Optimization of Liposome-mediated Transfection in Sf9 Insect Cells and Analysis of Integration Pattern and Copy Number in Stably Transformed Cell Lines

by

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Date Jan 3, 2002
Abstract

With increasing application of DNA transfer technology in human gene therapy and heterologous protein production, various methods of introducing DNA into *ex vivo* or *in vivo* cells have been developed in a wide variety of cells. Recently, growing interest has focused on insect cells for production of heterologous proteins. Insect expression systems have been recognized as an easy and rapid system for production of a large quantity of recombinant or therapeutic proteins.

Liposome-mediated transfection is an effective non-viral delivery method. However, optimization is essential to achieve maximal production of the heterologous proteins. The goal of this project is to optimize the transgene expression in *Sf9* insect cells using lipofection. Transfection variables involved in the efficiency of transgene uptake by cells were examined for optimization. The type of cationic liposomes and the liposome to DNA ratios were the most significant determinants of efficient DNA uptake. The transient expression of the *B-galactosidase* reporter gene could be improved by 7.8 fold by optimization.

Southern blot analyses revealed that the integrated plasmids were predominantly arranged into head-to-tail concatemers in all the stably transformed cell lines except for one. The concatemeric integration was speculated to occur by extrachromosomal homologous recombination between plasmid DNAs and a subsequent illegitimate recombination into the chromosomal DNA by a double-strand break repair pathway.

Up to a 27-fold difference in the integrated copy number was observed among 19 stably transformed cell lines by quantitative southern blot analyses. The reporter gene expression in stably transformed cells could be correlated with the integrated copy number in general. In addition to the copy number, the positional effect of the integration loci is likely to play a role in controlling the expression level. A large amount of DNA uptake reflected on the high transient expression is necessary, but not sufficient for high expression in stable cells. However, there appeared to be higher probability of obtaining high-producer cell lines with high transient expression. Therefore, the use of a lipofection protocol, producing high transient expression, is advantageous in producing high producer stable cell lines.
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<tr>
<td>Ω</td>
<td>ohms</td>
</tr>
<tr>
<td>∞</td>
<td>infinity</td>
</tr>
<tr>
<td>AA prep</td>
<td>Ammonium acetate-modified alkaline lysis plasmid DNA preparation</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>AcNPV</td>
<td>Autograph californica polyhedrosis virus</td>
</tr>
<tr>
<td>B-gal</td>
<td><em>B-galactosidase</em></td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>But</td>
<td>Sodium butyrate</td>
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<tr>
<td>CC prep</td>
<td>Cesium chloride-modified alkaline lysis plasmid DNA preparation</td>
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<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
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<tr>
<td>CFTR</td>
<td>CF transmembrane conductance regulator</td>
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<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
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<td>COS</td>
<td>African green monkey cell line transformed with mutant SV40 genome</td>
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<tr>
<td>DDAB</td>
<td>Dimethyldioctadecylammonium bromide</td>
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<td>Dimethylaminoethyl</td>
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<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DOPC</td>
<td>Dioleoyl phosphatidyl choline</td>
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<td>DOPE</td>
<td>Dioleoyl phosphatidylethanolamine</td>
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<td>DOTAP</td>
<td>Dioleoyl trimethyl ammonium propane</td>
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<td>DSB</td>
<td>Double-strand break</td>
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<td>EDTA</td>
<td>disodium ethylenediaminetetra-acetate-2H₂O</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FDA</td>
<td>Food and drug administration</td>
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<td>GFP</td>
<td>Green fluorescence protein</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GFP::Zeo</td>
<td>GFP-zeocin fusion gene</td>
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<tr>
<td>GM-CSF</td>
<td>(human) Granulocyte-macrophage colony-stimulating factor</td>
</tr>
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<td>HEK</td>
<td>Human embryonic kidney cell line</td>
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<td>HMG</td>
<td>High-mobility group protein</td>
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<td>Drosophila HMG</td>
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<td>LacZ</td>
<td>B-galactosidase gene</td>
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<td>MCS</td>
<td>Multiple cloning site</td>
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<td>Alkaline lysis plasmid DNA preparation</td>
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<td>mOD</td>
<td>milli Optical density</td>
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<td>messenger RNA</td>
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<tr>
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<td>Mouse fibroblast cell line</td>
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<tr>
<td>occ +/-</td>
<td>occlusion phenotype plus or minus</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>ONPG</td>
<td>o-nitrophenyl-β-D-galactopyranoside</td>
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<tr>
<td>pA</td>
<td>polyadenylation signal</td>
</tr>
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<td>Pichia pastoris</td>
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<td>PBS</td>
<td>Phosphate-buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PolyEthlyene Glycol plasmid DNA Preparation</td>
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<td>polyhedrin</td>
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<td>Ribonucleic acid</td>
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<td>Schneider 2 (Drosophila cell line)</td>
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<td>SDS</td>
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<td>Spodoptera frugiperda</td>
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<td>Myeloma cell line</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SSB</td>
<td>Single-strand break</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
</tr>
<tr>
<td>Tn</td>
<td><em>Trichoplusia ni</em></td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>U</td>
<td>unit of endonuclease enzyme</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet light</td>
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<tr>
<td>Zeo</td>
<td>Zeocin</td>
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Chapter 1  Introduction

1.1  Usage of transgene expression research

DNA transfer techniques to introduce a desired gene into an expression system has become an essential tool. The applications of this technology exist in studies such as clinical gene therapy, production of therapeutic proteins, large-scale production of proteins for biophysical or biochemical analysis, gene regulation, and functional and localization studies of a gene or protein.

Gene therapy entails correction of a single gene defect in a hereditary disease or cancer. Various methods of gene therapy have been reported in clinical studies. The defect can be corrected by replacement of the defective gene with functional copy of the gene through recombination into the genome or ectopic expression. A gene can be introduced to complement the function of the default gene or correct the default biochemical pathway causing the disease. In gene therapy of human cancer, a gene or antisense gene is introduced to repress the expression of the dominant oncogene (Van Craenenbroeck et al., 2000). In other cases, genetically altered cells expressing the functional gene can be implanted into patients.

Gene therapy of cystic fibrosis (CF), which is caused by defective membrane chloride channel, CFTR, has been reported in a clinical study (Kunzelmann and Schreiber, 1999). The CF patients were given cationic liposome complexes with a complementary DNA encoding the CF transmembrane conductance regulator (CFTR) gene, and the defect could be lessened for several days (Caplen et al., 1995). In another study, implantation of genetically altered fibroblast cells, expressing factor VIII, to patients with Hemophilia A could reduce bleeding for 10 months (Roth et al., 2001). In addition to therapy of hereditary diseases, various cancers are treated by gene therapy. Phase I clinical trial of breast or ovarian cancer has been performed by intracavitary injection of cationic liposome-complexed with \( E1A \) gene, which is associated with antitumor activities (Hortobagyi et al., 2001).

Whereas the gene therapy technology is still in its infancy, the correction of hereditary or acquired diseases by administration of the protein(s), which are missing or
defective in the patients, has been an accepted form of therapy for a longer period of time. By 1990, seven recombinant human therapeutic proteins had received FDA approval - OKT3, tPA, EPO, insulin, growth hormone, α-interferon, and hepatitis vaccine - and a dozen more were in advanced human clinical trials (Ogez and Builder, 1990). There is increasing number of such therapeutic proteins. Administration of factor VIII, one of the protein cofactors essential for blood coagulation, leads to temporary treatment of hemorrhagic syndrome in hemophiliacs (Roth et al., 2001). Administration of tissue plasminogen activator (tPA) can treat a patient with an acute stroke (Wiebe et al., 2001) by dissolution of the blood clots (Pennica et al., 1983). Thus, acquiring a large quantity of the purified forms of the proteins such as factor VIII or tPA is a key interest in the pharmaceutical industry. These proteins are mainly obtained from heterologous expression systems including baculovirus-insect cell system (Farrell et al., 1999). The main commercial source of tPA is CHO cells (Wiebe et al., 2001). Expression and purification of factor VIII for treatment of hemophilic patients have also been reported in CHO (Chinese hamster ovary) cells (Eriksson et al., 2001).

These applications in clinical and pharmaceutical studies exemplify the importance of the recombinant DNA technology. In addition to the direct benefit of the gene transfer technique to human health, the use of recombinant DNA technology is well recognized in a variety of biological researches including biochemical and biophysical studies. For instance, an examination of the physiological consequences of expression of specific genes to study the mechanism of regulatory control requires a recreation of an artificial system in a model organism or cells. Subcellular localization study of proteins or complexes by epitope tagging or GFP fusion is commonly performed in various cell lines by transfection of the gene of interest (de la Iglesia et al., 1999). Determination of the structure of proteins or molecular chaperons is often done by X-ray crystallography or transmission electron microscopy. These biophysical studies require a large quantity of often biologically functional molecules, thereby necessitating the use of a heterologous expression system. Human serum transferrin, expressed at high levels in Pichia pastoris, has been used to solve the structure by X-ray crystallography (Bewley et al., 1999). Promoter analysis in cell lines, analysis of in vivo interaction of two proteins, analysis of a biochemical or signal transduction pathway by reconstituting part(s) of the pathway in a
heterologous system, and other numerous studies make use of the gene transfer technique.

These applications of gene transfer technique all require efficient introduction of the gene of interest to cells, expression of the transferred gene, and the maintenance of the expression level for a long time, preferentially indefinitely. For clinical studies, efficient delivery of the gene to specific tissues or cells would be more important than the high expression level required for in vivo production of large quantity of therapeutic protein. The choice of the expression system, in other words, the type of cell line for recombinant protein production is very important. Cellular uptake of the transgene and its intracellular fate is always important. The method of gene delivery determines the efficiency of uptake and the subsequent processing for gene expression; therefore, it is a crucial element in improving the efficiency.

Various cell cultures and delivery systems, referred to as transfection systems, have advantages and disadvantages depending on the purpose of the production of heterologous protein. I will describe and discuss some of them from the perspective of gene transfer technology and protein expression.

1.2 Various model systems for transgene expression

Bacterial expression system
Bacterial recombinant protein expression was the first to be explored in the early years of recombinant DNA technology. *Escherichia coli* is the most frequently explored organism. The usefulness of bacterial expression system comes from the ease of culturing at high density on inexpensive growth medium without the requirement of special skills or equipment. Accumulated information on *E. coli* genetics and cell biology over many years of research is also a readily available resource, making its use convenient. Despite these advantages, its use for expression system is limited in some cases. A major factor hindering the production of functional eukaryotic proteins in bacteria is its inability to carry out post-translational modifications (Prinz et al., 1997). Lack of endomembrane systems such as ER and golgi and the proteins responsible for reduction of disulfide bridges results in the inability to glycosylate and form structural
disulfide bonds, respectively (Baneyx, 1999). In addition, differences in codon usage (Forman et al., 1998) and segregation of product into insoluble aggregates known as inclusion bodies (Thomas et al., 1997) can be sources of complication. For example, the arginine codons AGA and AGG are rarely found in E. coli genes, whereas they are common in eukaryotes (Baneyx, 1999). Purification of recombinant proteins as inclusion bodies can result in biologically inactive product from improper in vitro refolding of the proteins (Baneyx, 1999). These problems are being studied; however, expression of heterologous proteins in bacteria is limited to use of certain mutant strains, and glycosylation is likely to remain as a problem of bacteria (Baneyx, 1999).

**Yeast expression system**

Saccharomyces cerevisiae and Pichia pastoris are the most frequently used yeast expression systems. Their simplicity in molecular genetic manipulation and the high level of recombinant protein production are features that allow yeast to compete with bacterial system. The major advantage of yeast over bacteria is the capability to perform many of the eukaryotic post-translational modifications such as secretion signal processing, disulfide bond formation, and O- and N-glycosylation. Over 200 heterologous proteins ranging from viral proteins to human pharmaceutical proteins have been expressed in P. pastoris alone (Cereghino and Cregg, 2000).

Despite such a wide range of uses, the difference in post-translational modifications to mammals still remains as a limitation. The O-linked glycosylation in mammals occurs by adding a variety of sugars (Cereghino and Cregg, 2000). On the contrary, lower eukaryotes add O-oligosaccharides composed solely of mannose residues. In addition, frequent hyperglycosylation by the addition of varying length of mannose residues in N-linked glycosylation process can make the heterologous protein antigenic in pharmaceutical uses and can interfere with protein folding due to the bulky oligosaccharide chain (Goochee et al., 1991). These features of yeast become problematic when the carbohydrate residues play an important structural or functional role for the heterologous protein.

**Mammalian expression system**
Problems associated with bacterial and yeast expression systems can be resolved by using mammalian cell culture. The heterologous proteins of mammalian origins expressed in various mammalian cell lines are most of the time, functional with correct structure containing post-translational modifications similar to those of the native proteins. The production of heterologous proteins in cell lines such as CHO cell line is very efficient (Birch and Froud, 1994). However, mammalian cell culture requires more careful attention in maintenance conditions, and its cost is much higher than the former two systems. For example, the most commonly used systems, such as CHO, human embryonic kidney (HEK), myeloma cell line (Sp2/0), and African Green Monkey cell line transformed with mutant SV40 genome (COS) cell lines, require far more strict growth conditions (Birch and Froud, 1994). Although some cell cultures adapt to non-serum medium, most require the medium to be supplemented with serum. In addition to the cost of serum, the addition of serum, whose composition is generally not known in detail, can complicate protein recovery and increase the risk of contaminating the cultures with animal virus (Birch and Froud, 1994).

Insect expression system
Recently, insect cell culture has become an attractive alternative to mammalian cell cultures. The most frequently used cell lines are Sf21 and Sf9 derived from Spodoptera frugiperda and S2 derived from Drosophila embryonic cells. The maintenance conditions of insect cells are much more lenient than mammalian cell lines. For example, insect cells do not require CO2, and they can be easily adapted to serum-free medium for simpler protein purification. In addition, insect cells grow at room temperature and thus are not hosts for most mammalian pathogens. The availability of many expression shuttle vectors containing insect-specific baculovirus strong promoters is also an advantage. Moreover, the amount of heterologous protein recovered from baculovirus-mediated transfection in S. frugiperda cell lines is comparable to that obtained generally with mammalian expression system. In the case of hu-LIF (human Leukemia Inhibitory Factor) expression, CHO, Sp2/0, MEL, and COS-1 cell lines expressed 11-17, 19-25, 1-3, and 4-5 mg/liter of recombinant protein respectively (Geisse et al., 1996). The baculovirus-infected Sf9 cell culture could produce 12 mg/liter of product making it a
better expression system than MEL and COS-1 cells. Similar to mammalian cell cultures, large-scale production of proteins is feasible with insect suspension cell culture (Goosen, 1991). One major advantage of insect system over mammalian system is that the time required to produce large quantity of recombinant proteins from stably expressing cell lines is generally shorter than that of mammalian cell lines (Geisse et al., 1996). Amplification of the integrated gene in the stable mammalian cell line is a rare event and has to be selected in multiple rounds of selection (Wurm and Petropoulos, 1994). The time required from transfection to production of a large quantity of proteins is approximately 9 months and 6 – 9 months for CHO and Sp 2/0 cells respectively, whereas baculovirus insect expression system takes 3 months (Geisse et al., 1996).

Although the features above make the insect cell expression system as good a system as the mammalian system and a better system in some aspects, the recurring problem of complex post-translational modifications in the bacterial and yeast expression systems is not completely resolved in insect cells either. The importance of the post-translational modifications such as phosphorylation, N-glycosylation, O-glycosylation, addition of fatty acids, and formation of disulfide bonds is considerable if these modifications attribute significantly to the function of protein. For example, the alpha subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF) loses its ability to bind GM-CSF when the COS cells expressing the alpha subunit are treated with N-glycosylation inhibitor, indicating that N-linked oligosaccharides are essential for its function (Tu et al., 1998). The \( tPA \), one of the components responsible for dissolution of clot clots, has 17 disulfide bonds, which are important for \( tPA \) enzyme activity. Coexpression of an enzyme, which catalyzes disulfide bond isomerization could allow expression of active authentic \( tPA \) in \( E. \ coli \) where normally disulfide bond formation is very inefficient (Qiu et al., 1998). In some cases, these post-translational modifications may have no effect on the biological activity of proteins. Two of the human alpha-interferon subtypes are glycosylated in one residue whereas the other seven subtypes are not glycosylated (Nyman et al., 1998). The unglycosylated alpha-interferon expressed in bacteria was fully active, demonstrating that the glycosylation is not required for its function (Zhao et al., 1987). Since the effect of the post-translational modifications on the protein function varies depending on each
protein, the feasibility of insect expression system has to be considered for individual protein with potential glycosylation signals.

In most cases, insect expression systems have been reported to provide post-translational modifications like the mammalian system, far more accurate than the bacterial and yeast system. Generally, phosphorylation, O-glycosylation, N-glycosylation, lipid addition, and disulfide bond formation are properly performed in insect cells (O'Reilly et al., 1992). However, the addition of complex oligosaccharides (N-glycosylation pathway) such as GlcNAc, Gal, or sialic-acid, onto the shorter core oligosaccharide structure does not appear to occur in some insect cells (O'Reilly et al., 1992). Instead, the addition of complex oligosaccharide is substituted by some fucosyl transfer. In one study, however, a heterogeneity of N-glycosylation pattern on human plasminogen, that is, the presence of proper N-glycosylation residues in some products, (Davidson and Castellino, 1991) has been found. This might imply that insect cells have the ability to properly N-glycosylate certain proteins, however, that may be inefficient in the processing (Geisse et al., 1996). It may also suggest that insect cells of different genus and tissue-type differ in their post-translational capability. Therefore, if the glycosylation of the protein of interest plays an important functional or structural role for its activity, there is a possibility that the protein expressed in insect cells may not be biologically active.

In summary, having recognized the restricted capability of each expression system, the choice of an expression system would depend largely on the purpose of the heterologous expression and on the status of the protein's post-translational modifications and their biological importance in protein function. Numerous heterologous expression studies have been done in bacterial and yeast systems; nevertheless, only recently the insect expression system using baculovirus has received attention in its ability to compete with the mammalian expression systems. Although baculovirus-mediated gene transfer into insect cells has been intensely studied, other gene transfer methods in insect cells are only beginning to emerge as common research tools. If the limited capability of N-glycosylation is kept in mind, insect cell cultures can be a good alternative to mammalian system in expression of many heterologous proteins due to ease of cell culture, large production level, and short time-frame required for establishing stably transformed cell
lines. Therefore, relevant studies of gene transfer in insect cells and development of efficient insect expression system are important and active areas of research.

1.3 Methods of DNA transfer into *ex vivo* cells

The wide use of gene transfer technique has led to improvement and development of new vehicles for efficient delivery of transgenes to cells. The available methods can be divided into two broad categories - viral and non-viral delivery methods. Viral vectors use the viral mechanism of entry into cells alleviating the concern for inefficient uptake of the transgene. Although non-viral vectors or delivery methods are often less efficient than the viral method, they are attractive alternatives to viral methods mainly because of the safety concerns and the lack of specific immune response when the application extends to therapeutic use.

1.3.1 Baculovirus-mediated transfection

The most extensively used vector for mediating introduction of foreign DNA into insect cells is baculovirus. There are over 300 genes expressed using the baculovirus expression system (O'Reilly et al., 1992). The best-characterized member of the Baculoviridae family is Autograph Californica Nuclear Polyhedrosis Virus (AcNPV). As many as 23 different lepidopteran cell lines can be infected by AcNPV (Hink et al., 1991). Therefore, the use of the natural infection mechanism to introduce foreign DNA into insect cells is an efficient means of transfection.

The genome of baculovirus is functionally characterized by genes that are expressed early, late, and very late during infection (O'Reilly et al., 1992). The very late genes such as *polyhedrin* and *p10*, that are not essential for infection or replication of the virus (Williams et al., 1989) are expressed in high amounts, thus making the promoters of these late genes (expressed 20 to 24 hours post-infection) useful for heterologous expression in insect cells. Therefore, infection of insect cells with recombinant baculovirus DNA whose *polyhedrin* or *p10* genes are replaced with a transgene is an efficient means of producing a large quantity of heterologous protein.
In order to prepare the recombinant virus, the gene of interest is cloned into a transfer plasmid vector, containing viral DNA flanking the gene, and cotransfected with linearized wild type baculovirus DNA into insect cells. This cotransfection is carried out by calcium phosphate coprecipitation, DEAE-dextran precipitation, or liposome-mediated transfection. The double-crossover recombinant containing the gene of interest and the interrupted viral gene can be identified by its occlusion phenotype. The functional polyhedrin (polh) gene results in the occ+ phenotype, which is characterized by the formation of polyhedral occlusion bodies in the infected cell nuclei (Smith et al., 1983). The recombinant virus, whose polh gene is interrupted from targeted gene replacement, forms no such structures (occ- phenotype). Therefore, microscopic examination of each plaque is necessary to identify the recombinant virus. After several rounds of plaque identification and purification, the recombinant viral particles are amplified and used to infect insect cells to produce the recombinant protein. The infection resulting in the production of recombinant protein is lytic to insect cells, therefore, limiting its use to a transient expression. Another drawback is that the lytic infection leads to the release of many intracellular proteins, including degradative enzymes, which complicate the protein isolation and purification steps.

The most distinguishing feature of the baculovirus system is in the potential for high production of the recombinant protein, resulting from the burst of expression driven by the strong viral promoter (late genes, polh or p10). Most of the heterologous proteins expressed with baculovirus system reach production range from 10 mg to 100 mg per 10^9 cells (O'Reilly et al., 1992). The efficient foreign protein production is attributable to the specific transcriptional environment that its natural host, insect cells, provides for the transgene expression driven by the baculoviral promoter. Another advantage is in the large genome size of baculovirus. In most expression systems, the shuttle vectors can accommodate one, at most two transgenes of small sizes due to the constraint of vector size, thereby requiring co-transfection or separate transfection procedures. Up to three heterologous genes have been successfully expressed in a single recombinant virus (Chatterji et al., 1996), and the accommodation of more genes is conceivable.

Although the baculovirus system has been used widely, the viral manipulation in in vitro culture is tedious and cumbersome. It involves the purification of intact viral
DNA that is 80 - 220 kb in length, the construction of the transfer vector, the cotransfection of the transfer vector and wild type viral DNA, the isolation of the recombinant virus particles, and the infection of permissive cells with the recombinant virus particles. Despite many improvements made to the procedure such as lacZ-, PCR-, or antibody-based recombinant virus identification (O'Reilly et al., 1992), the method involves more work and technical difficulties than any other transfection methods with insect cells. The calcium phosphate coprecipitation, DEAE-dextran precipitation, electroporation, particle-bombardment, and lipofection methods require a single transfer of the shuttle vector plasmid containing the transgene into cells by different means of transfer. In addition to the many viral manipulation steps, the time frame it takes to produce recombinant protein is approximately 3 months from the time the transgene is cloned into the transfer plasmid (Geisse et al., 1996). Transient expression using other transfection method, however, takes as short as a few hours and often times, a few days depending on the nature of the recombinant protein from the time the transgene vector is ready for transfection.

In summary, the usefulness of the baculovirus expression system is proven by its high level of recombinant protein production. However, the long time frame it takes and the complexity of the protocol are driving the research to alternative transfection methods.

1.3.2 Non-viral delivery methods

Calcium phosphate precipitation
The calcium phosphate coprecipitation was first devised for an animal cell line (Graham and van der Eb, 1973) and adapted for use with insect cells by various groups (Burand et al., 1980; Carstens et al., 1980; Potter and Miller, 1980). When CaCl₂ in the transfection buffer is mixed with the sodium phosphate in the culture medium, calcium phosphate coprecipitates with DNA onto the monolayer of cell culture. The adsorption of DNA calcium phosphate coprecipitates is thought to induce endocytosis of DNA into cells. This method is often used with glycerol or DMSO shock treatment to increase the transfection efficiency (Burand et al., 1980; Potter and Miller, 1980). Use of this
transfection method in insect cell culture has been frequently reported with baculovirus DNA and the transfection assays performed by viral plaque formation (Burand et al., 1980; Carstens et al., 1980).

**DEAE-dextran precipitation**

Treatment of cells with polycationic diethylaminoethyl-dextran (DEAE-dextran) allows the adsorption of the chemical with the negatively charged cell surface. Subsequent treatment of cells with DNA in a small volume allows negatively charged DNA to be bound to the DEAE-dextran (Potter and Miller, 1980). The adsorption of DNA onto the cell surface promoted by polycationic DEAE-dextran (Maes et al., 1967) is thought to stimulate the uptake of DNA by pinocytosis (Pagano et al., 1967). Although the DEAE-dextran treatment has been shown to stimulate the infectivity of viral nucleic acids by many folds (McCutchan and Pagano, 1968; Pagano and Vaheri, 1965), it has not been extensively used for insect cells.

**Electroporation**

Electroporation uses electric field pulses to render cell membranes transiently porous to substances normally impermeable. Gene transfer mediated by an electric field was first adapted to a mouse cell line (Wong and Neumann, 1982). Plant cells have also been transfected with electroporation to render DNA entry across cell walls (Fromm et al., 1986). Electroporation has not found as much popularity with insect cells, because other efficient methods exist. One reported case of efficient gene transfer into Sf9 cells was found with electroporation to introduce recombinant baculoviral DNA (Mann and King, 1989).

The electroporation procedure requires a physical interaction between the electric field and cell membrane. Hence, the successful gene transfer depends on the cell type to a lesser degree than other methods that require direct interaction between the delivery vehicle and the membrane. To allow efficient DNA transfer across membrane, the right amount of electric field (voltage) should be applied for a proper amount of time (pulse length). These parameters determine the extent of cellular damage and subsequently the cells' ability to recover efficiently from the procedure to express the transferred DNA.
(Chu et al., 1987). Therefore, a balance between cell death and the degree of DNA transfer has to be achieved for optimal expression.

