EVALUATION OF TECHNIQUES FOR THE PRODUCTION OF TRANSGENIC ANIMALS

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(ABSTRACT)

A polymerase chain reaction (PCR) technique was used to detect transgene presence after pronuclear microinjection of mouse zygotes cultured to various stages of development. The transgene was detected in 88% of 1-cell, 88% of 2-cell, 44% of 4-cell, 40% of morula, and 29% of blastocysts. By comparison, the integration frequency for transgenic mice made using the same DNA construct was 22%. After 5 days of *in vitro* culture, the injected construct was detected in 83% of arrested 1-cell, 85% of arrested 2-cell, and 85% of fragmented embryos. Only 28% of zygotes cultured after microinjection of DNA developed to the blastocyst stage compared to 74% of noninjected zygotes. When DNA buffer alone was injected, 63% of zygotes developed to the blastocyst stage. These data suggest that pronuclear microinjection of DNA is highly detrimental to subsequent embryonic development. Also, most injected DNA that is either unintegrated or that will not be integrated into the genome has been degraded by the blastocyst stage such that it can no longer be detected by PCR.

The production of transgenic mice by cytoplasmic injection of DNA mixed with poly-L-lysine is also described. The effects of DNA concentration and stoichiometric ratio of positive charges provided by the polycation to negative charges provided by DNA on transgenic frequency and embryonic viability were studied. The highest transgenic frequency (13% of pups born were transgenic) was obtained when a polylysine/DNA complex having a stoichiometric charge ratio of one to one (equal positive charges as negative charges) at a DNA concentration of 50 μg/ml was used. The transgenic frequency by pronuclear injection of the same DNA construct was 22%. The percentage of zygotes, cultured *in vitro*, reaching the blastocyst stage which were injected cytoplasmicly was not different ($p>0.05$) than that of control zygotes that were not microinjected (65% versus 74%, respectively). The percentage of zygotes reaching the blastocyst stage after pronuclear microinjection with DNA at a concentration of 1.5 μg/ml was significantly lower ($p<0.05$) than control embryos (28% versus 74%, respectively). The overall transgenic pup production efficiency (percent of transgenic pups per embryos transferred) by cytoplasmic injection was 2.4% compared to 3.5% by pronuclear microinjection.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER IV.

LIST OF FIGURES

construct DNA that has been mixed with polylysine.

Figure 3. Southern blot of founder transgenic mice.

CHAPTER I.

LITERATURE REVIEW

INTRODUCTION

Transgenic animals are rapidly becoming an integral part of biotechnology useful for the study of disease and developmental biology, and possibly the development of more efficient and novel production animals. These genetically engineered animals will undoubtedly impact our society in a broad manner. For example, animal lines are being created with specially modified physiology for the study of particular disease states such as sickle cell anemia and various forms of cancer (1-3). In addition, new biochemistry can be introduced into milk production animals such as goats and cows (4). This technology enables the synthesis and secretion of recombinant (genetically engineered) proteins into the milk of transgenic animals (4-6). Because the mammary gland has a high capacity for the production of milk proteins, this approach has great promise as a more economical method for the large-scale biosynthesis of therapeutic proteins than is currently achieved by recombinant tissue culture methods. In addition, production livestock such as pigs and cattle can be genetically engineered to yield a higher protein content (7,8). Transgenic livestock and poultry with increased resistance to disease could also be produced by genetic engineering (8-10).

In all of the above applications, it is the biosynthesis of a foreign protein such as a growth hormone or enzyme made under the direction of a manipulated gene which is the salient characteristic of the transgenic animal. The manipulated gene or transgene consists of sequences of deoxyribonucleic acid (DNA) which specify regulation and synthesis of the protein of interest. The origin of either the regulatory or protein encoding DNA sequences may or may not be from the same animal species. Furthermore, gene function does not require either of these sequences to be native to the host animal that will possess the transgene.

DEFINITION OF TRANSGENIC ANIMALS

A transgenic animal is a result of the incorporation of a foreign gene such that it becomes an integral part of the natural chromosomal makeup of the animal. The gene is introduced into the fertilized egg in vitro, during early embryonic development and the embryo undergoes gestation in a surrogate mother. If the foreign gene is incorporated at the one-cell embryonic stage, the resultant animal will be hemizygous for the transgene because every cell contains the foreign gene. Since the germ cells also contain the gene, it will be transmitted in a Mendelian fashion to the progeny. However, the animal may contain the foreign gene at several different loci and in multiple copies. The integration site is thought to be random, with many copies inserting in tandem arrays after concatenation (11).

Frequently, the foreign gene does not integrate until after the first cell division, and the resultant animal will not contain the gene uniformly throughout its tissues. A founder animal of this type is termed "mosaic". This animal may only contain the foreign gene in a fraction of its germ cells. Thus, a non-Mendelian transmission of the gene to the progeny can result. However, any progeny from that founder that contain the gene are true hemizygotes and henceforth will transmit the gene in a Mendelian fashion to their progeny. As with hemizygous transgenic animals, the same phenomena with respect to randomness of the integration site and gene concatenation apply to mosaic founder animals.

Yet another type of transgenic animal can result when embryonic stem cells from one animal are fused into the developing embryo of another animal (12,28, reviewed in 9). For example, viral mutagenesis can be used to affect changes in the embryonic cells of one mouse, and these genetically altered cells are fused with a normal mouse embryo (typically at the blastocyst stage). The result is a chimeric mouse whose genetic makeup is partly represented by the mutated embryonic stem cell(s). Chimeric animals have also been created from the fusion of embryonic cells from a sheep and a goat resulting in an animal with the phenotypic characteristics of both species (12). In chimeric animals, only a fraction of the cells of the animal, including the germ cells contain exogenous genetic information. Thus, very few chimeras are able to transmit the traits derived from the embryonic stem

cell to their progeny (9).

In summary, there are three types of transgenic animals: hemizygous, mosaic, and chimeric. In most cases, a hemizygous transgenic animal presents a more desirable genotype usually due to a much higher production level of the protein encoded by the transgene. However, mosaics and chimeras may be suitable for some aspects of the fundamental study of disease and/or developmental biology.

METHODS OF MAKING TRANSGENIC ANIMALS

The most commonly used methods to make transgenic animals are microinjection and retroviral transfection (13,14). In addition, either of these techniques can be used to introduce transgenes into embryonic stem cells. Subsequently, these undifferentiated cells can be fused with early embryos to form a chimeric embryo (reviewed in 9). Currently, the most widely used technique for introducing new genetic constructs into animals is microinjection of single cell embryos (zygotes) followed by implantation into surrogate mothers. Typically, this procedure involves harvesting one-cell embryos from donor animals, then microinjecting the desired DNA construct into one of the two pronuclei (maternal or paternal) using a finely drawn glass capillary. The paternal pronucleus is usually much larger and therefore easier to microinject. Approximately 1-2 picoliters of buffered solution containing 200-500 copies of the genetic construct are typically

introduced into the pronucleus by microinjection. Embryos surviving the injection procedure are then surgically transferred into the oviduct of an oestrus-synchronized recipient animal. Typically, 70% of microinjected murine embryos survive through at least one cell division, and about 40 microinjected embryos are transferred to surrogate mothers. The litter sizes in mice average about 4-6 pups but can vary widely from 1 to greater than 10. On average, about 10-30 % of these pups will be transgenic animals.

The embryos of large animals such as pigs and cows require centrifugation prior to microinjection (15-16). This concentrates the cytoplasmic lipid away from the pronuclei such that they are easily visualized and microinjected. The zygotes from pigs and cows seem to be more robust with respect to the trauma caused by microinjection. However, the expense of experiments using large animals can be prohibitive. This is especially true in cattle since only one healthy offspring can be produced per recipient mother. Consequently,
relatively few transgenic animal studies have been attempted in cattle. Because pigs can naturally sustain litter sizes of 10 or more piglets, they are more easily used for transgenic animal studies. In experiments performed with pigs, 25-40 microinjected embryos are implanted into surrogate mothers (15,16). Superovulated pigs generally produce 10-20 embryos and therefore a ratio of 3 donors per recipient animal is usually employed. The pregnancy rates obtained using this procedure vary from

25-50% with litter sizes averaging about 4-7 piglets. The frequency of transgenic piglets varies widely, but is similar to that of mice with an average of less than 30% being transgenic (16). Transgenic sheep have also been produced using essentially the same procedure, but with the restriction of less than 4 progeny born per litter. The frequency of transgenic lambs is similar to that for other species (17).

Figure 1 schematically shows how microinjection is typically applied to large animals. Several modifications of this procedure are currently being developed in an effort to increase the efficiency of producing transgenic animals. These modifications integrate techniques such as polymerase chain reaction (PCR), which permits the detection of the transgene from a few cells which have been biopsied from an early embryo (18). The remaining embryo is further cultured and if shown to possess the transgene is transferred into a surrogate mother (See Figure 1, pathway B). Theoretically, only transgenic animals would result from implantation although some false positives have been observed in mice produced by this method (19). Embryo cloning which has been demonstrated in mice (31) and cattle (32) could also enable the cloning of positive embryos prior to implantation. Another improvement for efficient production of transgenic livestock involves in vitro maturation, fertilization, and subsequent microinjection of immature ova recovered from slaughterhouse ovaries (See Figure 1, pathway C). The development of this technology would allow large numbers of embryos to be obtained very inexpensively. If combined with the

detection methods afforded by PCR, the use of slaughterhouse embryos will undoubtedly facilitate transgenic research in large production livestock.

Another method of introducing DNA into the germ line of an organism is by retroviral transfection. The "life cycle" of the retrovirus requires integration into a host genome in order for subsequent replication of retroviral ribonucleic acid "genes" (RNA) to occur (22).

The infectious segment of the retroviral life cycle is encoded by RNA and thus the host genome is employed as a DNA intermediate through which this RNA is generated. By subverting the infectious element of the retroviral life cycle, the RNA-encoded stage can be used to transmit the genetic element of interest to the host genome. Here, the pathogenic elements of the retrovirus have been deleted and replaced with sequence information encoding the transgene. The main advantage of this technique is that the mechanism of host integration provides greater site-specificity and single copy insertion. In contrast to microinjection, this method almost exclusively generates mosaic animals because of the temporal restrictions imposed by the process of viral infection. An additional drawback is that the size of the transgene is limited when using the retroviral RNA as a vehicle for gene transfer. However, because of the difficulty encountered in trying to microinject chick embryos, retroviral transfection may be the best method for producing transgenic poultry (20,21).

Embryonic stem cells can also be used to produce transgenic animals. Typically, a biopsy of a morula or blastocyst stage embryo is allowed to grow into a monolayer by cell culture methods. DNA is then introduced into these cells by either conventional or retroviral transfection techniques. The cells containing the transgene are then selected by resistance to a specific antibiotic which has been placed in the culture medium. This resistance can be imparted by a "piggyback" transgene or free plasmid which is co-transfected into the stem cell.

The antibiotic resistance gene encodes an enzyme which specifically breaks down the antibiotic placed in the media. In this way, it is likely that only cells which contain both the primary transgene and resistance gene will survive in the culture medium. Once a pure culture of "transformed" stem cells has been obtained, several of these cells can be injected into a host blastocyst consisting of many cells. The fusion of the transformed stem and host cell masses results in a chimeric animal.

METHODS FOR DETECTION OF TRANSGENIC ANIMALS

Transgenic animals can be easily detected by the isolation and characterization of DNA from tissue samples taken from tail biopsy, etc. The isolation procedure involves a tissue digestion and solubilization step using proteinases (enzymes which degrade protein) and surfactants, followed by phenol extraction and ethanol precipitation of the DNA. The purified DNA is then specifically digested by

restriction endonucleases (enzymes), which result in cleavage sites that release all or parts of the foreign gene from the host genome. The gene or gene fragments can then be recognized with highly specific, labeled polynucleotides (short, single-stranded DNA sequences labeled with a radioisotope or an enzyme) which act as probes for the transgene. These synthetic probes are complementary to and specifically hybridize with selected segments of the transgene. The polynucleotide hybridization is performed upon an electrophoresed sample of DNA which has been transferred and immobilized onto a solid membrane (a technique termed a Southern transfer). Once the labeled probe has been hybridized to the immobilized DNA (procedure termed Southern blotting), the transgene is detected upon development of an X-ray film exposed to the membrane.

Polymerase chain reaction (PCR) has gained popularity as a facile and sensitive method for the detection of relatively few copies of the transgene (18,19). This method utilizes a DNA polymerase (enzymatic) amplification of all or parts of the transgene as identified by oligonucleotides which hybridize to specific segments of the foreign gene. When provided with free nucleotide triphosphate bases (the building blocks of DNA), the enzyme then synthesizes complementary copies of both strands of the sequences within the transgene delineated by the flanking oligonucleotide primers. By going through a series of denaturation (DNA strand separation), hybridization, and polymerization steps, each polymerase product provides a new template for subsequent

synthesis (23). Thus, amplification of the original gene copy(s) occurs in a geometric progression (i.e. two-strand makes four, four makes eight, eight makes sixteen, etc.). Each cycle takes approximately 6 minutes, such that more than one million copies can result after several hours, when starting with only a single gene copy. The amplified gene or gene fragment is easily detected by agarose gel electrophoresis of the PCR product followed by ethidium bromide staining. Since only a few cells provide sufficient DNA to be processed by PCR, the tissue (e.g. a tail snip from a mouse) or cells biopsied from an early stage embryo can be subjected to aa appropriate DNA isolation procedure. This solution can then be used directly in the DNA amplification procedure. Thus, the transgene can be detected in only 4 or 5 hours, versus the 5 day procedure required for detection by Southern blot analysis.

MOLECULAR BIOLOGY OF GENE INTEGRATION

The highest frequency of transgenic pups are obtained when the male pronucleus is injected with linearized DNA in Tris-HCl, EDTA buffer (11). However, the actual physical and chemical mechanisms of transgene integration have yet to be elucidated. Depending on the species of animal, one can expect from 10-30% of the progeny born from microinjected embryos to be transgenic (8,16). Mosaicism can still occur even in embryos which have been injected at the one-cell stage because the integration event may not occur until after cell division. Integration seems to be a random event which is mediated by enzymes responsible for the repair and

maintenance of the host chromosomal DNA. Because the integration is random, insertion of a desired number of copies of DNA at a specific chromosomal site will probably not occur. However, it appears that retroviruses contain elements which may be naturally capable of directing the site of integration as well as the number of copies inserted into the host genome (reviewed in 9).

Although the chromosomal integration site cannot be controlled, it is still possible to direct expression of the transgene to a specific tissue. This is done by fusing the recombinant DNA sequence encoding the protein of interest (reporter sequence) to a DNA promoter (regulatory) sequence derived from a gene which is activated in a tissue specific manner. For example, the regulatory elements of DNA associated with milk proteins are responsive to hormonal induction such that milk proteins are made only during lactation and primarily in the mammary tissue. Thus, the DNA promoter from the Whey Acidic Protein (WAP) of mice has been fused to a cDNA (reporter) sequence of human tissue Plasminogen Activator (tPA) to make a mammary tissue specific genetic construct (5). Transgenic mice containing the WAP-tPA gene have made human-tPA protein in their milk in a mammary tissue specific fashion similar to that of the native WAP protein. The analysis of 23 different tissues from these mice has shown that the human tPA is not made to a significant extent in any tissue other than the mammary gland.

About 5-10% of the animals carrying the transgene will express a detectable level of the desired gene product (24). Although the mechanisms of transgene expression are not well understood, two factors believed to be important are the site of integration and the structure of the regulatory elements fused to the reporter DNA sequence. For example, the site of integration may be such that expression is attenuated by the flanking host DNA. In addition, the regulatory elements from a given gene may be located far away from the DNA sequence encoding for the protein. Thus, it is sometimes difficult to identify and include the sequences necessary for significant expression of the fusion gene. These regulatory sequences may be far upstream, downstream, or located in the introns of eukaryotic DNA. Introns are intervening sequences of DNA which do not encode for protein and hence are untranslated. The expression levels of the foreign proteins made by transgenic animals have been compared for a given construct with and without introns (24). The animals containing introns possessed a higher frequency of detectable expression than those without introns. However, the exact regulatory function of introns is still not well understood.

