2003

# Cloning of Bovine Placental Lactogen and Production in Vitro

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# CLONING OF BOVINE PLACENTAL LACTOGEN AND PRODUCTION IN VITRO

By

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B.A. Mount Holyoke College, 2000

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Animal Sciences)

The Graduate School

The University of Maine

May, 2003

Advisory Committee:

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# CLONING OF BOVINE PLACENTAL LACTOGEN AND PRODUCTION IN VITRO

By Stephanie A. Doucette

Thesis Advisor: Dr. Charles Wallace

An Abstract of the Thesis Presented
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Bovine placental lactogen (bPL) is a hormone produced by the fetal portion of the placenta during gestation. Although there is little known about the structure and function of the protein, it is thought to play a role in the growth of the fetus and in mammary growth and differentiation. Attempts have been made to heighten the understanding of bPL through the use of recombinant DNA technology. Thus far, researchers have been able to produce the recombinant molecule in bacteria cells. Bacteria, however, are incapable of carrying out some of the post-translational modifications characteristic of mammalian proteins. For example, unlike native bPL, recombinant bPL is non-glycosylated. Other examples of post-translational modifications include disulfide bond formation and correct folding of the protein. The aim of this study, therefore, was to clone the bPL cDNA into a mammalian expression vector, amplify the recombinant molecule in *E. coli* cells, and transfect the rbPL into mouse fibroblast cells. The idea

behind this research was that the mouse cells would be able to produce the bPL complete with the post-tanslational modifications seen in the native protein.

The bPL cDNA was successfully cloned into the pcDNA3.1(+) vector and the recombinant molecule was transformed into *E. coli* cells. The bPL sequence isolated in this study was similar to that isolated by Schuler *et al.* (1988), who were able to divide the gene into five exons. The bPL gene in this study was missing the third exon, which contains a cysteine residue that is likely involved in the proper folding of the mature protein.

Attempts were made to transfect the pcDNA3.1-bPL into mouse L929 cells. Media was collected post-transfection and assayed (Wallace, 1993) for the presence of bPL. An average concentration of 1 ng/mL bPL was measured. It was also noted that the transfected cells, as compared to control non-transfected cells, grew at a slower rate. Initially, it was suspected that the L929 cells were infected with mycoplasma. Transfection of a new batch of mouse cells showed improved growth rate, but did not produce a higher transfection efficiency. Studies with ovine placental lactogen have shown that the 5' flanking sequence of the PL gene is important in the production of the protein. In this study, the 5' flanking region of the bPL gene was removed during the making of the recombinant bPL molecule. It is therefore hypothesized that the 5' flanking sequence of the bPL gene is important to the production of the protein due to the low transfection efficiency observed.

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# Chapter 1

# LITERATURE REVIEW

## Introduction

During pregnancy, a modification in the dam's endocrine production is responsible for keeping her offspring alive. Hormones secreted in this crucial period work to maintain the fetus and assist the dam in preparing for lactation (Senger, 1999). In 1905, Halban was the first to suggest that the hormonal changes noted during gestation could be attributed to the newly formed placenta instead of to some preexisting organ. Since then, a hormone referred to as placental lactogen (PL) has been identified in primates (Kaplan and Grumbach, 1964; Vinik *et al.*, 1973; Blank *et al.*, 1977), rodents (Blank *et al.*, 1977), and ruminants (Martal and Djiane, 1975; Chan *et al.*, 1976; Hurley *et al.*, 1977; Becka *et al.*, 1977; Currie *et al.*, 1990).

The focus of this study is on bovine placental lactogen (bPL). bPL has proven to be quite difficult to study since such low levels are found in the circulation of the cow during pregnancy (Byatt *et al.*, 1992b). The hormone has been purified from both placental homogenates (Murthy *et al.*, 1982) and secretory vesicles (Byatt *et al.*, 1986), a grueling process that produces relatively low yields. The aim of this research, therefore, is to produce bovine placental lactogen in the laboratory by means of genetic cloning techniques, so that we may gain a better understanding of the structure and function of the hormone.

**Biosynthesis of bPL**

The placenta is a temporary endocrine organ whose purpose is to mediate metabolic exchange between the mother and fetus during pregnancy (Anthony *et al.*, 1995). Surrounding the placenta is a layer of cells known as trophoblast cells, approximately twenty percent of which are binucleated (Schlafer *et al.*, 2000). These binucleated cells appear between days eighteen and twenty in the cow and are formed continuously throughout gestation (Senger, 1999). Biosynthesis of placental lactogen, along with other pregnancy maintenance hormones and growth factors, takes place within these cells. After production, the hormone is stored in the cells' membrane-bound secretory granules (Verstegen *et al.*, 1985; Duello *et al.*, 1986; Rice and Thorburn, 1986). It is not until day thirty-six that placental lactogen is detectable in the trophoblastic tissue (Buttle and Forsyth, 1976), and synthesis of the hormone continues throughout the duration of pregnancy.

Binucleate trophoblast cells are characterized by their ability to migrate into the maternal epithelium (Wooding and Wathes, 1980). It was hypothesized that they do so in order to transfer a large, non-diffusible molecule across the fetal-maternal microvillar junction to the maternal circulation. The possible stages involved in binucleate cell migration are diagrammed in Figure 1.1. The fetal binucleate cells are formed in the chorion, where they synthesize the granules needed for storing the placental lactogen. The cells move toward and make contact with the basement membrane of the uterine epithelium, crossing the microvillar junction en route. The granules remain in the fetal portion of the cell until the majority of the cell has made its way into the uterine epithelium (Wooding and Wathes, 1980). A hybrid cell is formed from the fusion of the

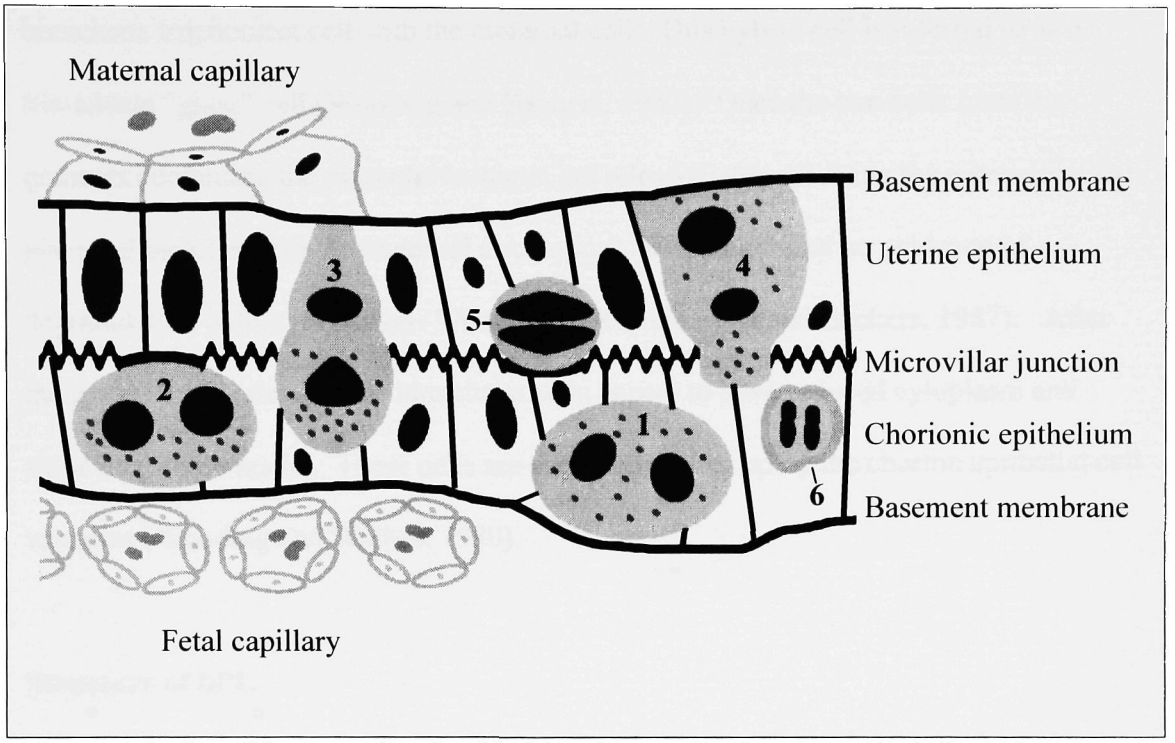


**Figure 1.1.** Possible stages involved in binucleate cell migration. (1) A fetal binucleate cell is formed in the chorionic epithelium and synthesizes the granules needed for storage of the placental lactogen. (2) The cell makes contact with the microvillar junction. (3) It crosses the microvillar junction and approaches the basement membrane of the uterine epithelium. The granules remain in the fetal portion of the binucleate cell. (4) The uterine epithelium contains the majority of the cell. The granules are now scattered throughout the entire cell or are localized in the maternal end of the cell. (5) The granules have been released into the maternal circulation. The binucleate cell appears to have reduced cytoplasm and shrunken, dense nuclei. (6) The binucleate cell is eventually taken up by a chorion epithelial cell vacuole. Diagram adapted from Wooding and Wathes (1980).

binucleate trophoblast cell with the maternal cell. This hybrid cell is referred to as a trinucleate "giant" cell (Wooding and Beckers, 1987). Once the two cells fuse, the granules containing the placental lactogen are released, thus allowing the entrance of the placental lactogen into the maternal circulation. Wooding found no evidence of degranulation before migration (Wooding, 1987; Wooding and Beckers, 1987). After the granules are released, the binucleate cells appear to have reduced cytoplasm and shrunken, dense nuclei. These cells are eventually taken up by the chorion epithelial cell vacuoles (Wooding and Wathes, 1980).

**Structure of bPL**

Ruminant placental lactogens are members of the growth hormone(GH)/prolactin (PRL) gene family. Despite their many similarities, there is a 10,000 molecular weight difference between bPL and the placental lactogens found within sheep and goats, oPL and cPL respectively. Ovine and caprine placental lactogens are single-chain polypeptides with molecular weights of 21,000 to 23,000 and intramolecular disulfide bonds (Chatterjee and Munro, 1977). In contrast, bPL exhibits a complex glycosylation pattern (Shimomura and Bremel, 1988; Byatt *et al.*, 1990), a factor that increases its molecular weight to 31,000 to 33,000 (Beckers *et al.*, 1980; Murthy *et al.*, 1982; Arima and Bremel, 1983). The amino acid composition of the placental lactogens as a whole resembles that of GH and pituitary PRL (Chatterjee and Munro, 1977; Hurley *et al.*, 1977, Vinik *et al.*, 1973). More specifically, the gene for bPL has been determined to translate into a 200 amino acid protein that is 50 and 22% similar to bPRL and bovine somatotropin (bST), respectively (Schuler *et al.*, 1988).

**bPL Levels Across Gestation**

Placental lactogen is found in both the fetal and maternal circulations during pregnancy. Among the ruminants, cows display the lowest levels of PL. In fact, the maternal concentration of PL is 100 to 1000 times greater in sheep and goats than in cows (Byatt et al., 1992b). In the cow, fetal serum concentrations of bPL decline throughout gestation (Holland *et al.*, 1997), while maternal concentrations peak at day 215 and remain high until just prior to birth. The highest reported concentration detected in the maternal serum of the cow was 2.93ng/mL (Wallace, 1993).

**Receptor Binding and Biological Activity**

In keeping the structural similarity of bPL to bST and bPRL in mind, it seems to follow suit that bPL would bind with high affinity to both bST and bPRL receptors. This has, in fact, proven to be the case (Staten *et al.*, 1993; Scott *et al.*, 1992; Gertler *et al.*, 1996). Growth hormone and PRL receptors belong to the cytokine receptor superfamily. A characteristic of this family of receptors is the need for receptor subunit homodimerization to initiate signal transduction and ensuing biological activity (Ihle *et al.*, 1994). bPL, however, does not appear to have the ability to dimerize these receptors (Staten *et al.*, 1993). This seems to suggest that homodimerization is not essential for some biological responses signaled through these receptors, since bPL displays both somatogenic and lactogenic activity. In fact, recombinant bPL (rbPL) has been shown to stimulate weight gain in mature female rats (Byatt *et al.*, 1991) and mammary differentiation in steroid-primed dairy heifers (Byatt *et al.*, 1994).

In 1995, Anthony *et al.* suggested several possibilities about the identity of the ruminant placental lactogen receptor using the growth hormone receptor as a model (Figure 1.2). Ruminant PLs may act by binding to one monomer of the growth hormone receptor (GHR), to one monomer of the GHR and an undefined monomer, to two monomers of a variant form of the GHR, or to a receptor that is specific for PL. There have also been specific binding sites for bPL identified in the bovine endometrium (Galosy *et al.*, 1991) and corpus luteum (Lucy *et al.*, 1994), suggesting that bPL plays a role in the maintenance of pregnancy.

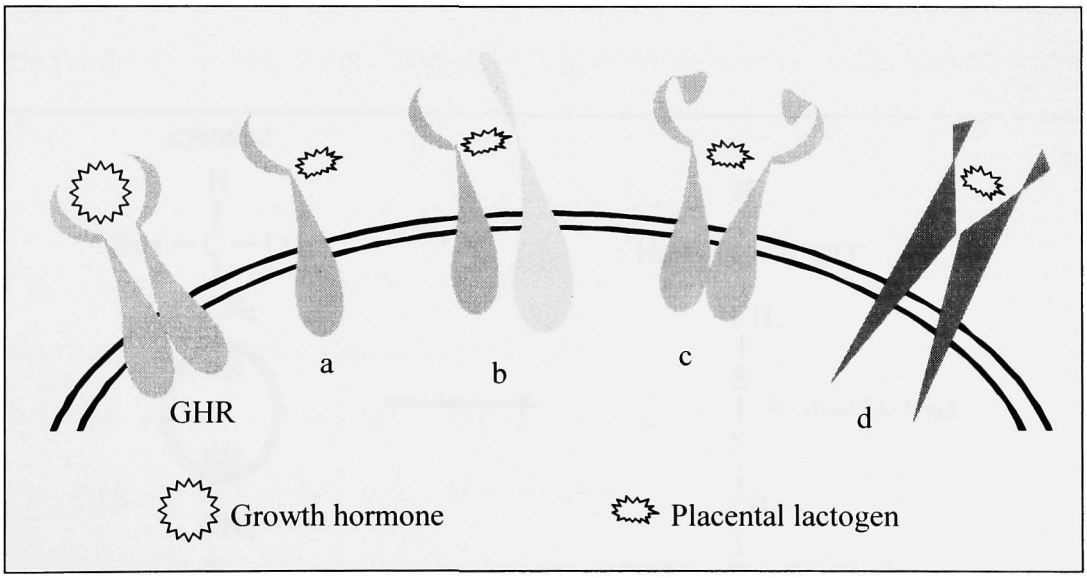


**Figure 1.2.** Possible identity of the ruminant placental lactogen receptor. Ruminant PLs may bind to: (a) one monomer of the growth hormone receptor (GHR), (b) one monomer of the GHR and an undefined monomer, (c) two monomers of a variant form of the GHR, or (d) a receptor that is specific for PL. Diagram adapted from Anthony *et al.* (1995).

**Recombinant bPL**

Recombinant analogues of bPL (rbPL), along with GH, PRL, and a few soluble extracellular domains of their receptors, have enabled researchers to study the structures of the hormones and their interactions with their receptors (Gertler, 1997). To date, rbPL has been produced in bacteria cells. Unlike the native protein, rbPL has not been glycosylated because bacteria cells are unable to carry out many of the post-translational modifications that are common in mammalian proteins. Other examples of post-translational modifications include the formation of disulfide bonds, proper folding of the protein, and addition of carbohydrate chains.

Disulfide bonds occur between the sulfhydryl groups (-SH) in two cysteine residues of a protein (Figure 1.3).