One drawback in considering these parameters is that a large quantity of DNA (up to 80 µg/ml) is necessary for efficient transfer of DNA in a small-scale transfection (Chu et al., 1987), whereas calcium phosphate, DEAE-dextran precipitation, lipofection, (Hilliard et al., 1996) and particle-bombardment methods (Burkholder et al., 1993) need a few micrograms of DNA for a similar scale of transfection efficiency. The procedure is probably the simplest of all the transfection methods mentioned here since it requires the cells to be exposed to a set of electric parameters in the presence of DNA. However, the limitation in electroporation is the availability of the equipment that can generate an appropriate type of electrical pulse for a broad range of voltage and capacitance.

**Biolistics method**

A particle bombardment technique was originally designed for transfection of plant cells (Klein et al., 1992). Since plant cell walls provide a secure barrier for introduction of DNA by conventional transfection methods, accelerated gold particles coated with plasmid DNA have been used for effective penetration of DNA across cell walls and membranes. Since then, many different cell types and tissues have been transfected using the biolistic method (Klein et al., 1992), including cell lines that are difficult to transfect using conventional techniques, such as differentiated neuronal cell lines (Biewenga et al., 1997).

Generally gold particles of either varying or uniform sizes between 0.5 and 5 µm are used to conform the size of the cells being transfected. Gold particles coated with DNA by salt precipitation such as calcium phosphate (Zelenin et al., 1991) or calcium-polyethylene glycol precipitation (Burkholder et al., 1993) are accelerated by compressed gas, gunpowder explosion, or electrical discharge (Takeuchi et al., 1992). Usually, helium gas is used for its inertness and low density, which allows high velocities to be reached by the accelerated particles. To minimize the resistance to acceleration or disruption by the presence of particles in the air, the cells are often placed in a vacuum chamber or overlaid with helium (Biewenga et al., 1997).
The major advantage of the biolistic method is that the efficiency is independent of cellular receptors or membrane dynamics unlike the calcium phosphate coprecipitation, lipofection, or virus-mediated methods. Thus, the biolistic method can be a good alternative to cell lines that are not receptive to most of the transfection techniques.

The efficiency of the technique, however, depends on how the bombarded cells survive the unnatural environment created by the procedure. The technique requires exposure of cells to physical conditions such as puncture of the plasma or nuclear membrane, vacuum, high pressure, and/or temperature changes. The survival of the bombarded cells depends on the extent of the cellular damage and the initial cell viability, thereby requiring optimization of the biolistic parameters. In addition, the requirement of equipment that can produce adjustable particle acceleration levels in a helium vacuum chamber becomes a disadvantage with its unavailability while other transfection techniques do not require specialized equipment.

1.3.3 Non-viral synthetic vector methods

Among various types of synthetic vector, cationic lipid- and polymer-based systems are the most extensively studied.

Lipopolyamines and other cationic polymers

Lipopolyamines were designed to bind DNA molecules and to condense them into particles coated with a cationic lipid layer (Behr et al., 1989). The condensed and coated DNA is then brought to the proximity of the cell membrane via electrostatic interaction of the cationic lipid layer with anionic residues present on the cell surface. Lipopolyamines, as the name implies, exert their cationic interaction with DNA via multiple amines on fatty acid chains.

Other DNA binding synthetic molecules that are different types of cationic molecules such as polylysine (Midoux et al., 1993), dendrimers (Hudde et al., 1999), and polyethylenimine (Choi et al., 2001) are also available. Polylysines are positively charged proteins made of lysine amino acids, whose amino groups interact with DNA. Polyethylenimines are a web of ethylenimine groups, and dendrimers are radial branches
of amine groups formed into spheres with the positive charges surrounding the surface of the sphere for interaction with DNA (Tang and Szoka, 1997). These different cationic molecules commonly carry protonatable amines. Different abilities and efficiency in transfecting various cell lines displayed by these vectors (Boussif et al., 1995; Loeffler and Behr, 1993; Tang et al., 1996) are suspected to be due to the difference in the type and the number of positively charged amines.

Liposome-mediated transfection (Lipofection)
The use of cationic liposomes was developed by Felgner et al. (1987), and its transfection efficiency is many fold higher than that of calcium phosphate or DEAE-dextran precipitation methods (Felgner et al., 1987). Cationic liposomes can be made from a variety of synthetic and naturally occurring phospholipid molecules with positively charged head group. The transfer or uptake of DNA into cells is achieved by formation of cationic liposomes and negatively charged DNA into DNA-liposome complexes, referred to as lipoplexes, and subsequent interaction of the lipoplexes with negatively charged cell surface. In addition to the many commercially developed proprietary liposome formulations, private research labs have developed empirically identified liposome formulations.

Liposomes form uni- or multi-lamellar vesicles depending on the method of preparation (Gregoriadis, 1973). Radler et al. (1997) determined the general structures of liposomes alone and of DNA-liposome complexes by X-ray diffraction. In the absence of DNA, liposomes composed of DOPC (dioleoyl phosphatidylcholine) or DOPE (dioleoyl phosphatidylethanolamine) as neutral lipid and cationic DOTAP (dioleoyl trimethylammonium propane) were between 0.02 to 0.1 μm in diameter (Radler et al., 1997). Upon addition of DNA, lipoplexes formed globules with sizes on the order of 1 μm. The globules consisted of multi-lamellar structure with DNA sandwiched between cationic bilayers, resembling a ball wrapped with alternating layers of DNA and lipid bilayer membrane.

The use of lipofection is limited by the availability of efficient liposome molecules for a given type of cell line. Although the basic principle in lipofection involves electrostatic interaction, there appears to be other factors involved in efficient
complex formation and interaction with cell membrane, such as size, stability, and charge
density of the lipoplexes (Koltover et al., 1998). In addition to the physical properties of
the lipoplexes, cell-specific membrane molecules such as heparansulfate proteoglycan
(Mounkes et al., 1998) also appear to play an important role in allowing efficient uptake
of lipoplexes by mediating an active endocytotic pathway. Therefore, the liposome
formulation appropriate for a given cell line has to be determined empirically. Often, the
reason that a cell line cannot be successfully transfected using liposomes may be that
appropriate liposomes are not available.

When an effective liposome formulation is determined for an expression system,
the transfection can be optimized for maximal production of heterologous protein for
individual cell lines. The parameters involved in optimization will be discussed in
Chapter 3. Unlike the time-consuming procedures in baculovirus expression system,
lipofection procedure requires formation of lipoplexes and incubation of the complexes
with cells, normally for a few hours, depending on the protocol. The production of
heterologous protein can initiate as early as a few hours post-transfection depending on
the strength and the type of promoter driving the transgene, and on the stability of the
transcript and the protein in the cell line.

1.3.4 Summary
Among the non-viral gene delivery techniques described here, highest transfection
efficiencies have been reported with synthetic vectors. The polyamine-based vectors
show varying transfection efficiency in comparison between themselves and to cationic
liposomes. For example, 20-fold higher transfection efficiency with lipopolyamines than
with cationic liposomes has been reported in mammalian cells (Legendre and Szoka,
1993). In another study, however, lipopolyamines were reported to be less efficient than
transfection by cationic lipid into a hepatoma cell line (Remy et al., 1995). It is difficult
to determine which is the more efficient carrier system because no comparative studies
have been done systematically, especially in insect cells. There may not be a single
universal delivery system that is optimal for all expression systems.

The use of lipopolyamines and other cationic polymers is often not as convenient
as liposomes. Since the structures of lipopolyamines, polylsines, polyethylenimines,
and dendrimers are more complex and more defined than those of liposomes, the
generation of these defined molecules is often more difficult than that of liposomes,
which naturally form into uni- or multi-lamellar vesicles upon mixing. Taking into
account that the liposomes are easier to make and to manipulate for formulation
optimization and that similar transfection efficiency is obtained, liposomes are the
preferred form of synthetic delivery vector for the gene transfer.

The most studied gene transfer method into insect cell lines has been baculovirus-
mediated transfection. However, the baculovirus-mediated method involves the use of
virus particles, and permissiveness of baculovirus to mammalian cells (Hofmann et al.,
1995) raises the concern of bio-safety. Use of non-viral methods can, of course, alleviate
this problem. Although baculovirus system results in high level of transgene expression,
the laborious experimental procedures and the long time-frame required for protein
production relative to other non-viral methods are the main drawbacks.

The ease of transfection technique, the rapid establishment of the expression
system, and the ability to produce a relatively high amount of heterologous proteins
within a short period of time make the liposome-mediated gene transfer a useful and
actively explored expression system.

1.4 Path of DNA delivery in liposome-mediated gene transfer

One of the most important attributes of viral-based vectors is their ability to promote
destabilization of the host cell membrane to allow the receptor-mediated entry of its
genetic material into the target cell and to derive cellular machinery for expression of
viral genes. These processes are controlled largely by the coat proteins on the surface of
viruses (Lollo et al., 2000) and the specific usage of the cellular transcriptional machinery
for viral gene promoter in the case of baculovirus, respectively. The lack of these active
mechanisms in the non-viral carriers may be the main reason for the lower efficiency of
transfection. Therefore, these have been the key obstacles of non-viral delivery systems
in improving the efficiency of transfection.

The efficiency of the liposome-mediated gene delivery relies on several events
during the transfection. Firstly, the formation of liposome-DNA complexes has to be
controlled so that other charged molecules in the medium, in which complex formation occurs, do not sequester the liposomes and DNA away from each other. Secondly, the complexes are required to interact with the cell surface to elicit entry into cells either via endocytosis or fusion. Thirdly, the release of the complexes or the DNA from the endosomes has to occur to allow transfer to nucleus. Lastly, the transferred gene has to travel the milieu of cytosol to reach the nucleus and to cross the nuclear membrane for entry into nucleus for expression of the gene.

**Efficient formation of liposome DNA complexes**

Interaction of liposomes and DNA molecules allow packaging of the DNA into condensed form with appropriate surface charge for its interaction with the cell membrane. The efficiency of the complex formation is dependent on a few variables, which were examined in this study. The ratio between the cationic and neutral lipids, the charge ratio between the liposomes and DNA molecules, and the medium in which the electrostatic interaction between liposomes and DNA occur are important factors contributing to efficient formation of complexes. The interaction of the complexes with cell surface is thought to be realized through electrostatic interaction. The cell membrane is negatively charged owing to the presence of glycoproteins and glycolipids containing negatively charged sialic acid residues (Chesnoy and Huang, 2000), thereby favoring the electrostatic interactions with the liposome-DNA complexes. In support of this, the net charge of the liposome-DNA complexes in optimal transfection has been shown to be positive (Arima et al., 1997). Removal of negatively charged proteins from serum by DEAE Sephacel column or neutralization of the serum with positively charged molecules could abolish the inhibitory effect of serum on lipofection (Yang and Huang, 1997). Therefore, a careful consideration of the medium, in which the complexation and the incubation of the complexes with cells are performed, has to be given for maximal electrostatic interaction between liposomes and DNA and between the complexes with cell membranes.

**Interaction with cell membrane for uptake**
Upon reaching proximity of the cell membrane, the liposome-DNA complexes enter cells mainly by endocytosis and partly by membrane fusion (El Ouahabi et al., 1997). Several electron microscopic studies (Labat-Moleur et al., 1996; Lappalainen et al., 1997; Zhou and Huang, 1994) have clearly shown the entry of the complexes mainly through endocytosis. In some studies, receptor-mediated endocytosis has also been reported to occur through heparansulfate proteoglycan receptor (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998). Folic acid-targeted lipid-based formulations have also been used to deliver DNA to tumor cells in vitro (Lee and Huang, 1996).

**Release of transferred gene from endosomes for cytoplasmic entry**

The efficiency of gene delivery depends not only on the amount of total DNA taken into cells, but also on how efficiently the transferred DNA molecules are processed within the cells. Upon entry into the cell by endocytosis or fusion, DNA molecules must dissociate from liposomes and from the surrounding endosome to avoid degradation by the lysosomal degradation pathway. One mechanism of dissociation proposes that uptake initially involves destabilization of either plasma or the endosomal membrane to cause subsequent membrane mixing reactions (Xu and Szoka, 1996). In this model, upon the destabilization of membranes and entrapment of complexes into endosomes, the cationic lipids induce a flip-flop of cellular anionic lipids (Xu and Szoka, 1996) that are located primarily on the cytoplasmic side of the membrane and endosomal membrane (Devaux, 1992). This leads to lateral diffusion of anionic lipids into the complex. The diffusion results in charge-neutralization between anionic and cationic lipids, displacing the DNA molecules from the complex and subsequently permitting the release of DNA into cytosol (Xu and Szoka, 1996). Xu and Szoka speculated that the anionic lipids should have displayed stronger affinity for cationic lipids to compete with negatively charged DNA molecules, such as hydrophobic interaction in addition to the electrostatic interaction. The initial destabilization, resulting in entrapment of complexes into endosomes, is speculated to be triggered by lipid structure with high curvature (Wilschut and Hoekstra, 1991). DOPE neutral lipid has been known for this property, and the increase in the transfection efficiency by DOPE has been documented (Farhood et al., 1995; Leventis
and Silvius, 1990). Hence, a careful selection of the cationic and neutral lipid molecules is necessary in considering the composition of the liposomes.

The time at which the reaction leads to the mixing of anionic lipids and the release of DNA, is not well understood. Individually labeled liposomes and DNA molecules accumulate predominantly in perinuclear endosomes (Zabner et al., 1995). This suggests that the entrapped complexes may not be released until the endosomes reach the perinuclear area, facilitating the transport of DNA through the entire path of cytoplasm from plasma membranes to nuclear membranes. On the way to the perinuclear area, the endosomes may aggregate or fuse with other vesicles causing the initiation of the anionic lipid mixing and release of DNA.

In addition to improved composition of liposomes, other methods have been evaluated to augment the release of DNA from endosomal compartment. Stimulation of osmotic swelling of endosomes by the addition of sucrose or a lysosomotropic drug chloroquine during transfection has been proposed (Guy et al., 1995). The use of pH-sensitive liposomes, which become destabilized under low pH of endosome has been demonstrated (Kono et al., 2001). Incorporation of peptides such as virus hemagglutinin (Stegmann et al., 1987) or synthetic analogues (Morris et al., 1997) that can initiate endosomal lysis or fusion has also been utilized to increase the efficiency of transfection.

Transfer to and across nuclear membrane

Upon the release of the DNA from the endosomes into the cytoplasm, DNA molecules then have to be delivered to the nucleus for transcription or integration into the genome. When liposome-DNA complexes were injected into the nucleus, there was no reporter expression, suggesting that DNA has to be dissociated from the liposomes prior to entry into the nucleus and to be available for transcription (Zabner et al., 1995). The nuclear membrane represents as much a barrier as the plasma membrane for efficient transfection. The aqueous channels of the nuclear pore complex allow free diffusion of small macromolecules less than 40 kDa (Lang et al., 1986), but larger macromolecules require an active process (Dowty et al., 1995). This nuclear membrane barrier was tested in two experiments. Injection of naked plasmid DNA into the nucleus of a mouse cell line led to protein expression in over 50% of cells, whereas injection into the cytoplasm
led to expression in less than 0.01% of cells (Capecchi, 1980). A similar study using *Xenopus* oocytes supported Capecchi’s study (Zabner et al., 1995). The lack of nuclear membrane during mitosis may be able to eliminate this barrier. In some studies, the efficiency of transfection has been observed to be dependent on cell cycle and to be higher in rapidly dividing cells (Nicolau and Sene, 1982; Wilke et al., 1996). However, the ability of the non-dividing muscle cells that maintain nuclear membrane integrity to be transfected *in vivo* contradicts the cell cycle-dependent transfection (Dowty et al., 1995).

Use of covalently-linked nuclear localization signals (NLS) derived from SV40 virus NLS was found to exhibit variable enhancement of the transfection efficiency dependent on cell type (Zanta et al., 1999). NLS-tagged plasmid DNA could promote transfection levels ranging from 10- to 1000-fold in comparison to untagged plasmid DNA for human macrophages and NIH 3T3 cells, respectively.

In addition to the nuclear membrane barrier, the transport of DNA molecules across the milieu of cellular degradative enzymes poses another challenge to the cells. The inefficiency of cytoplasmic delivery may be attributed to the cytosolic nucleases that cause instability of the plasmid DNAs in the cytoplasm. Lechardeur et al. (1999) demonstrated that the half-life of naked plasmid DNA after direct injection is very short (1-2hr). Neves et al. (2000) have also proposed that the disappearance of fluorescent-labeled plasmids microinjected into cytoplasm of NIH 3T3 cells was due to progressive degradation of the plasmids rather than diffusion from the site of injection (Neves et al., 2000). To avoid the cytoplasmic degradation and the nuclear transport, one group developed a cytoplasmic expression system, which required usage of a bacteriophage T7 promoter to drive the reporter gene and codelivery of T7 RNA polymerase (Gao and Huang, 1993). However, the mechanism by which the cytoplasmic expression system works is not well understood.

### 1.5 Gene expression from stably integrated versus transient ectopic transgene

Transgene expression in heterologous systems can be performed in either stably transformed cells or transiently expressing cells. The protein expression from stable and
transient expression systems differs in quantity because the amount of template for transcription differs. In transient expression, the DNA molecules transferred successfully into the nucleus will be available for transcription. Stable expression involves only those transgenes that become integrated into chromosomal DNA. The limited amount of integration opportunities such as those from double-strand breaks or recombinations restricts the number of templates available for transcription in stably transformed cell lines. Therefore, the expression level is often much lower in stable than in transient expression. Selection of stable cell lines that contain integrated transgenes in high copies and/or that have integrated into transcriptionally active loci of the genome ensures high expression of the transgene. Ex vivo expression of transgenes in cell cultures may not be affected significantly by unforeseen mutations as a result of integration since disadvantageous mutations can be selected out. However, there is a danger of causing mutations in transgenic animals, human gene therapy, or in ex vivo cell culture.

A transient expression produces a high level of the transgene product lasting for a few days at most. For continuous production of transgene, repeated transfection is essential for transient expression. On the other hand, a stably transformed high producing cell line can be cultured to produce the product continuously or can be frozen for later use. Although the establishment of stable cell lines to select for the high producing clonal lines can be a time-consuming step, an insect cell line requires much less time than a mammalian cell line, which has to be selected for rare amplification events of the integrated genes.

In many cases, transient expression level is evaluated as an estimate of the efficiency of stable transgene expression. The correlation between a large amount of transgene uptake by cells and high level of stable gene expression has been observed in various studies (Echalier, 1997). High level of transient ectopic expression indicates that a large quantity of the transferred transgenes was transferred into and transcribed in nucleus. The presence of a large amount of transferred transgenes in the nucleus may increase their probability to integrate into the chromosomes.

The correlation between integrated transgene copy number and stable gene expression (Kowolik et al., 2001) is often not an absolute relationship due to variable transcriptional activity of the chromosomal DNA into which the transgenes are integrated.
(Ramirez et al., 2001). The position effect of the integrated transgenes can be seen as variegating expression of transgenes in transgenic organisms. Special insulator DNA sequences have been shown to protect integrated genes from the influences of the chromosomal transcriptional activity, thus conferring position-independent, copy number-dependent expression of the transgenes (Nagaya et al., 2001).

The integrated copies of the transgenes are often found as concatamers of either random or uniform (head-to-tail) arrays. Others are found as dispersed single integration events. Not only the integration pattern, but the number of integrated copies of transgenes varies from a single copy to thousands of copies per cell genome. For example, DNA co-transfection experiment of mosquito Mos 20 cell line resulted in 3500 copies of the transgene integrated in complex arrangement (Lycett and Crampton, 1993). Electroporation of mouse erythroleukemia cells introduced few copies of transgene dispersed in the genome (Boggs et al., 1986).

Some correlation may be present between the method of transgene introduction and the integration pattern, depending on the type of stimulation the method can induce on cells. Transfection methods that do not harm the integrity or the structure of the chromosomal DNA such as microinjection into nucleus (Folger et al., 1982), lipofection (this study), and DNA co-transfection (Lycett and Crampton, 1993; Sinclair et al., 1985; Vulsteke et al., 1993) appear to produce complex integration pattern such as concatamers. On the other hand, methods that may affect the chromosomal DNA such as electroporation (Baer et al., 2000; Boggs et al., 1986) appear to produce simple integration patterns.

Various mechanisms and cellular processes have been described to explain different integration events. Both homologous and illegitimate recombination pathways are thought to be involved with integration events of the transfected DNA molecules. These recombination events are linked to repair of double strand breaks (DSB). Many reports suggest that DSBs caused by ionizing radiation, chemicals, or mistakes during DNA replication may be repaired by homologous or illegitimate recombinations (Karran, 2000). Because cells are commonly exposed to DSBs during regular cell cycle (Haber, 1999) and the damage, if not repaired, can result in chromosome fragmentation, loss and translocation (Kanaar et al., 1998), the repair is important to maintain the genome.
integrity. DSB damage is repaired either with the homologous pair of the chromosome by a homologous recombination mechanism (Kanaar et al., 1998), or by a non-homologous DNA end-joining mechanism (Tsukamoto and Ikeda, 1998). Where structural complexity hinders the search for homologous sequences such as that in higher eukaryotic cells, the majority of the repair is thought to be performed by illegitimate recombination involving end-joining of DNA sequences with microhomology of 1 – 10 bp (Pfeiffer et al., 2000; Tsukamoto and Ikeda, 1998). Illegitimate recombination is stimulated 10-fold more than homologous recombination when site-specific DSBs are induced by expression of I-SceI, a rare-cutting endonuclease from *Saccharomyces cerevisiae* (Sargent et al., 1997) in a mammalian cell line. On the contrary, DSBs in *Saccharomyces cerevisiae* are primarily repaired by homologous recombination (Osman and Subramani, 1998; Rouet et al., 1994). Together these findings indicate that the structural complexity represented by chromosomal DNA restricts homologous recombination in higher eukaryotic cells.

Upon entry into nucleus, the fate of the transgenes is left to the hands of cellular recombination machinery or mechanism. The human parovirus adeno-associated virus (AAV) targets integration to a specific site on human chromosome 19 (Yang et al., 1997). Unless the integration is targeted by viral infection with such a specific integration mechanism, illegitimate recombinations are the predominant form of DNA integration mechanism into genome. Most of the time, since transgenes carry bacterial vector sequence and/or foreign gene, there is no homologous sequence to stimulate homologous recombination. With lack of specific mechanism to induce transgene integration, the transferred DNA molecules would have to remain in the nucleus for an opportunity such as sporadically occurring DSBs. During the repair of DSBs by cellular machinery, the transgenes present in the proximity of the repair site may be utilized through end-joining illegitimate recombination mechanism, resulting in integration into the DSB sites.

Many components of illegitimate and homologous recombinations have been identified and their molecular mechanisms are being explored actively (Haber, 1999; Kanaar et al., 1998). With elucidation of the recombination mechanism, the different molecular mechanisms of integration may be resolved.
1.6 Use of Sf9 insect cell expression system with liposome-mediated transfection

As discussed earlier, each of the expression systems introduced here has its own advantages depending on the purpose of the study. One objective of this project is to maximize the heterologous protein expression for potential use in the production of therapeutic proteins. With the increasing use of insect cells as a heterologous expression system, the parameters involved in an efficient introduction of transgenes to insect cells for either transient or stable expression of the transgene need to be studied. Although numerous studies of mammalian expression systems have been reported, insect cells have not been as extensively studied. So far much studies with lepidopteran insect cells have been done with baculovirus-mediated transfection. However, with the growing concern of biosafety, an effective non-viral alternative method has not been studied in detail in insect cells. Liposome-mediated transfection appears to be convenient and effective method of DNA introduction into insect cells. Therefore, liposome-mediated transfection method was explored in lepidopteran insect Sf9 cells for heterologous protein expression. In order to optimize the heterologous protein expression, various factors involved in the lipofection procedure were examined in transient assays.

In addition to the transient expression, heterologous expression in stably transformed Sf9 cells was also analyzed. Determination of the integration pattern and the number of the integrated genes in stably transformed Sf9 cells would enable comparative study with other expression system as well as other transfection methods. The relationships between integration pattern, copy number, and stable transgene expression level was also explored. The analysis of the integration pattern in the stably transformed Sf9 cells could explain the likely mechanism of chromosomal integration of the transferred DNA molecules to be a combination of homologous and illegitimate recombination events.

This study could be used as a model for optimizing expression of other gene of interest in lepidopteran insect cells and possibly in other cell lines. Furthermore, the implications of this study may extend to help human gene therapy as similar barriers in transgene introduction to cells and similar concerns, regarding the integration of the
transferred genes into human chromosomes and their stable expression, are inherently present.
Chapter 2  Methods and materials

2.1 Plasmid DNA Purification Methods

2.1.1 Preparation of Plasmid DNA by Alkaline Lysis

A starter culture of 30 ml of bacterial culture was used to inoculate 400 ml of pre-warmed LB medium (bacto-trypton 10 g, bacto-yeast extract 5 g, NaCl 5 g, per 1 L, pH 7.0). The culture was incubated for 16 hours. The cell pellet from the 400 ml culture was collected by centrifugation using a Sorvall Type GSA rotor at 7000 rpm for 15 min at 4°C. The pellet was resuspended in 100 ml of ice-cold STE (0.1 M NaCl, 10 mM Tris pH8.0, 1 mM EDTA pH8.0) and centrifuged again using the above conditions. The pellet was resuspended in 10 ml of Solution I (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0). One milliliter of fresh lysozyme (10 mg/ml in 10 mM Tris pH 8.0) and 20 ml of fresh Solution II (0.2 N NaOH, 1% SDS) were added and mixed by inversion a few times. Following a 10 min incubation at room temperature, 15 ml of ice-cold Solution III (60 ml of 5 M Potassium Acetate, 11.5 ml Glacial acetic acid, 28.5 ml water) was added and mixed by inversion followed by incubation on ice for 10 min. The entire mixture was centrifuged at 4000 rpm for 15 min at 4°C using a Sorvall Type GSA rotor (with no brake). The supernatant was filtered through a 3M #1 Whatman paper directly into a centrifuge bottle. A 0.6 volume of isopropanol was added, the solution mixed gently, and incubated at room temperature for 10 min. The DNA was pelleted by centrifugation using a Sorvall SA-600 rotor at 12000 rpm for 30 min at room temperature. The DNA pellet was washed with 70% EtOH and centrifuged in the same rotor at 5000 rpm for 10 min at room temperature. The pellet was dried and dissolved in 3 ml of TE pH 8.0 (10 mM Tris Cl pH 8.0, 1 mM EDTA pH 8.0).