TRANSGENIC ANIMALS IN BIOTECHNOLOGY AND THE STUDY OF DISEASE

Prior to the advent of transgenic manipulations, animal models which possessed mutations that facilitated the study of some diseases were the result of laborious

inbreeding and serendipity (1,2). Because transgenic manipulations change the biochemistry in a directed fashion by the addition of specific genetic information, the study of disease is facilitated. The study of disease resistance is of particular interest to the agriculture industry. However, because of the long generation times of production animals such as cattle or pigs, most studies have been performed using mice. There have been several studies using mice in which biochemical intermediates known to regulate the immune response were affected by transgenic manipulation. For example, transgenes which encode for antigens associated with the major histocompatability complex (MHC) have been used to test the immune competence of mice which overproduce an antibody against a particular protein (25). The understanding of the effects of these modifications will likely lead to the genetic engineering of disease resistance by improved immune system responsiveness (2).

Due to the short generation time of poultry, there have been disease resistance studies performed in transgenic chickens. For example, the development of resistance to avian leukosis virus (ALV) has been studied using retroviral infection. In these studies, a retroviral strain which is noninfectious but produces a viral envelope capable of stimulating the production of antibodies against infectious versions of ALV was used to make transgenic chickens (20,21). These transgenic chickens possessed significant resistance to ALV which was transmitted to offspring.

Animal improvement with respect to desired phenotypes such as faster growth rates for commodity livestock is also a potential benefit of transgenic animal research. For example, pigs are marketed based upon reaching a given weight and not reproductive maturity (7,8,10). Expediting the time to reach slaughter weight and improving the yield of meat per pound of feed would be of great economic significance since feed costs can represent 50% of the overall cost to reach market size (7). Increases in growth rates for broiler poultry would also greatly impact production costs. However, the difficulties in making transgenic poultry except by retroviral infection may put these achievements further into the future (9).

Growth studies in rats and swine that were injected daily with pituitary extracts containing growth hormone resulted in significant increases in weight gain with a concomitant decrease in body fat (reviewed in reference 7). Analogous growth studies have also been performed using transgenic mice (26) and swine (8,16). The mouse studies employed a modified rat growth hormone gene such that it produced up to 100 times the normal amount of growth hormone than is naturally made by the pituitary gland. At the same age, these transgenic mice were twice the size of control mice who did not possess the transgene. Similar experiments in pigs produced swine with dramatically reduced backfat content (8). Both of these experiments employed a gene regulation system (metallothionein-I promoter) that is activated by zinc naturally occurring in tissue and plasma. The control of the growth hormone expression by zinc

was not optimal in transgenic pigs as several undesirable physiological changes such as stomach ulcers tended to occur in these animals. Therefore, much more research is needed in the area of gene regulation so that such adverse physiological changes can be minimized.

The need for improvements in meat quality in cattle are equally important. The minimization of trim fat while maintaining the degree of marbled fat content is desirable. Because cattle are marketed at sexual maturity due to the marbling phenomena associated with post-pubertal development, more rapid prepubertal weight gain is of less significance than in swine (7,8,10). Thus, the complex relationship between weight gain and fat deposition upon maturity presents a significant challenge to the current understanding of gene regulation.

The dairy industry currently seeks greater milk productivity per animal such that it can decrease herd size and thus production costs (reviewed in 7). Experiments with dairy cattle injected daily with growth hormone have shown that as much as 30% greater milk yield per animal can be obtained (29,30). Transgenic dairy cattle may provide an alternative to the administration of exogenous growth hormone. Again, gene regulation becomes the pivotal aspect of the biology needed to produce a cow that will have well-regulated expression of augmented growth hormone upon parturition.

Dairy livestock may also be capable of acting as bioreactors for the production of therapeutic proteins which can not be economically made by tissue culture technology (4). Transgenic biotechnology can potentially utilize the mammary tissue's natural role for producing protein at gram per liter levels. The mammary tissue of animals is well suited for production of a heterologous protein because it is purposefully a largely self-contained, secretory organ. Currently, recombinant tissue culture methods can produce only milligram per liter levels of functional protein. Thus, therapeutic proteins produced by tissue culture are limited in supply and as a consequence critical shortages can result. For example, antihemophiliac factor A is currently in short supply and the cost of annual therapy exceeds $25,000 per patient (27). Because of these constraints, hemophiliacs are unable to be treated in a prophylactic manner resulting in a dramatically reduced quality of life. The use of livestock as bioreactors for making therapeutic proteins could provide increased availability at decreased cost.

The use of the mammary gland as a bioreactor for therapeutic proteins will require a detailed understanding of gene regulation. Milk proteins are regulated by prolactin as well as other hormones. The manner in which milk protein genes are controlled by these hormones is being extensively studied using transgenic animals. These studies have shown that genetic regulatory elements of milk proteins are at least partially conserved in mice, pig, and sheep. For example, transgenic mice containing a

gene encoding ovine ß-lactoglobulin (BLG) including the flanking regulatory elements, expressed native BLG protein at levels comparable to those found in sheep (reviewed in 4). Transgenic mice containing fusion genes using the regulatory elements of the Whey Acidic Protein (WAP) gene have also been produced (5,27). In one of these studies, WAP promoter was fused to the cDNA of human issue plasminogen activator (tPA) and microinjected into mouse embryos (5). The resultant transgenic mice expressed human tPA protein in their milk in a well controlled, tissue specific manner. Furthermore, the regulatory elements of ovine BLG gene have been fused to the cDNA encoding human Factor IX (FIX) protein (6,17). Factor IX is currently isolated from human plasma and used to treat hemophilia B. Several of the transgenic sheep containing the BLG-FIX fusion gene were found to make human FIX protein in their milk. However, the milk FIX protein appeared to be a nonnative form of human FIX and was expressed at only 25 nanogram per milliter levels.

In summary, the conservation of regulatory structure for some milk proteins makes possible the expression of a heterologous protein in animals which do not necessarily produce the endogenous protein associated with those same regulatory elements. As a result, a genetic construct used to impart expression of a heterologous protein in the milk of mice can then be tried in a cow with a reasonable expectation of similar mammary tissue-specific expression. Future work will be undertaken in cows and goats once a better understanding of the regulatory control for milk proteins is

gained. In all of the above applications for transgenic biology, a better understanding of gene regulation is needed. The location and function of regulatory elements and how they complement the reporter element of the transgene is of primary importance.

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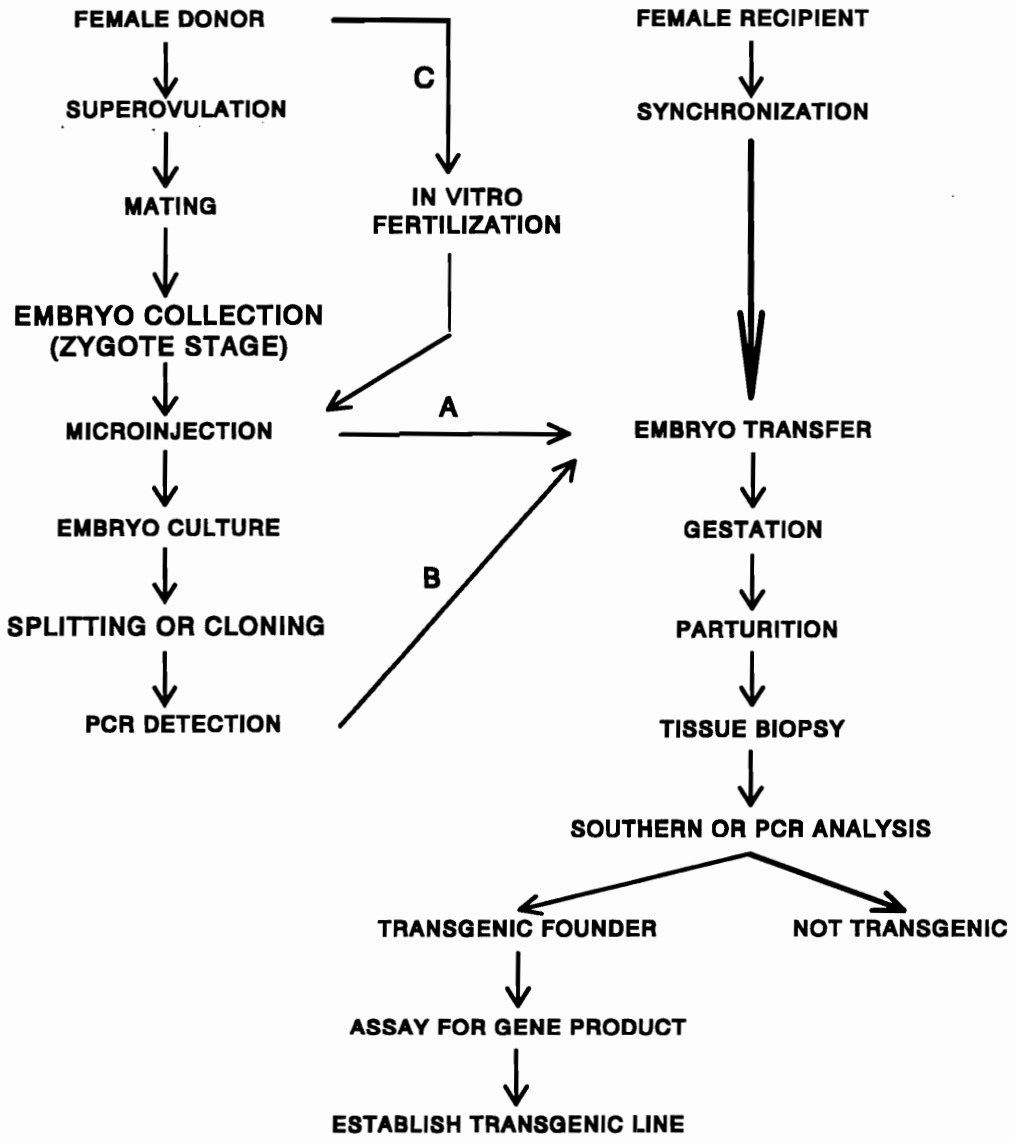

Figure 1. Schematic diagram of methods of making transgenic animals by pronuclear microinjection. A) General procedure for making a transgenic animal. B) Modification employing the early detection method using the Polymerase Chain Reaction (PCR) to amplify the DNA construct obtained from an early embryo biopsy. C) Alternative method for making large transgenic animals using in vitro fertilization of immature ova obtained from slaughterhouse ovaries.

CHAPTER II.

PRODUCTION OF BIOLOGICALLY ACTIVE HUMAN PROTEIN C IN THE MILK OF TRANSGENIC MICE

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INTRODUCTION

There are many therapeutic proteins derived from human plasma and their availability is dependent upon the limited supply of plasma (1). As a result, extensive efforts are being made to produce recombinant versions of these proteins (2-7). Recent advances in the understanding of gene expression have led to high level production of relatively simple proteins in both cell culture (8,9) as well as the mammary gland of transgenic mice (10-12) and livestock (13-16). However, these expression systems have not yet demonstrated the ability to perform many aspects of protein processing needed to synthesize highly complex enzymes (10-17). For example, proteolytic cleavage of propeptide sequences and amino acid derivatization are frequently essential for efficient secretion or biological activity (2,6). The present study has focused upon the vitamin K-dependent (VKD) carboxylation of glutamic acid which has not been shown previously to occur in mammary tissue to any significant level (6,17).

Multiple gamma-carboxylated glutamic acid (gla) residues are necessary for the membrane mediated pro- and anti-clotting activities of the VKD-proteins (18-21). The carboxylase activity necessary for gla formation has been directly or indirectly found to be limited in most tissues (22,23) and manipulated cell lines (2,4). As a result, biologically active VKD proteins have been produced in genetically engineered systems at levels less than 0.4 μg/ml/hour (2-6). Furthermore, the isolation of most

VKD proteins from human plasma is difficult because they occur at less than 10 μg/ml, with the exception of prothrombin which is found at approximately 120 μg/ml (24). Alternatively, if higher levels are to be produced in cell culture, greater levels of carboxylase activity may be needed for the efficient secretion of biologically active VKD proteins (2-4).

Human Protein C (hPC) is a member of the VKD-protein family and serves as the central regulator of hemostasis (21). Therefore, hPC has potential as a therapy for many disease states. Examples include: fibrinolytic therapy (21), vascular trauma (21, as occurs in surgical procedures such as hip and knee replacement), congenital deficiency of hPC (25), and blood poisoning (26). Protein C exists as the zymogen of a serine protease that undergoes a species-specific activation by thrombin (27). Activated Protein C inhibits further generation of fibrin clots by proteolytic cleavage of Factor VIIIa and Factor Va (28). While several different forms occur in human plasma, the most prevalent hPC form consists of a 62,000 M_r glycoprotein with four N-linked glycosylation sites, 12 intrachain disulfide bridges, and one beta-hydroxylated aspartic acid residue (29). The mature zymogen structure results from proteolytic cleavages of prepro-hPC which remove signal and propeptide sequences that have been predicted to be 33 and 9 amino acids in length, respectively (29). In 70-95% of plasma-derived hPC, a dipeptide at amino acids 156-157 has also been removed to yield a heterodimeric form of hPC consisting of a 41,000 M_r heavy chain and a 21,000

M_r light chain (27) linked by a single disulfide bridge (30,31). Within the first 29 amino acid residues of the light chain, there are 9 gla residues which are essential for the anti-clotting function of hPC (20,27,29).

The structure and function of hPC make it one of the most complex members of the VKD protein family and efforts to express Protein C in recombinant cell lines have had limited success (2,5-7). Of the many cell lines, including those derived from human liver[5] and mouse mammary tissue (6), only human kidney 293 cell line (2,32) has produced fully functional recombinant Protein C (rhPC) at 1-25 μg $(10^6 \text{ cells})^{-1}$ $(24 \text{ hours})^{-1}$. However, the rhPC produced by the 293 cells had both structural and functional properties which differed from those of plasma-derived hPC (2,32). We present a study which demonstrates the ability of murine mammary tissue to produce biologically active rhPC which closely resembles the native population of hPC.

MATERIALS AND METHODS

DNA construct. Plasmid containing the murine Whey Acidic Protein-human Protein C (WAPPC-1) hybrid gene was received as a gift from Christoph Pittius and Lothar Hennighausen (Molecular Genetics Laboratory, NIH, Bethesda, MD). The WAPPC-1 construct (Figure 1) was purified by digesting plasmid DNA with restriction endonuclease EcoRI. The WAPPC-1 construct was purified from the plasmid DNA using a GEN PAC FAX (Millipore Corp., Milford, MA) high performance liquid

chromatography (HPLC) column. The elution conditions for the WAPPC-1 product were determined by gradient chromatography over the range 0.5-1.0 M NaCl. An isocratic elution condition was chosen (25 mM Tris-HCl, 1.0 mM EDTA, and 0.63 M NaCl, pH 7.5). Approximately 15-20 μg of digested DNA was injected per run, and eluents containing the WAPPC-1 fragment from each injection were pooled, precipitated, and processed by HPLC a second time. The purity and concentration of WAPPC-1 were determined on a 1% agarose gel stained with ethidium bromide.

Transgenic Mice. Female CD-1 mice (Charles River Laboratories, Willmington, MA) 3-4 weeks of age were superovulated with interperitoneal injections of 10 I.U. Pregnant Mare's Serum Gonadotropin (Diosynth Inc., Chicago, IL) followed by 5 I.U. human Chorionic Gonadotropin (hCG; Sigma Chemicals, St. Louis, MO) 48 hours later and then placed with stud males. Embryos were then collected 21-23 h after hCG. One-cell embryos were microinjected with 1-3 pl of DNA solution (3.3 μg/ml DNA, 10 mM Tris-HCl, 0.25 mM EDTA, pH 7.4) according to Brinster et al. (33). Surviving embryos were transferred to pseudopregnant females (20-30 embryos per recipient).