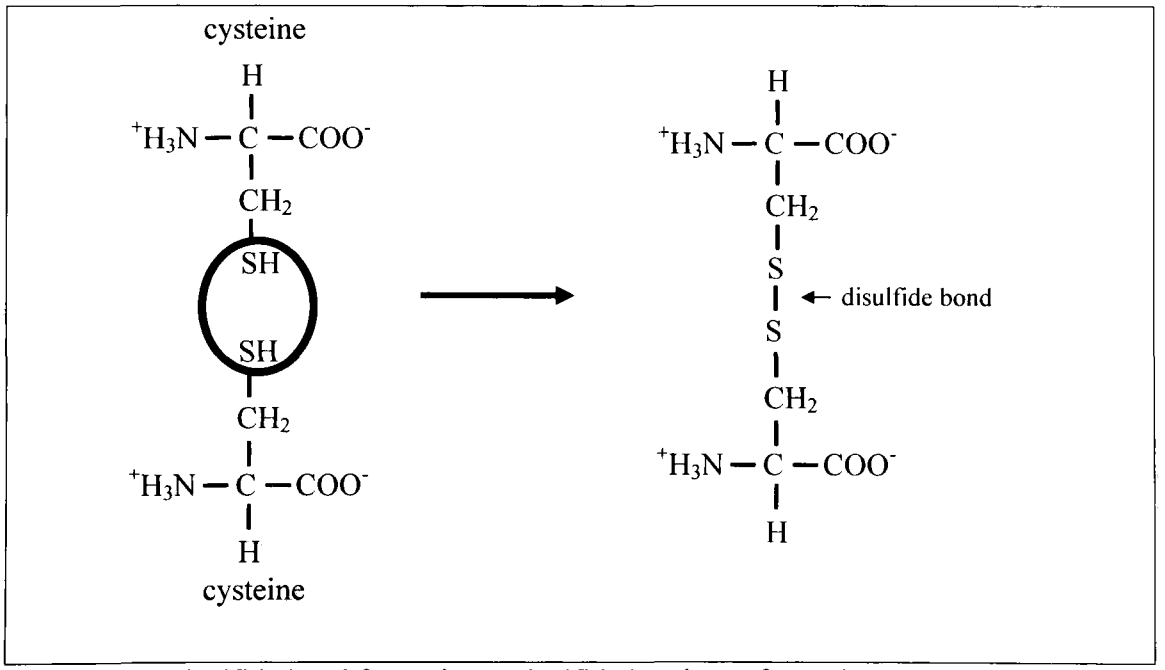


**Figure 1.3.** Disulfide bond formation. Disulfide bonds are formed between the sulfhydryl groups (-SH) of two cysteine residues.

The formation of disulfide bonds is important to the three-dimensional folding of a protein (http://www.biotech.vt.edu/classes/bion_4784/4-GeneExpression/Expression.htmL). Schuler *et al.* (1988) reported six cysteine residues in their bPL sequence assumed to form three disulfide bonds similar to those found in bovine PRL. The intracellular reducing environment of *E. coli* is not conducive to the formation of disulfide bonds. In order for disulfide bonds to be formed, it is necessary for the protein to be transported to the periplasm of the bacteria. This move needs to be directed by the protein itself, by means of a specific signal sequence in the primary amino acid sequence (http://www.biotech.vt.edu/classes/bion_4784/4-GeneExpression/Expression.htmL).

The proper folding of a protein is accomplished with the help of special proteins called chaperones. The correct chaperones needed for the folding of mammalian proteins may not be present within the bacteria cells. The incorrect folding results in the formation of insoluble aggregates called inclusion bodies (http://www.biotech.vt.edu/classes/bion_4784/4-GeneExpression/Expression.htmL). Helman *et al.* (1998) found that after transformation of their recombinant bPL molecule into *E. coli* cells, over 95% of the protein was found in inclusion bodies. A process has been established to make the protein in inclusion bodies soluble and to refold it, but this is a process that often fails (http://www.biotech.vt.edu/classes/bion_4784/4-GeneExpression/Expression.htmL).

A characteristic of bPL is that it is glycosylated, meaning that it has carbohydrate groups attached at specific amino acid sequences (Shimomura and Bremel, 1988; Byatt *et al.*, 1990). Bacteria are not able to add these carbohydrate groups. Studies have shown

that enzymatic removal of the N-linked oligosaccharides of bPL causes increased binding to growth hormone receptors (Byatt *et al.*, 1990). rbPL is therefore active, despite the fact that it is not glycosylated, but it does not resemble the native protein as closely as it could.

The key to producing bPL that is as close to the native protein as possible is to produce it in mammalian cells. The mammalian endoplasmic reticulum is a more suitable environment for disulfide bond formation. In addition, mammalian cells possess the appropriate chaperones needed for the proper folding of a mammalian protein, and they are able to add carbohydrates in the correct locations (http://www.biotech.vt.edu/classes/bion_4784/4-GeneExpression/Expression.htmL). The aim of this study, therefore, is to clone the bPL cDNA into a mammalian expression vector and amplify the recombinant molecule in E. coli cells. The final step will be to transfect the rbPL into mouse cells with the hopes that the mouse cells will be able to express the hormone complete with the post-translational modifications that are characteristic of native bPL. The following sections introduce the gene cloning techniques that will be used to accomplish such a feat.

**Gene Cloning**

The birth of genetics dates back over a century ago beginning with Gregor Mendel, who devised a set of rules explaining that inherited traits were controlled by factors called genes. Next came the discovery of chromosomes by Sutton in 1903 and the development of techniques for gene mapping by Morgan and his associates in the early 1920s. It was not, however, until the work of Avery, MacLeod and McCarty in 1944, and of Hershey

and Chase in 1952, that DNA was identified as the genetic material. Between the years 1952 and 1966 scientists began to understand the structure of DNA, the genetic code, and the processes of transcription and translation. Finally, in the years 1971-1973 came the advent of recombinant DNA technology, otherwise known as genetic engineering, the basis of gene cloning. Gene cloning involves the insertion of a fragment of DNA carrying a gene into a cloning vector. The cloning vector will then facilitate the entry of the recombinant molecule into a host organism, where the gene can be amplified. The next sections will explain the methods involved in the amplification of a gene and the subsequent in vitro production of the protein that it codes for (Brown, 1995).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Often times, RNA is isolated from a tissue and used as the starting material for genetic engineering studies. The cells of that tissue contain the DNA sequence needed to make up the whole organism, but actually express relatively few of the genes. Only those genes that are being expressed are transcribed into messenger RNA (mRNA). Extracting the RNA therefore ensures that the researcher is working with only those genes that are expressed in the tissue of interest (Brown, 1995).

mRNA cannot be ligated into a cloning vector, so it is necessary to convert it into DNA by complementary DNA (cDNA) synthesis. This is achieved through the process known as reverse transcription (RT) in which the enzyme reverse transcriptase is used to synthesize a DNA strand complementary to an existing RNA strand. The RNA strand is then degraded, leaving a single-stranded cDNA. It is then possible to proceed with the

ploymerase chain reaction (PCR), thus amplifying the actual gene of interest (Brown, 1995).

The first step of the PCR is primer design. Primers are generally 17 to 30 base pair oligonucleotides that correspond to the regions flanking the gene of interest. A primer that is too short may be able to anneal to a site other than the site directly before the gene, while a primer that is too long will hybridize at a slower rate, reducing the efficiency of the PCR. One PCR cycle is composed of three steps: (1) denaturation of the double-stranded DNA, (2) hybridization of the primers to the templates, and (3) DNA synthesis. The cycle is usually repeated 25-30 times, resulting in the production of several hundred million copies of the gene (Brown, 1995).

Each step of the PCR is carried out at a particular temperature. Denaturation of the double-stranded DNA usually takes place at 94°C. At this temperature, the base pairs are broken releasing single-stranded DNA that can act as templates. The temperature at which the primers hybridize or anneal to the templates is dependent upon the length and sequence of the primers themselves. By determining the melting temperature ($T_m$) of the primer template hybrid, it is possible to estimate the temperature at which a hybrid would form. For a primer of 21 base pairs or less the $T_m$ would be calculated as follows:

$$T_m = (4 \times [G + C]) + (2 \times [A + T])\ °C$$

in which [G + C] is the number of guanine (G) and cytosine (C) nucleotides in the primer sequence and [A + T] is the number of adenine (A) and tyrosine (T) nucleotides in the primer sequence. For a primer that is more than 21 nucleotides in length, the $T_m$ is calculated as follows:

$$T_m = 69.3 + (0.41 \times [G + C]\%) - (650/\text{primer length})$$

Where [G + C]% is the percentage of the primer that is made up of either G or C nucleotides and the primer length is the total number of nucleotides comprising the primer. The hybridization temperature is usually 1-2°C lower that the calculated $T_m$. DNA synthesis usually occurs at 74°C, which is just below the optimum temperature for *Taq* polymerase, the enxyme used to facilitate the production of the complementary strands. *Taq* polymerase is an ideal enzyme to use because, unlike most DNA polymerases, it can withstand the high temperatures used during the PCR. Figure 1.2 is a diagrammatic representation of a RT-PCR (Brown, 1995).

**Ligation and Transformation**

The DNA fragments resulting from the RT-PCR can now be ligated into a vector to produce a recombinant DNA molecule. The vector acts as a vehicle to transport the DNA fragment into a host bacterium. The introduction of the DNA into the living cell is referred to as transformation. Once inside the host cell, the vector multiplies, manufacturing numerous copies both of itself and of the DNA fragment that it carries. Copies of the recombinant molecule are passed on to the progeny of the host cell, where the vector and the DNA fragment can be replicated further. Eventually, a colony of host cells is produced, with each cell containing at least one copy of the recombinant molecule. It is then that the DNA fragment carried by the vector is said to be cloned (Brown, 1995).

There are two kinds of vectors that are used in gene cloning: plasmids and bacteriophages. This study will concentrate on plasmids, circular molecules of DNA that act independently within the bacterial cell. Plasmids often times carry special genes, for

example, genes that confer antibiotic resistance. Bacteria itself cannot grow in the presence of antibiotics. However, if the plasmid that it carries has an ampicillin resistance gene, the bacteria will be able to display resistance to that particular antibiotic. Therefore, culturing in growth media containing ampicillin would ensure that the colonies that grow contain the plasmid. Another characteristic of plasmids is that they have at least one DNA sequence that can act as an origin of replication. This allows the plasmid to multiply within the host independently of the host's chromosome (Brown, 1995).

**Transfection**

One problem with using bacteria as hosts for recombinant protein synthesis is that the bacteria may not process the recombinant protein correctly. Mammalian proteins are processed after translation by means of chemical modification of the amino acids. The post-translational modifications, glycosylation for example, which take place are often essential for the correct biological activity of the protein. Certain post-translational modifications such as glycosylation are extremely uncommon in bacteria, therefore the bacterial cells are unable to glycosylate the recombinant protein correclty. Transfecting the recombinant DNA molecule into mammalian host cells is a way to circumvent this problem.

Transfection is defined as the process of introducing nucleic acids into cells by non-viral methods. This can be achieved through both chemical and physical means. DEAE-dextran, calcium phosphate, and artificial liposomes are examples of chemical reagents often used to facilitate the transfer of nucleic acids into cultured mammalian cells.

Physical methods include direct microinjection (Cappechi, 1980), electroporation (Wong and Neumann, 1982), and biolostic particle delivery (Ye *et al.*, 1990). This study will focus on the use of artificial liposomes.

Tfx Reagents (Promega), the transfection reagents used in this study, employ cationic lipids in the delivery of nucleic acids to eukaryotic cells. They are a mixture of the synthetic cationic lipid molecule N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-di(oleoloxy)-1,4-butanediammonium iodide and the neutral lipid L-dioleoyl phosphatidylethanolamine (DOPE). The structures of these compounds are shown in Figure 1.4. Presumably, the cationic portion of the lipid molecule associates with the negatively charged nucleic acid that is to be transferred into the mammalian cell (Figure 1.5). This creates a lipid/nucleic acid complex with an overall net positive charge. The complex is then able to closely associate with the negatively charged cell membrane and is endocytosed by the cell. It has been suggested that DOPE works to release the complexes from the endosomes so that they may make their way into the nucleus (Farhood *et al.*, 1995).

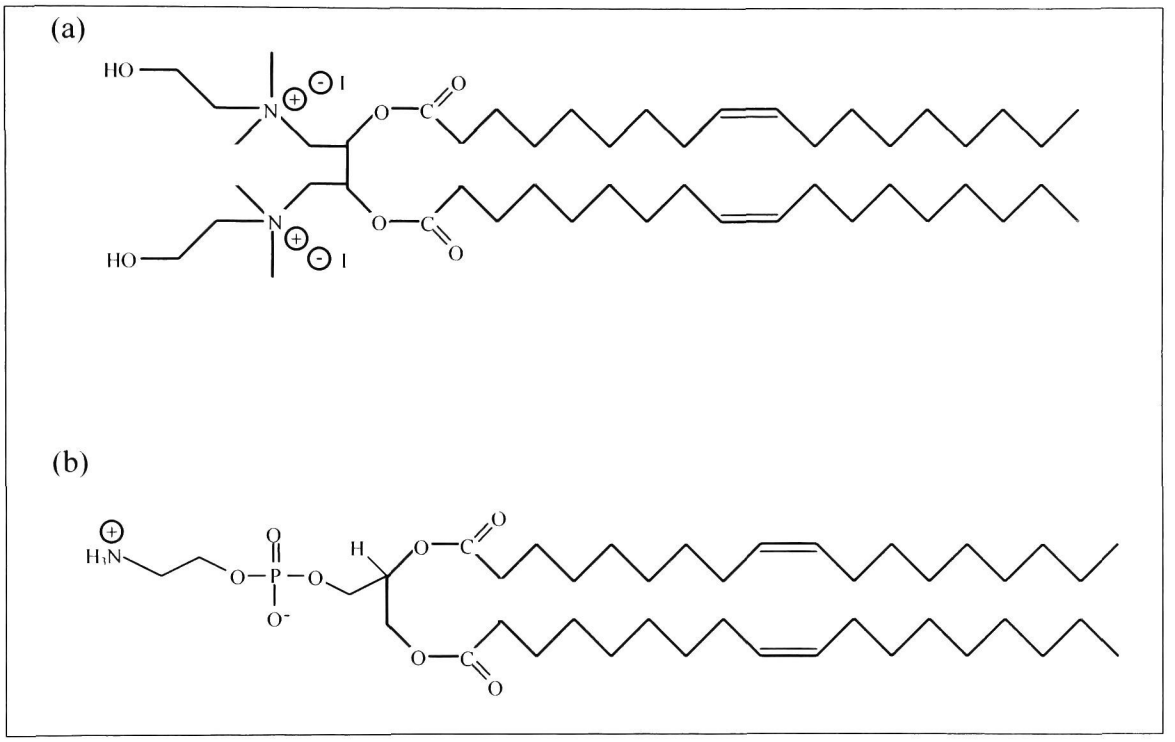


**Figure 1.4.** Components of the Tfx transfection reagents. The Tfx reagents (Promega) are a mixture of (a) the synthetic cationic lipid molecule N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-di(oleoloxy)-1,4-butanediammonium iodide and (b) the neutral lipid L-dioleoyl phosphatidylethanolamine (DOPE).