2.1.2 QIAGEN Maxi Plasmid Purification (Endotoxin-Free Kit)

DNA was prepared using the QIAGEN Maxi Kit according to the manufacturer’s directions. At the last step, the DNA pellet was dissolved in 1 ml of Endo-free Buffer TE (10 mM Tris pH 8.0, 1 mM EDTA).

2.1.3 Preparation of Plasmid DNA by Polyethylene Glycol Precipitation

Plasmid DNA was purified using the Alkaline lysis method as described in Section 2.1.1. The purified DNA was transferred to a 15 ml Corex tube and 3 ml of ice cold 5M LiCl solution was added to allow precipitation of high-molecular-weight RNA. The solution was mixed well and centrifuged at 10000 rpm for 10 min at 4°C using a Sorvall GS-600 rotor. The supernatant was transferred to a fresh 30 ml Corex tube and an equal volume of isopropanol was added and mixed well. The precipitated DNA was recovered by centrifugation at 10000 rpm for 10 min at room temperature using a Sorvall GS-600 rotor. The pellet was washed with 70% EtOH and air-dried for evaporation of all traces of EtOH. The pellet was dissolved in 500 µl of TE (pH 8.0) with RNAase (20 µg/ml) and transferred into a eppendorf tube. Following a 30 min incubation at room temperature, 500 µl of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG
8000) was mixed in well and centrifuged at 12000 xg for 5 min at 4°C. The pellet was dissolved in 400 µl of TE (pH 8.0) and extracted once with phenol, once with phenol:chloroform, and once with chloroform. The aqueous phase was transferred to a fresh microfuge tube and 100 µl of 10 M ammonium acetate was added and the solution mixed well. Two volumes of 95% EtOH was added and incubated at room temperature for 10 min to allow precipitation of DNA. The DNA precipitate was pelleted by centrifugation at 12000 g for 5 min at 4°C in a eppendorf tube. The DNA pellet was washed with 200 µl of cold 70% EtOH and centrifuged at 12000 g for 2 min at 4°C in a eppendorf tube. The pellet was air-dried and dissolved in 500 µl of TE (pH 8.0).

2.1.4 Wizard Plus Maxiprep DNA Purification System (Promega)

DNA was prepared using the Wizard Maxiprep Kit according to the manufacturer’s directions. The DNA pellet was resuspended in 1 ml of TE made with MilliQ water.

2.1.5 Ammonium Acetate Plasmid Purification (Large-scale)
Modified from Saporito-Irwin et al. (1997)

Bacterial culture was inoculated and incubated as described for the alkaline lysis method (Section 2.1.1) (adjusted to 250 ml LB culture). The cells were pelleted by centrifugation at 10000 g for 5 min at 4°C. The pellet was resuspended in 6 ml of Solution A (150 µl 1.0 M Tris pH 7.6, 120 µl 0.5 M EDTA pH 8.0, 600 µl 0.5 M Glucose, 12 mg hen egg lysozyme, 5.1 ml sterile water) and incubated on ice for 20 min. Twelve milliliters of fresh Solution B (600 µl 20% SDS, 480 µl 5.0 M NaOH, 10.92 ml sterile water) was added and the solution mixed by inversion. Following incubation on ice for 10 min, 9 ml of ice-cold 7.5 M Ammonium acetate pH 7.6 was added. The solution was mixed by inversion and the particulate broken up by vigorous shaking for 5 - 10 seconds prior to 10 min incubation on ice. The solution was centrifuged at 10000 g for 10 min at 4°C. The supernatant was transferred to a fresh tube and 0.6 volume of isopropanol was mixed in by inversion. Following incubation at room temperature for 10 min, the DNA precipitate was centrifuged at 10000 g for 10 min at 4°C. The pellet was resuspended in 4 ml of 2 M Ammonium acetate pH 7.4 by gentle rocking and incubated on ice for 10 min. The solution was centrifuged at 10000 g for 10 min at 4°C again and the supernatant was transferred to a fresh tube. The DNA was precipitated by addition of 4 ml of isopropanol followed by mixing by inversion and incubation for 10 min at room temperature. The DNA was pelleted by centrifugation at 10000 g for 10 min at 4°C. The air-dried pellet was dissolved in 2 ml of sterile water and incubated at 37°C for 20 min with addition of 10 µl of 5 mg/ml RNAase A. One milliliter of ice-cold 7.5 M Ammonium acetate pH 7.6 was added, the solution mixed by inversion, and incubated for 5 min at room temperature. The solution was centrifuged at 10000 g for 10 min at room temperature (to remove access salt). The DNA was precipitated by addition of 3 ml of isopropanol, followed by mixing by inversion and incubation at room temperature for 10 min. The precipitated DNA was pelleted by centrifugation at 10000 g for 10 min at room temperature. The DNA pellet was washed with 70% EtOH, air-dried, and resuspended in 1 ml of TE.
2.1.6 Purification of Plasmid DNA by Equilibrium Centrifugation in CsCl-EtBr Density Gradients

The plasmid DNA was purified using the Alkaline Lysis method as described in Section 2.1.1. One gram of solid CsCl was added for every 1 ml of DNA solution and dissolved completely at 30°C (if necessary). The DNA/CsCl solution was mixed immediately with 0.8 ml of EtBr (10 mg/ml in water) for every 10 ml. The DNA/CsCl/EtBr mixture was transferred to a “Beckman Quick-Seal” tube of appropriate size. The remainder of the tube was filled with more CsCl/EtBr solution of the same CsCl density. The top end of the tube was usually filled with light paraffin oil. The sample tube and a balancer tube were weighed precisely before sealing. Following centrifugation of the mixture at 60,000 rpm for 24 hr at 4°C using a Beckman vertical Ti70.1 rotor, two bands of DNA were visible in either ordinary light or under long-wave UV light in the CsCl density gradient. The lower band that consisted of closed circular plasmid DNA was collected as described in Sambrook et al (1989). The EtBr from the recovered DNA band was extracted by equal-volume-extraction with water-saturated-1-butanol. The mixture was incubated briefly at room temperature for separation of the aqueous and organic phases. The aqueous phase was extracted twice more or until the pink color of EtBr disappeared from the organic phase. The CsCl in the extracted DNA solution was removed by dilution with 3 volumes of water. The DNA was precipitated with 2 volumes of 95% EtOH. The DNA was pelleted by centrifugation at 10,000 g for 15 min at 4°C using a Sorvall SA-600 rotor. Following 70% EtOH wash, centrifugation in the same condition, and air-dry, the DNA pellet was dissolved in 1 ml of TE (pH 8.0).

2.1.7 Modified Alkaline Lysis Plasmid Purification (CC prep)

The plasmid DNA was purified as described in the Alkaline Lysis method (Section 2.1.1). To the DNA solution, 0.5 g of CsCl was added for every 1 ml of DNA solution and the CsCl dissolved completely at 30°C (if necessary). The solution was centrifuged at 10,000 g for 10 min at room temperature using a Sorvall SA-600 rotor. The supernatant was transferred to a fresh tube and 0.8 volume of isopropanol was added and mixed. The mixture was incubated at room temperature for 10 min and centrifuged at 10,000 g for 10 min at room temperature using a Sorvall SA-600 rotor. The DNA pellet was washed in 70% EtOH, centrifuged, air-dried, and dissolved in TE pH 8.0.

2.2 DNA Quantitation Methods

2.2.1 Spectrophotometric Quantitation

Five micro-liters of DNA solution was diluted into 95 ul of distilled water in an eppendorf tube (1/20 dilution factor). The optical density (OD) of the DNA solution was measured at 260 nm wavelength. The UV absorbance was scanned from 260 nm to 280 nm to determine the OD_{260}/OD_{280} ratio for quality evaluation of the purified plasmid DNA. One OD_{260} is equivalent to 50 µg of plasmid DNA/ml. Taking the dilution factor into consideration, the concentration of the undiluted plasmid DNA solution was
calculated. If DNA solution was too dilute or concentrated, less or more dilution was attempted to achieve optimal range of between 0.2 and 0.8 of absorbance measurement with the spectrophotometer.

2.2.2 Agarose Gel Quantitation

A series of linearized DNA diluted by known dilution factors was run on an electrophoresis gel with DNA standards of known concentrations and of similar length as the linearized DNA construct being quantified. The gel picture was captured into a digital image for NIHimage analysis.

2.2.3 Ethidium Bromide Dot Quantification

A serial dilution of the standard DNA and the DNA to be quantified was made. The initial series was made by diluting 1.5 µg of DNA standard, for example, a commercially available vector of similar size into 60 µl volume with TE (pH 8.0) in a 0.5 ml eppendorf tube. Uncut lambda DNA (New England BioLab), pZeoSV (+) (Invitrogen Cat # V850-01), and pEF/Myc/Mito (Invitrogen Cat # V892-20) were used as DNA standards. All dilutions were made in TE made with MilliQ water. The second serial dilution of DNA was made by transferring 30 µl of the initial dilution solution (60 µl) into a fresh tube containing 30 µl of TE (second dilution series). For the next dilution series, again 30 µl of second series was transferred to 30 µl of TE. The serial dilution was made up to 10th series. In all series, the final volume in the eppendorf tube was 30 µl, except for the last dilution, in which the final volume was 60 µl. The DNA to be quantified was similarly diluted from its original DNA solution. To minimize pipetting errors during serial dilution, the same pipette tip was used for transferring diluted DNA to pre- aliquoted 30 µl of TE. In addition, accurately calibrated pipetters were used as the volume measurement was critical for accurate quantitation. The eppendorf tubes were kept closed at all times to prevent evaporation. To each dilution series, 3 µl of 0.005 µg/ml EtBr was added, making the final concentration of EtBr 0.5 µg/ml. For the last dilution series, 6 µl of EtBr was added since there was twice as much volume as the other series. For no-DNA control, same amount of EtBr was added to 30 µl of TE with no DNA. More replicates were prepared for each dilution series as necessary.

A piece of good-quality SaranWrap big enough to cover the entire UV trans-illuminator was placed on the illuminator without any wrinkles or air pockets as these could interfere with UV reflection or with the shape of the liquid dots placed on the trans-illuminator. Video camera was zoomed to an appropriate size so that enough area could be seen on the computer screen to place all the serial dilutions and their replicates as necessary. This area on the trans-illuminator was then marked with a white or ‘Milky’ pen as a rectangular box, which would provide some guidance as to where about the dots need to be placed. The first dilution series (33 µl of volume containing DNA, TE, and EtBr) was placed on the trans-illuminator as 3 separate 10 µl volume dots in a vertical direction, using a same pipette tip for each dilution series. The second dilution series was placed similarly next to the first dilution dots. The rest of the dilution series was placed similarly filling the rest of the area within the drawn rectangular box. The no-DNA
control dots were placed next to final serial dilution dots. The UV source was turned on and the image was captured using NIH image software. The no-DNA-control dots emit only background fluorescence, therefore, this was subtracted while quantifying the fluorescence emitted by each dots. NIH image software was used to convert the fluorescence into densimetric values, which could be used to compare the intensity of the fluorescence. As evaporation could greatly reduce the volume, hence, the size of the dots during spotting on the trans-illuminator, the procedure should be done efficiently in a short period of time. The number of replicates and the number of DNA samples to be quantified would determine how fast the procedure can be done.

2.3 Cell Culture Maintenance

2.3.1 Cell Lines

*Sf9* cell line is a clonal cell line isolated from Sf-21AE in 1983 by G. Smith and C. Cherry. Sf-21AE was made from IPLB-SF-21 cells by adapting to TC-100 media, which were originally established from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda* (Vaughn et al., 1977).

2.3.2 Cell Line Passages in T25 Tissue Culture Flask

The doubling time of *Sf9* cells is approximately 18 to 22 hours in both monolayer and suspension culture (O'Reilly et al., 1992). However, we have observed that *Sf9* cells grown in ESF 921 medium or TC100 medium supplemented with 10% FBS have a cell cycle of approximately 30 hr. The cell cultures were grown at 26°C. One passage took approximately 7 days. For every passage, 10⁵ cells/ml of cells were seeded to 5 ml of fresh ESF 921 growth media (Expression System LLC) in a T25 tissue culture flask (Nunclon) to a final confluence of approximately 40%. At the end of a passage, *Sf9* cells grow up to approximately 7 x 10⁶ cells/ml concentration in a mono-layer and 10⁷ cells/ml in a suspension culture.

2.3.3 Cell Line Passages in Spinner Culture Bottle

*Sf9* cells were grown in suspension culture in a spinner culture bottle (BELLCO). At least 5x10⁵ cells/ml of cells were seeded to 20 – 30 ml of fresh ESF 921 media. Cells grew up to 10⁷ cells/ml of concentration at the end of a 3 - 4 day passage.

2.3.4 Liquid Nitrogen Storage of Cell Lines

In order to reserve cell lines as backup stocks or for future use, exponentially growing cells were harvested from either mono-layer or suspension culture. One and a half milliliters of approximately 5x10⁶ cells/ml cells were aliquoted into cryo-proof tube. Following addition of 120 µl of DMSO, cells were mixed gently. The aliquots were placed into an isopropanol chamber (NALGENE), which allows gradual decrease (approx. 1 °C for every hour) in temperature when left in -80°C. The chamber was left in -80°C for 1 - 7 days before the aliquots could be moved for storage in liquid nitrogen.
If cells are left in -80°C for more than 7 days, the efficiency of recovery of the cells when brought back to fresh medium is reduced.

2.3.5 Culture Media

*Sf9* cells were maintained in either TC100 (Life Technologies, GIBCO BRL Cat # 11600) with supplement of 10% FBS (Fetal Bovine Serum, Gibco BRL) or ESF 921. For most part of cell culture, ESF 921 growth media was used.

The transfection media tested in this project were PBS (Phosphate Buffered Saline), PMN (PBS-modified by NaCl), PMS (PBS-modified with Sucrose and NaCl), PS (PBS-modified with Sucrose), and Grace’s media (Life Technologies, GIBCO BRL Cat #11300). The composition of each medium is shown in Table 2.

In addition to the components listed in Table 2, TC100 and Grace’s media contain amino acids and vitamins. PBS, PMN, PMS, and PS media do not contain amino acids or vitamins.

2.3.6 Antibiotic Selection of Stable Cell Lines

In order to select for transfected cells, Zeocin (Invitrogen) was added to the cell culture 48 hr post-transfection in the 35 mm dish. The selection was started with a low concentration of 300 μg/ml zeocin. When the culture reached confluency in the 35 mm dish in the presence of zeocin, the entire 2 ml volume of cells were transferred to a T25 tissue culture flask with 4 ml of fresh ESF 921 growth media containing zeocin. The first passage in the T25 flask took more than 7 days as transfected cells had difficulty re-establishing the population with antibiotics. Therefore, cells were seeded twice as many (1 - 2x10⁶ cells/ml) as that of a normal seeding of an untransfected culture for the first few passages. The zeocin concentration was gradually increased up to 1 mg/ml as each passage resumed back to the normal 7-day-period. In addition, the number of seeding cells was reduced back to the normal number as transfected cells secured the cell population under zeocin selection. Normally, it took at least 5 - 6 passages to ensure establishment of a stable cell line. In general, when seeding cells, zeocin was added after cells had a chance (30 min to 1 hr) to adhered to the bottom of T25 flask in fresh media. This was to allow some recovery of cell damages caused by pipetting during passage.

2.4 Liposome-mediated Transfection Protocols

2.4.1 Lab-standardized Lipofection Protocol

*Sf9* cells were seeded at 5x10⁵ cells/ml concentration in 35-mm dish in 1 ml of Grace’s media. While the cells were incubated at room temperature for adhesion to the dish, the DNA and liposome mixture was made. In a 1.5 ml eppendorf tube, 1 μg of plasmid DNA purified using CsCl-EtBr method (Section 2.1.6) or QIAGEN method (Section 2.1.2) was mixed into 1 ml of Grace’s media by vortexing. Ten micro-liters of the lab-generated liposomes (0.5:1=DDAB:DOPE) (DDAB and DOPE lipids are described in Section 6) was added into the eppendorf tube by fast pipetting and the mixture was vortexed. The DNA-liposome mixture was incubated at room temperature for 30 min to allow DNA-
liposome complex formation. Following incubation of both cells and DNA-liposome mixture, Grace’s media was removed from cells and the DNA-liposome mixture was added gently onto cells. The cells were incubated at 26°C for 4 hr to allow uptake of DNA-liposome complexes. One milliliter of ESF 921 media was overlaid on cells for resumption of growth. This resulted in dilution of both the growth medium and the Grace’s medium containing the DNA-liposome complexes. The cells were incubated at 26°C for further 44 hr before they were harvested for either transient \( B\)-\textit{galactosidase} assay (Section 2.7), stable antibiotic selection (Section 2.3.6), or GFP fluorescent microscopy (Section 2.8).

### 2.4.2 Optimized Lipofection Protocol

Exponentially growing \( Sf9 \) cells (> 2\( \times \)10\(^6 \) cells/ml) were diluted to 1\( \times \)10\(^6 \) cells/ml concentration with ESF 921 media just before seeding. One milliliter of 1\( \times \)10\(^6 \) cell/ml \( Sf9 \) cells were seeded in 35 mm dish containing 1 ml of overlaid PMS medium (composition found in Table 2). The cells were incubated at room temperature for 30 min to allow adhesion to the dish. DNA-liposome complexes were made by injecting 0.6 \( \mu \)g of CC-prepared DNA (Section 1.7) and 30 \( \mu \)l of the lab-generated liposomes (0.375:1=DDAB:DOPE) into 1 ml of PMS media in 1.5-ml eppendorf tube, using pipettement. The DNA-liposome mixture was mixed by vortexing and incubated at room temperature for 30 min to allow for complex formation. The medium was gently removed from cells. The DNA-liposome mixture was added onto cells gently and 1ml of fresh ESF 921 media was overlaid immediately. The cells were incubated at 26°C for 48 hr before harvesting for assays or antibiotic selection.

### 2.4.3 Commercial Lipofection Reagent Protocols

#### 2.4.3.1 Effectene (QIAGEN)

\( Sf9 \) cells were seeded at 5\( \times \)10\(^5 \) cells for 35 mm dish in 1ml of ESF 921 or TC100 plus 10% FBS and incubated at room temperature for 30 min for adhesion. To prepare DNA-liposome complexes, 0.4 \( \mu \)g of CsCl/EtBr-prepared DNA was diluted in Buffer EC (DNA condensation buffer) to a total volume of 100 \( \mu \)l in a 1.5 ml eppendorf tube, and 3.2 \( \mu \)l of Enhancer was added. The mixture was vortexed for 1 second. The ratio between DNA (\( \mu \)g) and Enhancer (\( \mu \)l) was recommended to 1:8 by the manufacturer. Following incubation of DNA-Enhancer mixture at room temperature for 5 min, 10 \( \mu \)l of Effectene Transfection Reagent was added to the mixture by pipetting up and down 5 times. Varying amount of Effectene Transfection Reagent can be tested with a fixed amount of DNA to optimize DNA to Effectene ratio. While the mixture was incubating at room temperature for 10 min to allow complex formation, the medium was removed from cells, and the cells were washed once with PBS (40 mM KCl, 140 mM NaCl, 1 mM Na\(_2\)HPO\(_4\)·7H\(_2\)O, 11 mM KH\(_2\)PO\(_4\), pH 6.2). To the washed cells, 1600 \( \mu \)l of ESF 921 media was added. Following addition of 400 \( \mu \)l of ESF 921 to the DNA-liposome complex by pipetting up and down, the mixture was immediately added onto cells dropwise. The cells were incubated at 26°C for 48 hr to allow for gene expression before harvesting for transient assays.
2.4.3.2 SuperFect (QIAGEN)

SJ9 cells were seeded as described for Effectene. Two micrograms of CsCl/EtBr-prepared DNA was diluted with Grace's (manufacturer recommends serum-free growth medium) medium to a total volume of 100 µl. Following addition of 10 µl of SuperFect Transfection Reagent to the DNA-Graces' mixture by pipetting up and down 5 times, the mixture was incubated at room temperature for 10 min to allow complex formation. The ratio of the volume of SuperFect Reagent (µl) to the quantity of DNA (µg) is an important factor to optimization, therefore, varying amounts of SuperFect Reagent can be tested on fixed amount of DNA to determine the optimum ratio. While SuperFect-DNA complex formation took place, medium was removed from cells. The cells were washed with 1 ml of PBS (40 mM KCl, 140 mM NaCl, 1 mM Na₂HPO₄•7H₂O, 11 mM KH₂PO₄, pH 6.2). To the SuperFect-DNA mixture, 600 µl of ESF-921 growth medium was added by pipetting up and down twice, and the mixture was immediately added to the cells. Following incubation of the cells for 3 hr at 26°C, SuperFect-DNA mixture was removed from cells. The cells were washed once with 1 ml of PBS, lay with fresh ESF 921 medium, and incubated for 48 hr at 26 °C to allow gene expression.

2.4.3.3 FuGENE™ 6 (Boehringer Mannheim)

SJ9 cells were seeded as described for Effectene. In a 1.5 ml-eppendorf tube, 1 to 5 µl of FuGENE 6 reagent was diluted in a final volume of 100 µl of Grace's medium by adding the reagent directly into the medium. After a gentle mix of the solution, 0.6 µg of CsCl/EtBr-prepared DNA in a volume of 0.5 - 50 µl was added into the diluted FuGENE. The solution was mixed gently by tapping and incubated at room temperature for 15 min. After removing the medium from cells, the complex mixture was added to cells, dropwise and the dish was swirled for even dispersion of the mixture. The cells were incubated for 4 hours at 26 °C and TC100+10% FBS growth medium was added. Harvesting or antibiotic selection was performed 48-hour post-transfection.

2.4.3.4 CellFECTIN® (Gibco BRL Life Technologies™)

SJ9 cells were seeded in the same manner as described for Effectene protocol, using Grace’s medium. To make DNA-liposome complexes, firstly 1 µg of CsCl/EtBr-prepared DNA and secondly 10 µl of CellFECTIN reagent was diluted in 1 ml of Grace’s medium in 1.5 ml eppendorf tube with quick vortex in between additions. The mixture was incubated at room temperature for 30 min to allow complex formation. After removal of Grace’s medium from the dish, the DNA-CellFECTIN mixture was added onto cells gently. Following incubation of the cells at 26 °C for 4 hours, 1 ml of growth medium was overlaid to cells. Harvesting for B-gal assay or antibiotic selection was performed 48-hour post-transfection.

2.4.4 Preparation of DDAB:DOPE Liposomes

Modified from Campbell (1995)
To make 33.6 μmol/ml of DOPE stock solution, 25 mg of dioleoyl phosphatidyl ethanolamine (DOPE) was dissolved in 1 ml of 100% ethanol. Dimethyldioctadecylammonium bromide (DDAB) was similarly made into a stock solution of 33.6 μmol/ml. Various molar ratios of DDAB:DOPE liposomes were prepared by mixing the two stock solutions in appropriate ratios. For example, 0.5:1 molar ratio liposomes was prepared by mixing 0.25 ml (8.4 μmol) of DDAB and 0.5 ml (16.8 μmol) of DOPE into final volume of 1 ml with ethanol. This mixture was drawn up into a 1 cc Insulin Syringe and rapidly injected through 28 1/2-gauge needle into a 50 ml Falcon tube containing 9 ml of sterile distilled water while vortex-mixing. The liposome solution was aliquoted into 1.5 ml eppendorf tubes and stored at 4 °C.

2.5 Electroporation Methods

2.5.1 Electroporation Protocol using BioRad Gene Pulser II™

Sf9 cells grown in ESF 921 were spun down at 3000 rpm for 5 min and resuspended gently in either PMS or HBS electroporation medium to bring the final concentration of cells to 2 x 10^6 cells/ml. Aliquots of 400 μl of diluted cells were prepared in UV-sterilized cuvettes (either 0.2 cm or 0.4 cm gap). As cells settle down to the bottom of cuvette quickly, prompt actions are necessary for each step. One, two, or five micrograms of QIAGEN-prepared DNA was mixed into cells in cuvette. BioRad Gene Pulser II™ allows capacitance range of 0 - 960 μF, applied voltage range of 0.05 - 2.5 kV, and resistance range of 100 - 1000 Ω or ∞. Depending on the experiment, appropriate setting for each electrical parameter was used. Immediately after electric shock, 500 μl of ESF 921 growth medium was added to cells, and 10 μl sample from each cuvette was drawn for trypan blue exclusion assay to determine the cell viability. After 10-min incubation at room temperature, cells were plated in 35 mm dish and incubated for 2 hours at 26 °C. Following incubation, the medium was removed and 2 ml of fresh ESF 921 medium was added. Harvesting was performed 48 hours post-transfection.