DNA Extraction. Tail tissue was biopsied from pups which were approximately 20 days old and DNA was isolated from the tissue by a modification of the procedure developed by Marmur (34). Briefly, 840 μl of lysing solution (50 mM Tris-HCl, 0.15

M NaCl, 1 M Na_2ClO_4, 10 mM EDTA, 1% sodium dodecylsulfate, 1% 2-mercaptoethanol, 100 μg/ml Proteinase K, pH 8.0) was added to each tube containing a tissue sample which had been previously frozen in liquid nitrogen. The tubes were incubated overnight at 50 °C and then extracted with 250 μl of chloroform: isoamyl alcohol (25:1) by mixing for 10-15 seconds on a "Mini Bead-Beater" (Biospec Products, Bartlesville, OK), followed by centrifugation for 10 minutes at 15,000 x g. DNA was precipitated by adding 50 μl of isopropyl alcohol to 83 μl of the aqueous supernatant, then centrifuged and washed with 80% ethanol. The pellets were dried at 37 °C and suspended in 50 μl TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) and stored at -20 °C until assayed by Polymerase Chain Reaction (PCR).

PCR Analysis. Analysis was done by the general method of Saiki et al. (35). One μl of DNA solution was used as template in 25 μl reaction volumes [1X Taq buffer, 2.5 μM dNTP's, 0.5 μM oligonucleotide primers, 0.625 units Taq polymerase (Promega Corp., Madison, WI)]. Primers used to amplify a 402 bp target sequence in the transgene were: WAP-specific sense 5'-GTG GCC AAG AAG GAA GTG TTG, and hPC-specific antisense 5'-GTG CTT GGA CCA GAA GGC CAG. The WAP-specific antisense primer 5'-GAC TTG TTC CTC TAG GTT CTG was also added to amplify a 222 bp fragment contained within the endogenous WAP gene. Initial denaturation was performed at 96 °C for 1 minute, followed by 40 cycles of: 55 °C annealing for 2 minutes, 77 °C elongation for 75 seconds, and 96 °C denaturation for 15 seconds.

Amplification products from mouse tail DNA were run concurrently with those from plasmid DNA on 1% agarose gels stained with 0.5 μg/ml ethidium bromide.

Mouse Milk Collection and Preparation. Females were removed from their pups for approximately 1 hour prior to milking to allow for milk accumulation. These females were then anesthetized with Metofane (methoxyfluorothane; Pitman-Moore, Washington Crossing, NJ) and induced to let down milk by intramuscular administration of 5.0 I.U. oxytocin (Vedco Inc, St. Joseph, MO). Milk was collected into 1.8 ml screw cap microcentrifuge tubes using capillary tubes (Kimax brand, 2.0 mm I.D.) which were flame polished to prevent tissue damage. The capillary was partially inserted into a stoppered hand held receiving chamber containing the microfuge tube. The milk was collected from the capillary directly into the microcentrifuge tube while operating the receiving chamber at 12 cm H_2O vacuum. Upon collection of 150 to 500 μl of milk, the tubes were stored at -90 °C until the final whey preparation stage.

To maximize recovery of whey-soluble proteins (including Protein C), the whole milk was diluted with 3 volumes of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.2). Individual samples were then ultracentrifuged at 115,000 x g for 30 minutes at 4 °C. The buffer-expanded whey phase was pipetted from the heavy pelleted precipitate and lighter lipid layer, placed in a clean tube and immediately frozen to -90 °C.

Diluted control mouse whey was identically prepared.

Antigen Assay Using Metal-Dependent 7D7B10 Monoclonal Antibody. Each whey sample was diluted to 0.5 OD_{280} using TBS, which corresponded to a concentration of approximately 0.5 mg total protein per ml whey. Reference dilutions of plasma-derived hPC were made (over the range 0.003-2.0 μg hPC/ml whey) using control mouse whey prepared in TBS. Microtiter plate wells (96 well; Dynatech, Alexandria, VA) were coated with 7D7B10 Monoclonal antibody by overnight incubation at 4°C with 100 μl per well of 2 mg Mab /ml (0.1 M $NaHCO_3$, 25 mM EDTA, pH 9.3). The wells were then washed 4 times with TBS-TWEEN-EDTA (TBS, 0.05% Tween-80, 25 mM EDTA, pH 7.2). Whey samples consisting of 50 μl of either hPC standard or unknown were applied in triplicate to wells which contained 50 μl of TBS-PEG-EDTA buffer (TBS, 1 mg/ml 25K polyethylene glycol, 25 mM EDTA, pH 7.2), and then incubated for 3 hours at room temperature, and then washed 4 times with TBS-PEG-EDTA. The bound hPC or rhPC was then detected by sandwich enzyme linked immunosorbent assay (ELISA). A 100 μl aliquot of a 1:1000 dilution of anti-hPC polyclonal antibody (American Bioproducts Co., Parsippany, NJ) prepared in TBS-PEG-EDTA was incubated in each well for 3 h at room temperature. The microtiter plates were then washed 4 times with TBS-PEG-EDTA and similarly incubated with 100 μl of a 1:1000 dilution of rabbit anti-mouse IgG-horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, MO). The microtiter plates were again washed 4

times with TBS-PEG-EDTA and developed by addition of 100 µl of ortho-phenylene diamine (2.56 mg/ml) in OPD buffer (0.1 M Citrate-phosphate, pH 5.0, Abbott Inc., Chicago, IL). The reaction was stopped after 4 minutes with 100 µl of 3 N H_2SO_4 per well (36). The absorbance (OD_{490} nm) of the reaction product was read within 30 minutes of when the reaction was stopped.

Immunopurification. Immunoaffinity isolations of rhPC from whey and hPC from Cohn IV-1 paste were done using the conformation-specific, metal-dependent monoclonal antibody (Mab) 7D7B10 (1,24,37) which was immobilized on Affiprep-10 (Bio-Rad Laboratories, Richmond CA) at 1-2 mg Mab/ml gel (24). Expanded mouse whey, prepared in the manner described above, was thawed at 4 °C and filtered on ice through semi-crimped rapid filter paper (product 9-795, Fisher Scientific, Pittsburgh, PA). The total protein concentration was adjusted to less than 10 mg/ml with TBS-EDTA (TBS adjusted to a final concentration of 25 mM EDTA). The diluted whey was loaded batchwise onto the 7D7B10/Affiprep-10 immunosorbent for 3-4 hours at 4 °C. The gel was centrifuged at 3000 x g for 5 minutes and loaded onto a 1 cm x 10 cm column (Pharmacia, Piscataway, NJ). The column was washed with TBS-EDTA at a flow rate of 30 ml/hour. The immunosorbed rhPC was eluted with TBS plus 25 mM $CaCl_2$. The column was then stripped with a step change to 4 M NaCl followed by a step change to 2M NaSCN solution and then equilibrated with TBS-EDTA. The 25 mM $CaCl_2$, CaSCN, and NaCl eluates were dialyzed against deionized water at 4 °C

for 12 h and then lyophilized at 0.1 torr.

SDS-PAGE. Samples of immunopurified rhPC were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 0.1% SDS, 10% acrylamide) under either reduced or non-reduced conditions according to Laemmli (38) and stained (0.125% Coomassie Blue G-250, 50% methanol, 10% acetic acid). Identical SDS-PAGE gels were prepared and a Western immunoblot was performed according to the method of Towbin (39).

Activated Partial Thromboplastin Time (APTT) Assay. The biological activity was measured by delay in coagulation time obtained for rhPC and hPC samples prepared in hPC-deficient plasma (American Bioproducts Inc., Parsippany, NJ). APTT reagent (Organon Teknika Corp., Durham, NC) included Protac (*Agkistrodon Contortrix* venom, American Diagnostica Inc., Greenwich, CT) to specifically activate hPC or rhPC prior to initiating coagulation by adding $CaCl_2$ (40). Clotting times were recorded with an Electra 750A Coagulation Timer. A reference anticoagulation curve was prepared using normal plasma reference pool (NPRP) whereupon the specific activities of Protein C in the samples were calculated assuming a theoretical activity of 1 unit per ml of NPRP and 4 μg/ml hPC antigen in NPRP.

RESULTS

A total of 105 mice were born from recipients which had received embryos microinjected with the WAPPC-1 construct. Polymerase chain reaction analysis of tail DNA indicated that 30 mice contained the transgene (Table 1). Figure 2 demonstrates the facile nature and reliability of the detection of transgenic mice using the 3 primer PCR method described above. Amplifications using transgenic mouse DNA as template result in the formation of two amplification fragments. A predominant band appears at 402 bp while a minor band occurs at 222 bp. These are the expected amplification fragment sizes for the transgene and the endogenous WAP, respectively. In contrast, DNA samples obtained from control mice containing only the single copy endogenous WAP gene produce a single PCR product at 222 bp. Of 16 transgenic females generated, 6 were tested for expression and all 6 expressed hPC antigen in their milk at levels of 1.0-3.0 μg/ml (Table 2). These animals appeared to exhibit a normal physiology. The hPC antigen levels were relatively stable with respect to the day of lactation, but the highest concentrations were observed for days 8-12.

Collection of milk 3 times per lactation using oxytocin yielded an average of 1 ml milk per mouse. All milk samples from founder animals and their transgenic offspring were pooled and subjected to immunopurification using the 7D7B10 metal-dependent, conformation-specific monoclonal antibody. No hPC antigen was found to be specifically eluted by Ca^{2+}, NaCl or CaSCN from purifications using control mouse

whey pool (data not shown). Thus, no evidence of cross-reactivity between endogenous mouse milk proteins and the anti-hPC 7D7B10 Mab was seen during immunopurification. An overall hPC antigen yield of 46% was obtained for the Ca^{2+}-specific immunopurification from transgenic mouse whey pool (Table 3).

The immunopurified material was evaluated by SDS-PAGE (Figure 3) and compared to identical Western analysis (Figure 4 a,b). Both the nonreduced Coomassie blue stained SDS-PAGE and the Western immunoblot of rhPC and hPC showed the presence of multimeric bands of approximately 58,000 M_r. Protein C antigen assignment was made for the bands appearing on reduced SDS-PAGE stained with Coomassie blue by comparison with equivalent Western analysis. Hence, both immunopurified hPC and rhPC products were determined to be greater than 95% pure by densitometry of the stained SDS-PAGE. The apparent heavy chain forms of rhPC occurring at approximately 40,000 M_r on reduced Western blot were slightly faster than hPC in electrophoretic mobility. The rhPC heavy chain forms were similar in relative amounts to those of hPC as determined by densitometry of reduced and stained SDS-PAGE (densitometry data not shown). The slightly faster mobilities for rhPC heavy chains were estimated to be less than 1-2 kd M_r than for the heavy chains of hPC. A heavy chain form intermediate to apparent alpha and beta heavy chain species was visible in Western analysis of rhPC and hPC. This intermediate band was more pronounced and well-resolved from the apparent beta heavy chain of rhPC than for the

beta heavy chain of hPC. A ratio of 70% alpha to 30% beta-plus (intermediate plus beta form) of total heavy chains and less than 1% gamma form of the heavy chain was observed for both rhPC and hPC on stained SDS-PAGE (densitometry data not shown). The single chain form was present at less than 17% of total stained protein for both immunopurified rhPC and hPC (densitometry data not shown) although single chain rhPC appeared to be more immunoreactive than single chain hPC on reduced Western blot (Figure 4b). The light chain of rhPC, appearing at approximately 21,000 M_r, migrated slightly faster than that of hPC with the apparent M_r difference being less than 1-2 kd. The percentage of theoretical specific anticoagulant activity of the rhPC (74% ± 2%) was essentially equivalent to that of the hPC (84% ± 14%) reference material (Table 3) as measured by APTT assay.

DISCUSSION

WAP regulatory elements have been demonstrated previously to direct the expression of heterologous genes in the mammary tissue of mice (10) and pigs (13). Here, a WAP genomic clone was used to drive the expression of the cDNA for human Protein C into the milk of transgenic mice. These studies in mice were important for demonstrating the capability of the murine mammary gland to produce a biologically active VKD-protein throughout lactation. The biologically active rhPC was produced by transgenic mice at levels of 0.5-3 μg/ml milk with milk letdowns approximately every hour. While the amino acid sequence of the rhPC has not been determined, the

high specific anticoagulant activity of the rhPC is strong evidence for the presence of a functional serine protease catalytic site and properly carboxylated glutamic acid residues. These gla residues are indicative of a functional membrane binding domain (20). Our results strongly contrast the very low specific activity reported for the VKD-Factor IX expressed in the milk of transgenic sheep at only 25 ng/ml (17). Furthermore, the extent of proteolytic removal of the dipeptide appears to be similar to that in human plasma, since the ratio of single chain to two chain form for rhPC was within the published range of 5-30% for hPC (30,31). The multiple forms of heavy chain hPC species have been attributed previously to different sites of glycosylation (2,31). The ratio of apparent tri-glycosylated (alpha-form heavy chain) to di-glycosylated (beta-form heavy chain) forms of dimeric rhPC appears similar to that of the hPC and thus the glycosylation site selectivity of the murine mammary gland appears comparable to that of human liver. The very slight differences in M_r between the heavy chain forms of hPC and rhPC may be due to slight differences in branched carbohydrate structure. The similarity in light chain mobility observed of both rhPC and hPC is consistent with a comparable incidence of N-linked glycosylation which occurs at ASN97 (29,31). However, the extent of propeptide cleavage has not yet been determined for rhPC. Direct chemical analyses of the carbohydrate content and amino acid sequence of rhPC are currently being performed.

This study of the expression of rhPC in the milk of transgenic mice

demonstrates that the murine mammary tissue can perform a variety of complex post-translational modifications which had not been shown in previous studies. In addition, the present study provides valuable information about the functionality of the WAPPC-1 construct. Both the transgene and its ability to express rhPC appear to have been transmitted to offspring in a Mendelian fashion (data not shown). We are currently studying the differences in WAPPC-1 gene structure between generations of mice. In addition, we are awaiting results from the lactation of transgenic swine containing the WAPPC-1 transgene. Upon completion of the latter studies, more will be known about the apparent conservation of WAP regulation that exists between mice (10) and pigs (13). Most importantly, knowledge will be gained about the feasibility of using the mammary gland of animals as a bioreactor for production of complex therapeutic proteins.

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Table 1. Mouse embryo microinjection, transfer, and founder animal generation data.

Production Stage	Number
Embryos injected	2336
Embryos transferred	1808
Number recipients	58
Embryos per recipient	31
Number pregnant	24
Number pups alive	105
Number pups transgenic	30
Percent live pups transgenic	29
Number transgenic pups female	16
Number assayed for expression	6
Number expressing	6

Table 2. Expression levels of six transgenic founder female mice as detected by sandwich ELISA using 7D7B10 monoclonal antibody mass capture.
- Not analyzed.

MOUSE ID	Expression of rhPC Antigen (μg/ml) Day of Lactation			
	5-6	8-9	11-12	13-15
R3	1.90	2.88	3.01	-
R12	-	2.98	2.48	2.40
Y51	-	2.80	1.30	1.79
Y52	-	1.52	-	0.95
Y57	1.47	-	0.98	-
Y68	-	1.05	-	-

Table 3. Immunopurified recombinant protein C from the transgenic mouse whey pool, recovery data from immunoaffinity chromatography, anticoagulant activity, and comparison to anticoagulant activity of human plasma-derived protein C. A total of 40 mice (founder mice and their offspring) were milked and 30 ml of milk was pooled and used for immunopurification of rhPC.

*Theoretical APTT specific activity based upon 1 unit/ml for normal human plasma pool at 4 μg hPC antigen/ml plasma. All samples were preactivated with venom of *Agkistrodon contortrix* (40).
**Percentage of theoretical specific activity calculated on a per mg basis of hPC antigen as detected by ELISA.
+The milk of transgenic founder animals and their offspring were combined to form a single pool.

Material Assayed	hPC Antigen Total (μg)	hPC Antigen % Yield	% Theoretical Anticoagulant Activity**
Whole Milk Pool+	30	-	-
Whey Fraction	30	100%	57 ± 10
Immunopurified Product	14	47%	74 ± 2
Plasma Reference*	-	-	84 ± 14

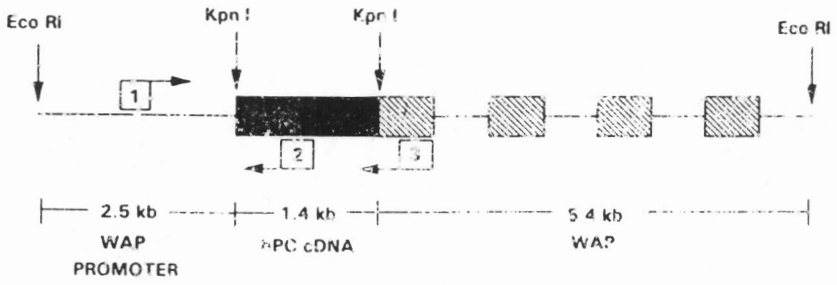

Figure 1. Schematic diagram of the Whey Acidic Protein-Human Protein C fusion gene. The cDNA for hPC was inserted into the KpnI restriction site present just before exon 1 of WAP. The boxes marked 1, 2, and 3 schematically represent the relative sequence positions of oligonucleotide primers. Solid lines represent non-coding WAP DNA and/or WAP introns, the solid box represents cDNA encoding hPC, and hatched boxes indicate WAP exons. Priming of DNA synthesis with three oligonucleotides as indicated permits simultaneous detection of both the transgene and the endogenous WAP gene in transgenic mice. Only the smaller target sequence (primed by oligonucleotides 1 and 3) is detected in non-transgenic mice.