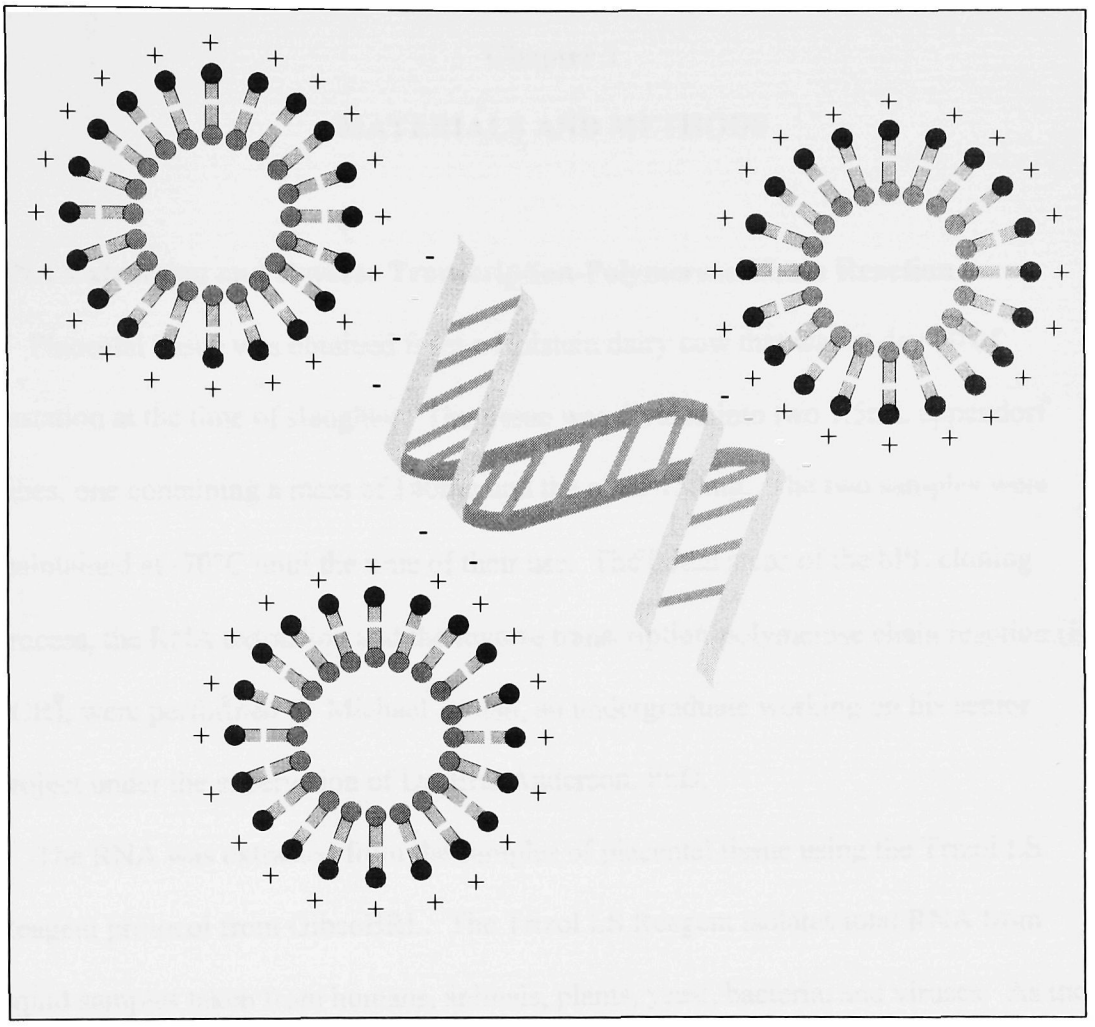

**Figure 1.5.** Schematic representation of a lipid/nucleic acid complex. The cationic portion of the lipid molecule associates with the negatively charged nucleic acid. This creates a lipid/nucleic acid complex with an overall net positive charge. The complex is then able to closely associate with the negatively charged cell membrane and is endocytosed by the cell.

# Chapter 2

# MATERIALS AND METHODS

## RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Placental tissue was obtained from a Holstein dairy cow that was at day 90 of gestation at the time of slaughter. The tissue was divided into two 1.5mL eppendorf tubes, one containing a mass of 140mg and the other 150mg. The two samples were maintained at -70°C until the time of their use. The initial steps of the bPL cloning process, the RNA extraction and the reverse transcription-polymerase chain reaction (RT-PCR), were performed by Michael Jordan, an undergraduate working on his senior project under the supervision of Dr. Eric Anderson, PhD.

The RNA was extracted from the samples of placental tissue using the Trizol LS Reagent protocol from GibcoBRL. The Trizol LS Reagent isolates total RNA from liquid samples taken from humans, animals, plants, yeast, bacteria, and viruses. As the samples were being homogenized, the Trizol LS Reagent disrupted the cellular components, while at the same time keeping the RNA intact. The solution was next separated into an aqueous phase (containing the RNA) and an organic phase by the addition of chloroform followed by centrifugation. Once the aqueous phase was collected, isopropyl alcohol was added to it to precipitate the RNA. The resulting pellet was washed with 75% ethanol, allowed to dry, and dissolved in 20mL RNase-free $H_2O$. The tubes containing the RNA were stored at -20°C.

Oligonucleotide primers were designed using a known bPL sequence obtained from GenBank, accession number M33268. This sequence, reported by Yamakawa *et al.*

(1990) is detailed in Figure 2.1. Also included in Figure 2.1 are the positions of the primer sequences, labeled bPL-F (forward) and bPL-R (reverse). The bPL-F primer (5' CCT CCA TTC AGT CCC TGT TGG 3') was a 21-mer with a melting temperature ($T_m$) of 66°C, while the bPL-R primer (5' GAG ACC CAT TAC ACC CAA ACA TG 3') was a 23-mer with a $T_m$ of 60.6°C. Both primers were obtained from Sigma.

Reverse transcription (RT) was performed on both of the sample tubes containing the extracted RNA. The reactions were set up as shown in Table 2.1.

**Table 2.1. Reverse transcription reactions**

| Tube | RNA template | bPL-R | RNAse out | $H_2O$ | Total volume |
|---|---|---|---|---|---|
| 1 | 2μl from 140mg sample | 1μl | 1μl | 6μl | 10μl |
| 2 | 2μl from 150mg sample | 1μl | 1μl | 6μl | 10μl |
| 3 | 0 | 1μl | 1μl | 8μl | 10μl |

The reverse transcription reactions were produced by mixing the RNA template, the bPL-R (reverse) primer (Sigma), RNAse out, which destroys RNAses (Sigma), and distilled water.

Each reaction was incubated at 72°C for two minutes, allowing the primer to anneal to the template. The RT reaction cocktail (Table 2.2) was then prepared. Reverse transcription reaction cocktail (Table 2.2) was added to each of the three reaction tubes at a volume of 5.8μl per tube. The tubes were then incubated at room temperature for 10 minutes, followed by an incubation of 1 hour at 42°C. The resulting products served as the templates for the polymerase chain reactions (PCR).

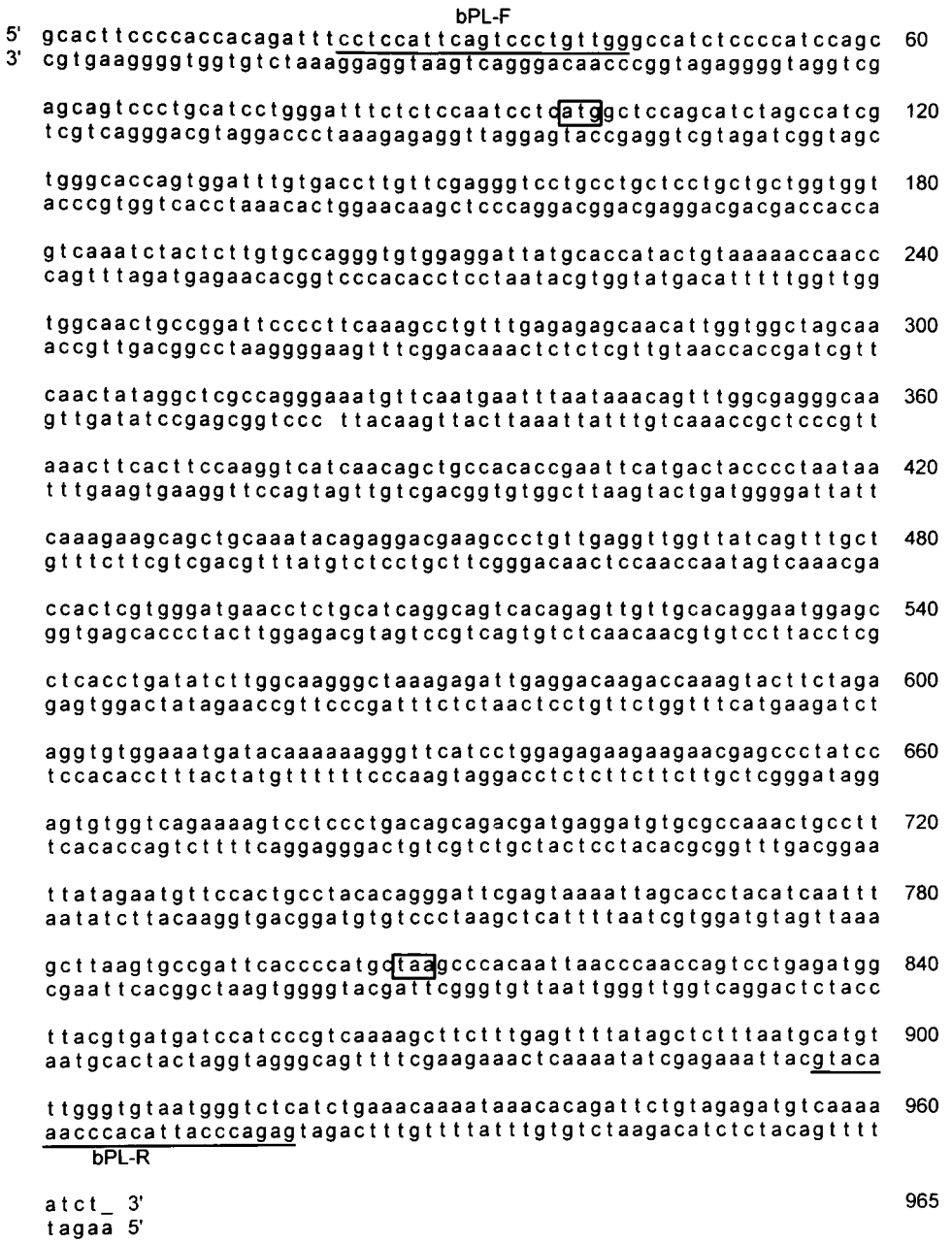


**Figure 2.1.** bPL sequence obtained from GenBank, accession number M33268. This sequence originally appeared in a paper written by Yamakawa et al. (1990). The initiation codon (ATG) and stop codon (TAA) are identified with boxes around them. The primer sequences (bPL-F and bPL-R) are underlined.

**Table 2.2. Preparation of the reverse transcription reaction cocktail.**

| Component | Volume per reaction tube |
|---|---|
| 5x RT buffer | 3µl |
| 0.1M DTT | 1µl |
| 10mM dNTP | 1µl |
| Superscript | 0.8µl |
| Total volume | 5.8µl |

Three clean microfuge tubes were obtained and 47.5 µl of the PCR cocktail (Table 2.3) was added to each. Next, 2µl of each template was added to its respective tube. The control tube (tube 3) received 2µl RNase-free $H_2O$. *Taq* polymerase was added at a volume of 0.5µl per reaction tube. The polymerase chain reactions were performed in the PTC-100 Programmable Thermal Controller (M.J. Research).

**Table 2.3. Preparation of the polymerase chain reaction cocktail.**

| Component | Volume per reaction tube |
|---|---|
| $H_2O$ | 27.5µl |
| $MgCl_2$ | 5µl |
| 10x buffer | 5µl |
| 2mM dNTP | 5µl |
| bPL-F | 2.5µl |
| bPL-R | 2.5µl |
| Total volume | 47.5µl |

**Gel Extraction of the PCR Product**

A sample of each PCR product (from tubes 1 and 2) was run on agarose (1%) gel electrophoresis and the bPL DNA fragments were excised using a scalpel. The QIAquick Gel Extraction Kit (QIAGEN) was used to extract and purify the DNA from each gel slice. The volumes of buffer QG and isopropanol used during the purification process

were based on the masses of the excised gel slices. The gel slices were weighed and 3 volumes of buffer QG were added per one volume of gel. Likewise, one gel volume of isopropanol was added at the appropriate time. The volumes used are outlined in Table 2.4. The optional step 9 was followed and the DNA was eluted with water.

**Table 2.4. Reagent volumes used in the gel extraction of bPL.**

| Mass of placental tissue | Mass of gel slice | Buffer QG | Isopropanol |
|---|---|---|---|
| 140mg | 110mg | 330µl | 110µl |
| 150mg | 120mg | 360µl | 120µl |

The mass of each gel slice was used to determine the volumes of buffer QG and isopropanol used in the gel extraction process.

The purified bPL insert originating from the 140mg sample of placental tissue was used in the following ligation step.

**Ligation of bPL with the pTarget Mammalian Expression Vector**

The purified bPL insert was next ligated into the pTarget Mammalian Expression Vector (Figure 2.2) from Promega. In order to accomplish this, three ligation reactions were set up (Table 2.5). Each reaction was brought to a total volume of 10µl with the addition of sterile $dH_2O$. The reactions were then kept at 4°C overnight (18 hours), resulting in the production of recombinant pTarget-bPL molecules.

**Table 2.5. Reactions set up for the ligation of the bPL insert into the pTarget vector.**

| Components | Ligation 1 | Ligation 2 | Ligation 3 |
|---|---|---|---|
| Vector | 1μl | 1μl | 1μl |
| bPL insert | 2.5μl | 5μl | 7μl |
| 10x ligation buffer | 1μl | 1μl | 1μl |
| Sterile $dH_2O$ | 4.5μl | 2μl | 0 |
| T4 DNA Ligase | 1μl | 1μl | 1μl |

The components of the three ligation reactions are listed above. The 10x ligation buffer and T4 DNA ligase were provided along with the pTarget vector (Promega).

**Transformation of Recombinant pTarget-bPL into *E. coli* Cells**

One tenth dilutions of each ligation product were made by adding 1μl recombinant DNA to 9μl sterile $dH_2O$. Three eppendorf tubes were placed on ice. A volume of 50μl competent JM109 *E. coli* cells was added to each tube, along with 1μl of the corresponding DNA dilution. The tubes were kept on ice for 30 minutes, placed at 42°C for 45 seconds, and then returned to the ice for 2 minutes. At the end of that time, 500μl SOC broth (sterile solution that supplies amino acids and other nutrients) was added to each tube. The tubes were then placed in the 37°C shaker (set at 220rpm) for 1 hour.

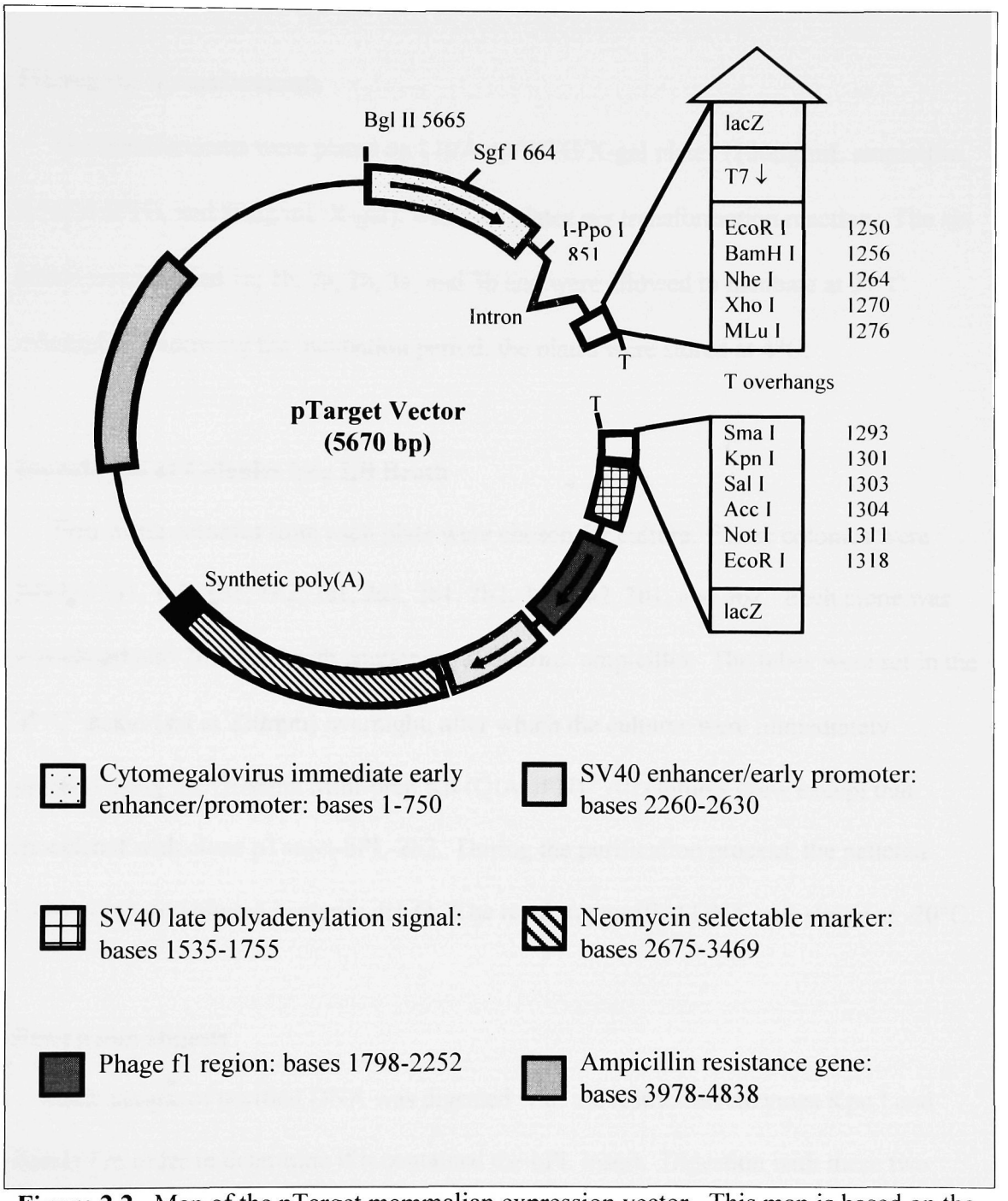


**Figure 2.2.** Map of the pTarget mammalian expression vector. This map is based on the map provided by Promega.

**Plating the Transformants**

The transformants were plated on LB/Amp/IPTG/X-gal plates (100μg/mL ampicillin, 0.5mM IPTG, and 80μg/mL X-gal), with two plates per transformation reaction. The six plates were labeled 1a, 1b, 2a, 2b, 3a, and 3b and were allowed to incubate at 37°C overnight. Following the incubation period, the plates were stored at 4°C.