2.5.2 Electroporation Protocol using BTX TransPorator™ Plus

Sf9 cells were adjusted to 2 x 10^6 cells/ml as done in above protocol (Section 5.1). Aliquots of 400 μl of cells were made to 0.2-cm cuvettes and 1 μg of QIAGEN-prepared DNA was added into the cells. The BTX bacterial electroporator allows applied voltage range of 0 - 2.5 kV, and other electrical parameters are fixed at 5 msec for pulse length and 1.4 Ω for resistance. Immediately following exposure of cells to an appropriate voltage, 500 μl of growth medium was added. At this point, 10 μl of sample was drawn for assessment of cell viability by trypan blue exclusion. The electroporated cells were incubated at room temperature for 10 min and transferred into 35 mm dish for further incubation at 26 °C for 2 hours. The medium was replaced with 2 ml of fresh growth medium and cells incubated at 26 °C for 48 hours before harvesting.
2.6 Chemicals used for Post-Transfection Chemical Treatment

2.6.1 Etoposide (VP-16)

To make 0.01 M stock solution, 25 mg of VP-16 (Sigma Chemical Co., USA) (MW 588.57) was dissolved in 4.25 ml of 100% DMSO (Dimethyl Sulfoxide). This stock solution was diluted in DMSO to make appropriate concentrations for use in post-transfection chemical treatments.

2.6.2 Sodium Butyrate

Sodium butyrate (Sigma Chemical Co., USA) was dissolved in water and diluted to make appropriate concentrations.

2.6.3 Zeocin™

Zeocin (100 mg/ml, MW 1535) was purchased from Invitrogen LifeTechnology and was stored at −20 °C. Appropriate dilutions were made in water.

2.6.4 HMGD

*Drosophila* HMGD protein was expressed and isolated from bacteria by Sarb Ner (Dr. Grigliatti lab). The expressed dHMGD gene contained the full-length coding sequence.

2.7 β-galactosidase Assays

2.7.1 *B-gal* Assays for Transient Transfection and Transformed Stable Cell Lines

**Recovery of Cells**

Following 48 hour-incubation of cells after transfection, the cells were pelleted down at 4000 rpm for 3 min and resuspended in 200 μl of 0.25 M Tris-HCl (pH 7.5) in 1.5 ml eppendorf tube by vortexing. The cells were frozen at -70 °C for 5 min and thawed at room temperature water bath for 2 min. The freeze/thaw was performed twice more. The cells were pelleted at 14,000 rpm for 1 min and the supernatant was transferred to a fresh tube.

**B-gal Assay**

Depending on how concentrated the supernatant is, 5 μl of the cell lysate can be tested first and appropriate volume can be formally tested later. Usually 30 μl of cell lysate was aliquoted first into each pre-warmed cuvette. Pre-warmed mixture of 66 μl of 4 mg/ml ONPG and 204 μl of Z-buffer was then added to the cuvettes. When there were a large number of samples for the assays, a premix containing ONPG and Z-buffer was aliquoted to cuvettes, containing the cell lysate, using a repeater pipettor. The mixture was incubated at 37 °C until the absorbance reached between 0.2 and 0.8 O.D. at 420 nm wavelength. It was necessary for the experimenter to know the approximate brightness of the solution ahead of time. Once an appropriate colour was reached, the time was
recorded first, and the reaction was stopped by adding 500 μl of 1 M Na₂CO₃ into each cuvette. The solution was mixed well by pipetting several times. If the reaction had reached appropriate yellowness in a very short period of time such as a few seconds, less cell lysate was used for slower B-gal reaction, hence obtaining more accurate recording of the time and B-gal units. For the negative control reaction, the assay was carried out exactly the same way with 30 μl of distilled water. The reactions that took a long time to stop, including the negative control, was incubated for up to 2 hours before the addition of sodium carbonate. The negative control reaction was used to calibrate the spectrophotometer. The absorbance of all the separate reactions was measured at the same period after the last reaction had been stopped. The absorbance was measured at 420 nm using Beckman Spectrophotometer.

ONPG (o-nitrophenyl-β-D-galactopyranoside)
4 mg/ml of ONPG is dissolved in 0.1 M sodium phosphate (pH7.5)

0.1 M Sodium Phosphate (pH 7.5)
41 ml of 0.2 M Na₂HPO₄•2H₂O
9 ml of 0.2 M NaH₂PO₄•2H₂O
50 ml of distilled water

Z-Buffer
60 mM of Na₂HPO₄•7H₂O
40 mM NaH₂PO₄•H₂O
10 mM KCl
1 mM MgSO₄•7H₂O
50 mM β-mercaptoethanol

Protein Assay
The Bradfold (BioRad) protein assay was performed on the cell lysate to determine the total protein concentration. The Bradfold reagent was diluted to 1x with distilled water. Five hundred microliters of the diluted reagent was aliquoted into 1.0-ml disposable cuvette. Initially 3 μl of the cell lysate was added and the solution mixed well by pipetting several times. Upon the addition of the cell lysate to the reagent, the solution turned into blue or bright blue color depending on the protein concentration. The amount of the cell lysate was adjusted so that the intensity of the blue color would obtain an absorbance reading of between 0.2 and 0.8 O.D. at 595 nm. The diluted Bradfold reagent without any cell lysate was used to calibrate the spectrophotometer and all the samples were measured at once.

Protein Standard Curve
The protein standard curve was generated by plotting the absorbance reading measured with 1 - 15 μg of Bovine Serum Albumin (BM) protein against the protein amount. The best-fit line of the curve within the amount of BSA, which had a linear relationship with the absorbance was used as the standard formula (y = ax + b, where y = absorbance at 595 nm, x = protein amount (μg), and a = constant) for calculation of protein amount in the samples.
Data Analysis
The protein standard formula was used to convert the absorbance values (595 nm) into the total protein amount (μg) present in the volume of cell lysate used to perform the protein assay. The total amount of protein in the protein assay reaction was used to calculate the total protein amount present in the volume of cell lysate used to do the B-gal assay. The B-gal unit is a product of the absorbance value at 420 nm and the conversion factor of 380 per time (min) measured to stop the reaction per total amount of protein (mg) in the B-gal reaction. Formula 2.8.1 describes the calculation of B-gal unit.

\[ B\text{-gal unit} = \frac{380 \cdot \text{OD}_{420} \cdot \text{time(min)}}{\text{total protein(mg)}} \]  

Formula 2.8.1

2.7.2 B-gal Assay using microplate Reader

B-gal assay using 96-well plate reader was performed using the same reagents in the same manner as described above. The only adjustment was in the smaller volume of the reaction so that each reaction could be performed in one of the 96 wells in the microplate. The amounts of ONPG and Z-buffer were scaled down to make 200 μl of total reaction volume instead of 300 μl in cuvettes. The B-gal unit was determined by kinetic analysis of the production o-nitrophenol, the product of B-galactosidase reaction, thus it was not necessary to stop the reaction by addition of sodium carbonate. The B-gal unit was in mOD/min. The protein assay was also done using Microplate reader with smaller volume of the Bradfold reagent. The data analysis was performed using SOFTmax® Pro (Molecular Devices Corporation) software.

2.8 GFP Fluorescence Microscopy and Image Analysis

2.8.1 GFP Fluorescence Microscopy of Transfected Cells

The transfected cells were examined under microscope for expression of the GFP reporter after 48 hours of incubation for reporter gene expression. The examination was done directly from the 6-well plate, in which the transfections were carried out to avoid transferring cells onto glass slides. This was important to avoid cellular damage or cell lysis during pipetting, which could interfere with B-gal assay performed on the same cells after examination. The growth medium was removed from each well for microscopy and saved in a tube. The 6-well plate was placed on the lowered stage, and the stage was brought up carefully so that the walls of the wells did not crash onto the objective lens.

Both the bright-field and the GFP images were captured from the same field of view under the total magnification of either 400 x or 100 x, using SPOT32 camera and software. When capturing the bright-field images, it was important to adjust the contrast so that the difference between the cells and the background in their contrast was prominent. This was necessary to produce reliable images for their processing later. A FITC filter with the excitation and the emission spectra of 485 nm and 518 nm was used. The images were saved as JPEG format and post-processed in Adobe Photoshop®.
2.8.2 Processing of Images using Adobe Photoshop®
Modified from Steenstrup et al. (2000)

Determination of Average Number of Pixels in one Sf9 cell from the Captured Images
Three equally sized boxes were randomly selected from the captured bright-field image and the total number of cells in each box was counted from the computer screen. These portions of images were cut and pasted into three different files and the following procedure was performed separately on each image. The image was adjusted for intensity to eliminate the background with the Levels function under Image menu and the Adjust function. The Actual Pixel option was selected under View menu. The image was inverted in color by using Inverse function under Select menu after conversion into 2-bit image. To select the area containing the pixels that should be counted, the Color Range function under Select menu was used. In the Color Range panel, Shadows option was selected if the cells for pixel counting were in black. If the cells were in white and the background in black, Highlights option was selected. The number of pixels was determined using the Histogram function under Image menu. The average number of pixels per cell (conversion factor) was determined by dividing the total number of pixels in the image by the number of cells manually counted. The two other images saved into separate files were similarly processed. The average number of pixel, calculated for each image was pulled to determine a more reliable conversion factor (number of pixels per cell).

Determination of Total Number of Cells from the Bright-field Image
The total number of pixels, representing all the cells in the bright-field image was determined using the same method described above on the entirety of the image. The number of pixels was divided by the conversion factor for determination of the total number of cells in the entire bright-field image.

Determination of Total Number of Fluorescent Cells from the GFP Image (same field of view as above bright-field image)
The amount of fluorescence was detected evenly in the cell. Therefore, the conversion factor determined above could be used to determine the number of fluorescent cells in the GFP photograph. The GFP images were similarly converted into 2-bit images to eliminate differences in the pixel intensity and the same procedure was performed as described above. The total number of fluorescent pixels was divided by the conversion factor to calculate the number of fluorescent cells. The transfection efficiency was calculated as the proportion of cells that were fluorescent from the total number of cells present in the image.

2.9 Cloning of Reporter Constructs

2.9.1 pBacTracer1 LacZ\textsuperscript{New}
The LacZ gene from pZeoSV LacZ (Invitrogen) was cloned into pIZT/V5-His (Invitrogen) construct. A Kpn I/Not I fragment of pZeoSV LacZ, containing the LacZ gene, the 3' and 5'-untranslated regions, and the SV40-polyadenylation signal, was cloned into the corresponding sites of pIZT/V5-His in the MCS (Fig 1A) to make pBacTracer1 LacZ (Fig 1B).

2.9.2 pBacTracer1 LacZ smRS GFP

The soluble-modified red-shifted GFP (smRS GFP) was substituted into pBacTracer1 LacZ to replace the fluorophore region of the wild type GFP in the construct. The p2Zop2F smRS-GFP construct was a gift from Dae-Kyun Ro (Department of Botany, UBC). In order to replace the GFP fluorophore region in the pBacTracer1 LacZ with the red-shifted version, four cloning steps were performed.

Firstly, the Hind III fragment (1.7 kb) of pBacTracer1-LacZ was cloned into the Hind III sites of pBKS (4.5 kb) cloning vector (Step 1, Fig 2) and was called pBKS/Tracerl707.

Secondly, 149 bp of Kpn I/Nde I fragment of pBKS/Tracerl707 was replaced with 271 bp of Kpn I/Nde I fragment of p2Zop2F-smRS-GFP construct, containing the fluorophore region (Step 2, Fig 2). This construct was referred to as pBKS/Tracer 1707/smRS GFP.

Thirdly, 1716 bp of Nco I/Sac II fragment of pBKS/smRS GFP was inserted into the Nco I and Sac II sites of the 2952 bp pBKS/Tracer 1707 fragment (Step 3, Fig 2). This construct was called pBKS/Tracer1707/smRS GFP(Nc/S) since it became the original pBKS/Tracer1707 sequence except for its 64 bp of Nco I/Nde I fragment which now contained the red-shift fluorophore instead of the wild type fluorophore.

Lastly, 1707 bp of Hind III fragment of pBKS/Tracer1707/smRS GFP construct was put back with 4809 bp of Hind III fragment of pBacTracer1 LacZ (i.e. rest of the pBacTracer1 LacZ construct) (Step 4, Fig 2). This final construct was pBacTracer1 LacZ smRS GFP (Fig 1C), which was exactly the same construct as the pBacTracer1 LacZ (Fig 1B) except for the 64 bp of Nco I/Nde I fragment now containing the red-shifted GFP fluorophore region instead of the wild type fluorophore. The complete map of pBacTracer1 LacZ smRS GFP can be found in Fig 1B.

In general, the GeneClean gel purification kit or QIAGEN gel purification kit was used to purify DNA separated on an agarose gel. The purified DNA was quantified on a gel by EtBr staining and comparison to a standard DNA. The restriction enzyme and ligation reactions were performed using Gibco BRL or NEB restriction enzymes and ligase.
Figure 1  pBacTracer1 LacZ smRS GFP derived from pIZT/V5-His expression vector

pBacTracer1 LacZ construct (B) was made by cloning the bacterial LacZ (Kpn I/Not I) gene into pIZT/V5-His (A). The wild type GFP in the pBacTracer1 LacZ was substituted with soluble-modified, red-shifted GFP (smRS GFP) to make pBacTracer1 LacZ smRS GFP (C) construct by replacing Nco I/Nde I fragment containing the fluorophore region indicated by ( ). The polyadenylation signals (pA) are OpIE2 pA and SV40 pA from left to right. MCS is multiple cloning site. H = Hind III, Nc = Nco I, Nd = Nde I
Figure 2 Cloning of pBacTracer1 LacZ smRS GFP

In the first cloning step, the Hind III fragment (1.7 kb) of pBacTracer1 LacZ was cloned into Hind III site of pBKS (4.5 kb) cloning vector, making pBKS/Tracer1707. In the second step, 149 bp of Kpn I/Nde I fragment of pBKS/Tracer1707 was replaced with 271 bp of Kpn I/Nde I fragment of p2Zop2F smRS GFP construct, containing the fluorophore region, making pBKS/Tracer 1707/smRS GFP. In the third step, 1716 bp of Nco I/Sac II fragment of pBKS/smRS GFP was inserted into the Nco I and Sac II sites of the 2952 bp pBKS/Tracer 1707 fragment, making pBKS/Tracer1707/smRS GFP(Nc/S). In the last step, 1707 bp of Hind III fragment of pBKS/Tracer1707/smRS GFP construct was put back with 4809 bp of Hind III fragment of pBacTracer1 LacZ, making pBacTracer1 LacZ smRS GFP. This new construct was exactly the same as pBacTracer1 LacZ except for the 64 bp of Nco I/Nde I fragment now containing the red-shifted GFP fluorophore region. (Abbreviations: H = Hind III, K = Kpn I, Nd = Nde I, Nc = Nco I, S = Sac II). ( ), ( ), and ( ) bars indicate sequences originating from pBKS, pBacTracer1 LacZ, and p2Zop2F smRS GFP. ( * ) indicates the red-shifted fluorophore sequence.
2.10 Purification of Genomic DNA from Transformed Stable Cell Lines

Three milliliters of logarithmically growing S/9 cells (~3x10^6 cells/ml) were collected in a 10-ml falcon tube and spun at 2000 rpm for 5 min using a bench-top centrifuge. The cell pellet was resuspended in 0.5 ml of TE (pH 8.0) and mixed with 3 ml of HB (homogenizing buffer, 10 mM Tris Cl pH 8.0, 0.1M EDTA pH 8.0, 20 μg/ml pancreatic RNAase, 0.5% SDS) by inversion. The resuspended cells were extracted in 1 volume of 1:1 ratio of phenol:chloroform for 15 min in a rotor. The aqueous and organic phases were separated using a bench-top centrifuge at 2000 rpm for 5 min. The aqueous phase containing DNA was transferred to a new tube and extracted twice more. To the last aqueous phase, 1 volume of isopropanol was added and left at room temperature for 10 min. The solution was spun at 2000 g for 5 min for precipitation of DNA. The pellet was washed in 70% EtOH, centrifuged, and dried by leaving in 70°C water bath with open top for 10 min. The pellet was resuspended in 200 μl of TE/RNaseA and incubated at 37°C for 1 hour for RNaseA digestion. The DNA solution was further incubated at room temperature overnight for complete resuspension of DNA. The absorbance of DNA was measured at 260 nm and 280 nm. The 260/280 ratio should be greater than 1.75. A lower ratio is an indication that significant amounts of protein remain in the preparation. The genomic DNA was stored at 4 °C to avoid DNA shirring from freeze-thawing.

2.11 Southern Blot Analysis

2.11.1 Treatment of Genomic DNA for Southern Blot Analysis

Approximately 10 μg of DNA was digested with 10 U of restriction endonuclease enzyme in a final volume of 50 μl overnight. The digested DNA was precipitated with 2.5 volume of 95% ethanol. The DNA was pelleted by centrifugation using bench-top centrifuge at full speed. The DNA pellet was resuspended in appropriate volume of TE (pH 8.0) for electrophoresis.

2.11.2 Conventional Electrophoresis

A 0.8% agarose was used to separate digested genomic DNA by electrophoresis. The gel was run in running buffer of 0.5 x TBE (Tris Borate EDTA) at < 1 volt/cm overnight. The gel was stained in 0.5 μg/ml of ethidium bromide for 30 min and visualized for DNA on UV trans-illuminator.

2.11.3 Pulsed-Field Gel Electrophoresis

A CHEF-DR™ II (BioRad) electrophoresis unit was used to separate EcoR I-digested genomic DNA in 1.0% agarose gel. The gel was run at 6 volts/cm (200 V) for 15 hours at 15 °C in 0.5 x TBE running buffer. The switch ramping time was 1 - 12 seconds at a ratio of 1.0. The gel was stained in ethidium bromide for visualization. Low range PFG markers (New England BioLab Inc.) ranging from 0.13 kb to 194 kb markers were used.
as standard DNA. Intact Lambda DNA (New England BioLab Inc.) was also used as standard DNA marker.

2.11.4 Capillary Transfer onto N+ Hybond

The electrophoresed gel was depurinated in 0.25 M of HCl with gentle agitation for 10 min at room temperature (optional when target sequence smaller than 10 kb). The gel was denatured in 1.5 M NaCl and 0.5 M NaOH for 25 min and neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), and 0.001 M EDTA for 30 min. The gel was washed in distilled water between solutions.

A capillary blotting apparatus was assembled in transfer buffer of 10 x SSC (1.5 M NaCl and 0.3 M Na3 citrate) using Hybond N+ (Amersham Pharmacia Biotech Inc.). The transfer was allowed overnight as described by Sambrook et al. (1989). After disassembly, the blot was washed in 2x SSC, dried on a piece of 3M Whatman paper for 1 hour, and UV-fixed for 2-5 min (312 nm recommended).

2.11.5 Preparation of Probe

To prepare 32P-labelled probe, 25 - 100 ng of agarose gel-purified DNA was denatured at 100 °C for 10 min in a final volume of 10 μl. To the denatured DNA, 2 μl of 10x concentrated random hexanucleotide mix (Roche), 3 μl of (dNTP-dATP) containing 0.5 mM of each nucleotide except for dATP, 2 Units of Klenow enzyme (Roche) were added in the order and the reaction was kept on ice. The volume of the reaction is made up to 17 μl with water, and 3 μl of [α-32P] dATP (30 μCi) was added last. The mixture was incubated at 37 °C for 1 hour. The labelled probes were separated from unincorporated dAT32P and dNTPs by MicroSpin S-300 HR Column method (Amersham Pharmacia Biotech Inc.).

2.11.6 Hybridization and Washing

The UV-fixed DNA blot was pre-hybridized in 0.25 ml/cm² of hybridization solution (5 x SSC, 0.5% SDS) containing 1% skim milk in a hybridization tube for at least 1 hour at 60 °C. Hybridization was performed at 55 °C or 60 °C overnight.

The blot was washed in 25 ml of wash solution (2x SSC, 0.1% SDS) at room temperature for 5 min with occasional gentle agitation. The solution was replaced with several 25 ml of pre-warmed wash solution (2x SSC, 0.1% SDS or 1x SSC, 0.1% SDS depending on the stringency required) and incubated at 65°C for 15 min. This step was repeated once more. Detection of probe was done by autoradiography using either X-ray film or phosphoimage screen. If necessary, removal of the probes from the blot was done by pouring boiled solution of 0.1x SSC and 0.1% SDS onto the blot. The blot was washed briefly once in 2x SSC after the solution cooled down to room temperature and stored either dry or wet.

2.12 Quantitation of DNA on Southern Blot using Phosphoimager Software
Hybridization of Southern Blot with *Sf9*-Actin Promoter Sequence and Quantitation of the hybridization signal

The DNA blot was hybridized in a similar conditions as described above using 1.7 kb *Sf9*-Actin promoter sequence, which was digested out of the pSfA4::LacZ construct with *BamH I* and *EcoR I*. The pSfA4::LacZ construct was created by Tara Colins (former Directed-Studies Student in our lab) by cloning the PCR-amplified *Sf9* actin promoter sequence and the *LacZ* reporter gene into pBKS vector. The 1.7 kb *BamH I/EcoR I* fragment contained the actin promoter and approximately 100 bp of the *LacZ* gene. The hybridized blot was exposed onto a phosphoimage screen for several hours or up to a few days depending on the intensity of the radioactivity signals. The southern images were scanned using STORM860 (Molecular Dynamics, part of Amersham Pharmacia Biotech). The images were saved as ImageQuant 5.2 gel file or tif file and analyzed using ImageQuant 5.2 software.

Use of ImageQuant 5.2 Software to Quantitate Signals

One of the signals from the actin promoter was used to normalize the amount of DNA loaded between lanes on the blot. The chosen actin signal was specified for quantitation by drawing a small box around it on the saved image. The same box was copied, pasted, and placed on the same actin signals in other lanes. A control box was also drawn for subtraction of the background.

Using the Background Correction function from Analysis menu, all the boxes for quantitation were highlighted under the Object list. From the same window panel, Object Average was chosen as the method of background correction and the box for background correction was chosen. By choosing the Object Average background correction method, ImageQuant calculates the average intensity value of the pixels in the selected background object box. The background value was automatically applied to all the objects selected for quantitation. Pixels below the background value was treated as background and the pixels above background were treated by subtracting the background values. The report of the signal intensities whose values were adjusted by background subtraction was viewed by selecting Volume Report from Analysis menu. The data were saved in Excel files and analyzed for visual presentation in Microsoft Excel software.

Hybridization of the Same Blot with Plasmid DNA Sequence and Quantitation of the hybridization signals

The same blot was stripped and hybridized in the same conditions as described above. The probe was either 1.9 kb, a *Kpn I/Sac I* fragment containing part of the *LacZ* gene or 1.3 kb, a *Bsm VI/Pst I* fragment containing the *GFP::Zeo* fusion gene from pBacTracer1 LacZ smRS GFP construct used to transfect *Sf9* cells. The quantitation procedure was done using the ImageQuant software as described above.

Quantitation of Relative Amount of Integrated Plasmids in Transformed Stable Cell Lines

The relative amount of DNA loaded between lanes on the southern blot was determined by calculating the relative intensity of the actin signal. The intensity of the chosen actin signal from each lane was divided by the weakest signal intensity from one of the lanes,
thereby setting the least intense signal to a value of 1. These relative values, thus, represented the relative amount of DNA loading between lanes.

These relative values were divided by their corresponding *LacZ* or *GFP::Zeo* signal intensity in each lane to normalize the signal intensity with DNA loading. The intensity of the normalized *LacZ* or *GFP::Zeo* signals were then divided by the weakest signal intensity from one of the lanes to calculate the relative amount of integrated plasmids.
Chapter 3  Optimization of transient transfection

Objective
The objective of this project was to improve the transfection efficiency of Sf9 insect cells for heterologous protein expression. The ability to transfect a cell line with relative ease and effectiveness is becoming valuable, whether for large scale commercial production of heterologous protein or for study of the function of specific genes or proteins in a model system. In addition, the findings of this study may have applications for those studying gene therapy by non-viral gene transfer method; as similar problems regarding the efficiency of cellular uptake and the expression of the transferred gene will be encountered for in vivo transfections.

Overview
There are a number of different methods of transfecting DNA into cells. I chose the liposome-mediated transfection method as the primary tool for transferring DNA into cells due to its effectiveness and relative ease with which it can be easily adapted to a wide variety of cell lines. The formation of DNA-liposome complexes, its cellular uptake, and processing of the transfected plasmid for expression in the cell all contribute to the efficiency of transfection. Variables affecting these steps have been examined for optimization. These variables were categorized into several groups: DNA, liposomes, cell density, transfection medium, time variables, and enhancing chemicals and proteins. In all cases, the expression of the LacZ gene was used as the reporter. Overall, the reporter activity could be improved by approximately 8-fold by optimizing the different variables studied in this thesis.

While I relied on the lipofection method as my principal transfection methodology, an attempt to compare the lipofection to the electrotransfection method was made. However, comparative study was not done extensively, as electroporation, in general, was found to be 3 orders of magnitude less efficient for transfecting Sf9 cells than the lipofection method.

Format of presentation
This thesis involves examinations of many different variables, which affect the uptake of DNA by cells and the expression of the transfected gene, in order to maximize the transient or stable reporter expression. Therefore, for the sake of clarity and to benefit the reader, this section of the thesis is organized to present an introduction of each variable being considered followed by the data, discussion of the significant data, and the summary or conclusion for each variable.

3.1 DNA variables

Several aspects of DNA contribute to the efficiency of transfection. The quality of the DNA product that is recovered from various DNA purification methods and the topology of DNA used in transfection were the variables I examined.

3.1.1 EtBr Dot Quantification of DNA

Rationale
In order to compare the efficiency of the DNA products purified from various DNA purification methods, equal amounts of DNA must be used for transfection. Therefore, the quantification of DNA is a crucial step for meaningful comparison of DNA preparations for transfection efficiency. To measure concentration of DNA accurately, two different methods of DNA quantification were examined.

