubit et al., 1995; Geredes and Kaether, 1996). 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 emittence 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 (Chafie 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, 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 Trunson (1980) reported the embryos failed to develop further, and were no longer viable. Eibs and Spielmann (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 effect embryo viability. These data suggest that if UV light is used appropriately in activation, the embryos will remain viable.

CHAPTER 3

THE DEVELOPMENTAL EFFECTS OF LINEAR AND GROUP ORIENTATION WITHIN THE ELECTROPORATION CHAMBER ON PRONUCLEAR STAGE MURINE EMBRYOS

ABSTRACT

Reversible electroporation allows the resumption of normal electrical function of cell membranes following disarrangement by electroporation. The purpose of this study was to evaluate the effects of embryo orientation (group or linear) within an electroporation chamber using zygotes which received one of two voltages (250 V or 400 V) at a pulse duration of 10 ?sec. Zygotes that received 250 V had development scores 1.35 greater than those that received 400 V ($P < 0.01$). No significant difference was found between the control (4.28 ? 0.12) and the mannitol (4.36 ? 0.18) media treated groups. These data validate the use of mannitol as the electroporation medium, and shows that electrical stimulation of zygotes can reduce early embryo development, but low amounts of stimulation may allow for potential gene transfer in transgenic experimentation.

INTRODUCTION

Electroporation, also termed electropermeabilization, is the application of an external electrical field, which can induce structural modifications in the cell membrane to which this field is applied. These modifications make the membrane transiently permeable allowing for materials to be introduced or released from the electromanipulated cell. Lipid peroxidation plays an important role in electroporation induced cell permeability. The enhancement of hydroperoxide content in the lipid bilayer following electroporation is associated with an increase in membrane fluidity, which may be the chemical process leading to the opening of pores within the membrane (Maccarrone et al., 1995). The cellular response to the electric shock may also be mediated by oxidoreductases, enzymes that lead to the formation of hydroperoxide from polyunsaturated fatty acids of the cell membrane (Radi et al., 1993). Exposure to this electric field also creates a charge separation across the membrane, resulting in a transmembrane potential difference (Somiari et al., 2000). The opposite electrical charges placed on the membrane attract each other, which exert pressure on the membrane, causing the membrane to become thin. Once a critical point is reached, this thinning results in the creation of small pores throughout the membrane (Nakamura and Funahashi, 2001). This state occurs when the energy applied exceeds the energy holding the lipid bilayer intact, resulting in membrane permeability (Hui, 1995).

Reversible electroporation occurs when the altered state of the membrane is transient; however, excessive electrical stimulation can cause cell death due to an irreversible change in the membrane resulting in permanent pore formation (Weaver,

1995). Therefore, the appropriate parameters for reversible electroporation for the specific cell type in use must be determined in order to ensure cell survivability and a transient state of permeation.

Electroporation has a broad range of applications in science, medicine, and biotechnology. Electroporation has been used successfully in facilitating gene transfer in a variety of cell types including plant, animal, bacterial, and yeast cells. The successful transfer of proteins, viruses (Novoa, 1997), bacterial cells, DNA (Catalado et al., 1998), and enzymes (Rols et al., 1995) into plant and animals has also been accomplished through the use of this technique (Somiari, 2000).

More recently, the application of electroporation to avian embryos has been found to aid in the transfer and establishment of regulated gene expression (Nakamura and Funahashi, 2001). Living animal tissues, including skeletal muscle, have been shown to harbor and transcript foreign DNA due to the application of electroporation (Mathiesen, 1999; Mir et. al., 1998; Rizzuto et al., 1999). Targeted transient gene expression has been successfully achieved in rat embryos through the use of electroporation, DNA injection into neural tube, and the whole embryo culture system (Osumi and Inoue, 2001). This finding makes the facilitation of gene transfer by electroporation a valuable tool for the study of the embryo in developmental biology.

Despite the wide range of applications involving electroporation, the molecular events occurring during the application of the electrical field and during the recovery period have not yet been elucidated and require extensive investigation for in depth understanding. Several difficulties have arisen in the study of this technique including the extremely rapid process of permeabilization, the instability of this permeated state,

and the variability in the parameters required for the electroporation of different cell types (Hibino et al., 1993). Biological and physical parameters must be determined for each individual cell type. As a general rule, 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). However, due to the unique, sensitive nature of the embryo as a cell type, traditional protocols used successfully for other cell types become impractical.

During nuclear transfer, blastomere-oocyte units must be manually aligned parallel to the two electrodes of the electroporation chamber for proper fusion to occur (Krisher et al., 1995). Therefore, the effect of embryo orientation within the electroporation chamber may effect the overall efficiency of electroporation. In addition, embryo crowding could provide protection from voltage application, resulting in inadequate electroporation. There is a paucity of data regarding the application of electroporation in embryos.

Nemec (1986) researched the effects of electroporation on the survivability of murine embryos, evaluating three levels of voltage (200, 400, 600) and two pulse durations (10 µsec and 20 µsec) at two different temperatures (5ºC and 25ºC). She reported that embryos receiving 200 V for 10 usec at 25ºC resulted in the best pregnancy rates following embryo transfer. However, differences in transfer numbers, significant experimental error, and lack of significant differences among treatments made these data difficult to interpret and apply. Therefore, previous data collected in our laboratory was used to select the pulse duration and voltages applied during this experiment (Dunlap-Brown, personal communication).

The objective of this experiment was to assess some of these parameters by evaluating media effects and the effect of embryo orientation within the electroporation chamber using a 10 ?sec pulse duration at three separate voltages (0 V, 250 V, and 400 V) on embryo development.

MATERIALS AND METHODS

Superovulation Procedure

Intraperotineal (i.p) injections of 10 IU of equine chorionic gonadotropin (eCG, Diosynth, Chicago, IL, USA) were administered to groups of 10 to 15 weaned female mice (strain CD-1) weighing 19.5 to 22.5 g and between 25 to 30 d of age. These females were grouped in cages of 3 or 4 each and received an i.p. injection of 5 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, Mo; Catalog #C5297) 46 to 48 h later. The females were then individually placed in cages with a single male. Female mice were be examined 21 to 24 h later for vaginal plugs. Those females with plugs were sacrificed by cervical dislocation, and their ovaries and oviducts removed for embryo harvesting (Hogan et al., 1986).

Ovary Collection and Embryo Harvesting

Mice were dissected, and the ovaries and oviducts collected and placed in 37.5?C M2 medium (Sigma, Catalog #M-5910) at pH 7.4. Zygotes were collected at the

cumulus stage, and removed from the cumulus cells in M2 medium containing 1 mg hyaluronidase (320 IU/mg; Sigma, Catalog #H-3506). Embryos were washed and manipulated in M2 medium until electroporation and long term culture (Hogan et al., 1986).

Electroporation

Development of electroporated embryos was examined after a single electrical pulse at 0, 250, or 400 V for a duration of 10 usec (BTX Inc., ECM®200; San Diego, CA). Embryos were placed in either a linear or group formation within the electroporation chamber. Embryos were electroporated in 0.22 M d-mannitol (Sigma, Catalog #M-4125) containing 0.01% polyvinyl alcohol (PVA) (Sigma, Catalog #H-6185) in Hepes (35 mM; Sigma, Catalog #H-6147) at a physiological pH of 7.2. The electroporation microslide chamber was part #453 (BTX Inc.), and was sterilized with 70% ethanol before each use. The two electrodes on the microslide form a 3.2 mm gap, which allowed for 0.7 ml medium volume. Approximately 20 to 40 embryos were electroporated at a time. This number was based on the total number of embryos collected containing the male pronucleus, and their subsequent division into experimental groups. No more than 40 embryos were electroporated at a time, due to the 40 embryo maximum per 10 ul long-term culture CZB drop (Chatot et al., 1989). Electroporated embryos were then washed in CZB long-term culture medium, and placed into 10 ul CZB drops for long-term culture.