**Inoculation of Colonies into LB Broth**

Two white colonies from each plate were chosen for culture. These colonies were labeled 1a1, 1a2, 1b1, 1b2, 2a1, 2a2, 2b1, 2b2, 3a1, 3a2, 3b1, and 3b2. Each clone was inoculated into 2mL LB broth containing 100μg/mL ampicillin. The tubes were set in the 37°C shaker (set at 220rpm) overnight, after which the cultures were immediately purified using the QIAspin Mini-prep Kit (QIAGEN). All cultures grew except that inoculated with clone pTarget-bPL-2b2. During the purification process, the pelleted DNA was reconstituted in sterile $dH_2O$. The resulting purified DNA was stored at -20°C.

**Restriction Digests**

Each sample of purified DNA was digested with the restriction enzymes Kpn I and BamH I in order to determine if it contained the bPL insert. Digestion with these two enzymes would effectively separate the insert from the vector since the BamH I recognition site was located 5' of the insert, while the Kpn I recognition site was located 3' of the insert. Another important reason for using these two enzymes was that there

was only one recognition site for each in the entire recombinant molecule. Table 2.6 gives the components of one digestion reaction and a cocktail recipe for 12 digests.

**Table 2.6. Components of a double digest using Kpn I and BamH I.**

| Component | Volume per digest |
|---|---|
| 10x NEBuffer I | 2μl |
| BSA (1mg/mL) | 2μl |
| RNAse-free $H_2O$ | 9μl |
| Kpn I | 1μl |
| BamH I | 1μl |

Each digest was carried out in a 0.5mL eppendorf tube. Digest mix was added to each of the 11 eppendorf tubes at a volume of 15μl per tube. Next, 5μl purified DNA was added to the respective tubes. The digests were incubated at 37° for 2 hours. In order to stop the action of the enzymes, 4μl loading dye was added to each of the digests.

**Gel Electrophoresis of Digested DNA**

The digests, along with a 100bp ladder from GibcoBRL, were run on a 1% agarose gel stained with ethidium bromide. Each of the digests was loaded into the appropriate well and the gel was run at 275V for 15 minutes. Photographs of the gel were taken using the ChemiImager 4400 Low Light Imaging System.

**DNA Sequencing**

Each clone appeared to contain the bPL insert, and was therefore sent to the University of Maine DNA Sequencing Facility to be sequenced. The sequences returned were analyzed using the MegaBLAST program provided by the National Center for Biotechnology Information (NCBI).

**Digest of pTarget-bPL-1b2 and pTarget-bPL-3a1 and Isolation of the bPL Gene**

The results of the MegaBLAST showed that the bPL gene, although present, was in the incorrect orientation. This made it necessary to remove the gene from the pTarget vector for insertion into another mammalian expression vector, the pcDNA3.1(+) vector from Invitrogen (Figure 2.3). Clones pTarget-bPL-1b2 and pTarget-bPL-3a1 were chosen at random to digest. The double digest using the restriction enzymes Kpn I and BamH I (Table 2.6) was again employed. The two digests were run on a 1% agarose gel stained with ethidium bromide and the bPL DNA fragments were excised and purified using the QIAquick Gel Extraction Kit (QIAGEN).

**Digest and Purification of the pcDNA3.1(+) Vector**

The pcDNA3.1 vector was also digested with Kpn I and BamH I. The reaction was set up as shown in Table 2.7.

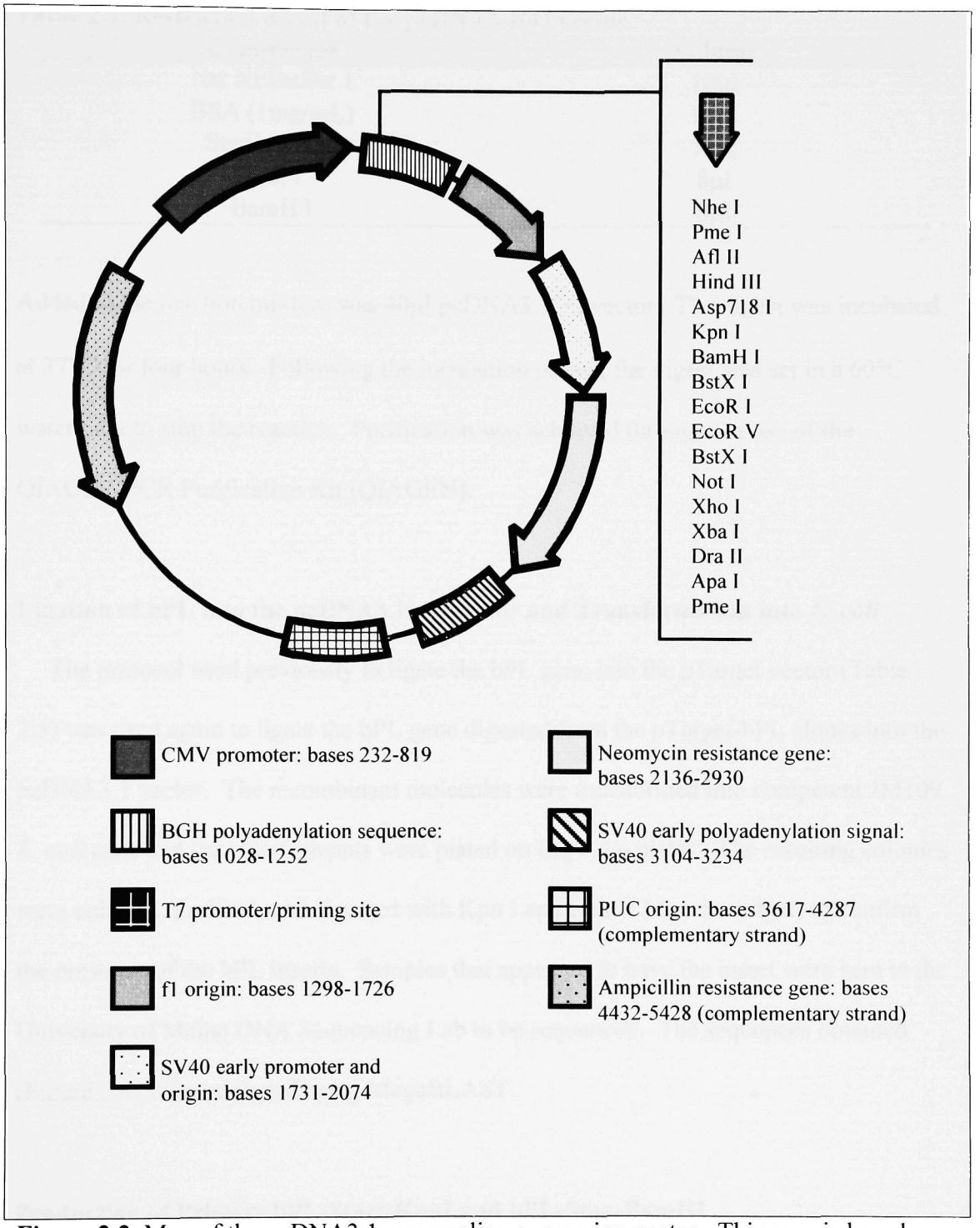


**Figure 2.3.** Map of the pcDNA3.1 mammalian expression vector. This map is based on the map provided by Invitrogen.

**Table 2.7. Restriction digest of the pcDNA3.1(+) vector.**

| Component | Volume |
|---|---|
| 10x NEBuffer 1 | 16µl |
| BSA (1mg/mL) | 16µl |
| Sterile $dH_2O$ | 72µl |
| Kpn I | 8µl |
| BamH I | 8µl |

Added to the reaction mixture was 40µl pcDNA3.1(+) vector. The digest was incubated at 37°C for four hours. Following the incubation period, the digest was set in a 60°C water bath to stop the reaction. Purification was achieved through the use of the QIAGEN PCR Purification Kit (QIAGEN).

**Ligation of bPL into the pcDNA3.1(+) Vector and Transformation into *E. coli***

The protocol used previously to ligate the bPL gene into the pTarget vector (Table 2.5) was used again to ligate the bPL gene digested from the pTarget-bPL clones into the pcDNA3.1 vector. The recombinant molecules were transformed into competent JM109 *E. coli* cells and the transformants were plated on LB/Amp plates. The resulting colonies were cultured, purified, and digested with Kpn I and BamH I (as described) to confirm the presence of the bPL inserts. Samples that appeared to have the insert were sent to the University of Maine DNA Sequencing Lab to be sequenced. The sequences obtained (Figure 2.4) were analyzed using MegaBLAST.

**Production of Primers bPL-Start-KpnI and bPL-Stop-BamHI**

According to the pcDNA3.1 product manual, the insert must contain a Kozak translation initiation sequence for proper initiation of translation to occur. It is critical to have a G or A at position –3 and a G at position +4 (positions are relative to the start

codon). The pcDNA3.1-bPL-3a1-2-1 sequence did have a G at position +4, but had a C in place of the required G or A at position –3. In order to rectify this, new primers, bPL-Start-KpnI and bPL-Stop-BamHI, were designed. Primer bPL-Start-KpnI (5' GTTCATGGTACCGCTATGGCTCCAGCATCTAGCC 3') also incorporated a Kpn I recognition site (GGTACC) prior to the start codon. Primer bPL-Stop-BamHI (5' GTACCTGGATCCTTAGCATGGGGTGAATCGGC 3') incorporated a BamH I recognition site (GGATCC) following the stop codon. The placement of these primers can be seen in Figure 2.4.

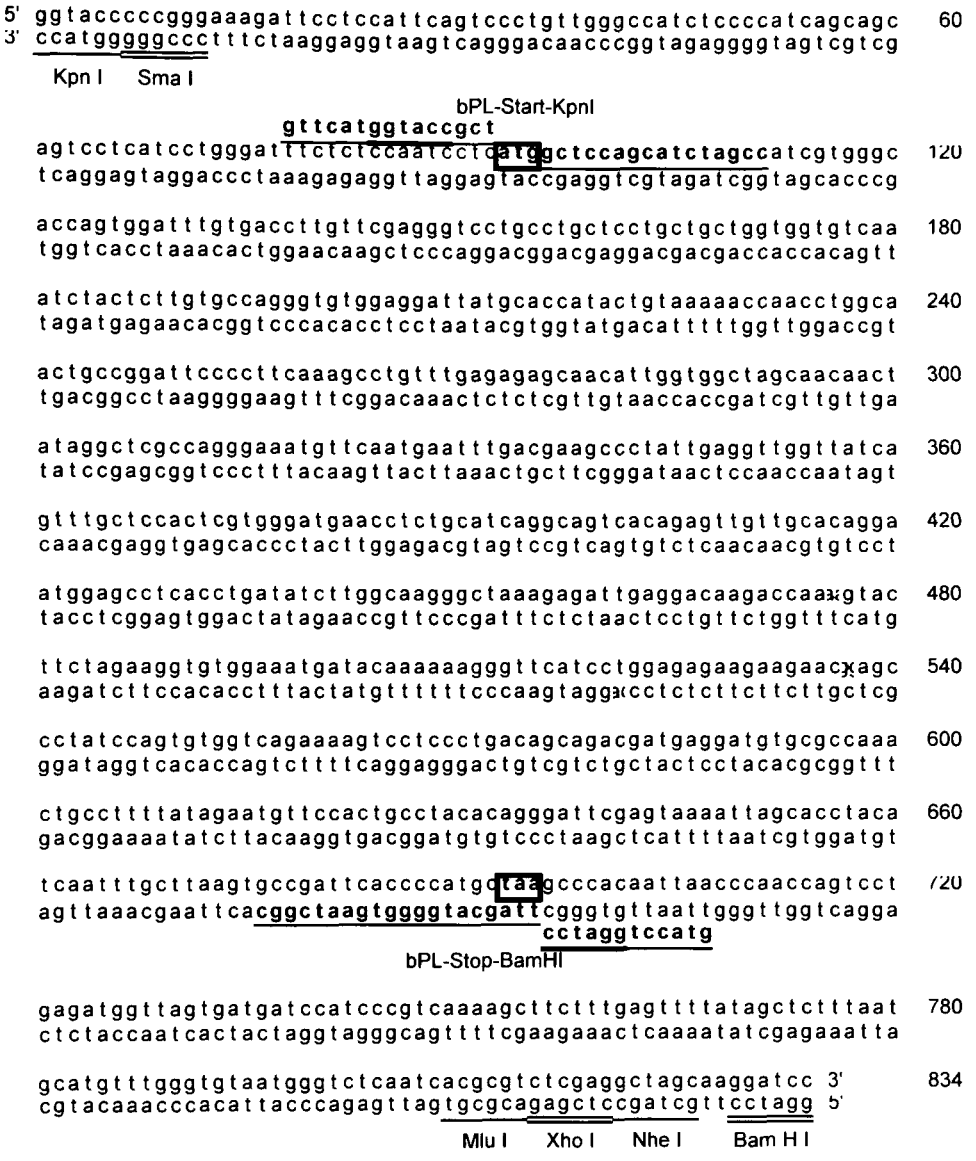


**Figure 2.4.** pcDNA3.1-bPL-3a1-2-1 sequence. The placement of the new primer sequences (bPL-Start-KpnI and bPL-Stop-BamHI) are indicated in bold and underlined. The cut sites incorporated into the sequence from the pTarget vector are underlined either once or twice

## PCR

A PCR was set up using pcDNA3.1-bPL-3a1-2-1 as the template and bPL-Start-KpnI and bPL-Stop-BamHI as the primers (Table 2.8).

**Table 2.8. PCR using pcDNA3.1-bPL-3a1-2-1 as the template.**

| Component | Volume |
|---|---|
| PCR buffer | 88μl |
| *Taq* polymerase | 2μl |
| bPL primer pair (5μM each) | 4μl |
| pcDNA3.1-bPL-3a1-2-1 | 8μl |

In order to make the bPL primer pair, 10μl of each primer (100μM each) was added to 180μl $H_2O$. The mixture was divided into four PCR tubes (25μl each) and the reaction was run for 35 cycles using a 55°C annealing temperature in the PTC-100 Programmable Thermal Controller. The PCR product was run on a 1.5% gel stained with ethidium bromide at 172V for 13 minutes. The remaining 90μl of PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN) and the DNA was eluted with 40μl buffer EB (provided in the purification kit).

## Restriction Digest of the PCR Product

A double digest using the restriction enzymes Kpn I and BamH I was performed on the PCR product (Table 2.9).

**Table 2.9. Restriction digest of the PCR product using Kpn I and BamH I.**

| Component | Volume |
|---|---|
| PCR product | 20µl |
| Buffer NEB 1 | 10µl |
| 100x BSA | 1µl |
| Kpn I | 4µl |
| BamH I | 4µl |
| Sterile $dH_2O$ | 6µl |

The reaction was incubated at 37°C for 30 minutes. In order to stop the action of the enzymes, 10µl 6x dye was added to the PCR product digest. The sample was run on a 1.5% agarose gel stained with ethidium bromide at 172V for 13 minutes. The band corresponding to the estimated size of the insert was excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN). The DNA was eluted in 20µl buffer EB (provided in the kit).

**Ligation of the bPL Insert into the pcDNA3.1(+) Vector**

A different protocol was used to ligate the new bPL insert into the pcDNA3.1(+) vector. This protocol involved a 1:1 ligation of insert:vector with 500ng DNA total. Table 2.10 shows the reaction components. The mixture was incubated at 15°C overnight (16-18 hours).