Conventionally, the concentration of DNA in a solution is measured spectrophotometrically by comparing the absorbance at two wavelengths, 260 and 280 nm. The absorbance at 260 nm is attributed to DNA principally while the absorbance at 280 nm is attributed to proteins. However, other molecules possibly present in the DNA preparation, such as contaminating amino acids, carbohydrates, and lipids from purification procedure can also absorb UV. Hence, quantification of crude DNA preparations can result in overestimation of DNA quantity in the sample. In order to avoid such problems, I devised an Ethidium Bromide Dot Quantification method (EtBr Dot Quantification).
Principle of EtBr Dot Quantification

Ethidium bromide (EtBr) intercalates in between the bases of DNA and RNA. More specifically, it intercalates in double-stranded DNA or RNA once in every five nucleotides resulting in a 21-fold increase of fluorescence (Le Pecq, 1971). Therefore, the increase in fluorescence emitted by EtBr is proportional to the amount of double-stranded structure. This property of EtBr is the basis of a fluorometric quantification of nucleic acids.

The amount of nucleic acid is directly proportional to the amount of fluorescence emitted within the linear range of sensitivity. Before determining this range, the minimum amount of EtBr required should be determined, as the quantity of double-stranded nucleic acid is proportional to the fluorescence emitted only if the EtBr concentration is in excess of the nucleic acid concentration. Therefore, two major parameters, the linear range of DNA and the saturating concentration of EtBr were examined. Then the EtBr Dot Quantification was used to determine the concentration of DNA solutions from various DNA purification methods.

Determination of saturating concentration of ethidium bromide

Rationale
The fluorescence of EtBr bound to DNA is 21-fold higher than that of the unbound EtBr. It is known that 5 μg/ml of EtBr is suitable for DNA concentrations ranging from 0.01 to 1 μg/ml (Le Pecq, 1971). However, the lowest EtBr concentration required for saturation is recommended when high sensitivity is required since excessive unbound EtBr can cause high background fluorescence.

Method
In order to determine the lowest concentration of EtBr, which could saturate a fixed amount of DNA, various amounts of EtBr were mixed with either 20 or 80 ng of standard pEF/Myc/Mito DNA (Invitrogen) in a final volume of 20 μl. The 20 μl mixtures containing 0 to 2.5 μg/ml of EtBr and the DNA standard were placed on a piece of SaranWrap on the UV trans-illuminator as liquid drops. The emitted fluorescence from
the drops was captured using a digital camera (Fig 3A) and analyzed using NIH image 1.61. The fluorescence level from each dot was converted into densimetric values. Background fluorescence emitted by EtBr alone was subtracted from the counterpart fluorescence containing DNA.

Data
The fluorescence converted into densimetric units displayed an inverse logarithmic relationship with DNA quantity (Fig 3B). The saturating concentration of EtBr was reached at 0.2 μg/ml for 20 ng of DNA. However, the saturation of fluorescence was not recognized as clearly with 80 ng of DNA (Fig 3C) due to large variation in the fluorescence. This variation could be attributed to either the change in the size of the liquid dots placed on the trans-illuminator, resulting from evaporation of the liquid or the sensitivity of fluorescence reflected from uneven surface on the trans-illuminator. Note that the three dots that represent 80 ng of DNA solution each containing 2.5 μg/ml of EtBr are smaller in size than the other dots. The EtBr aliquots containing 80 ng of DNA were deposited first starting from the left and then the 20 ng dots. By the time all 144 dots were placed, the dots that had been placed first had partially evaporated, resulting in the difference in the size of the dots. Despite the large variation and the decreasing trend of fluorescence with increasing EtBr, the maximal fluorescence was achieved at approximately the same concentration of EtBr as with 20 ng of DNA, at 0.2 μg/ml of EtBr.

Therefore, the saturating concentration of EtBr was found to be 0.2 μg/ml with DNA of 20 - 80 ng.
Figure 3 Effect of EtBr concentration on fluorescence of DNA samples

Varying amounts of EtBr were mixed with either 0 ng, 20 ng or 80 ng of pEF/Myc/Mito standard DNA and placed on UV trans-illuminator (A). The fluorescence emitted by each dot was quantified by densimetric analysis. The background fluorescence from no DNA dot was subtracted from the fluorescence emitted by 20 ng of DNA and 80 ng of DNA and plotted against concentration of EtBr in (B) and (C), respectively. The error bars are standard deviations of 3 replica dots.
Determination of the linear range of DNA quantity and fluorescence emitted

Rationale
The EtBr Dot Quantification method relies on the use of standard curve formula obtained from a plot of a known set of concentrations of DNA sample and the fluorescence emitted. Therefore, the conversion of the fluorescence values measured from DNA of unknown concentration into DNA quantity is accurate only when the fluorescence value is within the valid range to which the standard curve formula can be applied.

Method
To determine the range of DNA amounts which produces a linear relationship with the fluorescence, three different DNA standards of known concentration were used in EtBr Dot Quantification with 0.2 μg/ml of EtBr (Fig 4A). The circular plasmids, pEF/Myc/Mito (Invitrogen) and pZeoSV (Invitrogen), and Lambda phage DNA (BM) are 5.5 kb, 3.5 kb, and 48.5 kb, respectively. The EtBr Dot Quantification was performed in triplicate for each DNA standard in the 0 to 100 ng range. The background-subtracted densimetric values of each dot were plotted against the DNA concentration (Fig 4B – D).

Data
The amount of fluorescence increased with the amount of DNA due to the increased amount of EtBr intercalation. The standard curve extrapolated from fluorescence of various DNA quantity was reproducible with different standard DNAs as seen in Fig 4E. With all three DNA standards, DNA amounts of 5 to 40 ng resulted in a linear relationship with fluorescence (Fig 4F – H). The best-fit line of the data points within the 5 - 40 ng linear range was determined using Cricket Graph 1.2.3 software.

In conclusion, having examined the minimal saturating concentration of EtBr and the relationship between DNA quantity and the emitted fluorescence, I chose 0.2 μg/ml of EtBr and 5 – 40 ng range of DNA for use in EtBr Dot Quantification.
Figure 4  Effect of DNA concentration on fluorescence of DNA-bound EtBr

Increasing amounts of three different standard DNAs, pEF/Myc/Mito, pZeoSV, and uncut Lambda DNA of known concentration, were mixed with 0.25 μg/ml of EtBr and placed on UV trans-illuminator (A). The fluorescence produced from 5 – 100 ng of each DNA was plotted with standard deviations from 3 replica dots - (B) pEF/Myc/Mito, (C) pZeoSV, (D) Lambda DNA. The three graphs were superimposed for comparison (E). A range of DNA amounts, which resulted in a linear relationship with the fluorescence amount, was determined and the standard curve formula of its best-fit line was calculated using Cricket Graph 1.3.2 (F-H). The standard curve formula are $y = 37.9 + 16.9x$ for pEF/Myc/Mito (F), $y = 163.6 + 14.5x$ for pZeoSV (G), and $y = 106.3 + 13.4x$ for Lambda DNA (H).
Quantification of various DNA preparations by EtBr Dot Quantification

Method

Having determined the proper protocol for EtBr Dot Quantification, the concentration of DNA product from each DNA purification method (CsCl, QIAGEN, PEG, Wizard, AA, CC, and Miniprep) was determined. Various volumes of the plasmid DNA, pBacTracer1-LacZ-smRS-GFP was combined with 0.2 µg/ml of EtBr in a 20 µl volume and placed on the UV trans-illuminator for quantification (Fig 5B). A standard, containing various amounts of pEF/Myc/Mito DNA within the linear range determined above, was used (Fig 5A) at the same time for determination of the best-fit line formula (Fig 5C). The fluorescence values as measured by densimetric unit were adjusted by subtracting the background of EtBr only dots. For each dot of each DNA preparation method, the densimetric values were converted into DNA quantity (ng) by the use of standard curve formula generated. The concentration of the DNA solution was then calculated by dividing the DNA amount (ng) by the volume of DNA added into the 20 µl volume dot for each dot. Only the DNA concentrations whose densimetric values were within the linear range were pooled to produce the mean concentration of the DNA solution.

One representative EtBr Dot Quantification is shown in Fig 5. Similar quantification was performed seven times with the same DNA standard. Replications of samples were avoided as increased number of dots required longer time to perform the procedure, resulting in evaporation of the liquid dots and hence an inaccurate fluorescence measurement. The data from all seven EtBr Dot Quantifications were pooled for calculation of the average DNA concentration for each DNA preparation.
Figure 5  EtBr Dot Quantification of plasmid DNA purified from various DNA preparation methods

Varying amounts of reporter plasmid, pBacTracer1-LacZ-smRS-GFP were mixed with 0.25 μg/ml of EtBr and placed on UV tran-illuminator for detection of fluorescence emitted by the DNA samples (B). The first dot in each row contained no DNA serving as EtBr background. A known volume of DNA from CsCl, QIA, PEG, Wiz, AA, CC, or Mini purification methods were used in each dot. 0 – 25 ng of pEF/Myc/Mito standard DNA served as an internal standard (A). The standard curve formula, y = 226.4 + 50.9x, was calculated from the best-fit line of the pEF/Myc/Mito DNA graph (C).
Comparison of DNA concentrations determined by EtBr Dot Quantification with those by Spectrophotometric Quantification

Data

DNA concentrations determined by EtBr Dot Quantification method were compared to those determined by the spectrophotometric quantification method (Table 1). The concentrations determined by either method were within their standard deviations, for CsCl, QIAGEN, PEG, Wizard, and AA DNA preparation methods. However, when the spectrophotometric quantification method was used, the concentration was overestimated by a factor of 6 and 24 for CC and Mini-prepared DNA, respectively. It appeared that the spectrophotometric method produces a reliable quantification of DNA prepared by methods known to purify DNA with fewer contaminants. On the contrary, the spectrophotometric method appeared to overestimate DNA quantity in a crude DNA preparations as contaminating molecules such as amino acids, carbohydrates, or lipids from bacteria also absorb UV light within the tested range of wavelength. Interestingly, the overestimation of the concentration could not be attributed directly to such contaminants since the OD$_{260}$/OD$_{280}$ ratios of the various DNA preparations were within a range of 1.8 and 2.0.

Therefore, all subsequent DNA preparations were quantified using the EtBr Dot Quantification method.

Table 1  Comparison of DNA concentrations determined by EtBr Dot Quantification and Spectrophotometric Quantification methods

<table>
<thead>
<tr>
<th>DNA Preparation Method</th>
<th>EtBr Dot (ng/μl)</th>
<th>Spectrophotometric (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl</td>
<td>161 ± 83</td>
<td>95</td>
</tr>
<tr>
<td>QIAGEN</td>
<td>207 ± 79</td>
<td>140</td>
</tr>
<tr>
<td>PEG</td>
<td>441 ± 218</td>
<td>220</td>
</tr>
<tr>
<td>Wizard</td>
<td>219 ± 64</td>
<td>150</td>
</tr>
<tr>
<td>AA</td>
<td>35 ± 15</td>
<td>23</td>
</tr>
<tr>
<td>CC</td>
<td>327 ± 125</td>
<td>1910</td>
</tr>
<tr>
<td>Mini</td>
<td>329 ± 99</td>
<td>7800</td>
</tr>
</tbody>
</table>
3.1.2 Effect of DNA purification methods on reporter gene expression

**Determination of optimal DNA quantity for each DNA preparation for maximal reporter expression**

*Rationale and method*

Different DNA isolation methods are known to purify DNA to various degrees of quality. The quality of DNA product can determine how efficiently it can be transferred into cells. Thus, DNAs prepared by different procedures differ in their efficiency for cellular uptake or for integration into the genome. The efficiency of DNA prepared by seven different methods was examined by transfection of Sf9 cells.

DNA products prepared by various DNA purification methods differ not only in the quality, but also in the actual amount of DNA recovered. In addition, preparations may differ in the composition ratio of plasmid DNA to residual genomic DNA as well as other contaminants. To determine the best or most suitable DNA preparation method for transfection, equal amounts of plasmid DNA from each method must be tested. However, neither the EtBr Dot Quantification nor the Spectrophotometric Quantification method can distinguish the amount of plasmid DNA versus genomic DNA in a given DNA solution. Therefore, I tested various amounts of total DNA based on EtBr Dot Quantification in transfections to determine the amount of total DNA required for optimal reporter activity (Fig 6A - G). By doing this, one can empirically determine the optimal amount of total DNA to use in a transfection without having to know the exact amount of plasmid DNA in a DNA preparation. The B-gal assay was performed with the use of a kinetic microplate reader.

*Data*

The optimal DNA amount needed for the maximal reporter activity was achieved with between 0.4 and 0.6 μg of DNA in most DNA preparations except for the 'Mini' preparation method (Fig 6A – G).
Figure 6  Effect of DNA purification method on the reporter activity 48-hour post-transfection

Varying amounts of DNA (pBacTracer1 LacZ construct) prepared by CsCl (A), QIAGEN (B), PEG (C), Wizard (D), AA (E), CC (F), and Mini (G) DNA preparation methods were used to transfect Sf9 cells with a standard lipofection transfection protocol. The transfection efficiency was assayed by B-gal reporter assay using Kinetic Microplate Reader. The B-gal activity is presented in mOD/min/mg. The error bars represent standard deviations of three transfections.
Comparison of the maximal reporter activities obtained by various DNA preparations

Data
Another set of transfections were performed and evaluated by regular B-gal assay. The commonly used DNA purification methods such as CsCl, QIAGEN, and PEG showed high reporter activity (Fig 7). This was not unexpected. Surprisingly, however, the CC purification method modified from a crude ‘Mini’ DNA preparation resulted in a similar level of reporter activity relative to that of CsCl DNA preparation (Fig 7). Though this was not observed in the first set of transfections (Fig 6), the same trend was observed in the subsequent transfections including those results presented in Fig 8.

Rationale for using another construct for transfection
To further examine the effect of DNA preparation methods on the reporter activity, a new DNA construct, which contained the improved red-shifted GFP gene for enhanced fluorescence was used. The amount of GFP fluorescence detected from the transfected cells was used as another measure of the reporter activity.

Using GFP assay, I could determine the number of cells within the population that acquired DNA. This approach provides information regarding the percentage of cells transfected from the entire population as well as an assessment of the total amount of DNA taken up by those cells in the population that was transfected, by B-gal assay.

Four DNA preparation methods were chosen for comparison using the new plasmid construct. The criteria for choosing those methods were based on the reporter activity achieved so far, and the efficiency of time and cost of performing the DNA purifications. The method that produced the most reporter activity (CsCl method), which was the most time-efficient (QIAGEN method), and the most cost-efficient (CC method), were selected for further comparison using the new construct.
Figure 7  Comparison of reporter activities from transfections performed using DNA purified from various preparation methods (pBacTracer1-LacZ construct)

The transfection efficiency of DNA prepared by various DNA preparation methods are shown. The DNA amounts previously determined to produce maximal reporter activity for each DNA preparation method were used for transfection and they are indicated below each bar. The error bars represent standard deviations of three transfections.
Transfection efficiency of DNA prepared by four different DNA preparation methods were compared for pBacTracer1-LacZ-smRS-GFP construct. The DNA amount determined previously to produce maximal transfection efficiency for each purification method was applied for CsCl, QIA, CC, and Mini preparation methods. The $B$-gal activities (□) are found on the primary y-axis and the number of fluorescent cells (●) on the secondary y-axis as measured by GFP reporter gene in the same vector construct. Control is transfection performed without DNA and liposome. The error bars represent 95% Confidence Intervals (C.I.) from 6 transfections. The C.I. of the control transfections is from 2 transfections.
Data

In these transfections, CC DNA resulted in a significantly higher reporter activity than the CsCl and QIAGEN DNA preparations (Fig 8). In CC preparation protocol, DNA was first isolated using the ‘Mini’ preparation method and the DNA solution was treated with CsCl salt by dissolving the salt in DNA solution, centrifuging the mixture, and recovering the DNA in the supernatant by ethanol precipitation. The CsCl salt treatment was aimed at removing contaminating proteins that could become precipitated in the presence of high CsCl salt concentration. This additional treatment step in CC preparation may have contributed to the improved reporter activity. The CsCl treatment step did not appear to enhance reporter activity by removing all the contaminants since the quantification by Spectrophotometry resulted in overestimation of DNA concentration.

The number of fluorescent cells detected 48-hr post-transfection for each DNA preparation method displayed the same trend of the transfection efficiency as that determined for the \( B\)-gal reporter gene (Fig 8). Consistently, transfections performed with the CC-prepared DNA resulted in the most \( B\)-gal activity and the highest number of fluorescent cells. From the GFP detection assay, the increase in the \( B\)-gal activity after transfection with CC-prepared DNA could be attributed to increased number of cells taking up the plasmid.

Conclusion

Because of the high reporter activity, the cost efficiency, and the simplicity of the CC purification procedure, CC DNA was used for further experiments.

3.1.3 Effect of DNA topology on reporter gene expression

Rationale

The topology of the transfecting plasmid DNA is an important factor in a transient expression system (Cherng et al., 1999). Plasmid DNA can exist either in supercoiled, open-circular, or linear form. DNA of different topologies can affect transfection efficiency differently due to change in i) the efficiency of lipoplex formation, ii) the stability of plasmid DNA in the cell, or iii) the transcriptional efficiency of DNA of
different topologies. Therefore, it is important to examine different forms of plasmid DNA to determine the best form of DNA to obtain the maximal reporter activity.

Method
Since linear plasmid DNA was rarely seen with CC DNA when run on an agarose gel, DNA taken from the stock solution was assumed to be supercoiled/circular DNA. Supercoiled and open-circular DNA were examined as one category of DNA topology because of the difficulty obtaining them separately. Transfections were performed using either supercoiled/open-circular plasmid or linearized plasmid (Sac II-digested plasmid). The enzyme, Sac II cleaves pBacTracer1-LacZ once in the 3'-untranslated region of the LacZ gene (Fig 1B).

To be able to attribute the change in the reporter activity obtained from transfection of linear DNA to the topology of the plasmid only, a control transfection was also performed with heat-inactivated Sac II enzyme and supercoiled/open-circular DNA.

It was noticed that when the endonuclease enzyme was heat-inactivated at 65°C for 25 min according to the manufacturer's recommendation, the enzyme still retained partial activity. When the heat-inactivated enzyme was incubated with intact plasmid and the digestion reaction run on an agarose gel, a portion of the separated plasmids was in a linear form, indicating that the treatment at 65°C for 25 min was not sufficient to completely inactivate the enzyme. Therefore, heat-inactivation was performed at 100°C for 25 min. For the control transfection with the inactive enzyme, DNA was added to inactivated enzyme mixture before allowing formation of DNA-liposome complexes in order to perform the procedure in the same manner as the experimental transfection with linearized DNA, which had been incubated with active Sac II enzyme for digestion.

Data
The transfection performed with linear DNA produced only background B-gal activity (Fig 9). The decrease in the reporter activity was attributed to the linearization of the plasmid DNA, not the presence of the restriction endonuclease as the addition of either 65°C- or 100°C-heat-inactivated enzyme resulted in a similar or elevated reporter activity.
than that of the positive control, respectively. Therefore, the most efficient form of plasmid DNA for transfection was determined to be supercoiled/circular.

Figure 9  Comparison of reporter activities obtained from transfections using supercoiled/circular vs. linear form of plasmid DNA

Transfection of Sf9 cells were carried out with 0.6 μg of CC-prepared DNA either untreated supercoiled/circular or Sac II-linearized form of pBacTracer1-LacZ construct in a standard transfection protocol. Either 65°C- or 100°C-heat-inactivated Sac II enzymes were used for mock transfections. The positive and negative control transfections were carried out in the absence of enzyme with or without supercoiled/circular DNA, respectively. The error bars represent standard deviations of three transfections. The positive and negative control transfections were each done once.
Discussion

The B-gal assay could not determine whether the decreased reporter activity of linear DNA was due to its effect on DNA-liposome complex formation, the stability of DNA in the cell, or the transcription efficiency. DNAs of different topologies do not differ significantly in the condensation of DNA by liposomes or in the complex formation (Cherng et al., 1999). On the other hand, linear DNA may be less stable than other forms of DNA because it is more prone to cytoplasmic degradation (Weintraub et al., 1986). The topology of the plasmid DNA contributes to the transcriptional activity due to the differential accessibility of a given promoter to the transcription factors due to the compactness of the DNA (i.e. supercoiled versus linear plasmid) (Weintraub et al., 1986).

In agreement with the data presented here, other groups have demonstrated that the most efficient conformations of plasmid DNA for the maximal transfection efficiency are supercoiled and open-circular DNA (Cherng et al., 1999). Bergan et al. (2000) showed that either form of DNA transfects with similar efficiency. Linear DNA, on the other hand, displayed significantly reduced transfection efficiency (Cherng et al., 1999). Although formal evidence is not present, it has been speculated that the linear DNA is more susceptible to degradation by nucleases than supercoiled or circular DNA (Weintraub et al., 1986).

Although it is clear that linear DNA is inefficient in transient transfection, it has been a preferred form of DNA for creating transformed stable cell lines as linear DNA integrates more frequently into the genome than the circular form (Folger et al., 1982). Moreover, transformation with different DNA topology could result in different integration pattern, which may result in a different level of stable reporter expression.

Conclusion

The most efficient form of plasmid DNA for maximal reporter activity was determined to be supercoiled/circular DNA. Linear plasmid DNA resulted in near-background reporter activity. The reason for the dramatic reduction in the reporter activity when transfecting with linear plasmid DNA was speculated to be due to the reduced stability of the linear DNA in the cell and probably not due to the reduced efficiency of the DNA-liposome complex formation.
3.2 Liposome variables

Rationale and background

Although liposomes and other non-viral gene transfer methods can transfect a wide range of cell lines, some liposomes have been shown to be more efficient in transfection of one cell line than others (Rose et al., 1991).

The method of liposome preparation can produce either uni-lamellar or multi-lamellar liposomes even with the same lipid formulations. One form of liposomes may be more effective than others because of the different physical and chemical properties of DNA interaction with liposomes to form DNA-liposome complexes or interaction between the complexes and cell membranes.

From here on, DNA-liposome complexes will be referred to as lipoplexes. Liposomes can be generated by combining different lipids in different molar ratios. The lipid type and their ratio are crucial in determining how effectively the liposome will deliver DNA to a specific cell line. Moreover, the lipid composition of the cell membrane could affect how well the membrane will interact with lipoplexes and allow entry. Membrane phospholipid composition and fatty acid composition and saturation differ between insect cell lines, Sf9 and Trichoplusia ni (Tn) and mammalian BKH cell lines (Marheineke et al., 1998). A threefold higher level of phosphatidylinositol and a lower amount of fatty acid saturation were observed for insect cells over BKH cells (Renkonen et al., 1972). Cells of different origins may differ in lipid composition; therefore, different cell types may differ dramatically in their ability to interact with liposomes that vary in lipid composition or lipid ratio. Moreover, the difference in fluidity and cell surface charge, possibly resulting from the membrane lipid composition, may also hinder or enhance some liposome interaction than others.

The tolerance to various liposome formulations may differ between different cell types. The severity of the liposomes' toxic effect on cells will effect the efficiency of the transgene expression. In addition, the presence of cell type-dependent receptors that can interact with liposomes to stimulate uptake of lipoplexes could be an advantage for
liposome-mediated delivery. Cell surface heparin/heparan sulfate-bearing proteoglycans have been shown to mediate the lipoplex entry (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998).

In order to identify the best liposome system for transfection of Sf9 cells, various liposome types, liposome compositions, lipid-to-DNA ratios have been examined.

### 3.2.1 Effect of various liposome types on reporter gene expression

Several commercially available liposome-based transfection reagents and cationic polymers were examined along with the lab-generated liposomes in order to determine the best system for transfection of Sf9 cells. Effectene, SuperFect, FuGENE, and CellFECTIN were chosen (See Methods and Materials for manufacturer sources).

CellFECTIN® is a 1:1.5 (M/M) liposome of cationic lipid N, N\textsuperscript{1}, N\textsuperscript{11}, N\textsuperscript{1M}-Tetramethyl-N, N\textsuperscript{1}, N\textsuperscript{11}, N\textsuperscript{1M}-tetrapalmitylspermine (TMTPS) and dioleoyl phosphatidylethanolamine (DOPE). SuperFect is an activated-dendrimer molecule with a defined spherical architecture, which interacts with DNA phosphate groups. Effectene and FuGENE are based on a proprietary non-liposomal lipid with the former used with additional reagent to enhance DNA condensation. Although the latter three reagents are not liposome-based transfection reagents, they rely on electrostatic interaction of the cationic delivery molecule with negatively charged phosphate group of DNA molecules to form complexes which then interacts with cell membrane for internalization. This principle is similar to the liposome-based reagents like CellFECTIN and the lab-generated liposomes.

**Method**

Varying amounts of each lipid-based transfection reagent were examined with a fixed amount of DNA in transfection in order to determine the maximal reporter activity obtainable with each reagent. The transfection protocol for each transfection reagent can be found in Chapter 2. The maximal reporter activity obtained with each reagent was then compared to identify the most efficient type of lipid for transfection of Sf9 cells.
Data

The maximal reporter activities of FuGENE and SuperFect were approximately 20-fold and 4-fold lower than that of the lab-generated liposomes, respectively (Fig 10). Effectene displayed levels of \( B\)-\( gal \) activity that were slightly lower than those of the lab-generated liposomes. When Effectene was tested again for determination of the maximal \( B\)-\( gal \) activity in comparison to the lab-generated liposomes, it resulted in the lower reporter activity consistently (Fig 11). On the other hand, CellFECTIN\textsuperscript{®}, when tested in several independent transfections, produced an average of 2.5 fold higher \( B\)-\( gal \) activity than the lab-generated liposomes (Fig 24) when the optimized protocol was used. Interestingly, this demonstrated that both liposome-based systems, CellFECTIN and the lab-generated liposomes are superior in transfecting \( S9 \) cells to the non-liposomal delivery molecules, SuperFect, Effectene, and FuGENE.
Commercial transfection reagents were tested with 0.6 μg of CC prepared pBacTracer1-LacZ DNA construct following the manufacturer's recommendation. Varying volumes of each reagent were examined with a given amount of DNA for comparison of optima of each reagent. 1 – 5 μl of FuGENE (●●●●), 5 – 15 μl of SuperFect (●●●●), 5 – 15 μl of Effectene (■■■■), and 5 – 20 μl of the lab-generated liposomes (0.5:1 molar ratio of DDAB:DOPE) (▲▲▲▲) were tested. A single transfection was carried out for each volume of reagent.