In Vitro Evaluation

The development of electroporated mouse embryos was evaluated at d 2 and d 4 post manipulation.

Statistical Analysis

The embryo data were analyzed using the general linear model analysis of variance in the SAS system (SAS Institute, Inc., 1985). Developmental data were classified using a numerical scoring system. Embryos were assigned a 1 = degenerate or lysed, 2 = 2-cell, 3 = 4-cell, 4= 8-cell, 5 = 16-cell, 6 = morula, and 7 = embryos that developed to the blastocyst stage. Embryos were evaluated on d 2 and d 4. Evaluation of development on d 2 was performed to record embryos that had degenerated, or arrested at the one-cell stage of development, as these embryos can be mistaken on d 4 for later stages of development. Each embryo was assigned one numerical score on d 4 based on its final stage of development.

Six treatment combinations were tested: control, mannitol media control, 250 V-group orientation, 250 V-linear orientation, 400 V-group orientation, and 400 V-linear orientation. Eleven experimental repetitions were performed. The embryo data were analyzed using the general linear model analysis of variance in the SAS system (SAS Institute, Inc., 1985). The model included repetition and treatment. Linear contrasts were used to test control verses mannitol media control, group orientation verses linear orientation, 250 V verses 400 V, as well as, the interaction of voltage and orientation.

RESULTS

This experiment included 1,528 embryos. Electroporation medium control embryos (4.36 ? 0.18; mean ± SE) and unexposed control embryos (4.28 ? 0.12) had the highest 4 d development scores (Table 1). The electroporation medium control had 17.3% develop to the morula stage and 21.1% develop to the blastocyst stage (Table 2). The unexposed control had 17.4% develop to the morula stage and 22.6% develop to the blastocyst stage. Embryos in the 400 V group orientation treatment (2.06 ? 0.12) and 400 V linear orientation treatment group (1.97 ? 0.13) had the lowest overall development. The 400 V group orientation treatment had only 4.8% develop to the morula stage and 5.1% develop to the blastocyst stage. The 400 V linear orientation had only 2.7% develop to the morula stage and 3.8% develop to the blastocyst stage.

Embryos exposed to the 250 V group orientation treatment (3.42 ? 0.12) and 250 V linear orientation treatment (3.32 ? 0.12) had the highest development ($P < 0.01$) of the voltage treated embryos. Embryos in the 250 V group orientation had 12.3% of the embryos develop to the morula stage and 10.4% develop to the blastocyst stage. The 250 V linear orientated group had 15.4% develop to the morula stage and 8.1% develop to the blastocyst stage. No significant difference was found between group and linear orientation at either 250 V or 400 V (Table 1).

A significant voltage effect on embryo development ($P < 0.01$) was exhibited, however no significant difference between embryos assigned to the group or linear formation within the microslide chamber occurred at either the 250 or 400 voltage. Exposure to the Mannitol electroporation medium had no significant affect on embryo

development ($P > 0.05$), and was not significantly different from unexposed control embryos (Table 1).

Table 1. Least squares mean ± SE for d 4 development score, effect of voltage and orientation on development

Treatment		Score[1]	
Voltage	Orientation	LSMean	SE
None	Unexposed Control	4.28[a]	0.12
None	Mannitol Medium Control	4.36[a]	0.18
250 V	Group	3.42[b]	0.12
250V	Linear	3.32[b]	0.12
400 V	Group	2.06[c]	0.12
400 V	Linear	1.97[c]	0.13

Score[1]

1 = degenerate and lysed embryos
2 = 2 cell embryos
3 = 4 cell embryos
4 = 8 cell embryos
5 = 16 cell embryos
6 = morula stage embryos
7 = blastocyst to hatched blastocyst stage embryos

[abc]Means with different superscript differ significantly ($P<.0001$).

Table 2. Percentage of cultured embryos at each stage of development on d 4

Treatment									
Voltage	Orientation	N	Degenerate	2 Cell	4 Cell	8 Cell	16 Cell	Morula	Blastocyst
None	Control	270	18.9	13.0	5.6	7.8	14.8	17.4	22.6
None	Mannitol Control	185	22.2	6.5	6.0	11.9	15.1	17.3	21.1
250 V	Group	268	30.2	14.2	8.2	10.8	13.8	12.3	10.4
250 V	Linear	272	28.3	19.5	10.3	7.7	10.7	15.4	8.1
400 V	Group	272	61.8	17.6	4.0	1.0	5.9	4.8	5.1
400 V	Linear	261	59.4	22.2	3.1	2.3	6.5	2.7	3.8

DISCUSSION

This study evaluated the effects of embryo orientation within the electroporation chamber using a 10 ?sec pulse duration at two separate voltages (250 and 400 V). The pulse duration and voltage choices were selected from previous experiments performed in our laboratory (Dunlap-Brown, personal communication). The 250 V did not result in a significant decrease in embryo development, while the 400 V treatment caused the greatest decrease in embryo development. The 10 ?sec electroporation pulse length was used because excessive instability was shown in embryo development when electroporation duration lasted for 20 ?sec pulse length. Nemec (1986) also reported that the use of 20 ?sec was excessive and resulted in embryo lysing, irrespective of voltage applied (200 V, 400 V, or 600 V). She found that the length of the pulse duration appeared to be more detrimental to embryo viability than the amount of voltage applied.

The application of the 400 V treatment had a significant detrimental effect on embryo viability, which was most likely due to irreversible membrane damage resulting in the loss of membrane integrity. The negative effect of the 400 V exposure may also be due to the large cell size of the embryo. Cell diameter contributes to the efficiency of electroporation. Cells with a smaller diameter, as compared with large diameter cells, can survive electroporation with higher voltage and capacitance (Chu et al., 1987; Potter, 1988). Since a large cell can withstand a low voltage more easily than a high voltage, the 400 V treatment may have been extreme considering the large size of an embryo as compared to other cell types. The lowest embryo development ($P < 0.01$) occurred at the 400 V treatment regardless of orientation within the electroporation chamber. Therefore,

embryo crowding appears to have no protective benefit to increase embryo viability, as no significant difference was seen between group and linear orientation (Table 1).

Considerable testing was conducted when formulating the embryo activation medium used during the electroporation process (Data not shown). This medium needed to be validated to determine if it was an appropriate choice for future experimentation. No significant difference in development was seen between untreated control embryos and the Mannitol treated embryos. These data suggest that exposure to the Mannitol medium is not detrimental to embryo viability and the formulated medium is appropriate for use in embryo electroporation.

Because electroporation has been validated as an efficient method for facilitating the movement of various molecules from the external cellular environment into the cellular matrix (Nakamura and Funahashi, 2001; Mathiesen, 1999; Mir et al., 1999; Rizzuto et al., 1999), the evaluation of electroporation as a tool to help facilitate transgene integration warrants further investigation. While the use of cytoplasmic injection alone is an inefficient method of transgenic animal production (Page et al., 1995a), coupling cytoplasmic injection with electroporation may result in increased integration efficiency. Since molecules are known to move into the cell from the external environment upon electroporation, this electrical stimulation may also result in the movement of molecules from the embryonic cytosol into the nuclei of the embryo.

The development of the control embryos was used as the standard to assess the treatment effects of voltage and orientation. However, this criterion did not serve as a definitive test to assess membrane permeability and may not reflect the actual movement of molecules across the membrane. In future studies the use of a marker or dye may be a

valuable tool to evaluate the amount of membrane permeation and molecular movement that occurs at each voltage application.