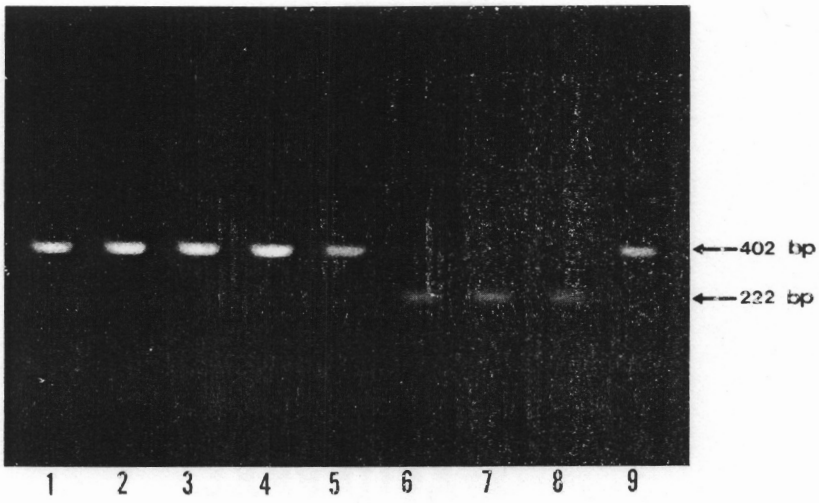

Figure 2. Example of detection of the WAPPC-1 transgene in mouse tail DNA by Polymerase Chain Reaction. Lanes 1, 5, and 9 are 402 bp amplification products of plasmid DNA containing the WAPPC-1 construct. Lanes 2-4 show the 402 bp target fragment indicating the presence of the WAPPC-1 transgene in mouse tail DNA. Lanes 6-8 are amplifications of control mouse tail DNA's showing only a 222 bp fragment within the endogenous WAP gene. DNA templates consisted of either 10,000 plasmid copies for the standards (Lanes 1, 5 and 9), or 10,000 genomes for the transgenic mice (Lanes 2-4) and control mice (Lanes 6-8).

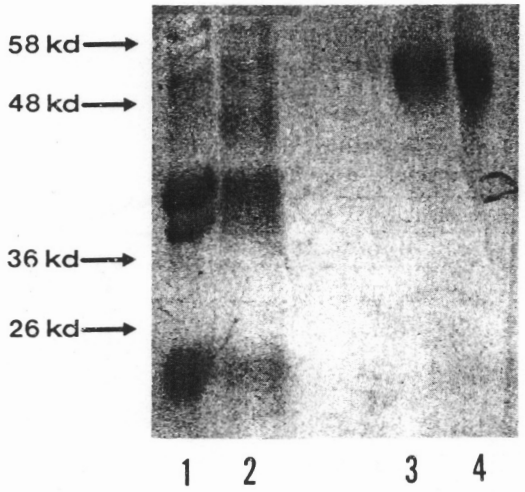

Figure 3. Sodium dodecylsulfate (0.1%) polyacrylamide gel (10%) electrophoresis of rhPC which was immunopurified from transgenic mouse whey pool. Lanes 1 and 4 contain 5 μg of rhPC reference, reduced and nonreduced, respectively. Lane 2 and 3 contain 5 μg of hPC reference, reduced and nonreduced, respectively.

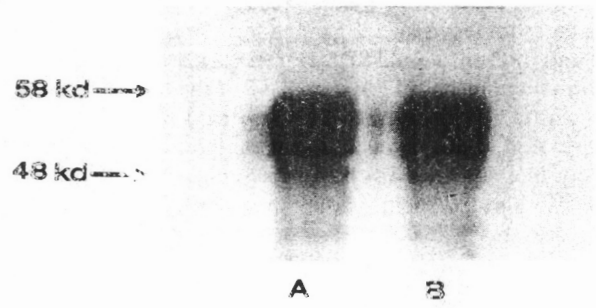

Figure 4A. Western immunoblot of nonreduced rhPC which was immunopurified from transgenic mouse whey pool. Lanes A and B contain 250 ng of reference hPC and rhPC, respectively.

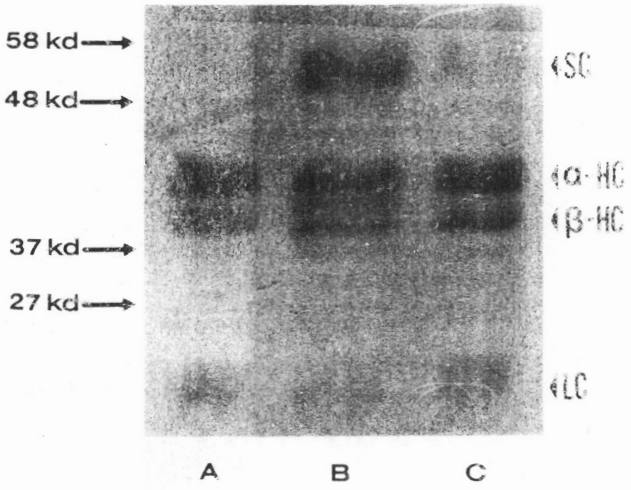

Figure 4B. Western immunoblot of reduced rhPC which was immunopurified from transgenic mouse whey pool. Lanes A and C contain 250 ng of reference hPC. Lane B contains 250 ng of rhPC. The relative locations of single chain (SC), α- and β-heavy chain (HC), and light chain (LC), are indicated on the right side.

CHAPTER III.

ANALYSIS OF TRANSGENE DETECTION DURING EARLY EMBRYONIC DEVELOPMENT AFTER PRONUCLEAR MICROINJECTION OF DNA

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ABSTRACT

A polymerase chain reaction (PCR) technique was used to detect transgene presence after pronuclear microinjection of mouse zygotes cultured to various stages of development. The transgene was detected in 88% of 1-cell, 88% of 2-cell, 44% of 4-cell, 40% of morula, and 29% of blastocysts. By comparison, the integration frequency for transgenic mice made using the same DNA construct was 22%. After 5 days of *in vitro* culture, embryos that were either developmentally arrested or fragmented were tested for the presence of the injected DNA. The injected construct was detected in 83% of arrested 1-cell, 85% of arrested 2-cell, and 85% of fragmented embryos. Only 28% of zygotes cultured after microinjection of DNA developed to the blastocyst stage compared to 74% of noninjected zygotes. When DNA buffer alone was injected, 63% of zygotes developed to the blastocyst stage. These data suggest that pronuclear microinjection of DNA is highly detrimental to subsequent embryonic development. Also, most injected DNA that is either unintegrated or that will not be integrated into the genome has been degraded by the blastocyst stage such that it can no longer be detected by PCR.

INTRODUCTION

Transgenic animals have become a powerful tool for biotechnology and biological science. Applications in biotechnology include the production of farm animals which produce useful recombinant versions of therapeutic proteins in the milk

and blood. Transgenic animals also have been used as models to help elucidate the role of genes and gene products in disease processes. Transgenic technology can potentially improve food and agricultural products.

Pronuclear microinjection of foreign DNA has been the most commonly used technique to produce transgenic animals (Gordon et al., 1980). Several factors thought to influence the efficiency of transgene integration have been studied. Specifically, the effects of injection buffer composition, DNA concentration, molecular form of the DNA (linear or supercoiled), form of the ends of linear DNA (similar, different, or blunt), and injection site (male or female pronucleus, and cytoplasm) upon integration efficiency have been assessed (Brinster et al., 1985). The integration efficiencies were optimal when about 750 copies of linear DNA fragments in 10 mM Tris-HCl and 0.1 mM EDTA at pH 7.5 were injected into the male (larger) pronucleus of mouse zygotes. While the EDTA was not absolutely essential for transgenic animal production, embryonic viability was best when 0.1 to 0.3 mM EDTA was used. This study also showed that the presence of $MgCl_2$ was detrimental to embryonic viability when used above 1.0 mM and it did not appear to affect integration frequency of foreign DNA at 1.0 mM.

Even with optimized injection conditions, the integration frequency by pronuclear microinjection produces transgenic animals in only about 10-30% of live

births. In addition, many founder transgenic animals contain a mosaic pattern of integration; having less than one copy of the construct per cell. *In vivo* recombination events have been studied in attempts to elucidate the process by which foreign DNA is integrated into an animal's genome (Bishop et al., 1989; Ninomiya, et al., 1989; Lin et al., 1987; and Brinster et al., 1985). Most studies are consistent with the model that integration involves the disruption of host DNA with subsequent repair resulting in insertion of the foreign DNA.

Burdon and Wall (1992) employed the polymerase chain reaction (PCR) DNA amplification technique to detect the presence of DNA in isolated blastomeres of embryos that were microinjected at the single cell stage and cultured to various stages of development. Due to the low amounts of foreign DNA that must be detected in a small quantity of genomic DNA, it was not possible to perform southern hybridization experiments at this early stage in development to determine at which stage the foreign DNA becomes integrated into the genome. When whole embryos were assayed by PCR, the foreign DNA was detected in 100% of 1-cell, 2-cell, and 4-cell embryos. When blastocysts were assayed after 5 days of *in vitro* culture, the detection frequency of the transgene (26%) was higher than that for live born mice (17%) transgenic for the same DNA construct. In addition, the foreign DNA was present in only one or two of the blastomeres isolated from an eight-cell embryo. The results of this study suggest that integration of foreign DNA occurred after the first cell division, and

maybe as late as the eight-cell stage.

A gene expression assay was employed by Takeda and Toyoda (1991) in order to study similar events. The assay used a plasmid DNA construct containing the *lacZ* coding sequence driven by the SV40 constitutive promoter. The purpose of this study was to determine the point in early mouse embryonic development where the individual blastomeres become capable of transcribing, and translating foreign DNA after pronuclear microinjection. Since transient expression of foreign DNA is possible in mammalian cells (Wu and Wu, 1987), it was not possible to determine whether or not the foreign DNA was integrated. These studies found no detectable expression in 2-cell embryos, while 7.4% of 4-cell embryos expressed the *lacZ* gene. In addition, expression was seen in 38.1% of the 8-16 cell (morula-stage) embryos. Blastocyst-stage embryos had a lower expression frequency of 20.9%. While the above data characterized the expression frequency in viable embryos, the expression frequency in arrested embryos was significantly higher with 69.2% and 46% expression in arrested morulae and blastocysts, respectively. These studies showed that microinjection of the SV40-*lacZ* transgene into the pronucleus may be detrimental to further development of the embryo, but does not inhibit DNA transcription and mRNA translation. All of the embryos analyzed for *lacZ* expression using histochemical staining exhibited a mosaic pattern of transgene expression. In summary, these results are consistent with the mosaicism found by Burdon and Wall (1992) who assayed individual blastomeres for

presence of the transgene.

While the above studies focused on the chronology of transgene integration, the ability to detect the presence of specific DNA sequences in small numbers of cells has other advantages. This application is particularly of interest for determining the sex of preimplantation embryos. For example, the bovine embryo transfer industry would benefit from being able to select embryos for production of calves having a preferred sex for a specific application. Many embryo sexing protocols have been developed (Aesen et al., 1990; Bradbury et al., 1990; Kunieda et al., 1992; Strom et al., 1991). For example, successful sex determination of mouse embryos using PCR amplification of the *Sry* and *Zfy* Y chromosome-specific DNA sequences from blastomeres isolated as early as the 2-cell stage has been shown (Kunieda et al., 1992).

The prenatal detection of genetic defects in embryos fertilized *in vitro* from couples having known genetic disorders is desired. For example, detection of genotypes identified with risk of maternal alpha-1-antitrypsin deficiency has been shown by analysis of the first polar body of oocytes (Verlinsky et al., 1990). The first polar body contains genetic material arising from the first meiotic division, therefore, has a haploid number of chromosomes. If genetic crossover has not occurred during meiosis I, then the genotype of the first polar body is opposite that of the oocyte. Therefore, oocytes whose polar bodies are positive for the genetic defect can be used

for in vitro fertilization and transfer, resulting in offspring free from the abnormal genotype.

In order to accurately perform any of the analyses discussed above, a reliable detection assay for the presence of specific DNA sequences in either single cells or small cell numbers is needed. Possibly one of the most important parameters determining whether DNA from single blastomeres can be reproducibly amplified by PCR is the treatment of the embryo or blastomere prior to amplification of DNA. For successful amplification of target DNA sequences, the genomic DNA must be released from the cell and freed from binding proteins (nucleosomes). Several treatment methods have been described and used. However, most of them have limitations in reproducibility and therefore reliability when small numbers of cells are involved. The efficiency of PCR may be enhanced by repeated heating and cooling cycles of the cells simply placed in reaction buffer in the absence of Taq polymerase (Kuneida et al., 1992; Mercier et al., 1990). However, the target sequences were only efficiently amplified using two-step PCR which requires additional steps and takes longer to complete. When single-step PCR was used, amplification of these multiple copy *Sry* and *Zfy* sequence was not obtained. Some investigators have simply plunged the embryo, blastomere, or biopsy (Aasen et al., 1990; Bradbury et al., 1990; Holding et al., 1989; King et al., 1990; Kunieda et al., 1992; Verlinsky et al., 1990) into a tube of sterile H_2O and relied on osmotic shock to lyse the cells. Visual inspection of the one-

cell mouse embryo lysis due to osmotic shock conducted in our laboratory showed that this procedure was slow (requiring at least 10 min) and was variable from embryo to embryo. Rapid repetitions of freezing and thawing seemed to accelerate the process, but the pronuclear structures containing the DNA were still slow to disappear. This may explain why DNA amplification using this technique for whole cells is somewhat inefficient and unreliable (Aasen et al., 1990; Bradbury et al., 1990; King et al., 1990; Verlinsky et al., 1990).

Perhaps the most efficient embryo preparation procedure used to date is the complete digestion of all protein and solubilization of other cellular material using mild nonionic detergents (Granada, Inc. Sexing Protocol, courtesy of Rebecca Krisher). The disadvantage of this protocol was that large amplification volumes were used (100 μl) which is expensive for a small laboratory needing to process many samples and it provides much excess amplification product over what is needed to determine the PCR results using ethidium bromide stained agarose gels. Also, the DNA sequences that were amplified were present within multiple copy genes (approximately 1000 copies per genome). Therefore, it is not known whether this protocol can be used for small reaction volumes (25 μl) where a single copy gene must be detected from only one or a few cells.

We have compared three different embryo preparation protocols to determine

the method which gives the most efficient PCR amplification of the single copy Whey Acidic Protein (WAP) gene in mouse blastocysts. First, embryos were simply be plunged into H_2O and frozen. Second, the embryos were dehydrated using 100% ethanol and then plunged into tubes containing H_2O and frozen. Third, embryos were subjected to protein digestion with proteinase K in a nonionic detergent mixture and heated to inactivate the proteinase K before adding the reaction mixture. The preparation method yielding the most reliable detection of the single copy WAP gene in blastocysts was then tested on mouse embryos at various stages in development.

We also have applied the optimized PCR DNA amplification technique to detection of foreign DNA which was microinjected into the pronuclei of mouse zygotes. Microinjected zygotes were cultured *in vitro* to specific points in development and then subjected to PCR analysis for transgene detection. The transgene detection frequencies obtained for 1-cell, 2-cell, 4-cell, morula, and blastocyst stage embryos were compared. Embryos that had arrested in development also were analyzed for transgene detection and the detection frequency was compared to that of viable embryos. This comparison was done to assess the potential toxic effects of pronuclear microinjection of DNA since a much lower percentage of DNA microinjected embryos have been reported to survive to the morulae/blastocyst stage in culture (81% versus 100%, Brinster et al. 1985).