**Table 2.10. Ligation of the bPL insert into the pcDNA3.1(+) vector.**

| Component | Volume |
|---|---|
| PCR product | 20μl |
| BSA (2mg/mL) | 1μl |
| Fresh 4mM ATP | 2μl |
| Sterile $dH_2O$ | 10μl |
| T4 Ligase | 1μl |
| bPL insert | 0.3μl |
| Cut pcDNA3.1(+) vector | 3.7μl |

**Transformation of the Recombinant DNA into Competent *E. coli* cells**

A different transformation technique was also employed. To a chilled tube was added 100μl cold 100mM Tris HCl (pH 7.4), 20μl ligation reaction, and 200μl competent DH5α *E. coli* cells. The tube was incubated on ice for 20 minutes, heatshocked for two minutes at 42°C, and then allowed to stand at room temperature for 10 minutes. At the end of the incubation period, 2mL LB broth was added. The mixture was incubated at 37°C in the shaker (set at 220rpm) for one hour, followed by centrifugation at 6000×g for five minutes. The supernatant was decanted and the pellet resuspended in 200μl LB broth. Samples (5μl and 50μl) were plated on LB/Amp plates with the concentration of ampicillin at 50μg/mL. Three colonies were chosen for culture in LB broth containing 100μg/mL ampicillin. The DNA was purified using the QIAquick Spin Mini-prep Kit (QIAGEN) and the purified DNA was digested with Kpn I and BamH I (Table 2.6). The digests were run on a 1% agarose gel stained with ethidium bromide and the gels were photographed using the ChemiImager 4400 Low Light Imaging System. Samples appearing to contain the bPL insert (based on apparent molecular weight) were sent to the University of Maine DNA Sequencing Facility for sequencing and sequences were analyzed using MegaBLAST provided by NCBI.

**Culture of the Mouse L929 Cells**

Mouse L929 cells (ATCC) were obtained from Dr. Charles Moody and were maintained in Medium 1640 (Sigma) supplemented with 10% calf serum and 1% glutamine-penicillin- streptomycin (Sigma) in 25cm$^2$ cell culture flasks. In order to pass the cells, the media was removed and the cells were washed with enough Dulbecco's Phosphate Buffered Saline (DPBS from Sigma) to completely cover the monolayer. The cells were then incubated in trypsin-EDTA (Sigma) for one minute until they began to round up. The trypsin-EDTA was then removed and the culture vessel was struck against the palm in order to dislodge the remaining adherent cells. The cells were then resuspended in 5mL complete media and counted using a hemocytometer. Cells were seeded in a new 25cm$^2$ flask at $5\times10^4$ cells/mL with a final volume of 5mL complete media per flask. The flasks were kept in a 37°C incubator at 6% $CO_2$ until they were confluent and needed to be passed again.

**Optimization of Transfection**

Mouse L929 cells were transfected with the pEGFP-N3 vector (Clontech) (Figure 2.5) in order to determine the optimal transfection reagent, Tfx-20 or Tfx-50 (Promega), and concentration of DNA to use with the cells. The day before the transfection was to take place, two 24-well plates were seeded with $5\times10^4$ cells/well in 1mL complete media. The plates were kept at 37°C and 6% $CO_2$ for 24 hours. Various amounts of purified vector DNA (0.25, 0.50, 0.75, and 1.00 μg per well) were tested at two different charge ratios of Tfx Reagent:DNA (2:1 and 4:1). One plate was used for transfection with Tfx-20 and the other for transfection with Tfx-50. The transfections were set up as shown in Table 2.11.

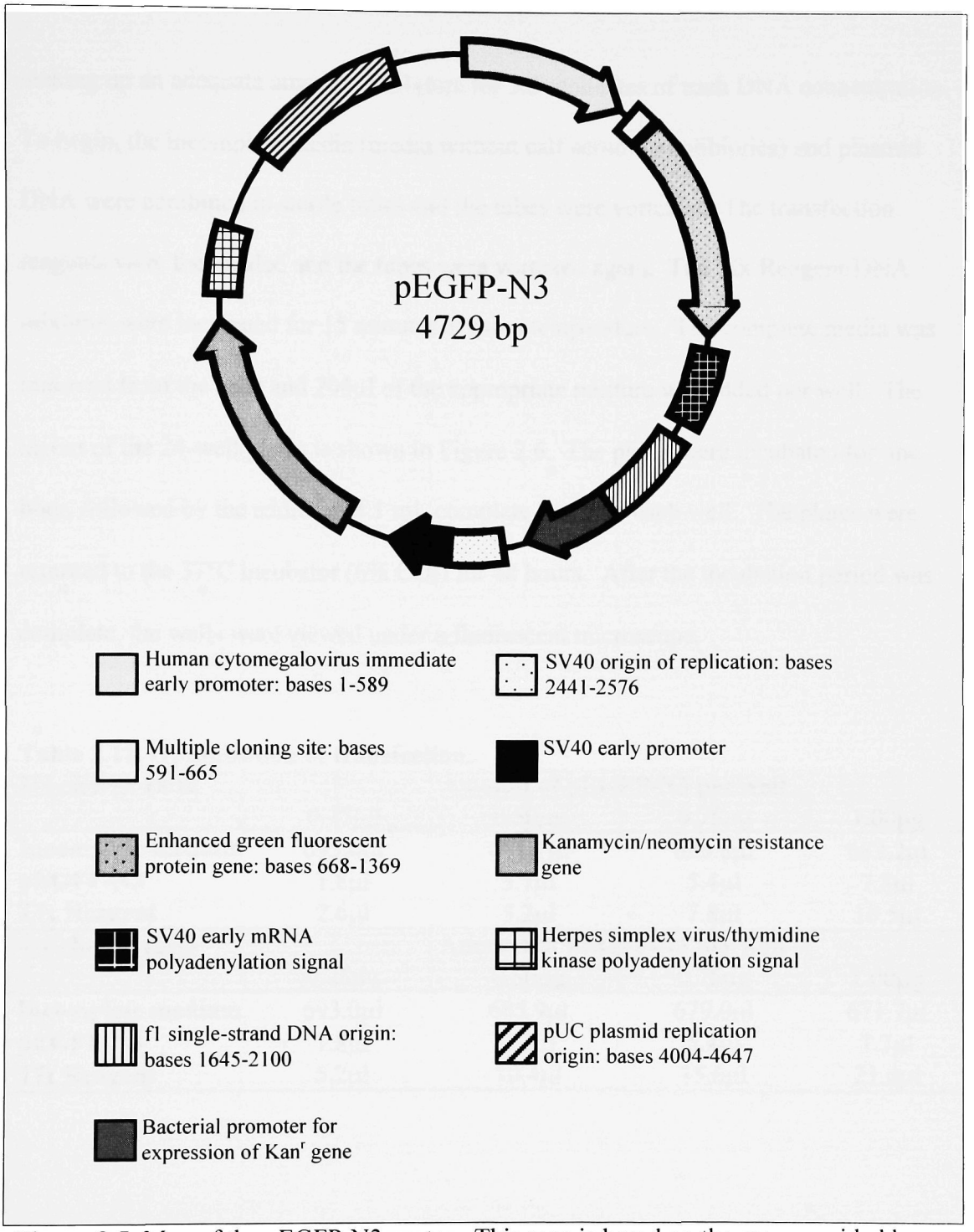


**Figure 2.5.** Map of the pEGFP-N3 vector. This map is based on the map provided by Clontech.

making up an adequate amount of mixture for 3.5 replicates of each DNA concentration. To begin, the incomplete media (media without calf serum or antibiotics) and plasmid DNA were combined in sterile tubes and the tubes were vortexed. The transfection reagents were then added and the tubes were vortexed again. The Tfx Reagent/DNA mixtures were incubated for 15 minutes at room temperature. The complete media was removed from the cells and 200µl of the appropriate mixture was added per well. The layout of the 24-well plates is shown in Figure 2.6. The plates were incubated for one hour, followed by the addition of 1 mL complete media to each well. The plates were returned to the 37°C incubator (6% $CO_2$) for 48 hours. After the incubation period was complete, the wells were viewed under a fluorescent microscope.

**Table 2.11. Optimization of transfection.**

| **2:1 charge ratio** | **Amount of pEGFP-N3 per well** | | | |
|---|---|---|---|---|
| | 0.25µg | 0.50µg | 0.75µg | 1.00µg |
| **Incomplete medium** | 695.6µl | 691.1µl | 686.8µl | 682.2µl |
| **pEGFP-N3** | 1.8µl | 3.7µl | 5.4µl | 7.3µl |
| **Tfx Reagent** | 2.6µl | 5.2µl | 7.8µl | 10.5µl |
| **4:1 charge ratio** | **Amount of pEGFP-N3 per well** | | | |
| | 0.25µg | 0.50µg | 0.75µg | 1.00µg |
| **Incomplete medium** | 693.0µl | 685.9µl | 679.0µl | 671.7µl |
| **pEGFP-N3** | 1.8µl | 3.7µl | 5.4µl | 7.3µl |
| **Tfx Reagent** | 5.2µl | 10.4µl | 15.6µl | 21.0µl |

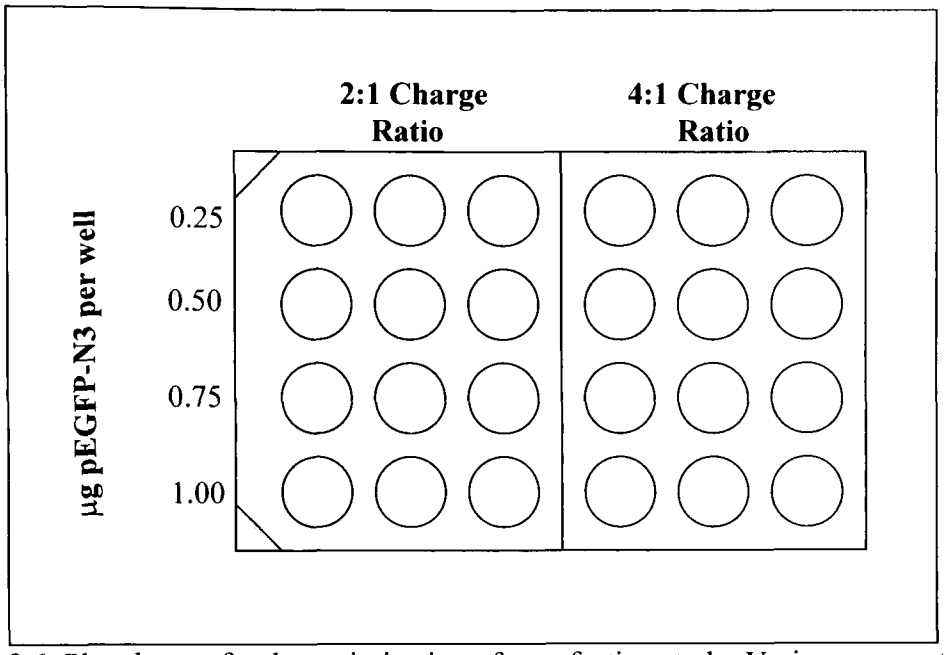


**Figure 2.6.** Plate layout for the optimization of transfection study. Various amounts of purified pEGFP-N3 DNA (0.25, 0.50, 0.75, and 1.00 µg per well) were tested at two different charge ratios of Tfx Reagent:DNA (2:1 and 4:1). Two plates were set up in this manner, one for Tfx-20 and the other for Tfx-50.

**Transfection of the L929 Cells with Purified pcDNA3.1-bPL DNA**

From the optimization of transfection study, it was concluded that the most favorable results occurred with the Tfx-20 Reagent at a 2:1 charge ratio and 1.00µg DNA per well. It was therefore decided that this was the combination that would be used in the transfection of the mouse cells with the pcDNA3.1-bPL recombinant DNA. Twenty-four hours before the transfection was to take place, a 24-well plate was seeded with L929 cells at $5\times10^4$ cells/well in 1mL of complete media. The transfection was carried out as had been done for the optimization study, this time using 1.00µg pcDNA3.1-bPL and a charge ratio of 2:1 for every well. The media from the cells was collected and replaced

with new media every 48 hours for ten days. The collected media was tested for the presence of bPL using radioimmunoassay (Wallace, 1993).

In a second trial, twelve wells of a 24-well plate were transfected with 1μg pcDNA3.1-bPL per well with a charge ratio of 2:1 Tfx-20 Reagent: DNA. The remaining twelve wells were to act as the control, being monitored over the course of the study for growth. At 12, 24, and 48 hours post-transfection, the media was collected from three of the transfected wells and the cells were removed from the surface of the flask (using trypsin-EDTA) and were counted. In addition to performing a total cell count, the cells were stained with trypan blue in order to distinguish the number of dead cells. At each collection point, the cells from three of the control wells were removed and counted, once again staining with trypan blue.

**Transfection of a New Batch of Mouse L929 Cells**

A new batch of L929 cells was purchased from American Type Culture Collection. The cells were thawed and cultured according to the manufacture's directions. These new cells were transfected with pcDNA3.1-bPL. Media was removed from the cells 48 hours after the transfection reaction took place. The media was then assayed for the presence of bPL (Wallace, 1993).

# Chapter 3

# RESULTS

## Production of the Recombinant bPL Molecule

bPL cDNA was isolated from the placental tissue of a Holstein dairy cow at day 90 of gestation. The bPL gene was identified with the help of primers (bPL-F and bPL-R) that were designed using a known bPL sequence reported by Yamakawa *et al.* (1990). PCR techniques were then carried out in order to amplify the DNA fragment of interest. This fragment, beginning and ending with the primers, would be composed of 847 nucleotides. The fragment was cloned into the pTarget mammalian expression vector and the recombinant molecule was transformed into competent JM109 *E. coli* cells. The transformants were plated on LB/Amp/IPTG/X-gal plates, which allowed for blue-white color screening of the clones (Table 3.1).

**Table 3.1. Colonies resulting from the transformation of the recombinant pTarget-bPL molecule into competent JM109 *E. coli* cells.**

| Plate | # white colonies | # blue colonies | Total # colonies |
|---|---|---|---|
| 1a | 2 | 4 | 6 |
| 1b | 4 | 4 | 8 |
| 2a | 15 | 16 | 31 |
| 2b | 17 | 15 | 32 |
| 3a | 8 | 4 | 12 |
| 3b | 7 | 7 | 14 |

Each of the three ligation reactions involving the pTarget vector and the bPL insert were used in the transformation of the recombinant molecule into competent JM109 *E. coli* cells. Two plates were made from each reaction.

Two white colonies from each of the six plates were chosen for culture. The DNA from the eleven colonies that grew was purified and the purified DNA was then digested with the restriction enzymes BamH I and Kpn I. The digests were run on agarose (1%) gel electrophoresis (Figure 3.1).

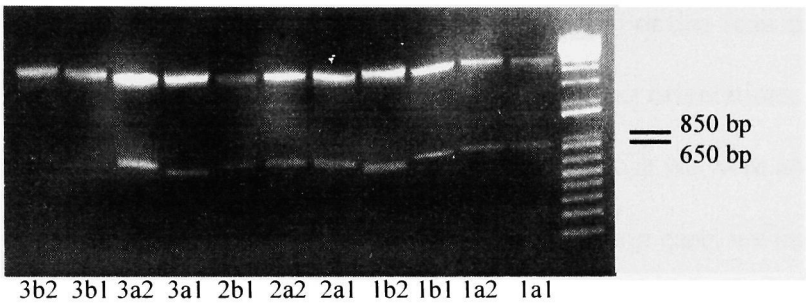


**Figure 3.1.** Gel electrophoresis of the pTarget-bPL restriction digests. Eleven clones were digested with Kpn I and BamH I and the digests, along with a 1kb plus ladder (GibcoBRL) were run on a 1% agarose gel stained with ethidium bromide. All eleven clones appeared to contain the bPL insert (band at 800bp).

Each of the clones appeared to contain the bPL insert and all were sequenced by the University of Maine DNA Sequencing Facility. The returned sequences (Figure 3.2) were analyzed using the Mega BLAST program. Mega BLAST was able to align the plus strands of our sequences with the minus strands of bPL sequences in the GenBank database. This told us that our inserts were ligated into the pTarget vector in the incorrect orientation (Figure 3.3). In order for proper transcription of the bPL gene to take place, it was necessary to have the initiation codon (ATG) of the gene on the plus strand following the transcriptional start site of the vector. In the pTarget-bPL recombinant molecule, the ATG was on the minus strand.