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CHAPTER 4

PRONUCLEAR MICROINJECTION OF GFP INTO MURINE EMBRYOS

ABSTRACT

Pronuclear injection is the most efficient method of transgenic animal production to date, making it an effective tool for the validation of prepared DNA constructs to be used for evaluation of new gene transfer methods. The objective of this experiment was to determine whether the enhanced green fluorescent protein (EGFP) construct could be used as a visual marker for protein expression following pronuclear injection. A total of 163 control embryos were cultured in this study, with 74.5% developing to the morula or blastocyst stage. The control embryos had a higher mean development score (5.72 ? 0.2) than the pronuclear injected group (3.85 ? 0.15). There was a significant treatment effect ($P < 0.01$) on development, with 47.2% of the injected embryos developing to the morula or blastocyst. Of the 296 pronuclear injected embryos, 114 expressed the EGFP (38.5%). Moreover, 66% of the morula and blastocyst stage fluorescing embryos were mosaic. These data confirm previous work that shows that the EGFP DNA construct can be used as a visual marker for protein expression for future experimentation to assess alternative gene transfer methods.

INTRODUCTION

Pronuclear injection continues to be the most commonly used and most efficient method of transgenic animal production (Krisher et al., 1994). The foreign DNA is injected into one of the pronuclei of a fertilized embryo using a finely pulled glass capillary, that is less than one micron in diameter and filled with injection buffer containing the foreign DNA solution. About 2 to 10 pl of the DNA solution containing tens to hundreds of copies of the gene are injected into the pronucleus; with proper injection a slow swelling of the pronuclear membrane occurs (Brinster et al., 1985; Simons and Land, 1987).

While pronuclear injection is the most efficient gene transfer method, the results are still unsatisfactory. The highest integration efficiency is achieved when injection occurs during the progression of the embryo from the G1/S boundary to the early S phase of the cell cycle (Chida et al., 1998). The injection process itself is very detrimental to the embryos; in the mouse only about 50 to 85% of the embryos survive the injection. This embryo loss is most likely due to the physical damage inflicted on the embryo during the injection process, improper embryo handling, or lethal genetic damage resulting from the insertion of the pipette during the injection procedure (Simons and Land, 1987). Of the embryos that survive the injection, 10 to 30% develop to term and only 10 to 30% of the live births carry the gene of interest in their genome (Wilmut et al, 1991).

While the search for new gene transfer methods continues, pronuclear injection remains the most successful, efficient method and an excellent method for testing newly prepared DNA for quality and effective integration. In order for a prepared DNA

construct to be used for further experimentation in the investigation of a new gene transfer method, it must first be validated using a known method of transgenic animal production.

The DNA construct used in this experiment codes for green fluorescent protein (GFP) and is considered an excellent marker to determine gene expression, as it has been used extensively for both transient and stable transfection analysis (Liu et al., 1999). The protein, when expressed, absorbs blue light and emits green light, requiring only brief UV light exposure for protein visualization (Chalfie et al., 1994). The specific GFP construct used in this experiment is an enhanced GFP (EGFP), which codes for the GFPmut1 variant, containing the amino acid substitutions Phe-64 to Leu and Ser-65 to Thr. This particular red-shifted variant of the wild type GFP has been optimized for higher expression and brighter fluorescence (Clonetech Vector Information, Palo Alto, CA; Catalog #6064-1). This mutation allows for increased protein folding and increased efficiency of chromophore formation.

The objective of this experiment was to validate the prepared EGFP construct as a visual marker for protein expression using pronuclear injection.

MATERIALS AND METHODS

Superovulation Procedure

Intraperotineal (i.p) injections of 10 IU of equine chorionic gonadotropin (eCG, Diosynth, Chicago, IL, USA) were administered to groups of 10 to 15 weaned female mice (strain CD-1) weighing 19.5 to 22.5 g and between 25 to 30 d of age. These females were grouped in cages of 3 or 4 each and received an i.p. injection of 5 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, Mo; Catalog #C5297) 46 to 48 h later. The females were then individually placed in cages with a single male. Female mice were examined 21 to 24 h later for vaginal plugs. Those females with plugs were sacrificed by cervical dislocation, and their ovaries and oviducts removed for embryo harvesting (Hogan et al., 1986).

Ovary Collection and Embryo Harvesting

Mice were dissected, and the ovaries and oviducts collected and placed in 37.5?C M2 medium (Specialty Media, Phillipsburg, NJ; Catalog #MR-015-D) at pH of 7.4. Embryos were collected at the cumulus stage, and removed from the cumulus cells in M2 medium containing 1 mg hyaluronidase (320 IU/mg; Sigma, Catalog #H-3506). Zygotes were washed and manipulated in M2 medium. Following pronuclear microinjection embryos were washed and stored in KSOM (Specialty Media; Catalog #MR-020-D) long term culture media (Erbach et al., 1994).

DNA Preparation

The construct cytomegalovirus-enhanced green fluorescent protein (CMV-GFP) was produced by removing the inter-ribosome entry site of enhanced green fluorescent protein (pIRES-EGFP) (Clonetech Laboratories, Palo Alto, CA; Catalog #6064-1) by *Bam*HI endonuclease digestion. The restriction products were separated, and the vector-containing the fragment was subjected to ligation. Ligation products were used to transform E. coli XL-Blu (Stratagene, La Jolla, CA) with selection on ampicillin plates (Chahaun et al., 1999). The plasmid was isolated and purified by column absorption (Mega prep; Qiagen Inc, Valencia, CA). The linear DNA construct was digested overnight with Xho1 (Promega Catalog Madison, WI; Catalog #R616A) at 37.5? C in a 10X Universal Buffer (Stratagene Catalog #50000005) and was isolated using a standard phenol/chloroform (50%/50%; v/v) extraction followed by ethanol precipitation (Davis et al., 1994). The final construct size was 4.8 kbp and had one sticky overhanging end and one blunt end left by the XhoI enzyme cut. The purified construct was reconstituted in 1 to 1.5 ml of buffer containing 10mM Tris HCl and 0.25 mM EDTA. The DNA concentration was determined by photospectroscopy at 260 nm, and diluted for pronuclear injection to 3 ug/ml and stored at –80ºC until use.

In Vitro Evaluation

The development of injected and control embryos was evaluated at d 2 and 4 post manipulation. Those embryos injected with EGFP were evaluated only on d 2 and 4 post

injection to minimize the exposure of the embryos to UV light and environmental stressors. Injected embryos were illuminated with blue light for several seconds at 488 nm, the excitation maximum for EGFP. Evaluation at these two times allowed for determination of those embryos that fluoresce early and then degenerate, or fluoresce early but did not express the transgene in later development. Emission of green fluorescence was examined in darkness at a wavelength of 509 nm.

Statistical Analysis

The embryo data were analyzed using the general linear model analysis of variance in the SAS system (SAS Institute, Inc., 1985). Developmental data were classified using a numerical scoring system. Embryos were assigned a 0 = degenerate or lysed, 1 = 1-cell, 2 = 2-cell, 3 = 4-cell, 4= 8-cell, 5 = 16-cell, 6 = morula, and 7 = embryos that developed to the blastocyst stage. Embryos were evaluated on d 2 and d 4. Evaluation of development on d 2 was performed to record embryos that had degenerated or arrested at the one-cell stage of development, as these embryos can be mistaken on d 4 for later stages of development. Each embryo was assigned one numerical score on d 4 based on its final stage of development.

Two treatments were tested: control and pronuclear microinjection of the EGFP construct. Four experimental repetitions were performed. The embryo data were analyzed using the general linear model analysis of variance in the SAS system (SAS Institute, Inc., 1985). The model included repetition and treatment.