The detection frequency determined in morula and blastocyst stage embryos compared to the known integration frequency for the same DNA construct in live mice should provide insight into the confidence of employing the PCR technique to screen demi-embryos or embryo biopsies for the transgene before transfer into recipients. Although this technique does not directly distinguish between integrated and episomal construct DNA, it may be used to at least, cull from transfer, embryos that definitely do not contain detectable copies of the transgene.

MATERIALS AND METHODS

Mice. The mice used in this study were CD-1 white Swiss mice (Charles River Laboratories, Madison, WI). Immature female mice 24 to 30 days old were superovulated by intraperitoneal injection of 10 I.U. Pregnant Mare's Serum Gonadotropin (PMSG, Dyosynth, Chicago, IL), followed by 5 I.U. of human Chorionic Gonadotropin (hCG, Henry Schein, Inc., Port Wahibgton, NY) 46 to 48 hours later. Mice were mated to males between 3 and 6 months of age by placing the females with males (one female per male) at the time of the hCG injection. Zygotes for pronuclear microinjection were collected 21 to 24 hours after the hCG injection according to standard methods (Hogan et al., 1986).

DNA Preparation. The DNA construct (designated WAPPC-3) was a hybrid construct consisting of the 1.6 kbp cDNA clone encoding human Protein C (hPC)

inserted into the start codon (ATG) of the mouse Whey Acidic Protein (WAP) gene. The WAP clone was the 7.2 kb fragment obtained from Lothar Hennighausen (Laboratory of Biochemistry and Metabolism, The National Institutes of Health). The construct was cloned using JM109 E. Coli in the plasmid pUC-19. The plasmids were isolated by alkaline lysis (Sambrook et al., 1989) and the construct was digested away from the cloning vector with the restriction endonuclease EcoRI. The WAPPC-3 construct was purified from the cloning vector using reverse phase HPLC. The DNA used for microinjection was precipitated, dried and reconstitute in microinjection buffer (10 mM Tris-HCl, 0.25 mM EDTA, pH 7.5) according to standard methods (Brinster et al., 1985). The concentration of WAPPC-3 for microinjection was 1.5 μg/ml which corresponds to about 200-500 transgene copies per microinjection. Pronuclei were microinjected with 1-2 pl of the DNA solution according to standard methods (Hogan et al., 1986).

Embryo culture. The embryos were washed in three successive 35 mm dishes of gas-equilibrated medium (at least for 4 hours) and then placed into 10 μl drops of CZB medium (Chatot et al., 1989) in groups of 10 to 20 embryos per microdrop. The drops were placed in 35 mm petri dishes and covered with paraffin oil (Sigma Chemical Co., St. Louis, MO) to prevent medium evaporation. The embryos were cultured at 37 °C in a humidified (95%) incubator (Haraeus Instruments, Inc., South Plainfield, NJ) maintained in an environment of 5% CO_2 in air. Culture times of 1 hour, 2, 3, 4, and

5 days were used for embryo developmental stages of 1-cell, 2-cell, 4-cell, morula, and blastocyst, respectively.

Embryo Preparation. Three different embryo preparation methods were tested using noninjected mouse blastocysts. This analysis was done to determine the most optimal method by which to treat embryos for detection of DNA fragments that are present in low copy number. (1) Blastocysts were placed into 5 µl of sterile H_2O in a 0.5 ml microcentrifuge tube, overlaid with 25 µl of paraffin oil, and the tubes frozen to -85 °C until the PCR analysis was done. (2) Blastocysts were dehydrated after washing in holding medium by plunging them into 100% ethanol. They were then pipetted into 5 µl of sterile H_2O in a 0.5 ml microcentrifuge tube, overlaid with 25 µl of paraffin oil, and the tubes frozen to -85 °C until the PCR analysis was done. (3) Blastocysts were placed into sterile 0.5 ml microcentrifuge tubes containing 5.0 µl of Embryo Lysing Buffer (ELB: 20 mM Tris-HCl, pH 8.3; 0.9% Tween-20; 0.9% nonidet P40; 400 µg/ml Proteinase K) using a sterile borosilicate glass pasteur pipette and overlaid with 25 µl of paraffin oil. The tubes were frozen to -85 °C until the PCR analysis was done.

Detection of microinjected DNA was done by PCR at various times during in vitro embryonic development using the best embryo preparation method. Embryos were removed from the incubator at the desired stage of development and rinsed with

3 ml of holding medium in three (3) successive 35 mm petri dishes. They were then placed into sterile 0.5 ml microcentrifuge tubes containing 5 µl of ELB using a sterile borosilicate glass pasteur pipette. The embryos were transferred with less than 1 µl of medium since the surface tension of the ELB was allowed to pull the embryo out of the pipette tip. The ELB containing the embryo was then overlaid with 25 µl of paraffin oil to prevent evaporation during the reaction. The microcentrifuge tubes containing the embryos were stored at -85 °C until used in PCR. analysis.

Polymerase Chain Reaction. Reactions were carried out in 25 µl volumes (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.1% Triton X-100; 0.2 mM each of dGTP, dATP, dCTP, dTTP; 1.5 mM $MgCl_2$; 0.5 µM each oligonucleotide primer; and 0.625 units of Taq Polymerase, Perkin Elmer Co., Rockville, MD). Each well in the thermocycler (Perkin Elmer Co., Rockville, MD, Model 480) was filled with 25 µl of paraffin oil to ensure efficient heat transfer between the microcentrifuge tube walls and the walls of the heating block wells. The tubes containing embryos in the ELB were placed into the thermocycler along with tubes containing serial dilutions (10000, 1000, 100, 10, and 1 genome copies; assuming 3 pg of DNA per diploid genome) of both control and transgenic mouse DNA preparations. The standard DNA samples were pipetted into microcentrifuge tubes containing 5 µl of ELB and overlaid with paraffin oil and treated in exactly the same manner as the embryos.

In treatments (1) and (2) listed above, the tubes were thawed and soaked at 98 °C for 10 min to inactivate DNases. The temperature was lowered to 85 °C for the addition of 20 µl of reaction mixture. In treatment (3), the tubes were thawed and soaked for 30 min at 55 °C to digest cellular and nuclear protein and therefore release the embryonic DNA into solution. The proteinase K was then heat inactivated by a soaking step at 98 °C for 10 min. The thermocycler was cooled to 85 °C for addition of 20 µl of reaction mixture to each microcentrifuge tube. The "hot start" PCR technique (Perkin Elmer Co., Rockville, MD) was employed in each treatment by lowering the temperature to 85 °C to prevent any nonspecific annealing of oligonucleotide primers to single stranded embryonic DNA as well as preventing any unwanted polymerase activity.

The tubes were subjected to 45 cycles of denaturation at 96 °C for 15 sec, annealing at 55 °C for 30 sec, and elongation at 75 °C for 30 sec. The G+C ratio for the particular target sequence used was 51%. Therefore, the denaturation temperature of 96 °C was needed in order to obtain adequate melting of the template DNA. Visualization of the amplification products was done using ethidium bromide-stained agarose gel electrophoresis. The 70 ml gels consisted of 1% agarose, and 0.5 µg/ml ethidium bromide in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.1). The electrophoresis was conducted using a 11 cm x 14 cm electrophoresis unit (Gibco BRL Life Technologies, Gaithersburg, MD, Model Horizon 11-14) at 74 volts for 60 min.

Gels were photographed using a 10 by 12.5 cm Polaroid camera using uv illumination.

Statistical Analysis. A Chi Square probability analysis was used to determine if values were significantly different. Each value for either developmental efficiency or PCR detection efficiency was compared to each other value within the given experiment.

RESULTS

For comparison of the three treatments, the PCR detection frequency of the single copy WAP gene in cultured mouse blastocysts was 100% (20 of 20), 100% (20 of 20), and 80% (18 of 20) for the ELB, ethanol, and water treatments, respectively. The PCR product band intensities were similar for ELB and ethanol and slightly greater than those for the water pretreatment (Figure 1). Although the ELB treatment method was not statistically better than the ethanol or water, it was chosen for analysis of microinjected DNA in cultured cells. Table 1 shows the detection of the single copy endogenous WAP gene in 1-cell, 2-cell, 4-cell, morula, and blastocyst stage embryos. The 442 bp WAP specific DNA fragment was successfully amplified in 78% of 1-cell, 88% of 2-cell, 94% of 4-cell, 95% of morulae, and 97% of blastocysts.

In pronuclear microinjected embryos, detectable copies of the transgene were found in 88% of 1-cell, 88% of 2-cell, 44% of 4-cell, 40% in morulae, and 29% of

blastocysts (Table 2). There was a significant decrease in detection frequency from 88% to 44% ($p<0.05$) between the 2-cell and 4-cell stages. There was also a further drop ($p=0.064$) in detection frequency from the 4-cell stage (44%) to the blastocyst stage (29%). An example of the detection of the WAPPC-3 transgene in blastocysts and degenerate embryos is shown in Figure 2. The detection frequency at the blastocyst stage (29%) was similar ($p<0.05$) to that for transgenic mice (22%) generated using the same DNA preparation (Table 3).

When the pool of microinjected zygotes was left in culture for the entire 5 day period, four populations of embryonic stages of development were present: 1-cell, 2-cell, degenerate, and blastocyst. These populations were collected and assayed by PCR individually to determine if any significant differences in transgene detection frequency would be obtained. The transgene was detected in 83% of 1-cell, 85% of 2-cell, 85% of degenerate, and 29% of blastocysts (Table 3). The degenerate embryos were those that had developed to some stage beyond 2-cell and fragmented making it difficult to determine the specific stage at which they ceased to develop.

The development of zygotes microinjected into pronuclei with solutions of various DNA concentration was also studied. The developmental and PCR detection results are summarized in Table 4. When buffer alone was injected, 63% (30 of 48) of the zygotes reached the blastocyst stage after 5 days of *in vitro* culture in CZB

medium. This developmental efficiency was slightly lower, but not different ($p>0.05$) than the efficiency of control (noninjected) embryo development to the blastocyst stage which was 74% (84 of 114). When DNA was injected into pronuclei at concentrations of 1.5, 15, and 50 μg/ml, 28% (89 of 323), 11% (7 of 63), and 9% (8 of 87) of zygotes developed to the blastocyst stage, respectively. The transgene was detected by PCR in 29% (26 of 89), 86% (6 of 7), and 88% (7 of 8) of blastocysts injected with DNA at 1.5, 15, and 50 μg/ml, respectively. A sample of the total population of degenerate embryos from injection of each DNA concentration was assayed for the presence of the transgene. The transgene was detected in 85% (78 of 92), 96% (27 of 28), and 97% (28 of 29) of the degenerate embryos injected with 1.5, 15, and 50 μg/ml, respectively. Also, the effect of zygote development to the blastocyst stage following injection of DNA buffer containing a higher EDTA concentration (1 mM) was evaluated. Development of zygotes to the blastocyst stage after pronuclear injection of buffer with 0.25 mM EDTA was 63% (30 of 48) compared to 59% (20 of 34) for injection with 1 mM EDTA. These results were not different ($p>0.05$).

DISCUSSION

A simple PCR-based method for reliably detecting single copy genes in preimplantation mouse embryos was developed. Embryonic DNA could be amplified by cell lysis with simple osmotic shock. However, either an ethanol or detergent and protease lysis procedures yielded a greater visible band intensity. The higher band

intensity observed with the ethanol and detergent-protease lysis was indicative of more efficient amplification of the embryonic DNA. The band intensities of PCR products from WAP gene amplification in later stage embryos were greater than those of earlier embryos suggesting a copy number dependent amplification efficiency. The detergent-protease based embryo lysis procedure was the method chosen to amplify construct DNA after pronuclear microinjection into mouse zygotes for analysis of the fate of construct DNA throughout preimplantation embryo development.

The gradual decrease in detection frequency to a level similar to that found in mice transgenic for the same DNA construct suggests that unintegrated copies of the microinjected transgene are degraded in a large population of embryos. These data suggest that by the blastocyst stage, nearly all DNA that is either unintegrated or that will not be integrated into the genome has been degraded. These results were consistent with those reported by Burdon and Wall (1992), although their detection frequency in blastocysts (26%) was slightly higher than in live mice (17%). The data from this study suggests that a single step PCR technique could be used to screen preimplantation embryo biopsies from blastocysts for transgene presence before transfer to reduce the number of nontransgenic embryos transferred to recipients. However, the degree of blastomere mosaicism present in embryos up to the 8-cell stage reported by Burdon and Wall (1992) suggests that several blastomeres need to be tested to prevent culling false negatives from transfer.

Mosaicism further complicates the diagnosis of transgenesis because a significant portion of the blastomeres in a preimplantation embryo form the fetal placenta. For example, only 7% (2 of 35) of the calves born from embryos testing positive by PCR analysis of an embryo biopsy were shown to be transgenic (Bowen et al., 1993). However, placental tissues were not analyzed for their transgenic status. In a previous study, we have shown that the transgene can be more frequently detected in placental tissue than in the fetus (Canseco et al., 1993). We have not determined if the presence of the transgene is less detrimental to placental tissue development and may present a lethal dominance in some tissues of the developing fetus. Thus, a preimplantation embryo biopsy from a microinjected embryo may be positive by PCR, but still produce a non-transgenic fetus. A recent statistical analysis of transgene mosaicism in microinjected embryos (Whitelaw, et al., 1993) demonstrated that even if chromosomal integration occurs after the first round of DNA replication, but before the second, a fetal mosaic frequency of 76% was predicted. This value was compared to an observed minimum mosaic frequency of 62%; which was based on germline mosaic animals generated in their laboratory. The prediction for mosaic frequency was made by assuming that in a 64 cell blastocyst, 15 cells comprise the inner cell mass, 45 cells comprise the trophectoderm, and 4 cells are not assigned to either the inner cell mass or trophectoderm. All cells within the inner cell mass were assumed to make up the fetus. The statistical mosaicism study by Whitelaw et al. supports the hypothesis that most chromosomal integration events may occur before the 4-cell stage

and still produce the observed percentage of mosaic fetuses. The fact that only 44% of embryos assayed at the 4-cell stage were PCR-positive compared to 29% for those assayed as blastocysts is consistent with the hypothesis that most integration events have occurred by this time and most unintegrated DNA is probably degraded.

The fact that the transgene was detected in approximately 85% of degenerate or non-developing embryos suggests two possibilities. First, the integration of foreign DNA into the genome of mouse embryos is detrimental to subsequent embryonic development in a large population (72%) of embryos. Second, the presence of DNA microinjected into the pronuclei is detrimental to subsequent development and the unintegrated DNA is never destroyed. We believe the former argument is preferentially supported by this study and the literature (Whitelaw, et al., 1993; Covarrubias et al., 1987; Brinster, et al., 1985). Specifically, the presence of increasing amounts of EDTA in the DNA injection solution has been shown to result in a higher frequency of transgenic fetus production, but with a decrease in the number of embryos resulting in live pups (Brinster et al., 1985). It was not determined whether the toxic effect of EDTA or DNA was responsible for the decreased viability. Pronuclear microinjection of buffer with EDTA at the same concentration (1 mM) used by Brinster et al. (1985) conducted in our laboratory had no deleterious effects upon subsequent *in vitro* embryonic development to the blastocyst stage. Microinjection of DNA had a strong deleterious effect on *in vitro* embryonic

development. This effect was DNA concentration dependent. Taken together, these data suggest that a higher degree of transgene integration is concurrent with a higher embryonic lethality, which is due to the microinjected DNA and not other factors alone such as EDTA. A definitive method to detect whether injected DNA is integrated into the genome in preimplantation stage embryos is needed to ultimately prove this hypothesis. Unfortunately, such a technique has not been developed.

It is well documented that pronuclear microinjection of DNA causes a great attrition in the development of embryos into fetuses that is not due to the injection procedure by itself (Brinster et al., 1985). The largest loss of injected embryos occurs during the early cleavage stages, and interestingly, large populations of these arrested embryos contain detectable amounts of the injected DNA. Our data support the hypothesis that the integration of DNA into the genome is not necessarily an uncommon event and therefore is not the limitation in transgenic animal production. Rather, integrated DNA may exert a selection pressure during subsequent development and therefore may be an inherent limitation for production of transgenic animals. These observations suggest that research emphasis be placed on attenuating the integration process possibly by controlling the site of chromosomal integration. Preimplantation embryo screening may still be an important tool in efforts to increase the efficiency of producing transgenic animals. For example, nuclear transfer technology could be applied to morulae generated from microinjected zygotes.