In order to rectify this, it was necessary to remove the bPL insert from the pTarget vector and ligate it into another vector, the pcDNA3.1(+) vector. Two of the clones, pTarget-bPL-1b2 and pTarget-bPL-3a1, were chosen at random to digest. The new vector was digested in such a manner that the insert had no choice but to ligate into the vector in the correct orientation (Figure 3.4). The pTarget vector came from the manufacturer already digested. This vector had been prepared by making a blunt cut and adding thymine (T) overhangs, so that each end was identical. For this reason, the insert was able to ligate into the vector in both the correct and incorrect orientations. The pcDNA3.1(+) vector was preferable to the pTarget vector because we were able to digest it ourselves with the enzymes appropriate for our research. In our case, we needed to digest with BamH I and Kpn I so that the bPL insert would be ligated in the correct orientation.

```
  1 cacccaaacatgcattaaagagctataaaactcaaagaagcttttgacgggatggatcat  60
 61 cactaaccatctcaggactggttgggttaattgtgggcttagcatggggtgaatcggcac 120
121 ttaagcaaattgatgtaggtgctaattttactcgaatccctgtgtaggcagtggaacatt 180
181 ctataaaaggcagtttggcgcacatcctcatcgtctgctgtcagggaggacttttctgac 240
241 cacactggatagggctcgttcttcttctctccaggatgaaccctttttgtatcatttcc 300
301 acaccttctagaagtactttggtcttgtcctcaatctctttagcccttgccaagatatca 360
361 ggtgaggctccattcctgtgcaacaactctgtgactgcctgatgcagaggttcatcccac 420
421 gagtggagcaaactgataaccaacctcaatagggcttcgtcaaattcattgaacatttcc 480
481 ctggcgagcctatagttgttgctagccaccaatgttgctctctcaaacaggctttgaagg 540
541 ggaatccggcagttgccaggttggtttttacagwatggtgcataatcctccacaccctgg 600
601 cacaagagtagatttgacaccaccagcagcaggagcaggcaggaccctcgaacaaggtca 660
661 caaatccactggtgcccacgatggctagatgctggagccatgaggattggagagaaatcc 720
721 caggatgaggactgctgctgatggggagatggccccaaccagggactgaatggaggaatc 780
781 tttcccgggggggtaccgtcgactgtggccgcgaattc                       817
```

**Figure 3.2.** pTarget-bPL sequence. This sequence is 817 base pairs in length and aligns with the minus strand of bPL sequences in the GenBank database.

The bPL insert was ligated into the pcDNA3.1(+) vector using ligation reaction 1 (Table 2.5). The recombinant molecules were then transformed into competent *E. coli* cells. Two plates were made from each reaction.

The five clones (Table 3.2) that resulted were cultured, and the DNA was purified and digested with BamH I and Kpn I. The digests were run on gel electrophoresis (Figure 3.5).

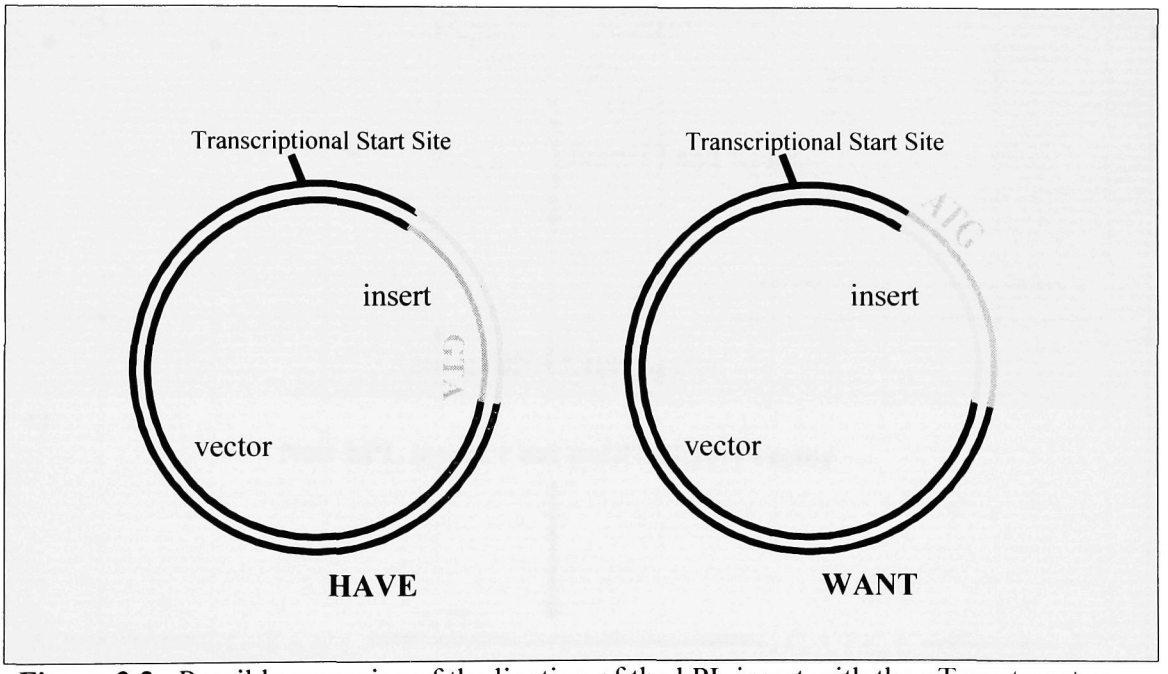


**Figure 3.3.** Possible scenarios of the ligation of the bPL insert with the pTarget vector. The ATG of the bPL insert is on the minus strand of the recombinant molecule. In order for proper transcription of the bPL gene to take place, it is necessary for the ATG of the gene to be on the plus strand following the transcriptional start site of the vector.

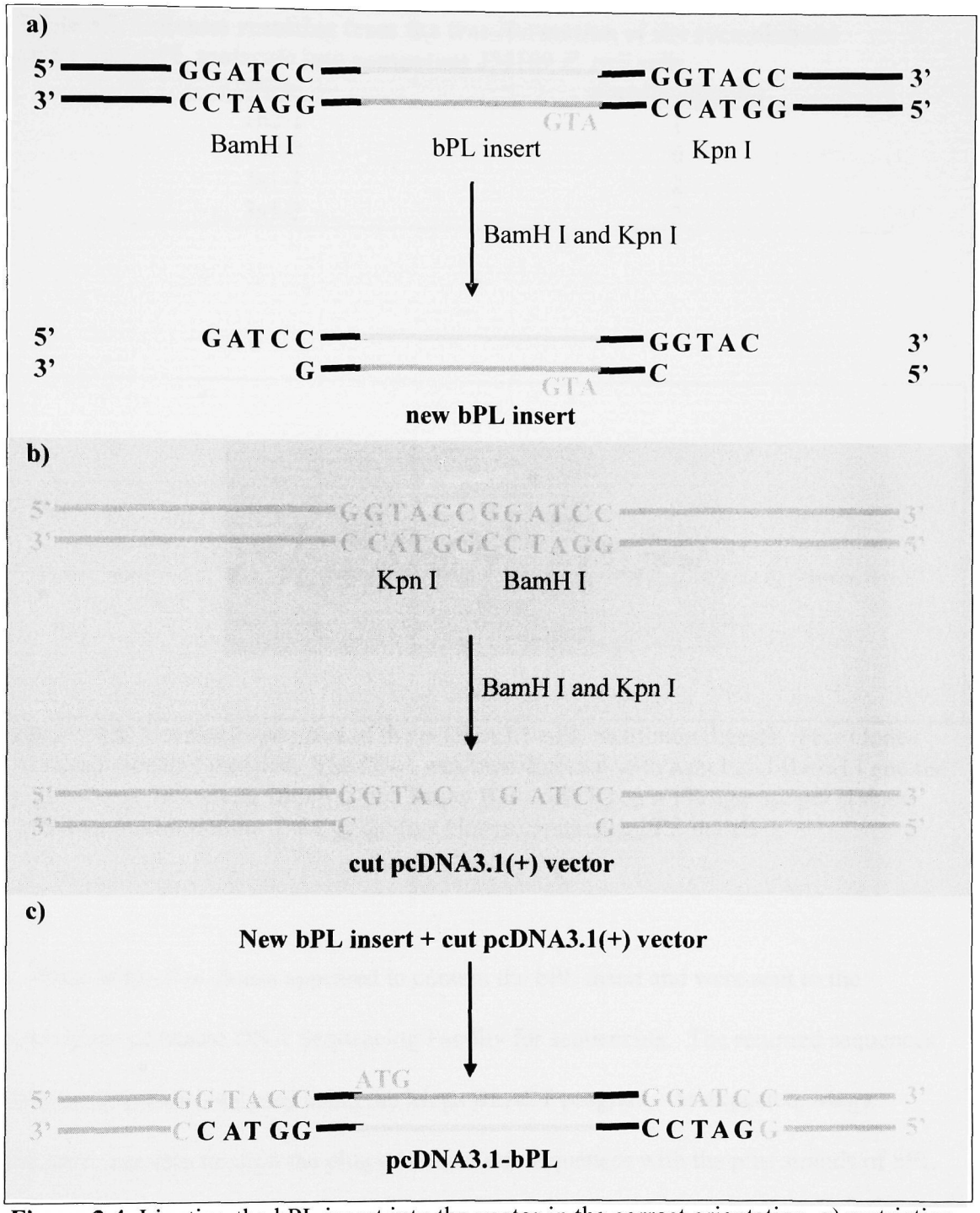


**Figure 3.4.** Ligating the bPL insert into the vector in the correct orientation. a) restriction digest of pTarget-bPL to remove the bPL insert. b) restriction digest of the pcDNA3.1(+) vector to prepare for ligation with the bPL insert. c) ligation of the bPL insert with the pcDNA3.1(+) vector to form the recombinant pcDNA3.1-bPL molecule. The insert has no choice but to ligate into the vector in the correct orientation.

**Table 3.2. Colonies resulting from the transformation of the recombinant pcDNA3.1-bPL molecule into competent JM109 *E. coli* cells.**

| Plate | # white colonies |
|---|---|
| 1b2-1 | 1 |
| 1b2-2 | 0 |
| 3a1-1 | 2 |
| 3a1-2 | 2 |

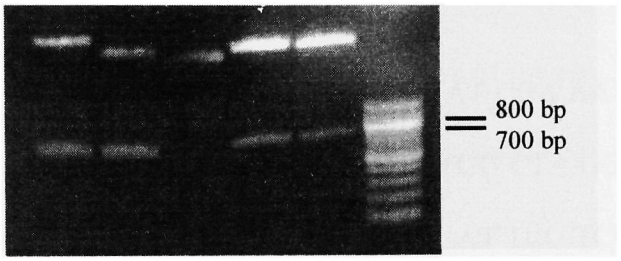


**Figure 3.5.** Gel electrophoresis of the pcDNA3.1-bPL restriction digests. Five clones were cultured and purified. The DNA was then digested with Kpn I and BamH I and the digests were run, along with a 100bp ladder (GibcoBRL) on a 1% agarose gel stained with ethidium bromide. Four of the five clones contain the bPL insert.

Four of the five clones appeared to contain the bPL insert and were sent to the University of Maine DNA Sequencing Facility for sequencing. The returned sequences (Figure 2.4) were analyzed using the Mega BLAST program. As expected, Mega BLAST was able to align the plus strands of our sequences with the plus strands of bPL sequences in the GenBank database.

In order for other studies to be performed using the recombinant pcDNA3.1-bPL recombinant molecule (vaccine production, etc.), it was necessary for the bPL gene to be in-frame with the transcriptional start site of the pcDNA3.1(+) vector. As can be seen in

Figure 3.6, the bPL insert was not in-frame with the transcriptional start site. Also, in order for proper initiation to occur, it was necessary to have a G or A at position –3 and a G at position +4. Our bPL gene had the G at position +4, but had a C at position –3.

Primers bPL-Start-KpnI and bPL-Stop-BamHI were designed so as to remedy these problems. As can be seen in Figure 3.7, the bPL insert was now in-frame with the transcriptional start site of the pcDNA3.1(+) vector and there was a G at position –3.

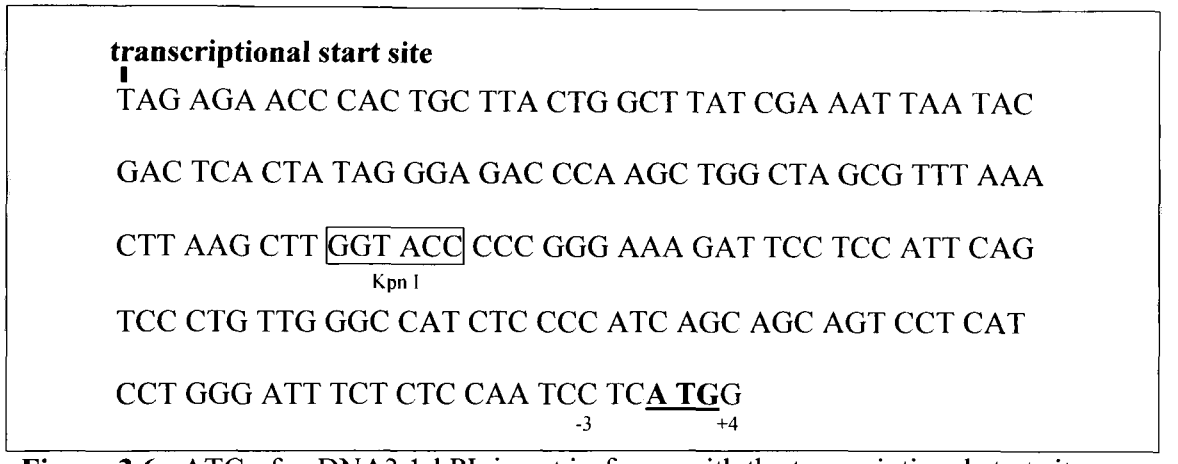


**Figure 3.6.** ATG of pcDNA3.1-bPL is not in-frame with the transcriptional start site. The start codon (ATG) of the bPL gene is not in-frame with the transcriptional start site of the pcDNA3.1(+) vector. In order for proper initiation of translation to occur, there must be an A or G at position –3 and a G at position +4. In this sequence, there was a C at position –3. The Kpn I recognition site is outlined for reference.

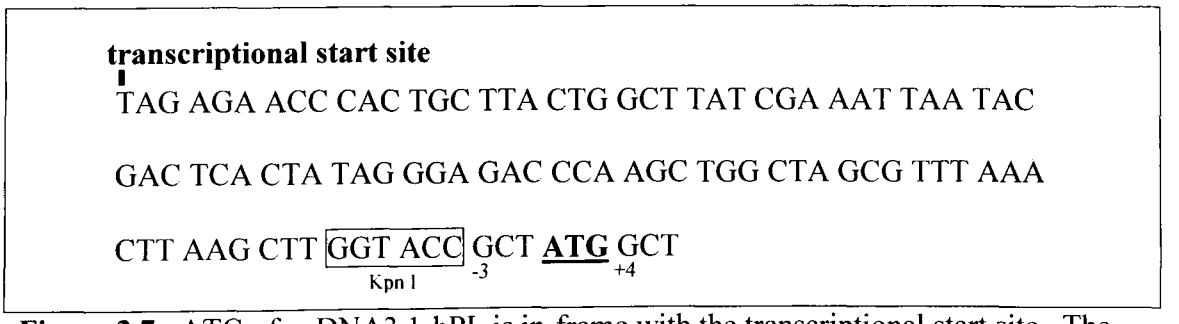


**Figure 3.7.** ATG of pcDNA3.1-bPL is in-frame with the transcriptional start site. The start codon (ATG) of the bPL gene was in-frame with the transcriptional start site of the pcDNA3.1(+) vector. The requirements for proper initiation have been met, with guanines at positions –3 and +4.

**Description of the bPL Gene**

The bPL cDNA isolated in this study (Figure 3.8) encoded an open reading frame from the beginning of the clone to the termination codon (TAA) at nucleotide positions 670-672. The sequence shown here includes a small number of nucleotides belonging to the vector sequence, including the Kpn I and BamH I recognition sites. There was a single ATG at nucleotide positions 10-12, which was thought to be the initiating methionine. This predicts a preprohormone of 200 amino acids.