RESULTS

This experiment included 459 embryos. Control embryos (n = 163) had a significantly higher ($P < 0.01$) 4 d development (5.72 ? 0.2; mean ± SE) than the pronuclear injected embryos (n = 296; 3.85 ? 0.15); (Table 3).

Table 3. Least squares mean ± SE for d 4 development of zygotes injected with EGFP DNA construct

Treatment	Score[1] Mean	SE
Control	5.72[a]	.20
Pronuclear Injection	3.85[b]	.15

[1]Score

0 = degenerate and lysed embryos
1 = 1 cell embryos
2 = 2 cell embryos
3 = 4 cell embryos
4 = 8 cell embryos
5 = 16 cell embryos
6 = morula stage embryos
7 = blastocyst to hatched blastocyst stage embryos

[ab]Means with different superscript differ significantly ($P < 0.01$).

A total of 123 control embryos (75.5%) and 140 of the pronuclear injected embryos (47.3%) developed to the morula or blastocyst stage of development. A significant treatment effect ($P < 0.01$) was observed for overall development. A total of 96 embryos (32.4%) of the 296 injected were fluorescent. Of the injected embryos, 61.1% at the 1-cell stage, 36.8% at the 2-cell, 38.5% at 8-cell, 16.7% at the 16-cell, and 22.9% at the morula and blastocyst stage were fluorescent (Table 4).

Table 4. Number and percentage of microinjected embryos that exhibited fluorescence and were mosaic at d 4 of in vitro development by stage of development

Stage of Development	N	Number Fluorescing	% Fluorescing	Number Mosaic	% Mosaic	Score[1]
1 cell	72	44	61	0	---	---
2 cell	38	14	37	9	64	1.79
8 cell	13	5	39	4	80	1.86
16 cell	6	1	17	1	100	1.33
Morula/Blastocyst	140	32	23	23	72	1.59

[1]Score

1 = 0% of cells fluorescing
2 = 25% of cells within embryo fluorescing
3 = 50% of cells within embryo fluorescing
4 = 75% of cells within embryo fluorescing
5 = 100% of cells within embryo fluorescing

Many of these fluorescing embryos were mosaic. A total of 37 of the fluorescing embryos (71%) exhibited mosaicism. The mean percentage of cells fluorescing within each embryo at each specified developmental stage is shown in Table 4. These figures are expressed as a percentage of total fluorescing cells within each treatment group, with a higher number meaning more cells fluoresced within that developmental segment. Embryos developing to the 16 cell stage exhibited the lowest score for total cells fluorescing, with a score of 1.33, while embryos at the morula and blastocyst stage exhibited the second lowest number of total cells fluorescing with a score of 1.59. However, 1-cell, 2-cell, and 8-cell embryos received scores of 2.94, 1.79, and 1.86, respectively (Table 4).

DISCUSSION

This study evaluated the prepared EGFP DNA construct as a visual marker for protein expression through the use of pronuclear microinjection and the subsequent effects of this manipulation on embryo development. Results from this experiment indicate that the prepared DNA construct is an effective visual marker for protein expression; however, the pronuclear microinjection event resulted in a significant decrease in embryo viability ($P < 0.01$).

The decreased embryo viability and cleavage rate observed in this study has been reported previously in mice (Gordon et al., 1980), pigs (Williams et al., 1992), and cattle (Krimpenfort et al., 1991; Krisher et al., 1995; Chauhan et al., 1999). The damage caused to the pronucleus of the zygote during the injection procedure may result in a reduction in cleavage rate. Superior initial embryo quality may allow some embryos to withstand the

manipulation, while embryos with inferior pronuclear formation, cytoplasmic integrity, and developmental competence cannot survive the manipulation and repair the resultant damage (Chauhan et al., 1999). The mechanical damage, however, is not the only cause of decreased embryo viability; DNA itself has been found detrimental to embryo development as compared to buffer-injected controls (Williams et al., 1992).

Most of the pronuclear injected embryos (71%) that fluoresced were mosaic. This percentage is consistent with previous data, reporting transgene mosaicism at 76%, and is a result of chromosomal integration occurring after the first round of DNA replication (Whitelaw et al., 1993). While the mechanisms resulting in DNA integration into mouse chromosomes following pronuclear microinjection are unknown, one possibility is that during the microinjection event chromosome breaks occur. These breaks become the integration sites of the foreign DNA construct through ligation reactions (Brinster et al., 1985). The number of chromosome breaks and the consequent integration sites are believed to be a limiting factor in proper integration of the transgene. If the injected DNA persists and is not degraded within the embryo, it remains available for integration at any of the subsequent divisions, resulting in a mosaic animal (Burdon and Wall, 1992). In addition, events occuring during transcription and translation may silence expression in some blastomeres and not in others, also resulting in mosaicism (Chahaun et al., 1999).

Page and associates (1995b) used PCR to detect frequency of the single copy whey acidic protein (WAP) gene injected into murine embryos using pronuclear microinjection. Of embryos developing to the blastocyst stage, 29% tested positive for the WAP gene. The integration frequency was 22% for the same DNA construct. These data indicate that the percentage of blastocysts testing positive for the gene using PCR is

a good indicator of the integration frequency seen following transgenic mouse production.

Studies on the expression of GFP in murine embryos have reported that the incidence of GFP expression relates well to the incidence of GFP transgenics in mice (Ikawa et al., 1995; Takada et al., 1997). Takada and associates (1997) used GFP to select transgenic embryos following microinjection. Of the 55 GFP-positive blastocysts transferred, 12 mice were transgenic. Analysis by PCR indicated that 11 out of the 12 mice were transgenic, while an additional southern blot analysis indicated that 8 of the 12 mice were transgenic. Chiocchetti (1997) and associates compared GFP to the commonly used *lac*Z reporter gene, which allows for the detection of transgene expression through low levels of ß-galactosidase activity, GFP was found to have a higher degree of sensitivity than *lac*Z in six transgenic lines analyzed. These studies suggest that visual selection of GFP positive embryos can be performed confidently in future experimentation.

These data confirm that the EGFP DNA construct can be used as a visual marker for protein expression, and show that the pronuclear injection of DNA has a detrimental effect on embryo development.

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CHAPTER FIVE

ASSESSMENT OF DNA INTEGRATION FOLLOWING CYTOPLASMIC MICROINJECTION OF CONDENSED DNA INTO MURINE EMBRYOS USING ELECTROPORATION

ABSTRACT

Cytoplasmic injection has, to date, been an inefficient method of producing transgenic animals, as DNA injected directly into the cytoplasm has no efficient means of initiating transgenesis. However, the benefits of this method to embryo viability due to decreased manipulation warrants further investigation of this technique. The objective of this experiment was to evaluate previously ascertained parameters for electroporation as a method of facilitating gene transfer into the pronuclei of murine embryos following cytoplasmic injection of linear and condensed DNA constructs and to evaluate the effects of this technique on subsequent embryo development. Non-manipulated control embryos had the highest d 4 development (5.57 ± 0.11), while manipulated control embryos had a significantly ($P < 0.01$) lower score (4.6 ± 0.18). Injecting DNA verses only inserting the injection needle had a significant impact on embryo development (2.42 verses 3.63; $P < 0.01$). A similar impact of 250 V electroporation verses no electroporation was also significant (2.23 verses 3.43, $P < 0.01$), as was the interaction of electroporation and DNA injection. The DNA construct (Linear or Condensed) had no significant impact ($P > 0.05$) on embryo development. Zygotes injected with the condensed DNA construct had

0.38% of the embryos fluorescing, while zygotes injected with linear DNA had 0.38% of the embryos fluorescing at d 4 of in vitro culture. When DNA injection was followed by electroporation (250 V, 10 µsec) 0.36% of zygotes injected with condensed DNA and 3.57% of zygotes injected with linear DNA fluoresced. These data suggest that electroporation of embryos following cytoplasmic injection of linear DNA decreases development score, but improves integration and protein expression following cytoplasmic injection and may improve overall integration efficiency since no mosaic embryos were found in this experiment.