Although the donor morula may be mosaic, the second generation morulae made from nuclei from the individual blastomeres which test positive by PCR should be truly hemizygous. Thus a PCR based screening technique may be useful in the generation of non-mosaic founder transgenic animals.

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Table 1. Detection of the single copy WAP gene by PCR amplification of whole mouse embryos at various developmental stages. Numbers with different superscripts were different ($p<0.05$).

Developmental Stage	Number Assayed	Number Positive	Percent Positive
1-cell	72	$56^{a,b}$	78
2-cell	17	$15^{b,c}$	88
4-cell	31	29^{c}	94
morula	21	20^{c}	95
blastocyst	78	76^{c}	97

Table 2. WAPPC-3 transgene detection percentage in pronuclear microinjected embryos cultured *in vitro* to the 5 developmental stages analyzed. Numbers with different superscripts were different ($p<0.05$).

Developmental Stage	Number Assayed	Number Positive	Percent Positive
1-cell	40	35[a]	88
2-cell	51	45[a]	88
4-cell	54	24[b]	44
morula	62	25[b]	40
blastocyst	89	26[c]	29

Table 3. Comparison of transgene detection frequency for expanded blastocysts cultured *in vitro*, degenerate embryos after 5 days of culture *in vitro*, and live mice from microinjection and transfer. Detection frequency among the various populations of embryos retrieved after 5 days of culture *in vitro*. Numbers with different superscripts are different ($p<0.05$).

Stage	Number Assayed	Number Positive	Percent Positive
Blastocysts	89	26[a]	29
1-Cell, Arrested	18	15[b]	83
2-Cell, Arrested	13	11[b]	85
Degenerate*	92	78[b]	85
Live Mice	23	5[a]	22

*Greater than the 2-cell stage by morphology.

Table 4. Effect of WAPPC-3 DNA concentration in the microinjection buffer on development to the blastocyst stage, and PCR detection frequency in the blastocyst and degenerate embryo populations. Zygotes in the control group were not microinjected. Numbers with different superscripts were different ($p<0.05$).

DNA Conc. (μg/ml)	Total	Blastocysts (%)	Blastocysts PCR Positive (%)	Degenerates PCR Positive (%)
control	114	84 (74%)[a]	**76/78 (97%)[a]	-
buffer only	48	30 (63%)[a]	-	-
1.5	323	89 (28%)[b]	26 (29%)[b]	*78/92 (85%)[a]
15	63	7 (11%)[c]	6 (86%)[a]	*27/28 (96%)[a]
50	87	8 (9%)[c]	7 (88%)[a]	*28/29 (97%)[a]

*Number positive/Number assayed.
**PCR positive contro. Amplification of the single copy WAP gene using noninjected control blastocysts as templates.
- not determined

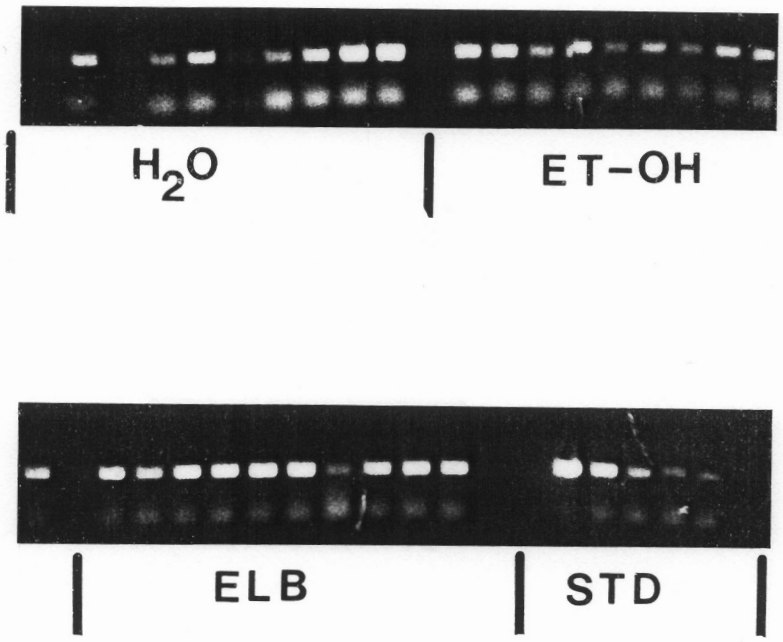

Figure 1. Signal intensity after polymerase chain reaction amplification of the single copy WAP gene in blastocysts using three different embryo preparation techniques. H_2O, water embryo pretreatment; ET-OH, ethanol embryo pretreatment; ELB, embryo lysing solution pretreatment; STD, 10000, 1000, 100, 10, and 1 genome copy of control mouse DNA, respectively.

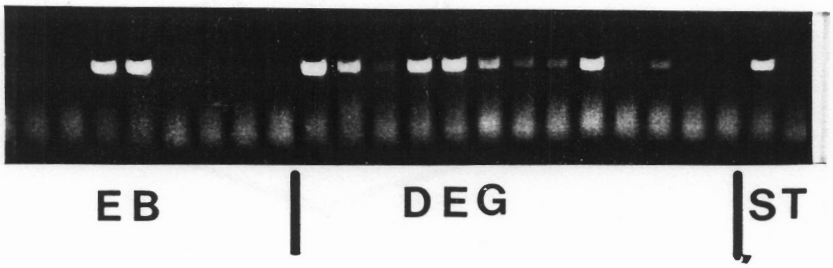

Figure 2. Sample gel showing detection of the WAPPC-3 transgene in blastocysts and degenerate embryos. EB, expanded blastocysts; DEG, degenerate embryos; ST, 100 and 10 genome copies of WAPPC-3 transgenic mouse DNA, respectively.

CHAPTER IV.

PRODUCTION OF TRANSGENIC MICE BY CYTOPLASMIC INJECTION OF POLYCATION/DNA COMPLEXES AND ANALYSIS OF THE TECHNIQUE

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ABSTRACT

The production of transgenic mice by a cytoplasmic injection technique is described. DNA was complexed with poly-L-lysine having a degree of polymerization of 51 and injected into the cytoplasm of mouse zygotes. The effects of DNA concentration and stoichiometric ratio of positive charges provided by the polycation to negative charges provided by DNA on transgenic frequency and embryonic viability were studied. The highest transgenic frequency (13% of pups born were transgenic) was obtained when a polylysine/DNA complex having a stoichiometric charge ratio of one to one (equal positive charges as negative charges) at a DNA concentration of 50 μg/ml was used. The transgenic frequency by pronuclear injection of the same DNA construct was 22%. The viability of embryos to the blastocyst stage after *in vitro* culture was greater for embryos having had cytoplasmic versus pronuclear injection of DNA. The percentage of zygotes, cultured *in vitro*, reaching the blastocyst stage which were injected cytoplasmicly was not different ($p>0.05$) than that of control zygotes that were not microinjected (65% versus 74%, respectively). The percentage of zygotes reaching the blastocyst stage after pronuclear microinjection with DNA at a concentration of 1.5 μg/ml was significantly lower ($p<0.05$) than control embryos (28% versus 74%, respectively). The detrimental effect of pronuclear microinjection on embryonic viability to the blastocyst stage was more pronounced when higher DNA concentrations were used (11% blastocysts at 15 μg/ml and 9% at 50 μg/ml). Microinjection of Brinster's buffer into the pronucleus and cytoplasm had no effect

($p>0.05$) on *in vitro* embryonic development to the blastocyst stage (61% and 65%, respectively) compared to development of non-injected embryos (74%). The polylysine mixed with construct DNA inhibited the effects of restriction endonuclease (BamHI), nonspecific endonuclease (DNase I), exonuclease (Exonuclease III), and DNA ligase (T4 DNA Ligase) on the construct DNA. The magnitude of enzyme activity inhibition was directly proportional to polylysine concentration. The overall transgenic pup production efficiency (percent of transgenic pups per embryos transferred) by cytoplasmic injection was 2.4% compared to 3.5% by pronuclear microinjection.

INTRODUCTION

The genetic transformation of mammalian cells cultured *in vitro* has been possible for many years. However, the transfer of DNA into whole mammalian organisms requires more specialized techniques. Transgenic animals have been made using retroviral infection (Jaenisch and Mintz, 1974), embryonic stem cells (Evans et al., 1981), and pronuclear microinjection (Gordon et al., 1980). Each of these techniques have inherent limitations in their usefulness and efficiencies. The limitations of stem cells are that the process is labor intensive and costly since cells must be cultured *in vitro* for long periods of time and resulting animals are mostly germline mosaic. In addition, stem cell technology has been successfully demonstrated only in mice (Anderson, 1992). Transgenic chickens (Salter et al., 1986) and mice

(Jaenisch and Mintz, 1974) have been produced by retroviral infection. The utility of transgenic animal production by retroviral infection is limited by the ability to package only small (5-6 kb) foreign DNA constructs into the retroviral genome (Jaenisch, 1976). The most popular and convenient method of making transgenic animals is pronuclear microinjection. Transgenic farm animals have only been obtained using pronuclear microinjection (Hammer et al., 1985; Wall et al., 1991; Velander et al., 1992; Ebert et al., 1991; and Wright et al., 1991).

About 5-30% of animals born from embryos that have undergone pronuclear microinjection of DNA are transgenic. With these low efficiencies, production of transgenic farm animals requires considerable effort and resources. Although the production of transgenic mice by pronuclear injection has been extensively studied, little is known about the mechanism of integration of foreign DNA into the genome. It is thought that integration may occur by a breakage-repair mechanism triggered by the injection event. The viability of pronuclear microinjected embryos is reduced compared to embryos that do not undergo microinjection (Brinster et al., 1985; Brinster and Walton, 1987; Chen et al., 1986; and Covarrubias et al., 1987). It also has been shown that when DNA is microinjected into the cytoplasm, there is not as much loss in embryonic viability as with pronuclear microinjection of DNA (Brinster et al., 1985). However, transgenic animals generally do not result from cytoplasmic injection of DNA alone. Specifically, only two transgenic mouse fetuses out of 58

pups born (3.4% transgenic frequency) were reported to arise from cytoplasmic injection (Brinster et al., 1985). In additional cytoplasmic injection experiments, no transgenic mice resulted even when up to 20,000 transgene copies were injected into zygotes. Both linearized construct DNA and supercoiled plasmid DNA were cytoplasmically injected with no transgenic fetus production.

The use of liposome technology has enabled the efficient genetic transformation of mammalian cells cultured *in vitro* (Wang and Huang, 1989; Rose et al., 1991). As a logical extension, researchers have attempted to use liposomes complexed with DNA to generate transgenic mammals. These attempts used microinjection of liposome encapsulated DNA into the blastocoel cavity (Reed et al., 1988) and perivitelline space (Loskutoff et al., 1986) of preimplantation embryos. Both of these experiments were unsuccessful in producing transgenic animals.

The normal cellular uptake process, receptor-mediated endocytosis, has been used to carry DNA into mammalian cells and enable the expression of exogenous genetic material by the host cells (Wu and Wu, 1988a; Wu and Wu, 1998b; Wu and Wu, 1987). This procedure involves covalently linking the ligand (asialoorosomucoid) for a specific cell surface receptor (asialoglycoprotein receptor) to polylysine, a polycation known to bind DNA by electrostatic interaction (Li et al., 1973). The polylysine-ligand complex was then mixed with and allowed to bind DNA. The cells

were incubated with the polylysine-ligand/DNA complex and the exogenous DNA was expressed by the transfected cells. However, the exogenous DNA was not incorporated into the host genome; thus only transient expression of the foreign DNA was observed. It is not known whether this exact type of approach will be successful in generating transgenic animals when done at the early embryonic stage. The success of this approach requires that the recipient cell possess a high density of surface receptors specific for the ligand to be used. Specifically, Wu and Wu (1987) used a hepatoma cell line which participates in active uptake of asialoorosomucoid by the asialoglycoprotein receptor. A similar approach has been used by linking transferrin to polylysine and transfecting hematopoetic cells with exogenous plasmid DNA (Wagner et al., 1990; Zenke et al., 1990; Cotten et al., 1990; Wagner et al., 1991). Hematopoetic cells take up large amounts of transferrin by receptor-mediated endocytosis (reviewed in Huebers and Finch, 1987). We are currently unaware of any direct evidence suggesting that zygotes participate in receptor-mediated endocytosis.

In the above experiments, the sole function of the surface receptor ligand was to facilitate entry of the DNA into the cell. It is unlikely the ligand-surface receptor complex facilitates entry of the exogenous DNA into the nucleus. The polycation was only used to bind the DNA so it could be carried into the cell along with the surface receptor-ligand complex. It is unknown if, once inside the cytosol, the polycation can play a role in integration of foreign DNA into the host genome, or if it facilitates

transport of the foreign DNA into the nucleus. The DNA bound to polylysine can however be expressed; whereas in a previous report (Capecchi, 1980), direct microinjection of DNA into the cytoplasm of cultured cells did not permit expression of the injected plasmid DNA. It also has been postulated that foreign DNA entering a cell by DEAE-dextran transfection may break down during its transit from the cytoplasm to the nucleus (Wong and Capecchi, 1985). DNA injected into the nucleus of cultured cell has been shown to remain largely intact (Folger et al., 1982 and Kopchick and Stacey, 1984).

The presence of a protective agent may allow foreign DNA to traverse the cytoplasm unmodified. DNA binding materials such as polycations may be able to act in such a capacity. One class of polycations, polyamines, have been shown to inhibit the digestion of DNA by restriction endonucleases (Kirino et al., 1990). These data showed that larger and more branched polyamines inhibited the endonuclease activity more efficiently. The endonuclease activity inhibition also was directly proportional to polyamine concentration. The transport of a substance from the cytoplasm into the nucleus in the absence of a facilitated transport system must rely on diffusion and phase partitioning into the nuclear envelope or transport through the nuclear pore complex. A polycation/DNA complex may be sufficiently charge-neutralized to allow association with the nuclear membrane. In addition, polycation coating of DNA will relax the DNA molecule into a rod-like structure which may have an opportunity to

enter the nuclear pore by axial diffusion. Taken together, these results and observations suggest that the polycation itself (i.e polylysine) may enable transport of intact foreign DNA into a cell's nucleus. However, although the foreign DNA was efficiently expressed in cultured cells, there was no evidence that it became integrated into the host cell's genome (Wu and Wu, 1987; Wagner et al., 1990).

Microinjections are usually done within a few hours of the time within the pronuclear stage when the pronuclei fuse and the nuclear envelope breaks down. The zygote cleaves shortly thereafter. With this sequence of events, the cytoplasmically injected material may contact the nucleoplasm. Therefore, the focus of the current study was to determine if transgenic animals could result from injection of DNA/polylysine complexes directly into the cytoplasm of zygotes. The effect of cytoplasmic and pronuclear microinjection procedures on *in vitro* embryonic viability also was studied. In addition, the effect of polylysine binding to DNA on *in vitro* enzyme activities was evaluated. Specifically, electrophoretic analyses of the products generated from restriction endonuclease BamHI, DNase I, Exonuclease III, and T4 DNA Ligase treatment of construct DNA were performed in the presence and absence of poly-L-lysine.