Figure 10  Comparison of reporter activities from transfections performed using commercial transfection reagents and the lab-generated liposomes
Figure 11  Comparison of reporter activities from transfections performed using Effectene reagent and lab-generated liposomes

Varying volumes of Effectene (→) were tested with 0.6 μg of CC-prepared pBacTracer1-LacZ DNA according to the manufacturer's instruction. The lab-generated liposomes (←) were also tested in various volumes with the same amount of DNA for comparison with Effectene. The error bars represent standard deviations of three transfections.
### Conclusion

In spite of the higher reporter activity obtained with the CellFECTIN liposome formulation, the lab-generated liposomes were chosen as the system for further optimization of Sf9 transfection for two reasons. Firstly, because the lab-generated liposomes could be easily and cheaply made in the lab by injection of a mixture of DDAB (dimethyldioctadecylammonium bromide) and DOPE lipids into water (Campbell, 1995). Secondly, the lipid ratios in the liposomes could be altered for optimization since the formulation is known, unlike the CellFECTIN reagent.

#### 3.2.2 Effect of lab-generated liposomes on Sf9 cell viability

### Rationale

Large amounts of liposomes in transfections have been reported to cause reduction in cell viability (Campbell, 1995; Choi et al., 2001) and adverse effects on normal cell physiology. Reduced viability will affect the efficiency of uptake of DNA and its expression in the transfected cells. The sensitivity of Sf9 cells to the lab-generated liposomes was examined to determine the maximal amount of liposomes, which meet the balance between the cell viability and the transgene expression level for production of the maximal reporter activity.

### Method

To assess the cytotoxicity of the lab-generated liposomes, a fixed number of cells were plated on a 35 mm dish in Grace's medium, as done in a regular transfection, and incubated with varying volumes of liposomes (DDAB:DOPE = 0.5:1 molar ratio liposomes) for 4 hours in the absence of DNA. The cell viability was evaluated by trypan blue exclusion assay after incubation of the cells with growth media for 24 hr.

### Data

Cells displayed no significant reduction in viability with up to 20 µl (20 nmol) of liposomes (Fig 12A). However, 40 µl (40 nmol) or more liposomes resulted in less than 20% cell viability and therefore, had a toxic effect. Cells stained with trypan blue after
incubation with various amounts of liposomes are shown in photos (Fig 12B-G).
Liposomes of no more than 40 μl were used for determination of optimal liposome to
DNA ratio.

3.2.3 Effect of lipid ratios of liposomes on reporter gene expression

Rationale
The liposome formulation used in our lab had been adapted from Campbell (1995), in
which DDAB and DOPE were mixed in 0.5 to 1 molar ratio to make liposomes for
transfection of several mammalian cell lines. Because different cell lines exhibit
different transfection efficiencies with a given liposome composition, variations of this
liposome formulation were tested on Sf9 cells to determine the best molar ratio of
cationic to neutral lipids.

Furthermore, it has been suggested that the ratio of the cationic and neutral lipids
or more precisely the net charge of the lipoplexes contributes to the intracellular
distribution of the transfected DNA which in turn determines the extent of reporter
expression level (Felgner et al., 1987). Therefore, it is important to determine the optimal
ratio between DDAB and DOPE to obtain the maximal reporter activity.

Method
Liposomes were generated by combining various amounts of DDAB with a fixed amount
of DOPE as described in Chapter 2. Lipid compositions in various liposomes were
0.06:1, 0.125:1, 0.25:1, 0.375:1, 0.5:1, and 1:1 (DDAB:DOPE).
Figure 12  Effect of the lab-generated liposomes on the viability of Sf9 cells

$10^6$ cells were exposed to 0 – 80 µl of the lab-generated liposomes (0.5:1 molar ratio) in the absence of DNA for 24 hr. The cell viability (%) was evaluated by counting non-viable and viable cells after staining with trypan blue (A). The standard deviations were calculated from the cell counting of three sampled areas of a fixed size within the image. The images (B - G) were captured under 100X magnification after addition of trypan blue. 0 µl, 10 µl, 20 µl, 40 µl, 60 µl, and 80 µl of liposomes were added in (B - G), respectively.
Figure 13  Effect of lipid composition of lab-generated liposomes on the reporter activity

Lab-generated liposomes made at various ratios of the cationic lipid, dimethyl dioctadecylammonium bromide (DDAB) and the neutral lipid, dioleoyl phosphatidyl ethanolamine (DOPE) were tested for transfection efficiency. The transfections were carried out using 0.6 μg of CC-prepared pBacTracer1-LacZ-smRS-GFP construct with 10 μl of each liposome and 1.5 x 10^6 cells/35 mm. The error bars represent standard deviations of two transfections.
Data

The molar ratios between 0.25:1 and 0.5:1 of DDAB and DOPE gave consistently high transfection efficiency as measured by the $B$-gal activity (Fig 13). Increasing or decreasing the cationic lipid in the liposome mixture resulted in lower reporter activities. The optimal molar ratio was 0.375:1. A lower ratio of the cationic lipid DDAB could have resulted in either a net negative charge or a lower positive charge of the lipoplexes. Therefore, the reduced reporter activity could be the result of insufficient amount of cationic lipid (DDAB) in the liposomes. A higher ratio of the cationic lipid is more likely to result in a higher net positive charge. However, higher than 0.375:1 cationic lipid ratio in the liposome resulted in reduced reporter activity. The higher net positive charge of the lipoplexes made with higher ratio of DDAB lipid (i.e. 0.5:1 and 1:1) could be attributed to the unfavourable intracellular distribution of the lipoplexes as described by Lappalainen et al. (1997). Although the mechanism involving such intracellular distribution of lipoplexes is not clear (Lappalainen et al., 1997), a fine balance between the ratio of lipids constituting the liposomes, and between the charge ratio of liposomes and DNA amount must be required for the maximal reporter activity.

Further transfection experiments were performed with 0.375:1 molar ratio of liposomes.

3.2.4 Effect of DNA to liposome ratio on reporter gene expression

Rationale

The nature of liposome-mediated transfection relies significantly on the efficient electrostatic interaction of the DNA molecules with liposomes to form compact lipoplexes. Therefore, a successful delivery of DNA and subsequent expression in the cell depends on the ratio of liposomes to DNA. In order to maximize the reporter activity, the optimal DNA to liposome ratio was determined.

Method

Three different volumes (10, 20, and 30 µl) of liposomes (0.375:1 molar ratio of DDAB and DOPE) were each mixed with varying amounts of CC-prepared DNA (0 - 1.5 µg) for
formation of lipoplexes. Each combination of liposome-to-DNA ratio was tested for its transfection efficiency using the \textit{B-gal} reporter gene. The liposome amounts were used within the range of minimal liposome toxicity (10 – 30 \textmu l) as previously shown (Fig 12).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure14.png}
\caption{Effect of DNA to liposome ratio on reporter activity}
\end{figure}

In order to optimize DNA to liposome ratio, varying amounts of DNA were examined with 10 \textmu l (\textbullet{}), 20 \textmu l (\textcircled{a}), and 30 \textmu l (\texttriangleleft{} ) of the lab-generated liposomes (0.375:1 = DDAB:DOPE). Transfections were carried out with CC-prepared pBacTracer1-LacZ-smRS-GFP construct. The error bars represent 95\% confidence intervals of two transfections.
Data

Ten-microliter volume of liposomes containing 10 nmol of lipids displayed maximal $B$-gal activity with 0.3 µg of DNA (Fig 14). Twenty- and thirty-microliter volume of liposomes each containing 20 nmol and 30 nmol of lipids showed maximal $B$-gal activity with 0.2 - 0.6 µg and 0.6 µg of DNA, respectively. Therefore, the optimum was achieved by using 30 µl of liposomes with 0.6 µg of DNA, which is a 50 nmol:1 µg ratio of liposomes and DNA. It is not surprising that more DNA can form into lipoplexes with more liposomes, hence resulting in a higher reporter activity. The amount of liposomes, therefore, is probably one of the limiting factors for transfection since $1 \times 10^6$ cells could not tolerate more than 40 µl (40 nmol) of liposomes as seen in the cell viability assay.

In comparison to a literature-described value of DNA to liposome ratio, 4-fold higher ratio was necessary to obtain optimal reporter activity in $S9$ cells than that determined (12 nmol lipid [0.5:1 molar ratio liposomes] : 1 µg DNA) for a murine Lewis lung carcinoma cell line (Campbell, 1995). This difference could be accounted for by the difference in lipid profile of cell membrane or the intracellular processing or sensitivity of lipoplex molecules between two cell lines.

Conclusion

The optimal liposome and DNA amounts for the maximal reporter activity was found to be 30 nmol of liposomes (0.375:1) and 0.6 µg of DNA (CC-prepared) in a ratio of 50 nmol to 1 µg. These amounts were used in subsequent transfection experiments.

3.2.5 Summary

The cationic liposome formulations, both CellFECTIN and the lab-generated DDAB:DOPE liposomes exhibited higher reporter gene expression than the dendrimer molecule SuperFect or the non-liposomal lipids FuGENE and Effectene. With further study of DDAB:DOPE liposome formulation, the maximal reporter activity was achieved with 0.375:1 molar ratio of cationic DDAB and helper DOPE lipids. The optimal liposome and DNA amounts were 30 nmol and 0.6 µg (50 nmol:1 µg), and 30 nmol of
liposomes was the maximal amount with minimal cytotoxic effect on SJ9 cells. Therefore, these conditions were used for further transfection experiments.

3.3 Cell density

Rationale
The number of cells transfected is important firstly since the toxic effect caused by intracellular accumulation of liposomes may be avoided by distributing the liposomes evenly among a given number of cells, in other words, by transfecting an appropriate number of cells. Secondly, as lipoplexes may enter cells by endocytosis or fusion with cell membrane, the total amount of cell surface available needs to be optimized. Thirdly, the number of lipoplex molecules a cell will take up, or is capable of taking up, may be limited by the mechanism of the uptake. For instance, a cell’s capacity to take up lipoplexes may depend on the number of membrane proteins such as proteoglycan, which has been reported to enhance uptake of certain liposomes (Mislick and Baldeschwieler, 1996). Different cell lines, which could utilize such receptor-mediated mechanisms specifically or generally to varying degrees, will differ in the optimal cell number for the maximal transfection efficiency. Therefore, a proper ratio between the number of lipoplex molecules in the transfection medium and the number of cells needs to be achieved for maximal transfection efficiency.

Method
In the first set, transfections were performed with a large range of cell numbers, from 5 x 10^5 to 1 x 10^7 cells/ml in a 35 mm dish. A constant amount of DNA and liposomes were used and the effect of varying cell density on the transfection efficiency was measured by B-gal activity. In a second set of transfections, a smaller range of cell numbers from 1 x 10^6 to 4 x 10^6 cells/ml was examined using the same transfection conditions.

Data
From the initial set of transfections, the optimum cell number appeared to lie between 1 x 10^6 cells/ml and 1 x 10^7 cells/ml (Fig 15). To determine an optimal cell number rather
than a wide range of cell numbers, a portion of that range was retested in the second set of transfections. However, the reporter activity did not vary significantly within the range. Therefore, a range of cell numbers between $1 \times 10^6$ and $4 \times 10^6$ cells/ml was determined to be the optimum. Although the optimal cell numbers may vary between cell lines being transfected or between the transfection methods utilized, similar cell numbers have been described and recommended in the literature and transfection protocols with commercial transfection reagents.

**Conclusion**

Within a range of cell numbers to choose from, I decided to use $1 \times 10^6$ cells/ml as this would result in approximately two cell-doublings ($4 \times 10^6$ cells/ml) 48-hour post-transfection. A population of Sf9 cells in this density would be in a logarithmic phase, thereby maintained in a healthy state of growth for use in either the $B$-gal assay or selection on antibiotics for establishment of stable cell lines.
Figure 15 Effect of cell number on reporter activity

A wide range of cell numbers, from $5 \times 10^5$ to $1 \times 10^7$ Sf9 cells per 35 mm dish was tested (−○−) in transfections carried out with 0.6 µg of CC-prepared pBacTracer1-LacZ construct DNA. A smaller range of cell numbers was tested in the second set (−△−) to determine the optimal range. The error bars represent the standard deviations of three transfections.
3.4 Transfection media

Rationale
The interaction between DNA and liposomes takes place in the transfection medium by electrostatic properties of DNA phosphate backbones and cationic lipids. The transfection medium plays a role not only in the lipoplex formation, but also in allowing an active interaction between the lipoplexes and the cell membranes as both electrostatic and hydrophobic interactions are thought to be involved. Consequently, the chemical properties of the transfection medium, such as ionic strength and pH, as well as the presence of charged macromolecules in the medium, contribute to the proper condensation and the formation of DNA into complexes with liposomes.

The ionic strength and pH of the medium determines the amount of positive charges of liposomes available for negatively charged DNA. The presence of certain buffering salts, proteins, or serum components in the medium also affects how much of the liposomes will interact with DNA as these components could sequester the positive charges of liposomes and hinder the interaction with DNA.

Commercially available Grace’s transfection medium contains vitamins and carbon sources such as glucose, organic acids, and sucrose in addition to inorganic salts, which allows buffering capacity (Grace, 1962). This medium can sustain cell viability for a short period of time while the transfection is performed. Although these components are necessary for maintaining minimal cell function during transfection, they could hinder the lipoplex formation to some extent, thereby lowering the reporter activity. Therefore, simpler media, which could allow maintenance of cell viability during transfection and produce higher transfection efficiency were explored.

Selection of various transfection media

Rationale
Phosphate-buffered saline (PBS) is commonly used to wash cells during transfection procedure in various transfection methods. When S9 cells were incubated in PBS medium for lipoplex uptake for 4 hours, abnormal cell swelling was observed, and the
subsequent change of medium could only partially restore the cell viability. The reduced cell viability and the physiological change were attributed to the lack of carbon sources and inappropriate osmolarity control. Therefore, modifications were made to the composition of the PBS medium so that the carbon source and salt content would resemble that of the Grace’s medium.

Method

The compositions of Grace’s, TC100 growth medium, and PBS were compared in order to make sensible modifications to PBS medium. The composition of these media can be found in Table 2. TC100 and Grace’s media contain 86 mM and 91 mM of total inorganic salts, respectively, whereas PBS contains twice as much (192 mM) inorganic salts of which the majority is NaCl. The major carbon source of the Grace’s medium is present as sucrose.

Table 2  Composition of transfection media

<table>
<thead>
<tr>
<th>Inorganic Salts (mM)</th>
<th>TC100</th>
<th>Grace’s</th>
<th>PBS</th>
<th>PMN</th>
<th>PMS</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl\textsubscript{2}</td>
<td>9</td>
<td>6.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KCl</td>
<td>39</td>
<td>55</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>MgCl\textsubscript{2}</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgSO\textsubscript{4}</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.6</td>
<td>140</td>
<td>40</td>
<td>40</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>NaH\textsubscript{2}PO\textsubscript{4}\textsubscript{•}H\textsubscript{2}O</td>
<td>7.3</td>
<td>7.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Na\textsubscript{2}HPO\textsubscript{4}\textsubscript{•}7H\textsubscript{2}O</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KH\textsubscript{2}PO\textsubscript{4}</td>
<td>0</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>85.9</strong></td>
<td><strong>91.1</strong></td>
<td><strong>192</strong></td>
<td><strong>92</strong></td>
<td><strong>92</strong></td>
<td><strong>192</strong></td>
</tr>
</tbody>
</table>

Other Components (mM)

| Fructose         | 0 | 2.22 | 0 | 0 | 0 | 0 |
| Fumaric Acid     | 0 | 0.47 | 0 | 0 | 0 | 0 |
| D-Glucose        | 6.1 | 3.89 | 0 | 0 | 0 | 0 |
| α-Ketoglutaric Acid | 0 | 2.53 | 0 | 0 | 0 | 0 |
| Malic Acid       | 0 | 5.0 | 0 | 0 | 0 | 0 |
| Succinic Acid    | 0 | 0.51 | 0 | 0 | 0 | 0 |
| Sucrose          | 0 | 77.9 | 0 | 80 | 80 | 80 |
| N2 Amine YT      | N/A | 0 | 0 | 0 | 0 | 0 |
| **Total**        | **6.1** | **92.52** | **0** | **0** | **80** | **80** |
Note: In addition to the components listed above, TC100 and Grace’s media contain amino acids and vitamins. PBS, PMN, PMS, and PS media do not contain amino acids or vitamins.

From these observations, PBS was modified into three varieties by addition or reduction of sucrose or NaCl, respectively (Table 2). PMN (PBS-modified with NaCl) was modified from PBS by reducing the NaCl molarity from 140 to 40 mM. PMS (PBS-modified with sucrose) was made similarly with reduced NaCl, but also with addition of sucrose in a similar amount (80 mM) found in the Grace’s medium. PS (PBS-sucrose) was made by addition of 80 mM of sucrose to PBS.

In the PMS medium, the addition of sucrose was expected to provide controlled osmolarity in place of the large quantity of NaCl removed in the PMS medium as well as some carbon source. In addition to these simpler media, two different growth media were examined as transfection media: one with serum (TC100+10%FBS) and the other without (ESF 921). These media were used for seeding of cells, lipoplex formation, and incubation of cells with lipoplexes.

Effect of different transfection media on reporter gene expression

Data
Although some commercially available liposomes allow transfection in a growth medium or in the presence of serum, the DDAB:DOPE formulation of our lab-generated liposomes did not allow efficient transfection in growth media as seen in Fig 16 (TC100 + FBS and ESF 921). Approximately 10-fold lower reporter activity was detected with both growth media relative to the Grace’s medium. PBS and PMN displayed only about 50 % of the reporter activity relative to the Grace’s medium. PS medium was tested in another set of transfections, however, resulted in a significantly lower reporter activity than the Grace’s medium and only slightly better than the growth media. On the other hand, the reporter activity obtained using the PMS medium was 30 % higher than that of the Grace’s medium.
Figure 16  Effect of various transfection media on reporter activity

Phosphate-buffered saline (PBS), PBS-modified with sucrose and NaCl (PMS), PMS-modified with NaCl (PMN), Grace's media, TC100 growth media containing 10% FBS, and ESF 921 growth media were used for seeding of cells and DNA-liposome complex formation. Transfections carried out using various transfection media were examined by $B$-gal assays. The error bars represent standard deviations of three transfections, except for PBS ($n = 1$) and PMN ($n = 2$).
Effect of modified transfection protocol on reporter gene expression

Rationale
In the transfections above, noticeable reduction in the cell viability was observed in all PBS-based media. In an attempt to reduce cell death, a slightly different transfection protocol was tested in this set of transfections. In our standard transfection protocol (depicted in Fig 17), the cells are exposed to the lipoplexes for 4 hours in the transfection medium, and at the end of this period, the growth medium is simply overlaid to the mixture to allow the gene expression. The addition of growth medium without removal of the transfection medium (Step 4, Fig 17), may affect the cell viability more severely than removing the transfection medium completely and adding the growth medium. Therefore, the removal of the transfection medium containing lipoplexes before the addition of the growth medium was examined. This perhaps could increase cell viability, but still allow a higher reporter expression when using the PMS medium than the Grace’s medium.

Method
To test the above strategies, two identical sets of transfections were performed with the only difference in the step where the growth medium is added. At the end of the 4-hour incubation period, the transfection medium was removed and the growth medium added for the first set. In the second set, the growth medium was simply overlaid to the transfection medium. The final volume of the medium in each case was the same. The cell viability was assessed by estimation at the time of harvest, 48-hour post transfection, by staining cells with trypan blue dye in order to determine whether the removal of the PBS-based media could indeed reduce cell death.

Data
The cell viability, estimated at the time of harvest, 48 hour post-transfection, was consistently higher if the transfection medium was removed: 60% vs. 85% for PBS, 60-70% vs. 80% for PMS, 55-60% vs. 70-80% for PMN, and 70% vs. 85-90% for Grace’s
medium (Fig 18). Thus, the viability could be improved by removing the transfection medium. However, the reporter activity did not follow the same pattern.

![Liposome-mediated transfection protocol (Standard protocol)](image)

**Fig 17** Liposome-mediated transfection protocol (Standard protocol)
Figure 18 Effect of transfection media and removal of DNA-liposome complexes from cells on the $B$-gal reporter activity

DNA-liposome complexes in various transfection media were either removed (•) upon addition of 2 ml of growth media 4 hr later or was left on (H) upon addition of 1 ml of growth media. The efficiency of transfections, performed using various transfection media with variation in the protocol, was measured by $B$-gal activity. The cell viability, estimated by trypan blue exclusion assay 48 hr post-transfection, is indicated above each bar. The error bars represent standard deviations of three transfections.
The replacement of the transfection medium with the growth medium did not improve the $B$-gal reporter activity for PMS, PMN, and Grace's; however, it did improve the activity for PBS. Therefore, improved cell viability did not always correspond with the improved reporter activity. Replacement of the PBS transfection medium with the growth medium could improve the transfection efficiency since the cells were exposed to PBS only for 4 hours instead of being exposed for 48 hours in the case where the growth medium was overlaid on top of PBS. In the latter case, despite the addition of the growth medium, the presence of PBS probably created an environment not suitable for a complete recovery of cells from the exposure to PBS. However, for the other media (PMS, PMN, and Grace's), the effect on the cell viability may not have been as severe as PBS. Since the reporter activities were higher when the growth medium was overlaid to the transfection medium, the presence of the transfection medium, containing lipoplexes, during the 48-hour incubation period probably allowed cells to take up more complexes. In other words, when the lipoplexes were removed along with the transfection medium after the 4-hour incubation, cells probably did not take up the lipoplexes in full efficiency, thus resulting in a lower reporter activity than when the lipoplexes were left on.

Consistent with the data from the previous set of transfections (Fig 16), a higher reporter activity was observed with PMS medium than with Grace’s medium. In contrast, PMN medium produced similar $B$-gal activity as that of PMS in this set (Fig 18). I could not resolve this inconsistency. Nevertheless, since PMS medium could produce higher reporter activity than the Grace’s medium consistently throughout the experiment, it was chosen for further transfection experiments and the transfection medium was not removed prior to the addition of growth medium for further experiments.

Discussion
The higher reporter activity seen with PMS medium, despite its slightly reduced cell viability effect, implied that a simple medium could provide a better environment for the efficient formation of lipoplexes and/or efficient transfer of lipoplexes across the cell membrane. It could be argued that the improved reporter activity is due to the absence of
interfering molecules, such as the carbon sources found in the Grace's medium. These molecules could potentially sequester the liposomes by charge attraction.

Interestingly, the presence of phosphate ions present in PMS has been implicated in improved transfection efficiency (Kariko et al., 1998). The report showed that the pre-incubation of liposomes with divalent phosphate anion, such as Na$_2$HPO$_4$$\cdot$7H$_2$O present in PMS, prior to lipoplex formation, enhances the reporter activity by 26-fold. The enhancement was dependent on the concentration of phosphate and the pH of the medium. The maximal reporter activity was reported at phosphate concentrations ranging from 0.03 M to 0.08 M and at pH in the range of 5.6 to 6.8.

The concentration of the divalent phosphate in PMS used in this project was 0.001 M at pH of 6.2. Although the phosphate concentration in PMS was much below the reported maximal range and the liposomes were not pre-treated in PMS, the presence of phosphate in the transfection medium could have contributed to the increased reporter activity observed (Fig 16 and Fig 18). Kariko et al. (1998) speculated that the divalent phosphate ions could bring about increased fusion between lipoplexes and cell membranes, subsequently resulting in increased reporter expression. It would be interesting to increase the divalent phosphate concentration in PMS medium and see whether further enhancement in the reporter expression can be obtained.

Contrary to PMS, lower reporter activity obtained with the PBS medium containing the same amount of phosphate ions could be attributed to the much reduced cell viability (60% when transfection medium not removed in Fig 18) after the 4 hour-incubation, perhaps masking the positive effect of the divalent phosphate ion.

**Conclusion**

In summary, using the PMS medium, in place of the commercially available Grace's medium, produced about 30% more $B$-$gal$ activity. The positive effect of divalent phosphate anion and the absence of the charged molecules in PMS medium, which could potentially interfere with liposome-DNA complex formation, are the likely explanation for the improved reporter activity. PMS medium is the preferred choice of transfection medium for $Sf9$ cells due to enhanced reporter activity, the ease of making, and the cost-
efficiency. Therefore, PMS medium was chosen as transfection medium for subsequent experiments.

3.5 Time variables

Rationale
The ability of cells to express transfected DNA depends on two time variables. The first variable is the time required for the cellular uptake of lipoplexes. The other is the time required for the expression of the transfected gene, which includes the time for transfer of DNA to the nucleus for transcription and translation back in the cytoplasm. Hereafter, the two variables are referred to as uptake period and expression period, respectively. The time required for cellular uptake of lipoplexes is determined by the delivery procedure. For example, the uptake of lipoplexes depends on how long the lipoplexes are incubated with cells before the addition of growth medium. On the other hand, the expression period depends on the transcriptional properties of the transfected plasmid construct and the cell type, which determines the quality and quantity of the gene product and its stability in the cell.