INTRODUCTION

The use of cytoplasmic injection for gene transfer and subsequent transgenic animal production has been extremely inefficient to date, as DNA injected into the cytoplasm provides no efficient mechanism for transgenesis (Page et al., 1995a). Experiments using cytoplasmic injection performed by Brinster and associates (1985) only produced two transgenic mouse fetuses and no transgenic mice. However, if the efficiency of this technique could be improved it would offer several advantages over pronuclear injection.

Pronuclear injection transiently restricts the process of zygote harvesting and the integration event, as replication of the genome can occur before the pronucleus has been injected. Injection at this time results in mosaicism. The use of cytoplasmic injection would allow for injection at the zygote phase, before the pronuclei are visible, increasing the chance that proper integration would occur; therefore, decreasing mosaicism (Page et al., 1995a). Cytoplasmic injection would also be advantageous in pig (Wall et al., 1985) and bovine (Biery et al., 1988) embryos, where the lipid bilayer makes it difficult to visualize the pronuclei without centrifugation.

Since the DNA injected into the cytoplasm provides no efficient method of transgenesis, coupling cytoplasmic injection with electroporation may allow for gene transfer into the pronuclei of the injected embryos. Continual application of an electrical field during and after the poration event may facilitate polynucleotide uptake and migration within the cell acting through electrophoresis, and hence may facilitate transport toward and through nuclear pores (Somiari et al, 2000). In skeletal muscle

tissue (Mir et al., 1998) and cutaneous transfection in murine and porcine models (Somiari et al., 2000) coupling naked DNA injection and electroporation resulted in a ten to one-thousand fold increase in expression of the reporter gene as compared to DNA injection alone. However, modest transgene expression has been reported in a number of tissue and cell types using the same methods, possibly due to the wide variability in efficient electroporation parameters required for gene transfer in each individual tissue and cell type (Somiari et al., 2000).

Electroporation was first investigated in the 1960s, when electrical fields were discovered to induce breakdown of cell membranes (Coster, 1965; Sale and Hamilton, 1967, 1968). According to BTX (San Diego, CA) cells within the electroporation chamber, when surrounded by the cell suspension medium, prior to application of the electrical field can be regarded as structures consisting of non-conducting membranes with aqueous solution on both sides. When voltage is applied it travels through the aqueous solution and leads to a charged separation across the membrane of the cell within the electroporation chamber. This transmembrane potential causes opposite charges on the membrane to attract one another, resulting in a thinning of the membrane and, at a critical point, the formation of small pores within the membrane. This state of permeability may result from the action of lipoxygenasse activity and the formation of hydrogen peroxide induced by the electrical shock, leading to a change in membrane properties (Maccarrone et al., 1995). Increasing the field strength has been reported to increase the area of the membrane made permeable, whereas increasing the pulse duration tends to increase the degree of perturbation making the membrane more permeable to DNA; therefore, facilitating the transport of DNA into the cell (Gabriel and

Teissie, 1997). This permeability and subsequent transport could, theoretically, continue into the pronucleus of a cell. While electroporation has a wide range of applications, more recently it has gained attention as a method of DNA transfer into cells.

The use of electroporation for gene transfer often results in a high cell mortality rate. These deaths result from the high pulse magnitude required for DNA transfer, resulting in death of over half the cells subjected to the voltage. An important factor may be the intra- to extracellular volumes, making cells electroporated in vivo less likely to be damaged than cell electroporated in vitro, where the extracellular volume is much larger. Large and relatively nonspecific molecular transport may cause chemical imbalances within the cell, from which the cell may not be able to recover. However, this is of little concern if the cells that do survive the manipulation are useful and carry the gene of interest. The breakdown of the membrane occurring in cells that are able to repair the damage due to the voltage effect is termed reversible electrical breakdown (Weaver et al., 1995). This repair causes the substance of interest, or gene of interest, to be trapped within the cell as the pores close, these substances may in turn exert a biological effect (Somiari et al., 2000)

Gene transfer aided by electrical pulses as a non-viral delivery method is believed to be a two step process, where permeabilization of the membrane is followed by transport of DNA into the cytosol by electrophretic forces, for this reason long duration pulses are believed more efficient in promoting gene transfer (Sukharev et al., 1992 ; Neumann et al., 1996). However, Gehl and Mir (1999) propose that perturbation of a large area of the membrane is not necessary for gene transfer, and use of a minimal

amount of perturbation is important to cell function and to increases the likelihood of gene expression within the cell.

The DNA construct used also contributes to the efficiency of transgene integration. The DNA of nearly all organisms is supercoiled, creating a complex tertiary structure. This folding and coiling is theorized to be required so that the DNA and the associated proteins can fit into the cell nucleus (Klug and Cummings, 1991). Condensation of the construct to be injected could improve integration of the transgene, as it would more closely resemble the state of DNA found within the sperm at fertilization, which is in a tightly condensed almost crystalline state (Balhorn, 1982). A DNA construct containing a cDNA encoding for human protein C was successfully condensed with polylysine and cytoplasmically injected into pronuclear stage murine embryos, resulting in a 12.8% integration efficiency (Page et al., 1995a).

DNA condensation is an important phenomenon, offering insight into DNA packaging in viruses and in cell nuclei, acting as a model for chromosome folding, and presenting an opportunity for the construction of artificial gene delivery vectors (Trubetskoy et al., 1999; Matulis et al., 2000).

The objective of this experiment was to evaluate the previously ascertained parameters for electroporation as a method of facilitating gene transfer into the pronuclei of murine embryos using linear and condensed DNA constructs and to evaluate the effects of this technique on subsequent embryo development.

MATERIALS AND METHODS

Superovulation Procedure

Intraperotineal (i.p) injections of 10 IU of equine chorionic gonadotropin (eCG, Diosynth, Chicago, IL, USA) were administered to groups of 10 to 15 weaned female mice (strain CD-1) weighing 19.5 to 22.5 g and between 25 to 30 d of age. These females were grouped in cages of 3 or 4 each and received an i.p. injection of 5 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, Mo; Catalog #C5297) 46 to 48 h later. The females were then individually placed in cages with a single male. Female mice were be examined 21 to 24 h later for vaginal plugs. Those females with plugs were sacrificed by cervical dislocation, and their ovaries and oviducts removed for embryo harvesting (Hogan et al., 1986).

Ovary Collection and Embryo Harvesting

Mice were dissected, and the ovaries and oviducts collected and placed in 37.5?C M2 medium (Specialty Media, Phillipsburg, NJ; Catalog #MR-015-D) at pH of 7.4. Embryos were collected at the cumulus stage, and removed from the cumulus cells in M2 medium containing 1 mg hyaluronidase (320 IU/mg; Sigma, Catalog #H-3506). Zygotes were washed and manipulated in M2 medium. Following cytoplasmic microinjection embryos were washed and stored in KSOM (Specialty Media; Catalog #MR-020-D) long term culture media (Erbach et al., 1994).