MATERIALS AND METHODS

Embryo Manipulation. Immature female mice (CD-1 white Swiss mice; Charles

River Laboratories, Madison, WI) 24 to 30 days of age were superovulated by intraperitoneal injection of 10 I.U. Pregnant Mare's Serum Gonadotropin (PMSG, Diosynth, Chicago, IL), followed by 5 I.U. of human Chorionic Gonadotropin (Henry Schein, Inc., Port Washington, NY) 46 to 48 hours later. The females were mated to CD-1 males (3 to 6 months of age). One cell embryos were collected 21 to 24 hours after the hCG injection by standard methods (Hogan et al., 1986). The embryos were removed from the oviduct by tearing the ampulla region. The cumulus mass that surrounded the embryos was dissolved by washing in 3 ml of M2 in a sterile 35 mm petri dish (Becton Dickson Labware, Linclon, NJ) containing 0.2 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO). The embryos were then washed in a fresh dish of medium and placed into a 300 µl drop of medium on the lid of a sterile 100 mm petri dish (Becdon Dickson Labware, Lincoln, NJ) which was covered with paraffin oil to prevent medium evaporation. The embryos were visualized using Hoffman Modulation Contrast Optics at 200 x magnification fitted to an inverted microscope (Carl Zeiss Inc., Hanover, MD, Model IM 35). The embryos were held in place using a heat-polished glass pipette made from filamented glass capillaries (World Precision Instruments, Inc., Sarasota, FL). A micropipette puller (David Kopf Instruments Inc., Tujunga, CA, Model 720A) was used to draw the pipettes and they were polished using a microforge (Narashige USA, Greenvale, NY, Model MF-83). Embryos were cultured in 10 µl drops covered with paraffin oil (Sigma Chemical Co., St Louis, MO) using CZB medium (Chatot et al., 1989) in a humidified environment consisting of 5%

CO_2 in air (Haraeus Instruments, Inc., South Plainfield, NJ).

DNA Preparation. The DNA preparation was a hybrid construct consisting of 1.5 kb of cDNA encoding human Protein C flanked by 2.6 kb of 5' noncoding DNA corresponding to the regulatory region of the mouse Whey Acidic Protein (WAP) gene, and 1.6 kb of 3' noncoding DNA also corresponding to the mouse WAP gene (Campbell et al., 1984). The construct (designated as WAPPC-3) was cloned using the plasmid pUC-19 (Gibco BRL Life Technologies, Gaithersburg, MD) and grown in JM109 E. coli cells (Gibco BRL Life Technologies, Gaithersburg, MD). The plasmid was isolated by alkaline lysis (Birnboim et al., 1979) and the construct was digested away from the cloning vector with the restriction endonuclease EcoRI . The construct was purified using High Performance Liquid Chromatography (Velander et al., 1992a), precipitated, reconstituted in sterile injection buffer (10 mM Tris-HCl, 0.25 mM EDTA, pH 7.5), and filtered using at 0.22 μm syringe filter. The concentration was estimated using ethidium bromide-stained agarose gel electrophoresis using 1 kb ladder and Hind III digest of phage λ DNA standards (Promega Corp., Madison, WI). The DNA was then diluted to a concentration two (2) times the final values to be used (e.g 30 μg/ml for an experimental concentration of 15 μg/ml) for the injection experiments. The DNA solutions were aliquoted and stored at -20 °C until used.

Polycation Preparation. The polylysine was a 51-mer bromide salt of a lysine

polymer (Sigma Chemical Co., St. Louis, Mo., Cat.# P6516, lot# 128F-5033). A 1 mg/ml stock solution of polylysine was made in the same injection buffer as was used for the DNA construct. The concentration of polylysine was adjusted such that the molar charge ratio of positive charges donated by lysine residues was fixed to a given ratio of that calculated for the negative charges donated by phosphate groups on the DNA. Specifically, one lysine monomer was assumed to donate one positive charge and one base pair of DNA was assumed to donate 2 negative charges. Three molar charge ratios were investigated, 0.5:1, 1:1, and 2:1. According to this calculation, polylysine solutions having a concentration two (2) times that needed for the appropriate stoichiometric charge ratio were made. The 2X polylysine solutions were aliquoted and stored at -20 °C until used.

The polylysine/DNA complexes were formed by mixing equal volumes of each 2X stock solution together and letting them stand at room temperature for at least 15 min prior to loading the solution into the microinjection needles. The degree and success of complexation was tested and shown by the absence of a band corresponding to the size of the DNA construct on a 1% ethidium bromide-stained agarose gel. The polylysine neutralizes the negative charge on the DNA and retarded its migration in an electric field. Both DNA and polylysine solutions were handled in sterile 0.5 ml microcentrifuge tubes (Denville Scientific, Inc., Denville, NJ).

The polylysine/DNA complex was back-loaded into a microinjection pipette using a finely drawn, sterile pasteur pipette. About 10 pl of the solution was injected into the cytoplasm of each zygote using a pneumatically controlled microinjector (Eppendorf North America, Inc., Madison, WI, Model 5242). Special care was taken to ensure that the injection needle did not puncture into either pronuclear membrane of the zygotes. DNA was isolated from the pups after they were weaned at 21 days of age by the methods described previously (Velander et al., 1992a). The DNA preparations were analyzed for the presence of the WAPPC-3 construct using PCR (Saiki et al., 1988 and Velander et al., 1992a). The positive mice were confirmed using southern hybridization (Velander et al., 1992b). The initial founder transgenic mice were evaluated for their ability to transmit the transgene to their offspring. The founder transgenic mice were mated to non-transgenic mice and DNA samples isolated from tissue biopsies from the offspring were analyzed for the WAPPC-3 transgene using PCR.

Restriction and Modifying Enzyme Activity Assays. Restriction digestions were performed using 20 µl reaction volumes containing: 200 ng WAPPC-3 construct DNA and 12 U (1µl) of BamHI restriction enzyme (Gibco BRL Life Technologies, Gaithersburg, MD), according to the manufacturers instructions. Separate reactions containing coated and uncoated construct DNA were carried out at 37 °C for 2 hours. DNase I treatments were done using 20 µl reaction volumes containing: 200 ng

WAPPC-3 construct DNA, 50 mM Tris-HCl (pH 7.5), 1.0 mM $MgCl_2$, 0.1 mg/ml BSA and 20 U (1µl) of DNase I endonuclease (Promega Corp., Madison, WI). Separate reactions containing coated and uncoated construct DNA were carried out at room temperature for 1 hour. Exonuclease III treatments were done using 20 µl reaction volumes containing: 200 ng WAPPC-3 construct DNA, 50 mM Tris-HCl (pH 7.5), 5.0 mM $MgCl_2$, 5.0 mM DTT, 0.05 mg/ml BSA, and 20 U (1µl) of Exonuclease III (Promega Corp., Madison, WI). Separate reactions containing coated and uncoated construct DNA were carried out at 37 °C for 1 hour. Ligation reactions were performed using 20 µl reaction volumes containing: 200 ng WAPPC-3 construct DNA, ligation buffer, and 1 U (1µl) of T4 DNA Ligase (New England Biolabs, Inc., Bevereley, MA). Separate reactions containing coated and uncoated construct DNA were incubated at room temperature overnight. For each of the above reactions, the products were treated with one µl of a 1% SDS solution to dissociate the polylysine from the DNA to permit migration in a 1% agarose gel. The gels were stained with ethidium bromide (0.5µg/ml) and photographed.

Statistical Analysis. A Chi Square probability analysis was used to determine if values were significantly different. Each value for either developmental efficiency or integration efficiency was compared to each other value within the given experiment.

RESULTS

Polylysine/DNA Binding Analysis. Figure 1 shows the electrophoretic mobility of construct DNA alone (Lane 3) relative to that in the presence of polylysine at the stoichiometric charge ratios (SCRs) of 0.25:1, 0.5:1, 1:1, 2:1, 3:1, and 5:1 at a fixed concentration of DNA (Lanes 4-9, respectively). The migration of construct DNA was retarded relative to untreated DNA when polylysine was present at SCRs of 0.25:1 to 5:1. The extent of retarded migration increased as the amount of polylysine present was increased. Then the SCR was 5:1, the majority of the DNA remained in the loading well (Lane 9).

Restriction digests, DNase I digests, Exonuclease III digest, and T4 DNA Ligase treatments were performed on DNA/polylysine mixtures having SCRs 0.5:1, 1:1, and 2:1. The reaction products were visualized using ethidium bromide-stained agarose (1%) gel electrophoresis (Figures 2A, 2B and 2C). Figures 2A, 2B, and 2C show untreated DNA (Lanes 3 and 12) and various reaction products for DNA alone and DNA mixed with polylysine (Lanes 4-11). The undigested DNA appeared as a single band at about 5.5 kb (Lanes 3 and 12). For DNA mixed with polylysine at SCRs of 0.5:1 (Figure 2A) and 1:1 (Figure 2B), no differences were observed for reaction products from treatments with BamHI (Lane 5) and Dnase I (Lane 7), as compared to DNA alone (Lanes 4 and 6). However, there were 6 bands observed among the BamHI products of the DNA/polylysine mixture at an SCR of 2:1 (Figure

2C, Lane 5), compared to three bands for DNA alone (Lane 4). With T4 DNA ligase treatment at these SCRs, only high molecular weight products were observed (Figure 2A and 2B, Lane 11, while T4 DNA Ligase treatment of DNA alone resulted in a lower molecular weight band as well as higher molecular weight bands (Lane 10). T4 DNA Ligase treatment had no effect upon the DNA/polylysine mixture with an SCR of 2:1 (Figure 2C, Lane 11), compared to untreated DNA alone (Lane 12). Products of Exonuclease III treatment of DNA/polylysine at an SCR of 0.5:1 showed no visible bands (Figure 2A, Lane 9) as well as did untreated DNA alone (Lane 8). However, for SCRs of 1:1 and 2:1, there was some undigested construct DNA remaining (Figures 2B, 2C, Lane 9), while there was no band present in the Exonuclease III treatment of DNA alone (Lane 8).

Microinjectablity of Polylysine DNA Mixtures. The efficiency of delivering DNA/polylysine mixtures by microinjection into the cytoplasm was tested by PCR analysis of 1-cell embryos which were frozen immediately after microinjection. The presence of polylysine at any of the SCRs studied did not interfere with amplification of the construct DNA applied directly to the PCR assay (data not shown). A sample of 5-10 zygotes from each of the DNA concentrations used and from each microinjection session were frozen and later analyzed by PCR. At SCRs of 0.5:1 and 1:1, the DNA construct was detected in about 100% and 50% of the injected zygotes tested, respectively. DNA was detected by PCR in only 2 of 30 zygotes which were

microinjected with DNA/polylysine having and SCR of 2:1.

These data suggested that the DNA/polylysine complexes were not efficiently and reproducibly microinjected. Injection needles with larger bore sizes were also tried. The larger bore size pipettes resulted in a higher DNA/polylysine complex injection efficiency at a 1:1 charge ratio, but not at 2:1. However, injections with larger needles caused large losses in embryos due to cell lysis shortly after the microinjection process. Microinjection capillaries coated with Sigmacoat® (Sigma Chemical Co., St. Louis, MO.) were also tried. The coating did not improve the delivery of the polylysine/DNA mixtures.

Effects on Viability of Cytoplasmic and Pronuclear Injection of DNA. Table 2 summarizes the effect of both pronuclear and cytoplasmic injection of DNA on *in vitro* embryonic development. About 74% of cultured embryos which were not microinjected developed to blastocyst stage. Pronuclear and cytoplasmic injection of 10 mM Tris-HCl, pH 7.5; 0.25 mM EDTA with no DNA resulted in 61% and 65% of cultured embryos reaching the blastocyst stage, respectively. In contrast, about 28%, 11% and 9% of zygotes injected into the pronucleus with DNA at 1.5, 15 and 50 μg/ml survived to the blastocyst stage, respectively. Cytoplasmic injection of 1.5, 15, and 50 μg/ml with no polylysine gave developmental efficiencies (to the blastocyst stage) of 64%, 66%, and 65%, respectively.

Transgenic Analysis. Potential founder mice from pronuclear or cytoplasmic injection of DNA were screened for presence of the transgene using PCR and then confirmed by southern hybridization. A southern blot showing the 5.5 kb band specific for a EcoRI fragment within the protein C cDNA indicates the presence of the transgene in the founder mouse DNA preparations (Figure 3). Not all of the founder mice identified as positive for the transgene by PCR (data not shown) were identified on southern blots. However, one founder mouse which was negative by Southern analysis but positive by PCR did transmit the transgene to one of its offspring.

The transgene integration efficiencies for different DNA concentrations and SCRs are shown in Table 1. No transgenic mice resulted from cytoplasmic injection of DNA in the absence of polylysine. Integration efficiencies yielded from cytoplasmic injection varied with SCR and DNA concentration; 11% and 13% were obtained for DNA at 50 μg/ml and SCRs of 0.5:1 and 1:1, respectively; 9% and 7% for DNA at 15 μg/ml and SCRs of 1:1 and 2:1, respectively; 4% transgenic efficiency was obtained from injections of DNA at 1.5 μg/ml and a SCR of 0.5:1. Pronuclear injection of DNA at 1.5 μg/ml gave a transgenic efficiency of 22%.

A summary of transgenic efficiency based on born mice produced by cytoplasmic injection as compared to that of pronuclear injection of DNA is given in Table 3. The cytoplasmic injection of DNA at 50 μg/ml and a SCR of 1:1 produced 6

transgenic pups of 47 born or a transgenic mouse production efficiency of 13%. No transgenic pups were detected in the 45 pups born from cytoplasmic injection of DNA at 50 μg/ml and no polylysine. Of 23 pups born from pronuclear injection of DNA, five were transgenic giving an integration efficiency of 22%. For the above cases, the overall efficiency of transgenic mouse production based upon total injected embryos (percent of transgenic pups per embryos transferred) was 3.5% for pronuclear injection DNA alone and 2.4% for cytoplasmic injection of polylysine/DNA.

Six founder animals (G_0) produced from embryos cytoplasmicly injectioned with DNA/polylysine were outbred with control mice to evaluate the germline transmission of the transgene. The tail DNA samples isolated from the G_1 mice were analyzed for the WAPPC-3 transgene using PCR. Three of the six founder mice tested transmitted the transgene to at least one of their offspring. The germline transmission frequency of the six founder mice tested was as follows; 1 of 14, 6 of 10, 11 of 11, 0 of 15, 0 of 11, and 0 of 12. An unusual pattern of transgene transmission for founder male 46 was observed in that each of the progeny was positive for the transgene when assayed by PCR (data not shown). The relative copy numbers were estimated by the band intensity from PCR amplification products viewed by ethidium bromide-stained agarose gel electrophoresis. Within a single litter, all 11 pups born contained the transgene but 4 of the 11 appeared to have a distinctly lower copy number.

Expression of the Transgene. The milk from outbred female G_1 and/or G_2 mice was assayed for recombinant human protein C (rhPC). The rhPC milk expression data are summarized in Table 4. The rhPC expression levels (130-260 μg/ml) were similar with those obtained for transgenic mice made by pronuclear microinjection (140-300 μg/ml).

DISCUSSION

Transgenic mice were produced by cytoplasmic injection of DNA/poly-L-lysine complexes, but not by cytoplasmic injection of DNA alone. While not optimized with respect to the efficiency of injecting large molecular complexes, transgenic frequencies as high as 13% were obtained. A transgenic frequency of 22% was obtained using pronuclear injection of the same DNA construct. The production of transgenic mice by cytoplasmic injection of DNA/polylysine complexes was a direct result of the presence of polylysine. Transgenic frequencies also were higher when higher DNA concentrations were used, suggesting a dependence on a concentration driving force phenomenon for nuclear import and/or enzymatic reactions involved in integration. Polylysine by itself is known to accumulate into the nucleus of cultured cells, and facilitate the aggregation of DNA. However, it is not possible from the present study to determine if the polylysine was important for nuclear transport or transgene integration.

In a previous study (Brinster et al., 1985), two transgenic fetuses were reported to have arisen from cytoplasmic injection of naked DNA. The transgenic efficiency was low (3.4% of fetuses were transgenic) and additional experiments using other DNA constructs at higher injection concentrations failed to produce transgenic fetuses. We failed to produce transgenic mice by cytoplasmic injection of DNA without polylysine at all DNA concentrations injected. Experiments by Kopchick and Stacey (1980) demonstrated that cytoplasmic injection of cloned viral DNA into chicken fibroblasts was 10- to 50-fold less efficient in producing expression of the foreign DNA than nuclear injection. Taken together, these data suggest that cytoplasmic injection of naked DNA is inefficient in transforming animals and cells with inconsistent positive results.