3.5.1 Determination of optimal uptake period for maximal reporter activity

Method
In order to determine the optimal incubation time for the uptake of lipoplexes, a time course study was performed in which growth medium was added onto cells at various time points after cells were exposed to lipoplexes. The addition of growth medium is necessary so that cells can resume their normal metabolism, which does not occur in minimal media such as PMS or Grace's media.

Since the PBS-based transfection media cause reduced viability (Fig 18), a modified procedure was examined in an attempt to lessen the reduction in viability if not eliminate it. In the normal transfection protocol, the growth medium is added on top of the transfection medium, while, in the modified protocol, the transfection medium
containing lipoplexes is removed from cells just prior to the addition of the growth medium.

In these transfection experiments, after the addition of lipoplexes, the cells were incubated for various time periods before the addition of the growth media. At various time points, the transfection medium containing lipoplexes was either removed (Series I) or left on (Series II).

Cells were treated with lipoplexes in the transfection medium in two different ways. Firstly, the transfection medium was removed (Series I) and the growth medium was added at time zero or after various incubation periods. For the zero time point, the transfection medium containing lipoplexes was added to cells and removed immediately, then the growth medium was added. Secondly (Series II), the growth medium was added on top (Series II) of the transfection medium immediately following the addition of lipoplexes (at time zero), or after various incubation periods. Upon the addition of the growth medium, all cells were incubated for a total of 48 hours, including the uptake period. In each case, the B-gal reporter assay was performed to determine the transfection efficiency.

Data and discussion
In the first time course experiment (Series I), where the transfection medium containing lipoplexes was removed before the addition of growth media, the B-gal activity steadily increased as incubation time increased (Fig 19). Therefore, the reporter activity was proportional to the amount of incubation with lipoplexes. The addition of the growth medium, during the early period of the time course, has a positive effect for the cell viability since cells are supplied with the growth medium in a short while. On the other hand, the removal of the transfection medium containing lipoplexes would have a negative effect on the transfection efficiency, since the lipoplexes are removed early in the time course. These data indicate that the positive effect of the longer incubation periods outweighs the negative effect of the absence of growth medium.

In the second time course experiment (Series II), the reporter activity was surprisingly higher with either a very short or a very long incubation (Fig 19). There was a distinct drop in the B-gal activity if the cells were incubated for 20, 40, and 60 min
prior to the addition of the growth medium. An opposite trend occurred after the 2-hour time point, resulting in a higher reporter activity with longer incubation time. Repeated time course experiments showed similar results.

Figure 19 Effect of various incubation periods of DNA-liposome complexes with cells on the B-gal reporter activity 48-hour post-transfection

Following addition of DNA-liposome complexes to cells, a time course experiment of various incubation periods was performed. At various time points, the DNA-liposome complexes were either removed (−○−) or left on (−●−) upon the addition of growth medium. The transfection efficiency was measured as B-gal activity. The error bars represent standard deviations of two transfections.
One would expect to observe an increasing reporter activity with increased incubation time during the entire time course experiment, as seen in Series I, since a longer incubation would result in a higher chance of cells to interact with lipoplexes for their uptake. In fact, Kariko and the colleagues (1998) have described a trend in which the uptake time and the reporter activity is proportional to each other.

I speculated that the unexpected higher reporter activity with a shorter incubation time during the first 2 hours could be explained by the osmolarity difference elicited by the addition of growth medium. The transfection medium, such as the PBS-based medium, and the growth medium are composed of vastly different molecules, which could alter the osmotic pressure cells experience. An osmotic shock induced by change in medium could bring about cell shrinking or swelling. In fact, Sf9 cells appeared enlarged shortly after the addition of the PMS medium and remained swollen during transfection. Change in cell size could possibly lead to sudden influx (or uptake) or fusion of membrane-bound lipoplexes or lipoplexes in the vicinity of cells.

The effect of osmotic shock, if it, indeed, plays a role in lipoplex uptake, did not appear to have the same effect on the reporter activity during the entire time course experiment of Series II. The osmotic shock would have been experienced equally by all cells in each time point when the growth medium was added. One possible explanation may be that the shock is reflected on the outcome of the lipoplex uptake only when the lipoplexes are in contact with cell membranes. This hypothesis requires the lipoplex molecules to interact or come into association with cell membranes immediately following their addition to cells. The electrostatic and/or hydrophobic attraction of the lipoplexes to cell membrane may be a very rapid process. It is conceivable that the rate limiting step in the lipoplex uptake may be the process of fusion or endocytosis of lipoplexes rather than the time required for the association of lipoplexes with cell membranes. The rapid association of lipoplexes with membranes and immediate osmotic shock induced by a medium change may have contributed to the high reporter activity at the time point zero in Series II. Interestingly when the lipoplexes, formed in the transfection medium, were pre-mixed with the growth medium just prior to addition to cells, a very low reporter activity was achieved. This indicates that the prior presence of lipoplexes on or in the close proximity to the cell membranes may be necessary at the
time of osmotic shock to allow the efficient uptake. However, this membrane association could be labile and consequently may be in equilibrium by the exchange of lipoplexes with other charged molecules in the transfection medium, such as salt and sucrose unless fusion or endocytosis occurs immediately to secure the interaction. This could explain the decreasing reporter activity with an increasing incubation during the 2 hour time period. During the remainder of the time course, lipoplex uptake may have occurred slowly by similar equilibrium between the association of lipoplexes with either membranes or charged molecules until the osmotic shock is induced by adding the growth medium.

**Conclusion**

In summary, among the various methods of lipoplex treatment described above, three of them resulted in similar levels of reporter activity - incubating cells in the transfection medium for 32 hr and either (i) removing the transfection medium, and then adding the growth medium or (ii) simply adding the growth medium on top of the transfection medium, or (iii) feeding the growth medium immediately following the addition of lipoplexes to cells. The method, in which the transfection medium was removed and the growth medium added at time zero, produced a near-background reporter activity. Although the former three methods resulted in similar reporter activity, the most efficient method was to feed the growth medium immediately following lipoplex addition (third case) since this would not necessitate a long waiting period in the transfection procedure. More importantly, the reduced viability effect of the PMS transfection medium could be essentially eliminated by adding the growth medium immediately following the addition of the transfection medium. Therefore, this method was chosen for further transfection experiments.

### 3.5.2 Determination of gene expression period for a maximal reporter activity

**Rationale**

As introduced earlier, the extent of gene expression level is dependent on the host cells and the expression cassette. Therefore, the optimal gene expression period would ideally
have to be determined on an individual basis for every gene expression cassette or cell line.

For example, a luciferase gene driven by human CMV promoter transfected into human osteosarcoma cell line using lipofectin transfection reagent has been reported to reach its maximal capacity approximately 5 hours after transfection (Kariko et al., 1998). Transfection of the same luciferase and CMV promoter into primary human keratinocytes using Effectene resulted in maximal reporter expression on Day 4 of transfection (Zellmer et al., 2001). Transfection of the same luciferase gene driven by SV40 promoter into human primary hematopoietic cell lines using DOSPA/DOPE liposomes required gene expression period of 2 days to reach its maximum (Harrison et al., 1995).

These data demonstrate that a significantly different optimal gene expression period is required for different cell lines. Moreover, the transfection reagent used, transcriptional property of the construct such as the strength of the promoter or enhancer driving the gene of interest, the stability of the gene product, or the effect of the protein product in the host system may also contribute to the optimal gene expression period. Therefore, it is important to optimize a gene expression period for each transfection system. I have optimized this time variable for transfection of Sf9 cell line using DDAB:DOPE lab-generated liposome and Opie2 baculovirus immediate early promoter driving the expression of the LacZ reporter gene.

**Method**

To determine the incubation time for the maximal transient reporter gene expression, transfected cells were incubated with growth medium for various time periods, up to 5 days. The cells were harvested at various time points after the addition of the growth medium. The collected cells were stored at −80 °C, and all B-gal assays were performed at the same time.

**Data**

A significant reporter activity was obtained at 24 hours after transfection (Fig 20). By 48 hours, approximately 50% of the maximal reporter activity was achieved. The optimal transfection efficiency was achieved 120 hours (5 days) after the initial exposure of cells.
to lipoplexes. For further experiments, I chose to measure the reporter activity at 48-hour post-transfection, since this is clearly sufficient time for a strong response (> 50% of the total accumulated $B$-gal protein).

Conclusion

In summary, the maximal $B$-gal reporter activity was reached 5-day post-transfection for $Sf9$ cells using the DDAB:DOPE liposome formulation. However, for the prompt processing of the experiment, all subsequent transfections were incubated for 48 hours before harvesting.

![Figure 20 Effect of various post-transfection incubation periods on reporter gene expression level](image)

Transfected $Sf9$ cells were harvested at various time points after incubation for expression of the reporter gene. The harvest was performed every 12 hr from 0 hr to 144 hr post-transfection. The transfection efficiency was measured as $B$-gal activity. The error bars represent standard deviations of two transfections.
3.6 Use of chemicals and proteins in transfection

Rationale

Some chemicals and DNA-associated proteins have been reported to produce an increase in the transient expression or an increase in the plasmid integration frequency in mammalian cell lines. In an attempt to improve the reporter gene expression further, I examined what affect, if any, these compounds might have on the transfection of insect cells. Molecules that have properties to cause chromosomal DNA damage or allow nuclear transfer of the transfected plasmid, such as Topoisomerase II inhibitor VP-16 (etoposide), zeocin, sodium butyrate, Drosophila high-mobility-group protein (HMGD), restriction endonuclease Sac II, and Bovine Serum Albumin (BSA) were chosen for investigation.

3.6.1 Chemical treatment of Sf9 cells in transfection

3.6.1.1 Background and rationale

Topoisomerase II Inhibitor

Topoisomerase II (Topo II) is a cell-cycle dependent nuclear enzyme known to alter the conformation of DNA. It has been found to play important roles in relaxation and condensation of chromatins for transcription (Wang, 1991), replication (Wang, 1991), and chromatid segregation (Shamu and Murray, 1992).

The enzyme creates transient DNA double-strand breaks by formation of covalent enzyme-DNA complex and a subsequent re-ligation completes the topo II activity. When the topo II enzyme was impaired by inhibitors such as etoposide (VP-16), teniposide (VM-26), ICRF-193, and m-AMSA, increase in non-homologous recombination (random integration) has been reported in various human cell lines (Aratani et al., 1996; Fujimaki et al., 1996). VP-16, VM-26, and m-AMSA inhibit the re-ligation activity of the topo II by stabilization of the double-strand breaks. Therefore, the increase in random integration has been attributed to the formation of the stable double-strand break and its subsequent repair induced by the host cell mechanism (Chen et al., 1984; Ross et al.,
During the DNA repair process, the repair machinery occasionally uses the closest DNA molecule present in the vicinity as an easy substrate to repair the damage, thereby incorporating potentially an incorrect piece of DNA. Transfected DNA molecules could potentially take the place of this incorrect piece of DNA. By inducing DNA repair mechanism through inhibition of topoisomerase II, the transfected DNA may be integrated more frequently in the genome of the insect cells, thereby resulting in higher stable transgene expression.

Zeocin

Zeocin is a member of the bleomycin family of DNA-intercalating antibiotics isolated from Streptomyces verticillus (Umezawa, 1973). It is a well known cancer therapy drug and has been implicated in enhancement of random integration of transfected DNA in human cell lines (Nakayama et al., 1998) and to have an effect on DNA replication (Dziegielewski et al., 2001). Similarly to topo II inhibitor VP-16, zeocin generates double-stranded DNA breaks through an oxidative cleavage of the C-3’ to C-5’ bond in the minor groove of DNA in the presence of Fe (II) and O$_2$ with preferential sequence of GpC and GpT.

Therefore, artificially induced DNA damage by zeocin or VP-16 is expected to provide more frequent opportunity for the transfected DNA to be integrated into the genome via DNA repair pathway, hence increasing the number of integrants in transformed stable cell lines. Although both zeocin and VP-16 have been shown to have no effect on the transient expression of the reporter gene in human cell lines (Nakayama et al., 1998), it has not been tested directly in Sf9 cells. Therefore, the transient reporter activity was analyzed on transfections performed with zeocin or VP-16.

Sodium butyrate

Another approach to generate increased transient expression and/or increased integration events of the transfected plasmids was to alter the conformation of the transfected plasmids or the host genomic DNA. By changing the conformation of DNA, the transcriptional machinery or the recombinant machinery would have easier access to the
plasmid or genomic DNA, respectively.

The use of sodium butyrate and trichostatin A, well-known inhibitors of histone deacetylase, results in increase in the acetylated state of histones in nucleosomes (Sealy and Chalkley, 1978; Yoshida et al., 1990). The increased promoter activity and hence, a higher gene expression observed with sodium butyrate treatment (Condreay et al., 1999; Garrison et al., 2000; Gorman et al., 1983) has been attributed to the increased accessibility of the transfected plasmid DNA by transcriptional machinery (Pazin and Kadonaga, 1997; Wu, 1997). Sodium butyrate has also been implicated in increasing random integration as measured by transduction with a recombinant baculovirus vector (Condreay et al., 1999). This could also be explained by increased accessibility of genomic DNA by recombinant machinery to allow more frequent integration of the foreign DNA into the chromosomal DNA.

Therefore, I speculated that the treatment of cells with sodium butyrate could either increase the promoter activity of the transfected reporter gene or increase the frequency of reporter gene integration into the Sf9 cell genome.

3.6.1.2 Effect of chemical treatment on viability of Sf9 cells

Rationale
Because VP-16 and zeocin cause DNA damage, they can bring about cell death. Similarly, butyrate if present in large amounts within the cell, could bring about adverse gene expression patterns. Therefore, the amount of the chemicals the cells can tolerate, while still producing efficient reporter expression or the random integration, needs to be determined. Before the effect of these chemicals on the reporter gene expression could be examined, various concentrations of chemicals were tested on Sf9 cell culture to assess the their effect on cell viability.

Method
To determine the effect of the chemicals on cell viability, Sf9 cells were incubated with various concentrations of chemical, and cell counting was performed for the course of 5
days. Each day, samples were drawn from cultures and assayed for viability by trypan blue exclusion.

Data

The control culture without any chemical reached one cell doubling after 2 days and declined in number from then (Fig 21C). With the lowest amount of each chemical tested (0.1 μM VP-16, 20 μM butyrate, and 20 μg/ml zeocin), cells could not reach one doubling (Fig 21A – C). There was a steady increase in the number of dead cells in all cases of chemical addition. The cells grew slowly in lower concentrations of chemicals, however, with the largest quantity of chemicals tested (10 μM VP-16, 2000 μM butyrate, and 1000 μg/ml zeocin), the cell population showed either no net increase or a steady decline in the cell number from day 1. Cell growth, although slower than that of control, could be maintained at concentrations of up to 1.0 μM, 200 μM, and 20 μg/ml for VP-16, butyrate, and zeocin, respectively.
Figure 21 Effect of chemical treatments on Sf9 cell viability

Three chemicals, VP16 (A), Sodium Butyrate (B), and Zeocin (C) were tested in various concentrations for evaluation of the cell viability. The number of viable cells (——) and non-viable cells (----) are plotted separately for each concentration. Negative control with no chemical treatment is shown in (C). The error bars represent standard deviations of three samplings of each chemical concentration. Only the upper error bars are shown.
Conclusion

In these viability assays, cells were continuously exposed to chemicals. However, the effect of these chemicals on the transfection efficiency was tested by exposing the cells to the chemical for 24 hours post-transfection. The temporary exposure to chemicals probably has less severe effect on the cell viability than a continuous exposure with the same amount of the chemical. When the chemical is removed after 24 hours of treatment, it was speculated that the cells would recover better than the cells exposed continuously to the chemical. Therefore, the same or a slightly higher concentration than that determined to have a moderate effect in the kill curve was expected to be tolerated by cells with lesser or similar effect on the cell viability, respectively. On account of this, the concentration of each chemical to be used in transfection was chosen to be 1.0 μM for VP-16, 2000 μM for butyrate, and 200 μg/ml for zeocin.

3.6.1.3 Effect of chemical treatments of Sf9 cells on reporter gene expression

The effects of these chemicals on transient transfections will be discussed here. The effects on integration pattern and copy number will be discussed in Chapter 4.

Method

Sf9 cells were transfected using the optimized transfection protocol and incubated for 1 hour before the addition of the chemicals – 1.0 μM of VP-16, 2000 μM of butyrate, and 200 μg/ml of zeocin. Following the incubation of cells with the chemicals for 24 hours, the chemicals were removed by aspirating the transfection medium. Fresh growth medium was added and the cells were incubated for another 24 hours. B-gal reporter assays were performed to determine the effect of these chemicals. Since VP-16 was dissolved in 1x DMSO, the effect of DMSO was also examined.

The transfections were performed with either circular or linear plasmid DNA. Linear plasmid DNA may be affected differently in the presence of these chemicals than the circular DNA in their transient expression level and/or in the integration pattern or frequency. The plasmid DNA was linearized by Sac II, and the entire digestion mixture was used to make lipoplexes.
Data

Effect of VP-16 and Zeocin

Transfections with linear plasmid DNA produced near background reporter activity regardless of the post-transfection chemical treatment (Fig 22) as observed previously in Fig 9. Although the transient reporter activity was negligible, it is still possible that the DNA constructs integrated into the genome (this will be discussed in Section 4.1).

With VP-16 or zeocin treatment, transfection of supercoiled/circular plasmid DNA produced lower reporter activity than that of the positive control transfected without the chemical treatment (Fig 22). This was not unexpected, since VP-16 and bleomycin have been reported to produce no effect (Fujimaki et al., 1996) or slightly reduced reporter activity (Nakayama et al., 1998) at concentrations of 0.15 μM and 0.1 μM, respectively. These concentrations are one and three orders of magnitude lower than those tested on Sf9 cells, respectively (1.0 μM VP-16 and 130 μM zeocin (= 200 μg/ml)).

DMSO only treatment resulted in a slight decrease in the reporter activity (Fig 22). Therefore, the decreased reporter activity seen with VP-16 treatment appeared to be the combined effect of DMSO and VP-16.

To ensure that the toxicity of the chemical was not masking the effect of these chemicals on the reporter gene expression, one order of magnitude lower concentrations of VP-16 (0.1 μM) and zeocin (20 μg/ml = 13 μM) were tested in a separate experiment. The lowered concentration of zeocin resulted in a reporter activity identical to that of the control transfection. However, the lowered concentration of VP-16 resulted in the same reduction in reporter activity. Therefore, these chemicals did not appear to have any significant effect on improving the transient reporter gene expression.
SJ9 cells were treated with various chemicals 1 hr post-transfection and their effect on reporter activity was examined 48 hr post-transfection. The $B$-gal activities obtained from transfections with untreated circular/supercoiled plasmid (■) and those with Sac II-linearized plasmid (□) are plotted on the primary and secondary y-axis, respectively. The concentrations of chemicals used to treat cells are indicated accordingly. The transfection treated with DMSO was used as a solvent control for VP-16. The positive and negative controls were transfections performed without any chemical treatment and without DNA-liposome complexes, respectively. The error bars represent 95% confidence intervals from two transfections.
Effect of Sodium butyrate

Unlike VP-16 and zeocin, sodium butyrate has been reported to cause a significant enhancement of reporter expression ranging from 3 to 44 fold in various cell types (Gorman et al., 1983). The enhancement in the reporter activity has been described from both transient and stable expression at concentrations ranging from 2 mM to 10 mM in various cell types (Garrison et al., 2000; Gorman et al., 1983; Lea and Randolph, 1998). Contrary to these data, Sf9 cells displayed approximately 3-fold lower transient reporter activity than the control transfection when treated with 2 mM of butyrate (Fig 22). Therefore, sodium butyrate did not improve transient the reporter activity in Sf9 cells.

Conclusion

In summary, the effect of VP-16 and zeocin treatment was in agreement with that reported by other groups, resulting in no effect or slightly reduced reporter activity. The butyrate treatment also resulted in lower reporter activity, however, this was not in agreement with the published data. Butyrate and VP-16 treatments were performed in similar concentrations as those tested in mammalian cell lines. Although zeocin was used in 100-fold higher concentration than that examined with mammalian cell lines, the viability curve was very similar to that of the no-chemical control. Therefore, the observed effect is not likely due to the reduced cell viability. These chemicals simply may not have any effect on improving the reporter activity in Sf9 cells.

3.6.2 Effect of DNA-associated protein on reporter gene expression

Rationale

In addition to chemicals, I looked for biologically active molecules that could enhance reporter activity. For instance, HMG protein, which has high affinity for chromatin may be perceived by cells as protein for nuclear transport. A restriction endonuclease was also examined since it has DNA binding affinity for specific sequences. Moreover, Sac II enzyme appeared to show increased reporter activity in a previous transfection experiment (Fig 9). Therefore, inactivated Sac II enzyme and Drosophila HMG
(HMGD) protein have been examined for the effect on the reporter activity. The plasmid DNA was treated with DNA-associated proteins so that the DNA-protein complex might be recognized by cells as a molecule destined for nuclear transport.

Effect of HMGD 1 protein

Background
HMG 1 protein is an abundant, non-histone, chromosomal protein that is highly conserved in all vertebrates (Bustin et al., 1990). It binds various forms of DNA including negatively supercoiled plasmid DNA (Mistry et al., 1997; Sheflin et al., 1993). It has been reported to be an effective DNA delivery agent without associated cell damage resulting in predominantly single copy integrations (Bottger et al., 1988; Mistry et al., 1997). It is not known whether the efficient transfection by HMG 1 protein is due to efficient uptake across plasma membrane or across nuclear membrane. However, because of its high affinity for supercoiled plasmid DNA and its abundance in nucleus, it is highly possible that HMG proteins would protect the plasmids from cellular degradation and target it to the nucleus.

Method
DNA was incubated with HMGD briefly to allow the association. The mixture was then added to the transfection medium for lipoplex formation and the standard transfection protocol was followed.

Data
The addition of HMGD protein did not have any effect on the reporter activity (Fig 23A). One order of magnitude lower or higher quantity of HMGD resulted in similar reporter activity (Fig 23B). Therefore, it did not appear to increase the uptake or prevent cellular degradation of transfected DNA as speculated.
Figure 23  Effect of protein-treatment of DNA on the B-gal reporter activity

The reporter activities obtained from transfections performed with 0.6 µg of DNA treated with either 10 units of heat-inactivated Sac II or Xho I or with 1 µg of BSA or HMGD proteins are shown in (A). In a separate set of transfections, 0.1 µg (square), 1.0 µg (■), 10 µg (□) of HMGD and of BSA proteins were examined (B). The positive and negative controls are transfections without protein-treatment or without DNA-liposome complexes, respectively. The error bars represent 95% confidence intervals from three transfections. Transfections in (B) were performed once except for positive control.
Contrary to my data, there has been a successful transfection using HMG 1 protein as the sole carrier of plasmid DNA (Mistry et al., 1997). They reported the use of up to 300 μg of HMG 1 proteins without a significant viability reduction in Caco-2 and CHO cell lines. The difference may be attributed to the combined effect of the presence of liposomes and the much smaller amount of HMGD 1 protein used in our system.

It is possible that HMGD protein and liposomes interfered with one another. For instance, the pre-treatment of DNA with HMGD protein may block further complexation with liposomes, or liposomes could displace HMGD from DNA. Or simply much more than 10 μg of HMGD 1 protein may be required to exert its effect on transfection.

Effect of Restriction Endonuclease Enzymes

Rationale
Restriction endonuclease enzymes bind to specific sequences on DNA to introduce site-specific double strand breaks. Two endonucleases were tested in transfection; each has a single recognition site on the transfected plasmid DNA, located outside the coding sequence or the promoter region.

Heat-inactivated enzyme was used for transfection for two reasons: i) firstly, the presence of heat-inactivated enzyme, Sac II, appeared to increase reporter activity in a previous transfection (Fig 9); therefore, further examination was pursued, ii) secondly, I did not want the enzyme to cleave the DNA, as the linearization of the expression construct decreased transient expression level dramatically (Fig 9).

It was speculated that the binding of endonuclease to DNA would either protect DNA from cellular degradation or deliver the plasmid DNA into nucleus where the enzyme could recognize more sequences on the chromosomal DNA. However, it was not certain whether the endonuclease protein would reform to its normal conformation after heat-inactivation to recognize its sequence and/or retain its endonuclease activity.
Method

Sac II and Xho I enzymes were heat-inactivated at 100 °C for 25 min and incubated with DNA briefly to allow the association. The mixture was then added to transfection medium for lipoplex formation and the standard transfection protocol was followed.

Data

Although inactivated Sac II appeared to increase the reporter activity to some extent in a previous transfection (Fig 9), in this set of experiments, Sac II (10 units) and Xho I (10 units) showed no effect or reduced reporter activity, respectively (Fig 23A). Considering the small amount of enzyme (20 units) and the inactive state of enzyme used, cytotoxicity is presumed to be minimal. In fact, 40 units of active restriction enzyme have been used with 50 to 80 % viability in human fibrosarcoma lines (Brenneman et al., 1996).

Therefore, restriction enzymes did not seem to have any effect on uptake or protection of DNA from intracellular degradation. It is possible that the inactivation of the enzyme abolished the binding ability of the enzyme to DNA since the tertiary structure may not properly come back after heat-inactivation, thus resulting in no association with DNA. It is also possible that more endonuclease may be required to exert its effect.

Effect of BSA

Rationale

BSA is albumin proteins purified from bovine serum extract and is generally used for protein quantification or in some restriction endonuclease reactions. Because of its ready availability in the lab, transfection with BSA protein was performed to determine if the presence of protein with no specific affinity for DNA would have any effect on the reporter gene expression.

Method

DNA was incubated with BSA briefly to allow the association. The mixture was then added to transfection medium for lipoplex formation and the standard transfection
protocol was followed.

Data
Unlike the endonucleases and HMGD, addition of BSA (1 μg) surprisingly resulted in 1.5-fold increase in reporter activity (Fig 23A). Later 0.1 and 10 μg of BSA were tested, but difference in amount did not produce a significant difference in the reporter activity (Fig 23B).