DNA Preparation

The construct cytomegalovirus-enhanced green fluorescent protein (CMV-GFP) was produced by removing the inter-ribosome entry site of enhanced green fluorescent protein (pIRES-EGFP) (Clonetech Laboratories, Palo Alto, CA; Catalog #6064-1) by *Bam*HI endonuclease digestion. The restriction products were separated, and the vector-containing the fragment was subjected to ligation. Ligation products were used to transform E. coli XL-Blu (Stratagene, La Jolla, CA) with selection on ampicillin plates (Chahaun et al., 1999). The plasmid was isolated and purified by column absorption (Mega prep; Qiagen Inc, Valencia, CA). The linear DNA construct was digested overnight with Xho1 (Promega Catalog Madison, WI; Catalog #R616A) at 37.5? C in a 10X Universal Buffer (Stratagene Catalog #50000005) and was isolated using a standard phenol/chloroform (50%/50%; v/v) extraction followed by ethanol precipitation (Davis et al., 1994). The final construct size was 4.8 kbp and had one sticky overhanging end and one blunt end left by the XhoI enzyme cut. The purified construct was reconstituted in 1 to 1.5 ml of buffer containing 10mM Tris HCl and 0.25 mM EDTA. The DNA concentration was determined by photospectroscopy at 260 nm, and diluted for pronuclear injection to 425 ug/ml and stored at –80ºC until use.

DNA Condensation

The EGFP was condensed with $MgCl_2$ to form rod shaped condensates. (Bloomfield, 1991). Linearized DNA in Tris was added to the sterilized condensing

medium. The condensing medium contained 25 mM $MgCl_2$ in 40% de-ionized water and 60% Isopropyl Alcohol. The EGFP was condensed during the 30 min incubation period, and during the 30 min centrifugation at 15,000 x g for 30 min at 4ºC. The supernate was removed and the condensed DNA re-suspended at 4ºC overnight in the proposed injection medium, physiological saline, which contained 160 mM KCl, 20 mM $MgCl_2$, and 5mM NaCl at pH 7. The absorbancy reading was determined on a spectrophotometer, in duplicate, and DNA was diluted with additional injection medium to reach the desired concentration (425 ug/ml). Condensates were stored in injection buffer at -80ºC until use (Butler, personal communication).

Electroporation

Development of electroporated embryos was examined after a single electrical pulse at 250 V for the duration of 10 usec (BTX Inc., ECM®200; San Diego, CA). Zygotes were examined after needle insertion followed by electroporation and electroporation after injection with linear or condensed GFP. Zygotes were electroporated in 0.22 M d-mannitol (Sigma; Catalog # M-4125) containing 0.01% polyvinyl alcohol (PVA) (Sigma; Catalog # H-6185) in Hepes (35 mM; Sigma Catalog #H-6147) at a physiological pH of 7.2. The electroporation microslide chamber was part #453 (BTX Inc.), and was sterilized with 70% ethanol before each use. The two electrodes on the microslide formed a 3.2 mm gap, which allows for 0.7 ml medium volume. Approximately 20 to 40 embryos were electroporated at a time. This number was based on the total number of embryos collected containing the male pronucleus and

their subsequent division into experimental groups. No more than 40 zygotes were electroporated at a time, due to the 40 embryo maximum per 10 ul long-term culture drop (Chatot et al., 1989). Electroporated embryos were then washed in KSOM (Specialty Media; Catalog # MR-020-D) long-term culture medium, and placed into 10 ul KSOM drops for long-term culture.

Cytoplasmic Microinjection

The mouse pronuclear stage embryos were placed at the tip of a narrow 18 ul drop of M2 medium at 37.5ºC, in the center of a 100 mm Becton Dickson petri dish (injection chamber, Falcon #1029; Franklin Lakes, NJ). Mineral oil (Specialty Media; Phillipsburg, NJ; Catalog #MR-8410) was gently poured until just covering the drops on the dish, decreasing loss of temperature due to exposure and medium through evaporation.

The EGFP construct was brought to room temperature and loaded into a microinjection needle. Needles were pulled using the KOPF Needle/Pipette Puller (Model 750, David Kopf Instrument; San Diego, CA). The EGFP construct was injected into the cytoplasm of murine zygotes at a concentration of 425 ug/ml, each embryo was injected with 10 pl, and the copy number injected ranged between 100 and 600 (Butler, personal communication).

In Vitro Evaluation

The development and fluorescence of electroporated, electroporated with needle insertion, electroporated injected, and control mouse embryos was evaluated at d 2 and 4 post manipulation. Those embryos injected with EGFP were evaluated only on d 2 and 4 post manipulation to minimize the exposure of the embryos. Injected embryos were illuminated with blue light for several seconds at 488 nm, the excitation maximum for EGFP. Evaluation at these two times allowed for determination of those embryos that fluoresce early and then degenerate, or fluoresce early but did not express the transgene in later development. Emission of green fluorescence was examined in darkness at a wavelength of 508 nm, photographs were taken of fluorescing embryos (Nikon Filter set # EF – 4 FITC HYQ, 480/(40) and 535/(50); Nikon, Japan).

Statistical Analysis

The embryo data were analyzed using the general linear model analysis of variance in the SAS system (SAS Institute, Inc., 1985). Developmental data were classified using a numerical scoring system. Embryos were assigned a 0 = degenerate or lysed, 1 = 1-cell, 2 = 2-cell, 3 = 4-cell, 4= 8-cell, 5 = 16-cell, 6 = morula, and 7 = embryos that developed to the blastocyst stage. Embryos were evaluated on d 2 and d 4. Evaluation of development on d 2 was performed to record embryos that had degenerated, or arrested at the one-cell stage of development, as these embryos can be

mistaken on d 4 for later stages of development. Each embryo was assigned one numerical score on d 4 based on its final stage of development.

The specific treatments tested included: control, needle insertion, needle insertion combined with electroporation, cytoplasmic injection of condensed DNA, cytoplasmic injection of condensed DNA combined with electroporation, cytoplasmic injection of linear DNA, and cytoplasmic injection of linear DNA combined with electroporation. Specific treatments were run on d 1 and d 2 of each repetition. Treatments run on d 1 included all non-voltage treated groups: control, needle insertion, cytoplasmic injection of condensed DNA, and cytoplasmic injection of linear DNA. Treatments run on d 2 included all of the voltage (10 μsec) treated groups: needle insertion combined with 250 V electroporation, cytoplasmic injection of condensed DNA combined with 250 V electroporation, and cytoplasmic injection of linear DNA combined with 250 V electroporation.

Development scores at d 4 were analyzed using the general linear model analysis of variance in the SAS system, using a model that included the day repetition subclass and treatment (SAS Institute, Inc., 1985).

RESULTS

This experiment included 2,207 embryos. The non-manipulated control embryos had significantly higher ($P < 0.01$) 4 d development (5.57 ± 0.11; mean ± SE) than manipulated control embryos (4.6 ± 0.18), where the injection needle was inserted into the cytoplasm and no DNA was injected (Table 5). Injection of condensed DNA (2.85 ±

0.19) and linear DNA (2.83 ± 0.19) caused a significant decrease in development ($P < 0.01$) as compared to manipulated and non-manipulated control embryos. Voltage treated embryos developed significantly less ($P < 0.01$) than non-voltage treated groups. No significant effect on development ($P > 0.05$) was observed due to the type of DNA construct injected, linear verses the condensed DNA construct, irrespective of voltage treatment. Addition of DNA injection and electroporation caused a significant ($P < 0.01$) decrease in development, irrespective of DNA construct, when compared to zygotes injected with a DNA construct alone. The mechanical effects of needle insertion coupled with electroporation were not significantly different ($P > 0.05$) from embryos injected with DNA alone, irrespective of construct injected (Table 5).