Polycations other than polylysine (e.g. protamines and histones) have been used to bind DNA for use in cell culture transfection experiments (Wagner et al., 1990). It may be suggested that polycations which have a specific biological function, such as nuclear targeting, may enhance the frequency of transgenic animal production if used in conjunction with cytoplasmic injection. The molecular form of the DNA in a physiological saline solution is altered by the presence of polylysine (Laemli, 1975; Wagner et al., 1991). In the experiment reported here, the DNA/polylysine complexes were made in the presence of injection buffer (10 mM Tris-HCl, 0.25 mM EDTA, pH 7.5), which has a low ionic strength. Extrapolating from data available in the

literature (Wagner et al., 1991), it is not possible to determine if the linear construct DNA/polylysine mixture used in our experiments resulted in condensation of the DNA into a toroid structure similar to that seen with transferrin-polylysine/DNA complexes. Wagner et al. (1991) postulated that the condensed toroid structure facilitated the cytosolic uptake of the transferrin-polylysine/DNA complexes by allowing the complex to fit within the diameter of coated pits. However, our study suggests that association of DNA with polylysine may play a role in either transport of exogenous DNA across the nuclear envelope or facilitate the recombination process as well. Although the exact function of the polylysine is not clear, our *in vitro* analyses with restriction endonuclease, DNA ligase, exonuclease, DNase, and gel migration retardation suggest that it is probably removed before recombination of the foreign DNA with the host chromosomal DNA. A detailed analysis of the fate of labelled DNA with and without polylysine injected into the cytoplasm of zygotes would lend valuable insight into the mechanism by which the polylysine facilitates transgenic animal production by cytoplasmic injection.

The transgenic mice produced using this cytoplasmic injection technique have genotypic and phenotypic characteristics similar to those produced by pronuclear microinjection. Specifically, germline mosaic mice, having less than a theoretical mendelian transmission of the genotype to the offspring, were produced. In addition, expression of the transgene in a tissue-specific manner indicated that integration did

not occur within genomic regions that suppress expression; and that the observed transgene integration was not into mitochondrial DNA. If the observed transgene integration had been into mitochondrial DNA, it would not be expected to be transmitted to the progeny through the germline, nor would it be expressed in the mammary gland. One cytoplasmic injection-derived mouse transmitted the transgene to 100% of its offspring. It is not known at this time how many chromosomal insertion sites were present, but at least two loci seemed to segregate in the G_1 generation.

The ability to make transgenic mice by cytoplasmic injection offers several advantages to pronuclear microinjection. First, there would be no need to centrifuge those embryos from species such as pigs (Wall et al., 1985) and cows (Hawk et al., 1989 and Biery et al., 1988) in which cytoplasmic lipid prevents pronuclear visualization. In species such as sheep (Hammer et al., 1985) where pronuclei are difficult to visualize and centrifugation does not help, a cytoplasmic injection technique may facilitate generation of transgenic animals. Second, the injection procedure may be performed at phases during the embryonic cell cycle other than when pronuclei are visible. This point may be important in large animal experiments (e.g. pigs, sheep, and goats) where it is difficult to obtain large batches of zygotes with all individuals having visible pronuclei due to the inherent asynchronous timing of ovulation and/or fertilization. In addition, the injection procedure may be performed

on oocytes before the addition of sperm for *in vitro* fertilization. Third, the increase in post-injection embryonic viability seen with cytoplasmic injection over pronuclear injection may enhance the efficiency of transgenic cow production where large losses are experienced due to use of *in vitro* matured/*in vitro* fertilized zygotes for microinjection (Krimpenfort et al., 1991).

The *in vitro* culture data suggest that higher DNA concentrations may be injected into the cytoplasm of zygotes without sacrificing embryonic viability. Pronuclear microinjection injection of DNA caused a dramatic reduction in the percentage of zygotes reaching the blastocyst stage at all DNA concentrations used. The reduction in embryonic viability was DNA concentration dependent. These results suggest that the rate of transgenic pup production may be improved by cytoplasmic injection of DNA/polylysine complexes having DNA present at concentrations higher than those reported here.

A limitation in the reproducibility of this cytoplasmic injection technique is the efficiency of DNA delivery to zygotes by microinjection. As long as stoichiometric charge ratios of 1:1 or less were used, DNA was delivered to the zygotes. However, it could not be determined by our PCR analysis technique if DNA/polylysine complexes were indeed being delivered into the zygotes. It may be argued that the DNA detected by PCR for injections at 0.5:1 and 1:1 stoichiometric charge ratios represent unbound

DNA molecules that could be efficiently injected. It is intriguing to postulate that the observed transgenic mice were made from only a few zygotes that actually received polycation/DNA complexes. If this phenomenon is true, the efficiency of transgenic animal production may be greatly enhanced by achieving efficient delivery of polycation/DNA complexes. The fact that a decreasing percentage of injected zygotes had detectable levels of construct DNA with increasing amounts of polylysine, suggests that the limitation of this technique may be with DNA delivery and not nuclear transport or chromosomal integration. This theory is supported by the fact that DNA/polylysine mixtures where polylysine was present at a 2:1 stoichiometric charge ratio could not pass through a 0.45 μm filter. This size approaches that of the bore of the tip of most microinjection needles. The use of larger injection needles is an unfavorable solution to this problem since a greater portion of injected embryos did not survive the injection procedure. A more practical solution to this DNA delivery problem may be to prewash the capillaries with a cationic solution which is capable of electrostatically neutralizing the $-SiO_2$ groups on glass surfaces without inhibiting capillary action.

This report describes production of transgenic animals by cytoplasmic injection of DNA/polycation complexes for the first time. Although the present experiments were less efficient at producing transgenic mice than pronuclear injection, we believe that continual refinement of the technique may result in improved transgenic

efficiencies. A more detailed investigation of problems in efficient DNA delivery and further optimization of the stoichiometric charge ratio of polycation to DNA will evaluate this hypothesis.

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Table 1. Effect of DNA concentration with and without polycation complexation on integration frequency for mice born from microinjection into the cytoplasm. Values for the integration frequency were compared to those obtained using pronuclear microinjection. The integration frequency is the percentage of pups born testing positive for the WAPPC-3 transgene.

	DNA Concentration (μg/ml)		
Charge Ratio	1.5	15	50
2:1	0/25 (0%)	4/58 (7%)	2/39 (5%)
1:1	0/35 (0%)	2/23 (9%)	6/47 (13%)
0.5:1	2/56 (4%)	0/54 (0%)	4/37 (11%)
naked DNA	0/8 (0%)	0/15 (0%)	0/53 (0%)
pronuclear	5/23 (22%)	*	*

*When microinjection into the pronucleus is done with DNA concentrations above about 5 μg/ml, the embryonic viability is reduced such that it is difficult to obtain live pups (Brinster, et al. 1985, also see Table 2). Therefore, the DNA concentrations of 15 and 50 μg/ml were not used to generate pups using pronuclear microinjection.

Table 2. Effect of cytoplasmic and pronuclear microinjection on *in vitro* embryonic development to the blastocyst stage after 96 hours of culture in CZB medium (Chatot et al., 1989). Between 15 and 25 embryos were placed in each 10 µl microdrop of culture medium in each treatment. Embryos were judged as blastocysts only when full development of the blastocoel cavity was detected by visual analysis using a stereomicroscope (Olympus Corp., Lake Success, NY, Model SZH).

	DNA Concentration (µg/ml)			
Injection Site	buffer only	1.5	15	50
Cytoplasm	43/66 (65%)	39/61 (64%)	62/94 (66%)	54/83(65%)
Pronucleus	50/82 (61%)	89/323 (28%)	7/63 (11%)	8/87 (9%)
Control- no injection			*84/114(74%)	

*The embryos in the control group were treated exactly the same as injected embryos except for the microinjection procedure.

Table 3. Comparison of the efficiency of producing transgenic mice by the cytoplasmic injection of either DNA alone (50 μg/ml) or a DNA/polylysine solution (50 μg/ml, 1:1 stoichiometric charge ratio) to pronuclear injection of DNA (1.5 μg/ml). All experiments were performed using dilutions of the same stock DNA preparation. The transgenic efficiency is the percentage of pups born testing positive for the WAPPC-3 transgene. The overall efficiency is the percentage of embryos transferred that result in pups testing positive for the WAPPC-3 transgene.

	Injection Site		
Parameter	Pronuclear (DNA only)	Cytoplasm (polylysine/DNA)	Cytoplasm (DNA only)
Embryos injected	262	451	293
Embryos transferred	143	246	205
Pups born	23	47	45
Transgenic pups	5	6	0
Transgenic Efficiency	22%	13%	0%
Overall Efficiency	3.5%	2.4%	0.0%

Table 4. Expression of recombinant human protein C in the milk of mice produced by cytoplasmic injection of DNA/polylysine complexes compared to those produced by pronuclear microinjection of the same transgene. The mice were milked between days 10 and 15 of lactation. The rhPC expression levels were determined by ELISA (Velander et al., 1992a).

Injection Site	Mouse Number	rhPC Expression Level (μg/ml)
Pronucleus	21-13	142 ± 1
	21-14	174 ± 8
	23-15	299 ± 7
	23-16	169 ± 20
Cytoplasm	7-3-2	255 ± 18
	7-3-9	135 ± 9
	46-2-2	240 ± 11
	46-2-4	230 ± 1

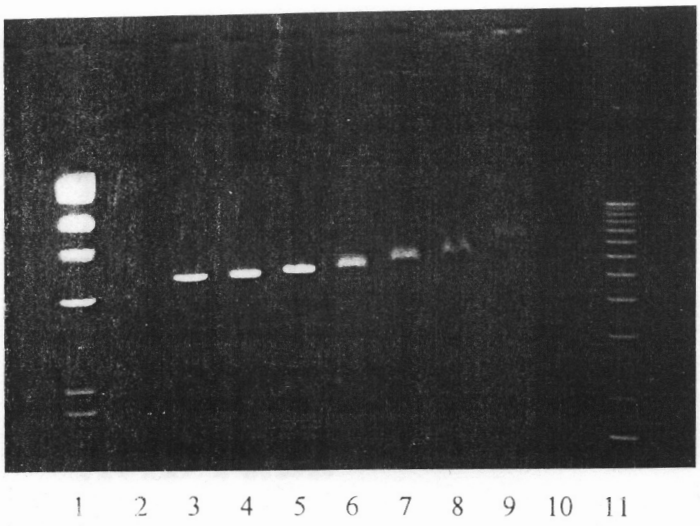


Figure 1. Effect of polylysine content on the electrophoretic mobility of construct DNA visualized using a 1% ethidium bromide-stained agarose gel. Lane 1, Hind III restriction digest of 0.5 μg of phage λ DNA molecular weight markers; Lane 2, blank; Lane 3, 100 ng of construct DNA with no polylysine; Lanes 4-9, 100 ng of construct DNA with polylysine at 0.25, 0.5, 1, 2, 3, and 5 to one stoichiometric charge ratios, respectively; Lane 10, blank; Lane 11, 0.25 μg of 1 kb ladder molecular weight standards.

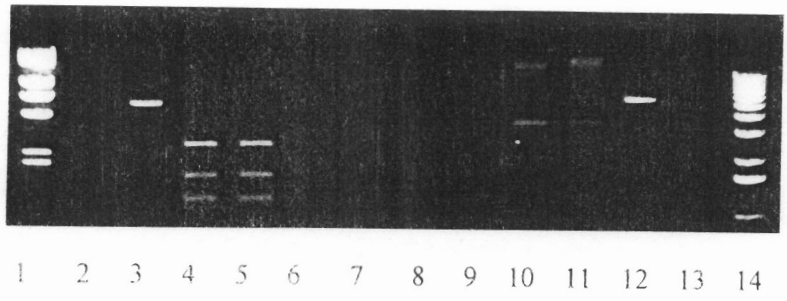


Figure 2A. Effect of DNA restriction and modifying enzyme treatments on construct DNA mixed with polylysine at a stoichiometric charge ratio of 0.5:1. Lane 1, Hind III restriction digest of 1 μg of phage λ DNA molecular weight markers; Lane 2, blank; Lane 3, 100 ng of construct DNA with no polylysine; Lanes 4-5, BamHI digest of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 6-7, DNase I digest of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 8-9, Exonuclease III treatment of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 10-11, T4 DNA Ligase treatment of 100 ng of naked DNA and polylysine coated DNA respectively; Lane 12, 100 ng of construct DNA with no polylysine; Lane 13, blank; Lane 14, 0.25 μg of 1 kb ladder molecular weight standards.

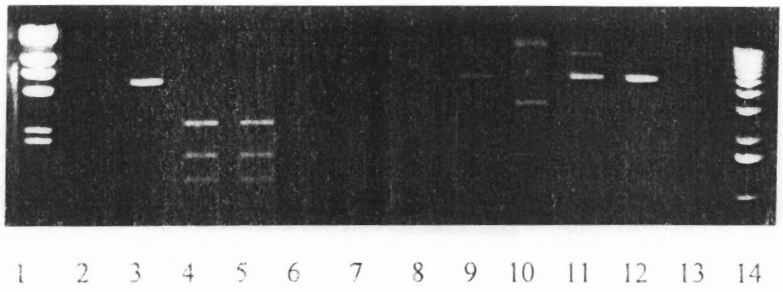


Figure 2B. Effect of DNA restriction and modifying enzyme treatments on construct DNA mixed with polylysine at a stoichiometric charge ratio of 1:1. Lane 1, Hind III restriction digest of 1 μg of phage λ DNA molecular weight markers; Lane 2, blank; Lane 3, 100 ng of construct DNA with no polylysine; Lanes 4-5, BamHI digest of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 6-7, DNase I digest of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 8-9, Exonuclease III treatment of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 10-11, T4 DNA Ligase treatment of 100 ng of naked DNA and polylysine coated DNA respectively; Lane 12, 100 ng of construct DNA with no polylysine; Lane 13, blank; Lane 14, 0.25 μg of 1 kb ladder molecular weight standards.

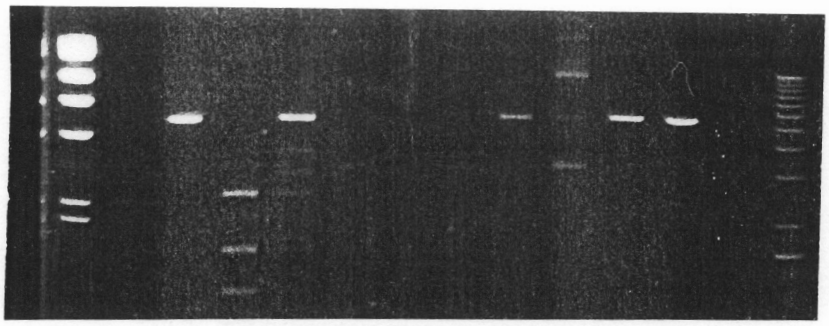
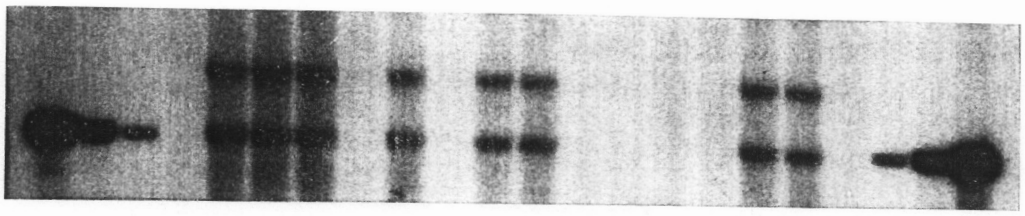


Figure 2C. Effect of DNA restriction and modifying enzyme treatments on construct DNA mixed with polylysine at a stoichiometric charge ratio of 2:1. Lane 1, Hind III restriction digest of 1 μg of phage λ DNA molecular weight markers; Lane 2, blank; Lane 3, 100 ng of construct DNA with no polylysine; Lanes 4-5, BamHI digest of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 6-7, DNase I digest of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 8-9, Exonuclease III treatment of 100 ng of naked DNA and polylysine coated DNA respectively; Lanes 10-11, T4 DNA Ligase treatment of 100 ng of naked DNA and polylysine coated DNA respectively; Lane 12, 100 ng of construct DNA with no polylysine; Lane 13, blank; Lane 14, 0.25 μg of 1 kb ladder molecular weight standards.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Figure 3. Southern blot of founder transgenic mice. Lanes 1-3, 250, 25, and 2.5 pg of construct DNA digested with EcoRI as a positive standard; Lane 4, 10 μg of control mouse DNA; Lanes 5-19, 5 μg of DNA from mice whose embryos had been injected with polylysine/DNA solutions into their cytoplasm; Lanes 20-22, 2.5, 25, and 250 pg of construct DNA digested with EcoRI.

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ABSTRACTS

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