Mega BLAST was able to align the plus strand of our bPL sequence with the plus strand of a bPL sequence reported by Schuler *et al.* (1988), which predicted a preprohormone of 236 amino acids. The comparison of the two preprohormone sequences is given in Figure 3.9. Schuler *et al.* (1988) were able to divide their bPL sequence into five exons. When aligned with the Schuler sequence, it is evident that the bPL sequence of this study was missing the entire third exon. Schuler and her colleagues (1988) were also able to identify the first exon as the signal peptide, with the site of cleavage following the glycine at amino acid 36. Their mature hormone would therefore be 200 amino acids in length. In comparison, our mature protein would be 164 amino acids.


**Figure 3.8**. pcDNA3.1-bPL sequence. The start codon (ATG) and stop codon (TAA) are underlined. The Kpn I and BamH I recognition sites are underlined twice for reference.

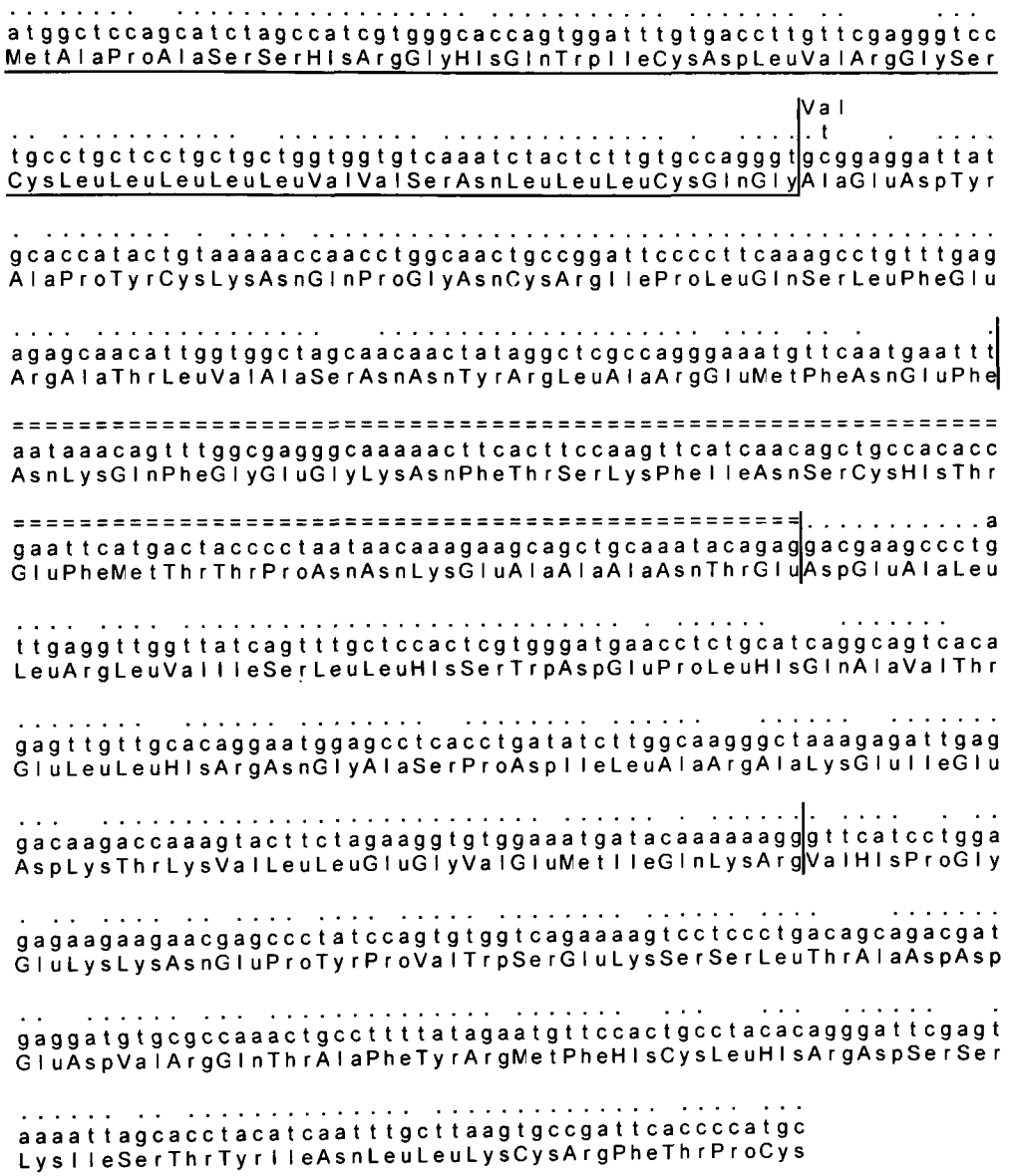


**Figure 3.9.** Comparison of two bPL preprohormone sequences. The sequence given is that reported by Schuler *et al.* (1988), along with the corresponding amino acid sequence. This sequence was made up of five exons, divided by the vertical lines. The dots represent nucleotides of the pcDNA3.1-bPL sequence that match the Schuler sequence, while the equal signs represent the missing third exon of pcDNA3.1-bPL.

The results of the optimization of transfection study are shown in Figures 3.10 and 3.11. As can be seen, the L929 cells that were transfected with a 2:1 charge ration of Tfx-20:DNA and 1.00μg pEGFP-N3 per well produced the most favorable results. As can be seen in Figure 3.10, a greater number of cells transfected with this combination could be visualized under the fluorescent microscope. This results from a greater transfection efficiency. For this reason, this combination of charge ration and concentration of DNA was used in the transfection of the cells with the pcDNA3.1-bPL.

The L929 cells were transfected with a 2:1 charge ratio of Tfx-20:DNA and 1.00μg pcDNA3.1-bPL per well. The media from the cells was collected and replaced with new media every 48 hours for ten days. Using radioimmunoassay (Wallace, 1993), it was determined that there was an average of 1.00ng bPL per milliliter of collected media. It had been hypothesized that the cells would be able to produce at least 100ng bPL per milliliter media (unpublished results using mouse L cells). It was noted throughout the collection of the media that the L929 cells were not growing at the expected rate. In order to determine the reason for this, another transfection of the cells with the pcDNA3.1-bPL was carried out, this time also maintaining nontransfected control wells. Media collection began 12 hours post-transfection, followed by collection at 24 and 48 hours post-transfection. At the time of media collection, the cells were trypsinized and stained with trypan blue in order to distinguish between the live and dead cells. The results of this study are shown in Table 3.3 and Figure 3.12.

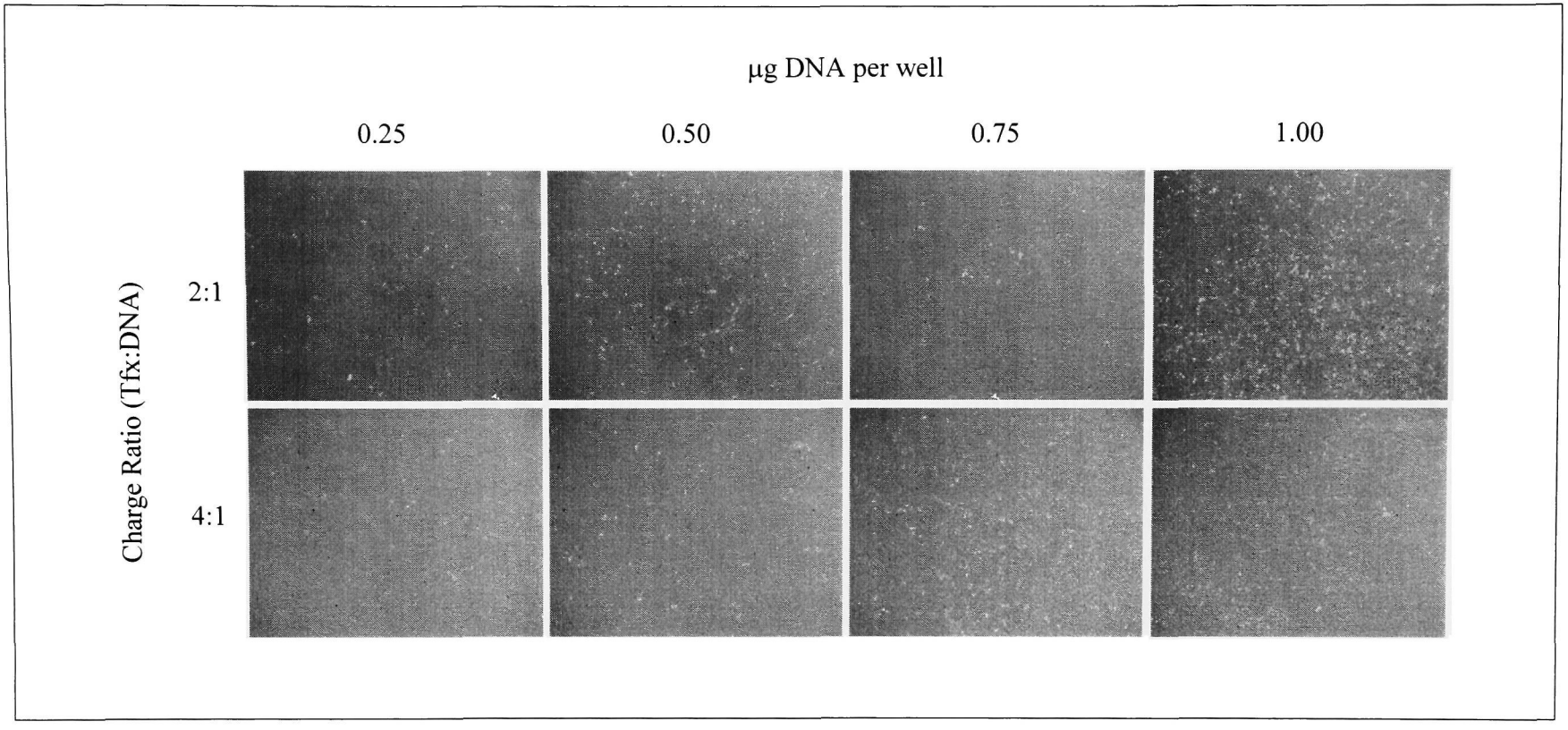


**Figure 3.10**. Optimization of transfection with Tfx-20. The combination of the 2:1 charge ratio and 1.00μg DNA/well produced the highest transfection efficiency.

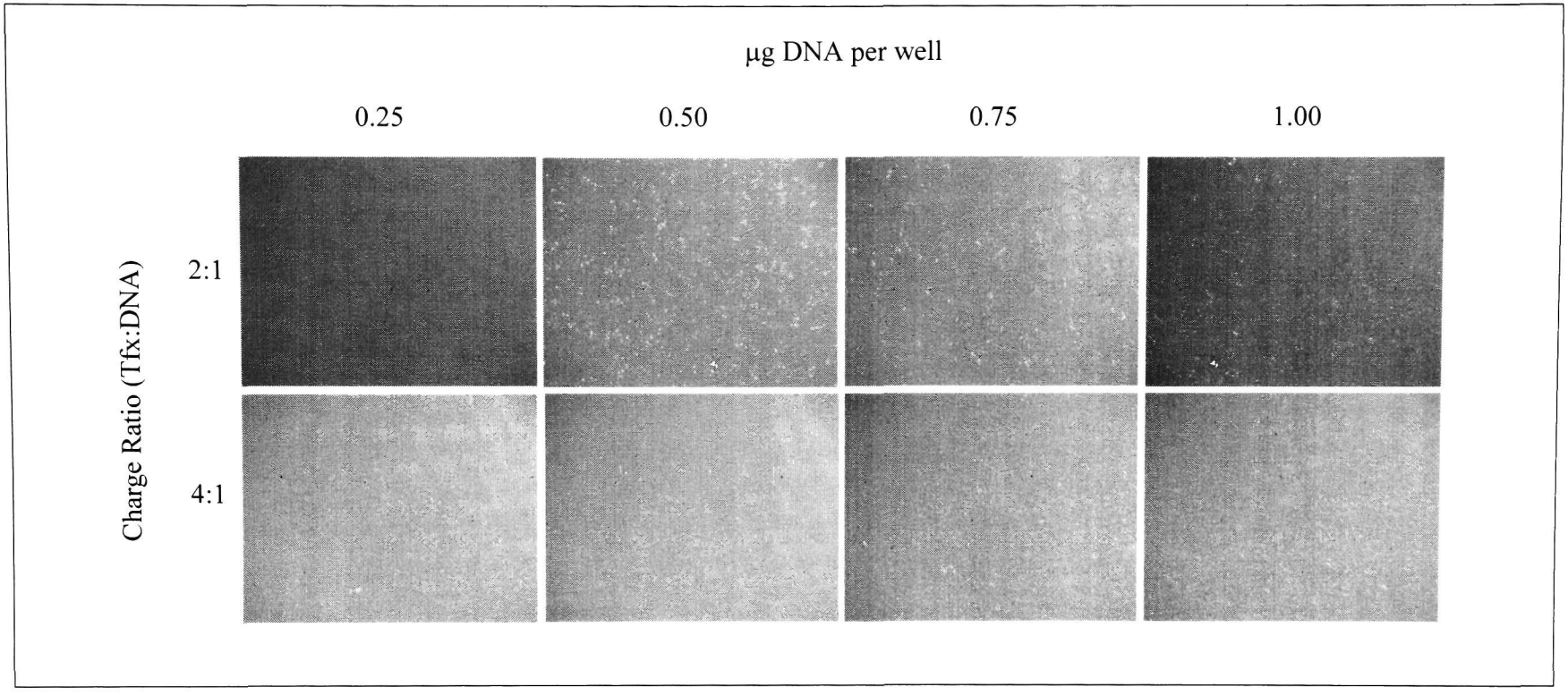


**Figure 3.11**. Optimization of transfection with Tfx-50. The combination of the 2:1 charge ratio and 0.50μg DNA/well produced the highest transfection efficiency.

**Table 3.3. Post-transfection cell viability results.**

| Hours post-transfection | Live cells (cells/mL) | Dead cells (cells/mL) | Total cells (cells/mL) | Cell viability (% cell/mL) |
|---|---|---|---|---|
| | | **Control wells** | | |
| 12 | 564,000 | 0 | 564,000 | 100 |
| 24 | 724,000 | 0 | 724,000 | 100 |
| 48 | 1,928,000 | 8,000 | 1,936,000 | 99.59 |
| | | **Transfected wells** | | |
| 12 | 456,000 | 8,000 | 464,000 | 98.28 |
| 24 | 388,000 | 8,000 | 396,000 | 97.98 |
| 48 | 576,000 | 4,000 | 580,000 | 99.31 |

At the indicated times, the cells from three wells of each of the groups (control and transfected) were trypsinized. The cells were pooled according to group, stained with trypan blue, and counted.

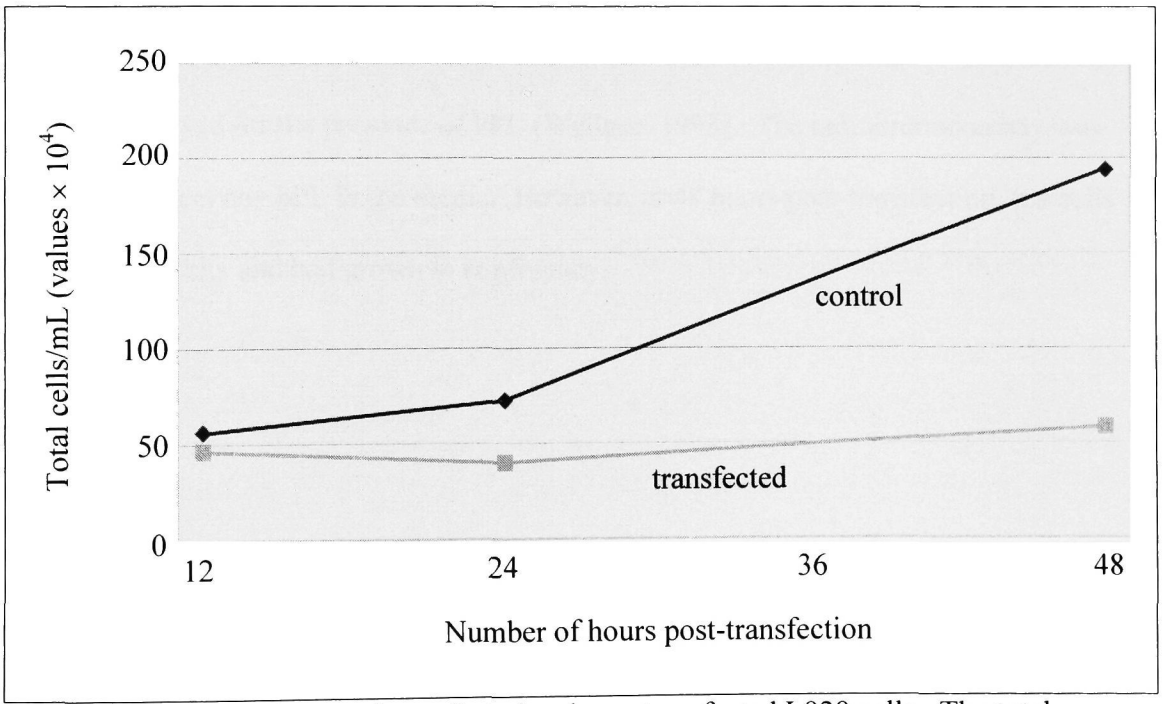


**Figure 3.12.** Growth rate of transfected and non-transfected L929 cells. The total number of cells per milliliter of media 12 to 48 hours post-transfection.