Table 5. Least squares means ± SE d 4 development scores for zygotes subjected to microinjection of DNA and electroporation

Treatment			Score[1]			
Injection Procedure	DNA Construct	Voltage Applied	N	LSMean	SE	% Morula and Blastocyst
Control	None	None	555	5.57[a]	.11	78
Needle Inserted Into Cytoplasm	None	None	302	4.60[b]	.18	59
Cytoplasmic Injection	Condensed DNA	None	266	2.85[c]	.19	28
Cytoplasmic Injection	Linear DNA	None	264	2.83[c]	.19	31
Needle Inserted Into Cytoplasm	None	250 V	264	2.65[c]	.19	27
Cytoplasmic Injection	Condensed DNA	250 V	276	1.97[d]	.19	19
Cytoplasmic Injection	Linear DNA	250 V	280	2.04[d]	.18	22

Score[1]

0 = degenerate and lysed embryos
1 = 1 cell embryos
2 = 2 cell embryos
3 = 4 cell embryos
4 = 8 cell embryos
5 = 16 cell embryos
6 = morula stage embryos
7 = blastocyst to hatched blastocyst stage embryos

[abcd]Means with different superscript differ significantly ($P < 0.01$).

Embryos were examined for fluorescence on d 2 and d 4 of development. No advanced stage mosaic embryos were observed in this experiment. In both the linear and condensed DNA injected, non-voltage treated groups, one fluorescent one-cell embryo developed. In the voltage treated group injected with the condensed DNA construct one embryo was fluorescent, developing to the one-cell stage. In the voltage treated group injected with the linear DNA construct, 13 embryos were fluorescent, ten embryos developed only to the one-cell stage, while three developed to the two-cell stage (Table 6).

Table 6. Embryos fluorescing on d 4 following cytoplasmic injection of EGFP and electroporation.

Treatment					
Injection Procedure	Voltage Applied	N	One cell	Two cell	% Fluorescent
Condensed DNA	None	266	1	0	0.38
Linear DNA	None	264	1	0	0.38
Condensed DNA	250 V	276	1	0	0.36
Linear DNA	250 V	280	10	3	3.57

Discussion

This study evaluated the effects of coupling electroporation with cytoplasmic injection of one of two GFP DNA constructs, linear or condensed (425 µg/ml). The 250 V, 10 µsec pulse duration combination was chosen from the first experiment in this study, as the 400 V, 10 µsec pulse duration treatment caused excessive cell death. The development of the non-manipulated control embryos was used as a standard to assess the effects of treatment, injection of linear or condensed DNA alone or coupled with electroporation, on subsequent embryo development. The group of embryos that had the microinjection needle inserted into the cytoplasm with no DNA injected served as a manipulated control, isolating the mechanical effects of the injection process. The group of embryos receiving needle insertion followed by voltage application, isolated the mechanical effects of the injection process when coupled with the voltage effects.

The needle insertion alone caused a significant decrease in development, suggesting that this mechanical event is a major factor in the decreased efficiency of the technique. Puncture and injection with hand-pulled glass instruments has previously been reported to cause changes in cell viability, behavior, and internal pH. Walton and associates (1987) also report that 30 to 32% of injected cells were lysed due to the injection event, even when a very small diameter pipette was used. Additionally, the required use of suction when using the holding pipette to stabilize the embryo during the injection process, resulted in additional cell lysis.

When these lysed embryos were examined using stereo electron micrograph pairs, holes were present among the microvilli, and open holes where found on the vitelline

surface. In injected, non-lysed embryos, patches, but not open holes, were found on the membrane. None of these characteristics were observed in non-injected control embryos. These observations suggest that cell death resulting from the injection process is a result of the cell's inability to repair the hole created by the pipette during the injection event. These holes cause an outflow of cellular material, clearly visible during the injection process, and a nonselective influx of ions, fluid, and dissolved molecules from the medium. Excessive perturbation of the actin network underlying the plasma membrane, could prevent proper sealing of the hole (Walton et al., 1987). These findings help explain the decrease in cell viability observed in the present study due to the mechanical events occurring during the injection event and the additional cellular disruption of the electroporation procedure.

The injection of DNA, irrespective of construct, caused a significant decrease in development indicating a major effect of the presence of exogenous DNA within the embryo on development (Page et al., 1995b). The pronuclear injection of buffer had no adverse effects upon subsequent in vitro embryonic development to the blastocyst stage. This data suggests that the presence of DNA, not the injection event itself, is the cause in the decrease in embryo development (Page et al., 1995b; Brinster et al., 1985). The coupling of electroporation and DNA injection, irrespective of construct, resulted in an additional significant detrimental effect on embryo development, suggesting that an additive effect of voltage application and DNA injection occurred. The high percentage of fragmented embryos, and embryos arresting at early stages of development, within these groups cannot be overlooked.

Recently, research has begun focusing on the role of apoptosis in degenerative early preimplantation embryos during in vitro culture. The Fas-Fas L system, a major pathway in the induction of apoptosis, has been found in 2-cell rat embryos, and apoptosis has been found responsible for fragmentation of oocytes and preimplantation embryos in both mice and humans. Additionally, the activation of the embryonic genome occurs at the 2-cell stage in mice, marking the transition from maternal RNA stores to newly transcribed embryonic RNA and proteins (Kawamura et al., 2001). During this transition, cellular checkpoints may initiate apoptosis, due to excessive cell damage resulting from mechanical events, and the presence of excessive amounts of exogenous DNA from the injection process. The exogenous DNA may trigger the cells to shut down, as it appears they did not undergo transcription properly.

Since the mechanical effects of needle insertion and voltage application did not have as severe of an negative impact on development as the injection or presence of DNA within the cell, the DNA itself may be the cause of the additional cell death and decreased development. This was observed in the DNA injected and DNA injected combined with electroporation groups, irrespective of construct injected.

While integration frequency is improved as DNA concentration increases, the high concentration (425 µg/ml) of the DNA constructs injected during the present experiment may have contributed to the decreased survivability. Pronuclear microinjection of DNA has a deleterious effect on in vitro development, and this effect is DNA-concentration dependent (Page et al., 1995b). Large amounts (45 ng/ul) of DNA have been reported as toxic to embryos, when injected into the pronucleus (Brinster et al., 1985; Page et al., 1995b). However, since the DNA constructs were injected into the

cytoplasm of the embryos during this experiment, the dilution effect, due to the increased volume of the cytoplasm as compared to the pronucleus, may have lessened this effect.

While the DNA construct injected had no significant effect on embryo development or protein expression with DNA injection alone, it did have an effect on protein expression when the injection of DNA was coupled with electroporation. The linear DNA construct resulted in 0.38% fluorescing embryos with injection alone, and 3.57% when coupled with electroporation. The injection of condensed DNA followed by electroporation (0.36%) did not result in an increase in protein expression over injection of condensed DNA alone (0.38%). Brinster and associates (1985) have previously reported that the integration frequency of linear DNA was superior to that of supercoiled DNA. However, the embryos that did fluoresce did not develop past the two-cell stage. Recently, the expression of GFP has been linked to apoptosis. Both biochemical and microscopic studies indicate that many cells expressing GFP have died, in four different cell lines, using three different GFP constructs, indicating this is a general phenomenon. Moreover, a minimal amount of GFP expression was sufficient to kill the cells (Liu et al., 1999). The embryos in the present study that did fluoresce were extremely bright, indicative of a high level of protein expression, possibly leading to cellular arrest and subsequent embryo death.

While the development of fluorescing embryos was unsatisfactory, these data suggest that electroporation of embryos following injection of linear DNA improves protein expression following cytoplasmic injection and may improve integration efficiency since no mosaic embryos were found in this experiment.

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CHAPTER 6

Conclusion

The purpose of this study was to assess in vitro development, transgene expression, and integration following pronuclear or cytoplasmic microinjection of condensed or linear green fluorescent protein DNA into murine embryos using group or linear orientation during electroporation.

Experiment 1, assessed the effect of the electroporation medium and embryo orientation (group or linear) within the electroporation chamber using a 10 μsec pulse duration at 250 V or 400 V. While a significant voltage effect was observed ($P < 0.01$), embryo orientation within the electroporation chamber had no significant effect on embryo development ($P > 0.05$). These data indicated that exposure to the 400 V treatment, irrespective of orientation, caused excessive instability and was severely detrimental to embryo development. Based on these observations, electroporation was conducted at the 250 V pulse strength for subsequent experimentation.

Experiment 2, confirmed previous work that shows that the EGFP DNA construct can be used as a visual marker for protein expression for future experimentation to assess alternative gene transfer methods. However, pronuclear injection of DNA has a detrimental effect on embryo development ($P < 0.01$).

The data from experiment 1 and 2, provided the validation of the construct and electroporation parameters to be used for the third experiment. Experiment 3, evaluated the previously ascertained parameters for electroporation as a method of facilitating gene transfer into the pronuclei of murine embryos using linear and condensed DNA

constructs and assessed the effects of this technique on subsequent embryo development. These data suggest that electroporation of embryos following cytoplasmic injection of linear DNA improves integration and protein expression following cytoplasmic injection and may improve overall integration efficiency since no mosaic embryos were observed in this experiment. However, the development of fluorescing embryos arrested at the one or two cell stage.

Further experimentation in this area is needed to evaluate the effect of DNA concentration on embryo development in order to evaluate whether embryo development is dependent on the DNA concentration injected into the cytoplasm, as development is concentration-dependent when DNA is injected directly into the pronucleus. Research assessing the dilution effect on DNA concentration upon injection into the cytoplasm, as compared to injection into the pronucleus, would aid in determining the appropriate concentration that should be used. Additionally, research assessing the toxic effects of green fluorescent protein expression following integration would be beneficial, in order to determine if protein toxicity is the cause of embryo arrest.

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APPENDIX

Table 7. Analysis of Variance Experiment 1, illustrating a significant effect of both treatment group and experimental repetition on development

Source	**df**	**Mean Square**	**F Value**
Repetition	10	19.10***	4.72
Group	5	242.58***	59.89
Error	1512	4.05	

Table 8. Analysis of Variance Experiment 2, illustrating a significant effect of treatment on development

Source	**df**	**Mean Square**	**F Value**
Repetition	3	0.96*	0.15
Group	1	365.68***	56.81
Error	454	6.44	

*** P > 0.05 ***P < 0.01**

Table 9. Analysis of Variance Experiment 3, illustrating a significant effect of treatment on development

Source	df	Mean Square	F Value
Repetition(Day)	15	1.25*	0.20
Group	6	652.00***	103.60
Error	2185	6.29	

Table 10. Contrasts for Experiment 1, evaluating the significance of specific treatment effects on development

Contrast	Df	Mean Square	F Value
Linear vs. Group Orientation	1	2.53*	0.62
250 V vs. 400 V Treatment	1	488.70***	120.65
Control vs. Mannitol	1	0.59*	0.15
Control vs. Treatment Groups	1	652.00***	160.97

Table 11. Contrasts for Experiment 2, evaluating the significance of specific treatment effects on development

Contrast	df	Mean Square	F Value
Control vs. Pronuclear Injection	1	365.68***	56.81

*** P > 0.05 ***P < 0.01**

Table 12. Contrast for Experiment 3, evaluating the significance of specific treatment effects on development

Contrast	Df	Mean Square	F Value
Needle Insertion vs. Condensed or Linear DNA Injected Treatments	1	599.14***	95.20
Voltage with needle insertion vs. Condensed or Linear Injected Treatments	1	72.95*	11.59
Condensed DNA vs. Linear DNA	1	0.05*	0.01
Voltage with Condensed DNA vs. Voltage with Linear DNA	1	0.64*	0.10
Voltage vs. Non-voltage	1	150.11***	23.85
Needle Insertion vs. DNA Injection	1	535.76***	85.13
Interaction (insertion and DNA injection)	1	117.90***	18.73
Control vs. Treatment Groups	1	3125.90***	496.70

*** P > 0.05 ***P < 0.01**

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OBJECTIVE

To obtain a position that allows me to interface my science and business backgrounds.

EDUCATION

1999-present

Master of Science in Dairy Science, Virginia Tech, Blacksburg, VA
Reproductive Physiology
Advisor: Dr. Frank Gwazdauskas

Thesis: Assessment of murine embryo development following electroporation and microinjection of a green fluorescent protein DNA construct

1995-1999

Double Major:

Bachelor of Science, Animal and Poultry Sciences, Virginia Tech, Blacksburg, VA
Production/Business option
Equine emphasis

Bachelor of Science, Agricultural and Applied Economics, Virginia Tech, Blacksburg, VA
Marketing and Management options

WORK EXPERIENCE

1999-present

Graduate Research Assistant, Virginia Tech, Blacksburg, VA
Conducting original research while teaching and assisting fellow researchers.

Responsibilities:

Technical training and supervision of undergraduate researchers and Fralin Scholars
Technical training and orientation of post-doctorate student
DNA isolation and purification
Superovulation
Embryo harvesting and manipulation
Pronuclear and cytoplasmic microinjection
In vitro maturation of zygotes
Oocyte grading following follicular aspiration
Electroporation
Small animal surgery
Maintenance of mouse colony for research purposes
Maintaining and ordering laboratory supplies

2000-2001 **Teacher's Assistant,** Virginia Tech, Blacksburg, VA
Anatomy and Physiology, Graduate level
Animals in Society, Undergraduate level

Responsibilities:

Assisted in teaching and laboratory instruction
Organized and setup each laboratory segment
Graded written assignments
Administered examinations

1999, Summer **Research Assistant,** The Pharmaceutical Engineering Institute, Virginia Tech Corporate Research Center**,** Blacksburg, VA
Assisted under Dr. William Velander in transgenic and in vitro fertilization research.

Responsibilities:

Porcine ovary collection
Oocyte aspiration
Aided in oocyte searching
Aided in sperm preparation

1998-1999 **Volunteer Lab Assistant,** Virginia Tech, Blacksburg, VA
Assisted under Dr. Frank Gwazdauskas in transgenic and reproductive physiology research.

Responsibilities:

DNA isolation and purification
Embryo harvesting and manipulation techniques
Prepared gels and media
Oriented new volunteers

1998-1999 **Volunteer Lab Assistant,** Virginia Tech, Blacksburg, VA
Assisted under Dr. Paul Siegel in a research project comparing the immune responses of high and low genetic weight lines of poultry to an *Escherichia coli* challenge.

Responsibilities:

Assisted in blood collection
Assisted in necropsy of animals
Assisted in heterocyte and lymphocyte counts

1996-1998 **Manager's Assistant,** Price Real Estate, Blacksburg, VA
Assisted in daily operation of main office.

Responsibilities:

Scheduled appointments
Completed balance sheets, late lists, and work orders
Created graphic designs for fliers and resident notices
Collected rent, deposits, and application fees

Responsibilities (continued)
Typed business letters
Showed apartments
Assisted in orientation of new employees
Prepared lease agreements

INTERESTS & ACTIVITIES

Elected member of Sigma Xi, The Scientific Research Society, 2001-present
Reproductive Physiology Journal Club, member and speaker, 2000-2001
Graduate Student Assembly, delegate for the Dairy Science Department, 1999-2001
Member of the Virginia Tech Block and Bridle Club, 1995-1999
Horse show committee member, Block and Bridle Club, 1997 and 1998