The cell viabilities of both the control and the transfected wells were comparable. The lowest cell viability noted in the control wells was 99.59% at 48 hours post-transfection and the lowest in the transfected wells was 97.98% at 24 hours post-transfection. Despite the similar cell viabilities, it is clear from Figure 3.12 that the cells of the nontransfected control wells were growing at a faster rate than the cells of the transfected wells. By 48 hours post-transfection, the number of control cells exceeded the number of transfected cells by 1,356,000 cells/mL. This seemed to point to the conclusion that the transfection was somehow altering the growth rate of the mouse cells.

Due to the slow growth rate of the L929 cells and the low transfection efficiency, it was believed that the cells were carrying a mycoplasma infection. Therefore, a new batch of L929 cells was purchased from American Type Culture Collection and the cells were transfected with pcDNA3.1-bPL. Media was collected 48 hours post-transfection and was assayed for the presence of bPL (Wallace, 1993). The radioimmunoassay was unable to detect any bPL in the media. However, at 48 hours post-transfection, the cells appeared healthy and had grown to confluency.

# Chapter 4

# DISCUSSION

Recombinant DNA technology has enabled advances in a myriad of research interests. Proteins that would have, at one time, been difficult to study, are now being produced in laboratories all over the world. Bovine placental lactogen, a hormone whose peak concentrations are reported at a mere 2.93ng/mL (Wallace, 1993), is no exception. The production of recombinant bPL (rbPL) has allowed researchers to delve into the structure and function of this mysterious protein, and many discoveries have been made. Site-directed mutagenesis of rbPL has made it possible to pinpoint exact locations on the protein that are important in receptor binding (Vashdi-Elberg *et al.*, 1996; Helman *et al.*, 1997). The N-terminal portion of bPL, which is not present in growth hormone, has been identified as being important in PRL receptor binding (Gertler *et al.*, 1992). bPL has also been labeled as a key factor in the regulation of mammary growth and differentiation (Byatt *et al.*, 1994; Byatt *et al.*, 1997) and in the stimulation of body weight gain (Byatt *et al.*, 1991). In addition, rbPL has been used to study the effects of the hormone on the growth and function of heifer ovaries (Lucy *et al.*, 1994).

Despite these developments, there is one possible downfall to the work that has been done with rbPL thus far. The rbPL used in the aforementioned studies was produced in bacteria, an expression system that may not be capable of carrying out all of the post-translational modifications that are important to the function of the protein.

In this study, we were able to isolate bPL cDNA from the placental tissue of a Holstein dairy cow at day 90 of gestation. We cloned the cDNA into the pcDNA3.1(+) vector and transformed the recombinant DNA into *E. coli* cells. The bPL sequence isolated in this study was similar to that isolated by Schuler *et al.* (1988). The Schuler sequence predicted a preprohormone of 236 amino acids and could be divided into five exons. Our sequence, however, predicted a preprohormone of only 200 amino acids. Upon further analysis of our sequence, it was evident that it was missing the entire third exon, as was the sequence isolated by Kessler and Schuler (1991). Kessler and Schuler noted that the third exon contains one of the six cysteine residues important to the conformation of the mature bPL protein. Although no further research has been reported on that particular variant, it was predicted that the missing third exon may change the folding pattern of the protein, thus rendering it inactive (Kessler and Schuler, 1991).

The initial transfection of the recombinant bPL molecule produced much lower yields of the protein than expected (as measured by radioimmunoassay (Wallace,1993)). It was noted during the collection of data that the transfected cells were not achieving confluence at the typical rate. Two hypotheses were established in order to try to explain the poor results: 1) the cells were experiencing some toxicity, caused either by the transfection agent or by the bPL and 2) the cells were not dividing properly. It was assumed that toxicity would result in low cell viability counts, whereas slow growth and division would result in low total cell counts, with high viability. The latter was the result that was observed. In the second transfection trial, when compared to the control group, the transfected cells were significantly less in number, but still maintained a high

viability level. This seemed to point to the conclusion that there was something slowing cell division in the transfected cultures.

In a similar study, Xia *et al.* (1997) observed decreased transfection efficiency and slow growth of apparently healthy COS cells. It was concluded that the results obtained were caused by a mycoplasma infection. Mycoplasma are parasitic prokaryotic organisms that are resistant to penicillin-type antibiotics (Rottem, 1980). This means that they are able to survive in cell cultures with media containing such antibiotics. The typical energy source utilized by mycoplasma is arginine. The utilization of arginine is mediated by the enzyme arginine deiminase (Sugimura *et al.*, 1992; Sugimura *et al.*, 1993). Arginine deiminase activity is thought to disrupt normal vesicular trafficking in cells, thus disrupting the liposomal-mediated transfection process (Xia *et al.*, 1997). Although the L929 cells were not tested for mycoplasma infection, it is assumed that this was the cause of the decreased growth rate of the transfected population. A new batch of L929 cells tested for the presence of mycoplasma before purchase were able to grow to confluence by 48 hours post-transfection.

Despite the fact that the new cells appeared to be free of mycoplasma, they still were unable to produce the bPL. This led us to believe that there was a problem with the bPL sequence that we were using in the transfection. As stated in Chapter 2: Methods and Materials, in order to prepare the sequence for the production of a vaccine also needed in Dr. Wallace's lab, the sequence 5' to the start codon was removed. Liang *et al.* (1999) in their study of ovine placental lactogen (oPL), concluded that the 1.1 kb 5'-flanking sequence , and most importantly, the proximal 383 bp of the oPL gene 5'-flanking sequence, was key to the transactivation of the oPL gene. In contrast to the lengthy 5'-

flanking sequence of oPL, the bPL gene 5'-flanking sequence reported by Schuler *et al.* (1988) was 78 bp. Wallis (1993) noted a rapid rate of molecular evolution of ruminant placental lactogens. According to comparisons of the amino acid sequences for ovine and bovine PLs (Calosi *et al.*, 1989; Schuler *et al.*, 1988), these two hormones differ in about 30% of all residues. The structural differences between the ruminant PLs deem it difficult to draw conclusions about bPL by making comparisons to oPL. For this reason, the next step in the quest for the successful transfection and production of bPL should be to fashion a bPL study after the oPL study done by Liang *et al.* (1999). Such a study would enable us to see if the 5'-flanking sequence of the bPL gene is required for the production of the bPL protein, and if so, which particular region(s) are of the most importance.

Ideally, a full-length bPL sequence should be isolated and cloned. The transfection of the full-length sequence, and thus production of the full-length protein, would enable comparisons to be made between the it and the proteins resulting from the alternatively spliced genes. Overall, the production of bPL in the laboratory through the use of transfection will enable researchers to further explore the importance of the different structures and functions of this protein.

## REFERENCES


Anthony, R.V., R. Liang, E.P. Kayl, and S.L. Pratt. 1995. The growth hormone/prolactin gene family in ruminant placentae. J. Reprod. Fertil. Suppl. **49**: 83.

Arima, Y. and R.D. Bremel. 1983. Purification and characterization of bovine placental lactogen. Endocrinology. **113**: 2186.

Becka, S., J. Bilek, J. Slaba, J. Skarda, and I. Mikulas. 1977. Some properties of the goat placental lactogen. Experientia. **33**: 771.

Beckers, J.F., C. Fromont-Lienard, P. Van der Zwalmen, P. Wouters-Ballman, and F. Ectors. 1980. Isolement d'une hormone placentaire bovine présentant une activité analogue à l'hormone de croisance. Ann. Med. Vet. **124**: 584.

Blank, M.S., J.S.D. Chan, and H.G. Friesen. 1977. Placental lactogens, new developments. J. Steroid Biochem. **8**: 403.

Brown, T.A. 1995. Gene cloning: an introduction. New York, NY: Chapman & Hall.

Buttle, H.L. and I.A. Forsyth. 1976. Placental lactogen in the cow. J. Endocrinol. **68**: 141.

Byatt, J.C., P.J. Eppard, J.J Veenhuizen, D.F. Curran, M.F. McGrath, and R.J. Collier. 1994. Stimulation of mammogenesis and lactogenesis by recombinant bovine placental lactogen in steroid-primed dairy heifers. J. Endocrinol. **140**: 33.

Byatt, J.C., P.J. Eppard, J.J. Veenhuizen, R.H. Sorbet, F.C. Buonomo, D.F. Curran, and R.J. Collier. 1992a. Serum half-life and in-vivo actions of recombinant bovine placental lactogen in the dairy cow. J. Endocrinol. **132**: 185.

Byatt, J.C., K. Shimomura, T.M. Duello, and R.D. Bremel. 1986. Isolation and characterization of multiple forms of bovine placental lactogen from secretory granules of the fetal cotyledon. Endocrinology. **119**: 1343.

Byatt, J.C., N.R. Staten, J.J. Schmuke, F.C. Buonomo, S.S. Galosy, D.F. Curran, G.G. Krivi, and R.J. Collier. 1991. Stimulation of body weight gain of the mature female rat by bovine GH and bovine placental lactogen. J. Endocrinol. **130**: 11.

Byatt, J.C., C. Warren, P.J. Eppard, N.R. Staten, G.G. Krivi, and R.J. Collier. 1992b. Ruminant placental lactogens: structure and biology. J. Anim. Sci. **70**: 2911.

Byatt, J.C., J.K. Welply, R.M. Leimgruber, and R.J. Collier. 1990. Characterization of glycosylated bovine placental lactogen and the effect of enzymatic deglycosylation on receptor binding and biological activity. Endocrinology. **127**: 1041.

Chan, J.S.D., H.A. Robertson, and H.G. Friesen. 1976. The purification and characterization of ovine placental lactogen. Endocrinology. **98**: 65.

Chatterjee, M. and H.N. Munro. 1977. Structure and biosynthesis of human placental peptide hormones. Vitam. Horm. **35**: 149.

Currie, W.B., C.E. Card, F.J. Michel, and G. Ignotz. 1990. Purification, partial characterization and development of a specific radioimmunoassay for goat placental lactogen. J. Reprod. Fertil. **90**: 25.

Duello, T.M., J.C. Byatt, and R.D. Bremel. 1986. Immunohistochemical localization of placental lactogen in binucleate cells of bovine placentomes. Endocrinology. **119**: 1351.

Farhood, H., N.S. Servina, and L. Huang. 1995. Biochim. Biophys. Acta. **1235**: 289.

Galosy, S.S., A. Gertler, G. Elberg, and D.M. Laird. 1991. Distinct placental lactogen and prolactin (lactogen) receptors in bovine endometrium. Mol. Cell. Endocrinol. **78**: 229.

Gertler, A. 1997. Recombinant analogues of prolactin, growth hormone, and placental lactogen: correlations between physical structure, binding characteristics, and activity. Mammary Gland Biol. Neoplasia. **2**: 69.

Gertler, A., J. Grosclaude, C.J. Strasburger, S. Nir, and J. Djiane. 1996. Real-time kinetic measurements of the interactions between lactogenic hormones and prolactin-receptor extracellular domains from several species support the model of hormone-induced transient receptor dimerization. J. Biol. Chem. **271**: 24482.

Halban, J. 1905. Die innere Secretion von Ovarion und Placenta und ihre Bedeutung für die Function der Milchdrüse. Arch. Gynaekol. **75**: 353.

Holland, M.D., K.L. Hossner, S.E. Williams, C.R. Wallace, G.D. Niswender, and K.G. Odde. 1997. Serum concentrations of insulin-like growth factors and placental lactogen during gestation in cattle I: Fetal profiles. Domest. Anim. Endocrinol. **14**: 231.

Hurley, T.W., S. Handwerger, and R.E. Fellows. 1977. Isolation and structural characterization of ovine placental lactogen. Biochemistry. **16**: 5598.

Ihle, J.N., B.A. Witthuhn, F.W. Quelle, K. Yamamoto, W.E. Thierfelder, B. Kreider, and O. Silvennoinen. 1994. Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem. Sci. **19**: 222.

Kaplan, S.L. and M.M. Grumbach. 1964. Studies of a human and simian placental hormone with growth hormonne-like and prolactin-like activities. J. Clin. Endocrinol. Metab. **24**: 80.

Lucy, M.C., J.C. Byatt, T.L. Curran, D.F. Curran, and R.J. Collier. 1994. Placental lactogen and somatotropin: hormone binding to the corpus luteum and effects on the growth and functions of the ovary in heifers. Biol. Reprod. **50**:1136.

Martal, J., and J. Djian. 1975. Purification of a lactogenic hormone in sheep placenta. Biochem. Biophys. Res. Commun. **65**: 770.

Murthy, G.S., C. Schellenberg, and H.G. Friesen. 1982. Purification and characterization of bovine placental lactogen. Endocrinology. **111**: 2117.

Post-translational protein modifications and the choice of expression system. http://www.biotech.vt.edu/classes/bion_4784/4-GeneExpression/Expression.html.

Rice, G.E. and G.D. Thorburn. 1986. Characterization of particle-associated choriomammotrophin and progesterone in ovine placentomes. J. Endocrinol. **111**: 217.

Schlafer, D.H., P.J. Fisher, and C.J. Davies. 2000. The bovine placenta before and after birth: placental development and function in health and disease. Anim. Reprod. Sci. **60-61**: 145.

Schuler, L.A., K. Shimomura, M.A. Kessler, C.G. Zieler, and R.D. Bremel. 1988. Bovine placental lactogen: Molecular cloning and protein structure. Biochemistry. **27**: 8443.

Scott, P., M.A. Kessler, and L.A. Schuler. 1992. Molecular cloning of the bovine prolactin receptor and distribution of prolactin and growth hormone receptor transcripts in fetal and utero-placental tissues. Mol. Cell. Endocrinol. **89**: 47.

Senger, P.L. 1999. Pathways to Pregnancy and Parturition. Pullman, WA: Current Conceptions, Inc.

Shimomura, K. and R.D. Bremel. 1988. Characterization of bovine placental lactogen as a glycoprotein with N-linked and O-linked carbohydrate side chains. Mol. Endocrinol. **2**: 845.

Staten, N.R., J.C. Byatt, and G.G. Krivi. 1993. Ligand-specific dimerization of the extracellular domain of the bovine growth hormone receptor. J. Biol. Chem. **268**: 18467.

Verstegen, J., D. Fellmann, and J.F. Beckers. 1985. Immunodetection of bovine chorionic sommatomammotrophin. Acta. Endocrinol. **109**: 403.

Vinik, A.I., S.L. Kaplan, and M.M. Grumbach. 1973. Purification, characterization and comparison of immunological properties of monkey chorionic sommatomammotropin with human and monkey growth hormone, human chorionic somatomammotropin and ovine prolactin. Endocrinology. **92**: 1051.

Wallace, C.R. 1993. Concentration of bovine placental lactogen in dairy and beef cows across gestation. Dom. An. Endocrinol. **10**: 67.

Wallis, M. 1993. Remarkably high rate of molecular evolution of ruminant placental lactogens. J. Mol. Evol. **37**: 86.

Wooding, F.B.P. and J.F. Beckers. 1987. Trinucleate cells and the ultrastructural localization of bovine placental lactogen. Cell Tissue Res. **247**: 667.

Wooding, F.B.P. and D.C. Wathes. 1980. Binucleate cell migration in the bovine placentome. J. Reprod. Fertil. **59**: 425.

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