Discussion
Why did the addition of BSA to the transfection medium augment DNA uptake and/or expression? One possible explanation is that BSA proteins like FBS (Fetal bovine serum) may be actively taken up by cells, thereby allowing co-transfer of lipoplex molecules into cells. Serum albumin, in general, is responsible for binding lipid molecules for cellular uptake for storage, metabolism, or as a secondary messenger in nucleus (Trigatti and Gerber, 1995). The majority of the long-chain fatty acids present in blood serum is bound to serum albumin for transport into various tissues (Trigatti and Gerber, 1995).

It is recognized that the presence of serum during transfection hinders the cellular uptake of lipoplexes or the DNA-liposome complex formation, hence lowers the transfection efficiency. Despite the general effect of the presence of serum, there have been reports demonstrating that cellular uptake of immunoglobulin antibodies (Hong et al., 2000) and poly-lysine-condensed DNA (Han et al., 1999) is improved by complexing them with BSA and galactosylated BSA, respectively, in cell culture.

It was uncertain whether the incorporation of all three components, DNA, BSA, and liposomes, into a complex occurred. If DNA could interact with either BSA or liposomes, but not both due to limitation in charge neutralization, then perhaps the presence of BSA itself in the medium may be able to enhance transfer of lipoplexes across the cell membrane. It may be of interest to see whether it is necessary for the plasmid DNA to be complexed with liposomes, if BSA were used.

I speculated that BSA could be recognized by cell surface receptors just as serum proteins in the FBS, which are used to supplement some growth media. Hence, if all
three components were formed into a complex, then lipoplexes that are associated with BSA might be taken up by cells through an adsorption-mediated endocytosis.

In fact, improved uptake of oleate fatty acid by adipocyte cell line has been shown in the presence of serum albumin, indicating the involvement of albumin binding or recognition site on cell surface for cellular uptake of fatty acids (Trigatti and Gerber, 1995). Interestingly, neutral lipid, DOPE, which is a component of the lab-generated liposomes, consists of dioleic acids. It is possible that the neutral lipid DOPE associated with BSA, whereas the cationic lipid DDAB could still interact with DNA. These interactions would create a tri-complex, which is then taken up by cells through albumin binding site on cell surface (hence adsorption-mediated endocytosis). Although the same receptors as those on the adipose cells may not be present in Sf9 cells, which were established from embryonic cells, similar receptors that are used in a lesser degree may have contributed to the uptake.

**Conclusion**

The objective of testing these DNA-associated proteins was to modify the plasmid DNA for improved nuclear uptake, thus bringing about higher reporter expression level. Restriction endonucleases and HMGD had no effect on transient reporter expression at levels tested here. Interestingly, a transfection performed with BSA protein, which was used primarily as a control, resulted in increased reporter activity of approximately 1.5-fold. The stimulatory effect of BSA was speculated on the affinity of BSA to DOPE neutral lipid, allowing the formation of BSA-lipoplex complex, which then could be recognized by the BSA recognition sites on cell surface for cellular uptake. The increased uptake, mediated through the BSA recognition, could then result in the enhanced reporter activity.

**3.7 Comparison of reporter activity obtained from standard and optimized transfection protocols**

*Rationale*
In order to investigate the combined effect (i.e. how many fold increase) of the optimized transfection variables, the optimum condition or formulation for each variable was amalgamated and tested in one transfection. The variables, which resulted in increased reporter activity were DNA preparation method, DNA conformation, liposome composition, DNA to liposome ratio, cell number, transfection medium, lipoplex uptake period, gene expression period, and pre-treatment of DNA with BSA. The variables, which had either no effect or decreased reporter activity were post-transfection chemical treatment of cells and pre-treatment of DNA with HMGD or endonuclease enzymes, and these were not included in the optimized transfection protocol.

Method
Multiple series of transfections were performed to determine which combination of the each optimized variable result in the highest reporter activity. Within each series of transfections, multiple replicates were performed. The optimized combination set is shown in comparison to the standard protocol in Table 3. In addition to the optimized and standard protocols, which were established using the lab-generated liposomes, the optimized variables were examined in conjunction with the CellFECTIN liposomes to determine the feasibility of similar optimization process with another type of liposomes. These transfections were assayed by B-gal reporter gene. Since B-gal values vary significantly from one transfection set to another set performed at different times, the comparison between different sets of transfections when pooling data was done based on the relative B-gal activities obtained from each protocol. The activity obtained from the standard protocol was set to a relative B-gal value of 1. The 95% confidence intervals from each series were also converted relatively. Circular/supercoiled DNA was used in these transfections.
Table 3 Conditions of transfection variables for various transfection protocols

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Data and Discussion

Four different series of transfections were performed using the optimized protocol (Fig 24). Three series were performed each for standard and CellFECTIN protocol, and 2 series for Modified CellFECTIN protocol (Fig 24). When the relative B-gal activities from each series were pooled to calculate the average, the mean activity obtained from the optimized protocol was 7.8-fold higher than that of the standard protocol (Fig 24). The CellFECTIN protocol was 21-fold more efficient than the standard protocol and approximately 3-fold higher than the optimized protocol.

Of those conditions which improved reporter activity, the pre-treatment of DNA with BSA was not included in the optimized transfection protocol. Although pre-treatment with BSA improved reporter activity by 1.5-fold when tested in a standard protocol, it resulted in lower B-gal activity when included in the optimized protocol. It is not clear why the BSA effect is contradictory in different protocols. It is possible that the combination of the optimized variables may create an environment unsuitable for the effect of BSA.

A similar explanation may be pertinent for the combined effect of other optimized variables. The improvements in reporter activity made individually by optimization of each variable are not arithmetically cumulative in the transfection performed with the optimized protocol. The cumulative sum of the improved reporter activities in all the optimized conditions for the variables was calculated to be 17-fold higher than the standard transfection protocol; however, the actual improvement was only 7.8-fold. Since it is not feasible to examine all possible combinations of different variables, they were examined independently. Hence, the effect of the first variable on reporter activity may not be equivalent at the end of the optimization process where conditions for other variables have been changed. For instance, the first variable investigated, CC DNA was determined to be the most efficient DNA preparation method for transfection when using the Grace’s medium as the transfection medium. However, after partial or complete optimization process, the unknown component(s) that allowed improved reporter activity in CC DNA may not have the equivalent effect on transfection in a changed environment such as in PMS transfection medium.
Figure 24 Comparison of standard and optimized transfection protocols for transient $B$-gal reporter activity

Four independent series of transfections were performed on different days and each series included transfections using four different transfection protocols (Optimized, Standard, CellFECTIN, and Modified CellFECTIN protocols). In each series, the $B$-gal reporter activities were converted into values relative to the reporter activity of the standard protocols. In Series 1 ( ■ ), Standard and Optimized protocols were performed. In Series 2 ( ■ ), Standard, Optimized, and CellFECTIN protocols were performed. In Series 3 ( ■ ), Optimized, CellFECTIN, and Modified CellFECTIN protocols were performed. In Series 4 ( ■ ), all four protocols were performed. The mean relative reporter activity of each protocol from all four series of transfections are indicated above each set of bars. The error bars represent 95% confidence intervals calculated from three transfections for Series 1 and 4 and from six transfections for Series 2 and 3.
Since a significant improvement in the $B_{-}gal$ activity with the lab-generated liposomes could be achieved by optimization in each of the variables, it was of interest to determine if a similar optimization process could be applied to the CellFECTIN protocol for improvement. Several combinations of modifications were tested with the CellFECTIN protocol in several independent transfections; however, these modifications resulted in a lower $B_{-}gal$ activity than the regular CellFECTIN protocol. One example of such modifications to CellFECTIN protocol is presented in Fig 24. Since the same optimized conditions cannot be applied to a different transfection system due to the unknown interaction between variables, it necessitates that each transfection system be individually optimized empirically.

Among the variables examined, some appeared to interact with one another more closely than others do, and to have larger influence on the transfection efficiency. Firstly, the type of liposome, that is, the carrier of DNA was found to have the most significant effect on transient transfection efficiency. Between the 5 different lipid formulations examined in this study, the CellFECTIN formulation was the most powerful transfection reagent for $Sf9$ cells. In comparison to the lab-generated liposome and to the least efficient formulation FuGENE, CellFECTIN was 3 times (Fig 24) and 60 times more efficient respectively. Secondly, the interaction of DNA with liposomes is based on charge attraction; therefore, the ratio between the two determines the formation of condensed lipoplexes. This is reflected upon the 10-fold higher reporter activity obtained by adjusting the ratio (Fig 14).

Conclusion

In summary, the transfection of $Sf9$ cells could be improved by 7.8-fold by combining the independently optimized conditions. The method of DNA preparation, liposome type, DNA to liposome ratio, cell number, transfection medium, and lipoplex uptake period contributed to the improved reporter activity. Interestingly, the treatment of DNA with BSA, which resulted in the enhancement of reporter activity with standard transfection protocol, did not have the same effect when the optimized conditions were combined. The optimization procedure applied to CellFECTIN did not have the same effect as that on the lab-generated liposomes for the transfection efficiency. These variabilities are
probably due to the complex interactions between the transfection variables. Therefore, examination of various lipofection variables is necessary for each transfection system as an optimization procedure for heterologous expression. Similar studies have also given emphasis to the importance of optimization for individual cell lines, especially with the type of liposomes (Griffiths et al., 1997; Zellmer et al., 2001) since it brings about the most significant improvement to the efficiency of transfection.

3.8 Comparison of electroporation and lipofection for reporter gene expression

Rationale
It was of interest to whether a different non-viral transfection method could allow more efficient or easier transfection of Sf9 cells than lipofection method. I chose to examine transfection by electroporation. Although electroporation, in general, is thought to be a simple and efficient method of transfection comparable to methods such as calcium-phosphate co-precipitation, DEAE-precipitation, or lipofection in various cell lines, it has not been performed extensively with insect cells. I have found only one report describing electroporation of Sf9 cells (Mann and King, 1989). They reported that the electroporation method was two orders of magnitude more efficient in transfection than the calcium phosphate co-precipitation method. However, there is no report of a direct comparison between lipofection and electroporation for Sf9 cell line.

Mann and King (1989) described the electrical parameters, which allowed the transfection of Sf9 cells with baculoviral DNA. Since the efficiency of transfection was measured by assaying the viral plaque formation, a direct comparison with our B-gal reporter gene expression obtained from lipofection could not be made. Therefore, the purpose of this investigation was to determine if electroporation could result in a similar or higher reporter activity than the liposome-mediated transfection in Sf9 cells.

Overall Method
The electrical parameters Mann and King (1989) reported for Sf9 cells were capacitance of 1300 μF, resistance of 13 Ω, pulse length of 7.7 msec, and field strength of 0.5 kV/cm using 0.2 cm gap cuvettes. These parameters could not be replicated in this project, as an
Two electroporators, BTX TransPorator™ Plus (bacterial electroporator) and BioRad Gene Pulser™ II were available and were used to examine electroporation as a method of transfection in Sf9 cells. The transfection efficiency was measured by the expression of the B-gal reporter gene.

3.8.1 Effect of electroporation with bacterial electroporator on reporter gene expression

Method
The bacterial electroporator allowed only the voltage parameter to be altered. The other parameters such pulse length and resistance were fixed to 5 msec and 1.4 Ω, respectively. Since the extent of physical damage to cells from electric shock can be severe depending on the parameters used, the reporter gene expression can be affected significantly. Therefore, for every individual electroporation tested at various voltage settings, the cell viability was assessed by the trypan blue exclusion assay. In addition to the electrical parameters, different electroporation media and DNA amounts were examined. These initial electroporation experiments were not as elaborate and systematic as those of lipofection because I wanted to obtain rough idea of how efficient electroporation could be with Sf9 cells in comparison to lipofection.

Data
Cell viability varied when different electroporation medium was used. The viability decreased to 40% at 0.10 kV in PMS buffer (Fig 25A), whereas near 100% viability was observed at the same voltage in ESF 921 medium (Fig 25B). Therefore, the use of growth medium as electroporation medium appeared to protect cells from the negative effects of the electric shock, to some extent. In ESF 921 medium, the viability decreased sharply between 0.10 and 0.20 kV, down to 10% (Fig 25B). Upon recovery of cells in the growth medium for 1 hour, the cell viability was improved in ESF medium (Fig 25B).

Although higher viability was observed when cells were electroporated in the growth medium, higher reporter gene expression was observed from the cells
electroporated in PMS buffer with smaller amount of DNA. The maximal $B$-$gal$ activity was obtained at 0.20 kV in PMS with 10-20% cell viability, whereas the maximum in ESF 921 medium was observed without any electric shock. The level of $B$-$gal$ activity observed in Fig 25B ($B$-$gal$ activity < 1 unit/min/mg) is outside the limits of the assay. Hence, Fig 25B is not likely to represent the relationship between applied voltage and reporter activity accurately. The proper relationship is described by decreasing cell viability with increasing voltage and peak transfection efficiency achieved with cell viability range of 20 to 50%. The important conclusion, however, is that electroporation using a bacterial electroporator does not produce significant levels of transfection in comparison to lipofection.
Electroporation using a bacterial electroporator was performed at various voltage settings with either 2.0 μg (A) or 5.0 μg (B) of DNA in electroporation medium of either PMS (A) or ESF 921 (B). Transfection efficiency was measured as B-gal activity. Cell viabilities accessed by trypan blue exclusion either 2 min (—•—) or 1 hr (—o—) after the electric shock are plotted on the secondary y-axis. The B-gal reporter activities (——) are plotted on the primary y-axis.

Figure 25 Effect of electric parameters on cell viability and the B-gal reporter activity after electroporation using bacterial electroporator
3.8.2 Effect of electroporation with BioRad Gene Pulser II on reporter gene expression

Method
An alternative electroporator, BioRad Gene Pulser II, was tested using a set of electrical parameters that were similar to the Mann and King protocol (1989). Since BioRad Gene Pulser II allowed capacitance (0 – 960 μF), voltage (0.05 – 2.5 kV), and resistance (100 – 1000 Ω or ∞ Ω) parameters to be altered, various combinations of these parameters were examined to determine the optimal electroporation parameters with this electroporator. Various capacitance values were examined first with fixed resistance and voltage. Secondly, various resistance values were examined with fixed capacitance and voltage. Lastly, different applied voltages were examined with fixed capacitance and resistance. One microgram of DNA was used in electroporation of S/9 cells with PMS medium. The cell viability and transfection efficiency was assessed by trypan blue exclusion assay and B-gal reporter assay, respectively.

Data
Since 0.10 kV of applied voltage allowed approximately 50% cell viability using bacterial electroporator in PMS medium, it was chosen for initial tests. Unlike the viability observed with bacterial electroporator at 100 V setting (Fig 25A), the viability for Gene Pulser was significantly higher (70 - 90%) at the same voltage (Fig 26A and B). This difference may be due to the difference in other parameter settings and/or in the shape of the electric pulse generated by the capacitor.

With similar cell viabilities, higher capacitance resulted in higher reporter activity (Fig 26A) at 100 V and 100 Ω setting. Similarly, higher resistance appeared to result in higher reporter activity when tested at the highest capacitance, 960 μF (Fig 26B) and 100 V. When different voltages were examined, I could not determine if difference in voltage could produce significantly different reporter activity at 960 μF and 100 Ω settings since the level of B-gal activity was outside the limits of assay. The cell viability observed in this instance was much lower overall than that in Fig 26A and B. This was attributed to
the electroporation medium (HBS medium) since the cell viability in HBS medium, before exposure to electric field, was already down to nearly 60%.

In summary, the capacitance and resistance appeared to have a direct relationship with reporter activity. The relationship between voltage and reporter activity could not be determined from above experiments. Although these relationships with reporter activity are interesting, the $B\text{-}gal$ activities obtained using the BioRad Gene Pulser II was almost negligible. The maximal activity was not more than 5 unit/min/mg. This value is 100 to 1000-fold lower than that is generally obtained using lipofection method, hence lipofection is a much efficient method of transfecting $Sf9$ cells.

Conclusion

Although this study on electroporation as a means of transfecting $Sf9$ cells was less extensive than those using lipofection, it was clear that the transfection by electroporation using bacterial electroporator or BioRad Gene Pulser II is much less efficient than the lipofection. In general, 100 to 1000-fold higher $B\text{-}gal$ activity can be obtained using lipofection method. I cannot rule out the possibility that further optimization of general transfection variables such as DNA amount, cell number, and electroporation medium can improve the reporter activity. However, it would necessitate a significant amount of improvement to be made to produce comparable or much higher reporter activity than the lipofection method.
Electroporation was performed using BioRad Gene Pulser II with 1.0 µg of DNA in PMS medium. Various capacitance values (0 – 960 µF) were examined at 100 Ω and 100 V (A). The electroporation efficiency (● or △) and the cell viability (∆) immediately following electric shock were evaluated by B-gal assay (primary y-axis) and trypan blue exclusion (secondary y-axis), respectively. Different resistance and capacitance were tested with 100 V setting in (B). The negative control was treated in the same condition with DNA except for the exposure to the electric field (B).
Chapter 4 Analysis of transformed stable cell lines

Rationale
The purpose of this study was to investigate the chromosomal integration pattern and frequency of transfected plasmid DNA and to determine the relationship between the pattern and the level of reporter gene expression in the stably transformed Sf9 cell lines. Firstly, this will provide insight into the potential mechanism of heterologous recombination of foreign DNA in Sf9 cells. The arrangement of transfected DNA in the chromosomal DNA and the orientation of the plasmids can help us understand the mode of integration. Secondly, this will also allow us to determine which integration pattern results in the highest transgene expression in the transformed stable cell lines. Thirdly, the effect of chemicals that are known to elicit DNA damage on the integration pattern and frequency could be studied, further providing indication of the mechanism of heterologous recombination. Transformed stable cell lines established from various transfection procedures described in Chapter 3 have been examined for the integration pattern and copy number of the integrated plasmid DNA by Southern Blot analyses. The transformed stable cell lines were established by selection with zeocin as polyclonal cell lines and their stable reporter expression levels have been determined by B-gal assay.

4.1 Results

4.1.1 Analysis of integration pattern

Rationale
Several possible integration patterns were speculated: plasmids may be integrated in i) concatameric arrangements, ii) several independent single integrations into different loci, or iii) in a mixture of both concatameric form and several single integrations. In each case, the interesting questions to address are the orientation and the intactness of the plasmids in the concatamer(s) or in individual single integrations.
Method

Stable polyclonal cell lines from five different series of transformations performed using pBacTracer1-LacZ-smRS-GFP construct were established. Series A and B were transformed independently using the optimized transfection protocol. Series C was transformed using the CellFECTIN protocol. The transfections described in Section 3.1.6.2 resulted in two stable lines, which were the positive controls transfected without chemical treatment. These were transfected with either circular (‘Circular’) or linear (‘Linear’) DNA. The second attempt to make chemical-treated stable transformants using lower concentration of chemicals resulted in ‘Zeo1’ and ‘But2’ stable cell lines. The positive controls transfected without chemical treatments were also established into stable cell lines (‘NoTreat1’ and ‘NoTreat2’). These transformants were transfected with circular DNA. Those transfected with linear DNA could not be established into stable cell lines in this case.

In order to determine whether the integration was in the form of concatamer(s) or multiple single inserts, the genomic DNA was digested with either EcoRI, KpnI, or BsmI for southern blot analyses. EcoRI does not cleave pBacTracer1-LacZ-smRS-GFP construct, whereas KpnI and BsmI cleave once and twice, respectively. The genomic DNA purified from cells, which had been selected on 1 mg/ml of zeocin for at least 5 sub-culturings after transfection was used for Southern Blot analyses. Entire plasmid DNA was used to probe the southern blots.

Data

Southern blot analysis of EcoRI-digested genomic DNA

When the genomic DNA was digested with EcoRI, a high molecular weight signal was detected from all of the stable cell lines in the Southern blot (Fig 27A). A signal of approximately 23 kb was detected after one day of exposure (Fig 27A). When the same blot was exposed for 6 days (Fig 27B), a few weaker signals of smaller sizes than the former could be detected. A weak signal in B2 stable line was slightly smaller than 6.5 kb and could be a single plasmid insert if the entirety of it had integrated. A signal
slightly larger than 4.4 kb was seen in ‘Circular’ stable line. A third signal in the ‘Linear’ stable line was much stronger than the former two and was approximately 3 kb.

The intensities of the 23 kb signal varied between cell lines although the genomic DNA cell lines with weak signal (B2, B3, Cir, and Lin) was not under-loaded in the EtBr-stained gel (Data not shown). Therefore, this suggested that the amount of integrated plasmid DNA may be different between different transformed stable cell lines. More detailed analysis on quantification of integrated plasmids will be presented in Section 3.2.2.

Untransformed Sf9 cells showed minimal background with no specific signals indicating that the detected signals were specific for the integrated plasmid construct. The signal detected in Kpn I-digested plasmid control lane corresponded in size to the signal of 6.5 kb-Lambda marker.

**Southern blot analysis of Kpn I-digested genomic DNA**

When the genomic DNA was digested with a single-cutting enzyme, Kpn I, the majority of the signal detected was approximately the same size as that of the plasmid control (Fig 27A). This indicated that the integrated plasmids were in concatameric forms in all of the stable cell lines since a single cutter could cut the insert into multiple single units of plasmid. The high molecular weight signal observed in EcoR I digest also supports the concatameric integration since the concatameric plasmids including the flanking genomic sequence would be cleaved out in the EcoR I digest. In addition to the signal from the concatameric integration, other weaker signals could be detected when exposed for 6 days (Fig 27B). Approximately 5 other signals could be seen in A3 cell line. One to two other signals could be detected in A2, A4, C1, ‘Circular’, and ‘Linear’ cell lines. All the weaker signals other than the 6.5 kb signal originating from the concatamer(s) were smaller than the size of one unit of plasmid construct. In the stable cell line transformed with linear DNA, the intensity of the lower molecular weight signal was greater than that of the common 6.5 kb signal in the same lane.
Figure 27  Southern blot of genomic DNA from transformed stable cell lines hybridized with transfected plasmid sequence

Stable cell lines obtained from various transfection protocols have been analyzed for integration of the reporter plasmid: Series A and B from optimized protocol, Series C from CellFECTIN protocol. The genomic DNA was digested with either EcoR I, Kpn I, or Bsm I. Plasmids digested with either Kpn I (Kpn I pl.) or Bsm I (Bsm I pl.) were run as positive controls. Hind III-digested lambda DNA and untransformed Sf9 genomic DNA (Sf9) were run as markers and negative control, respectively. The integrated plasmids were detected by hybridization with entire pBacTracer1-LacZ-smRS-GFP DNA probe. The blots were washed in 2x SSC, 0.1% SDS at 65°C and exposed for 1 day (A) and exposed for 6 days (B). Blots (C) was prepared with Bsm I-digested genomic DNA and exposed for 3 days under same washing conditions. The arrow heads indicate fragments of plasmid DNA digested with either Kpn I or Bsm I (A - C).
Southern blot analysis of \textit{Bsm} I-digested genomic DNA

\textit{Bsm} I cleaves the transfected construct twice giving 2.7 and 3.9 kb fragments. Therefore, the orientation of the plasmids in the concatameric inserts could be determined by the digestion pattern. If the plasmids were in the same orientation in the concatamer(s) such as head-to-tail arrangement, \textit{Bsm} I would cut in every 2.7 and 3.9 kb of the plasmid (Fig 28A). On the other hand, if the orientation was random, then at least two signals other than the 2.7 and 3.9 kb signals would result (Fig 28B).

In all the stable cell lines examined two signals were consistently detected and they corresponded to the signals (2.7 and 3.9 kb) of the \textit{Bsm} I-digested plasmid control run in the last lane (Fig 27B and C). This indicated that the orientation of the plasmids in the concatamer(s) is uniform.

A third signal could be seen (Fig 27C) in some lanes depending on the amount of DNA loaded as it varied between lanes in the EtBr-stained gel (Data not shown). Its size corresponded to that of the \textit{Kpn} I-digested plasmid control (Fig 27B), which is the size of a single plasmid unit. It may be from an independent insert containing a combination of partial and complete plasmids in tandem, which could coincidentally result in 6.5 kb signal after \textit{Bsm} I cleavage. Another possible explanation is that a very small proportion of the integrated plasmids is present in the opposite orientation than that of the majority of plasmids in the concatamer. And the rarely arranged plasmids could produce fragment exactly the same size as the entire plasmid. Since the sites of linearization for integration is not known, such possibilities cannot be excluded. However, it is unlikely that such fortuitous integration events would occur equally in all cell lines examined. On the other hand, it could simply be due to incomplete digestion of the genomic DNA, resulting in partial digestion in some of the inserts.
Fig 28 Orientation of plasmids integrated into transformed stable cell genome

Possible orientation of plasmids in the integrated concatamers is shown. The plasmid and genomic DNA are represented as $\rightarrow$ and $\bigtriangledown$, respectively. Digestion pattern of EcoRI, Kpn I, or Bsm I enzymes are indicated with predicted sizes of the digested DNA. The plasmids in the concatamer are present in a uniform head-to-tail orientation in (A). The plasmids are present in a random orientation in (B). Two of the sizes of Bsm I-digested DNA are predicted to be 2.7 kb and 3.9 kb (A & B). Two other sizes of Bsm I-digested DNA in the randomly oriented concatamer are unknown and are indicated as $x$ and $y$ kb (B).
Interpretations

The observations that the majority of the signals is i) of high molecular size in EcoR I digest and ii) of size of a single plasmid unit in Kpn I digest, implied that the integrated plasmids were present as concatamers in all transformed stable cell lines examined. Although a single 23 kb signal was speculated to be a concatamer, this did not necessarily indicate a single concatamer insert. It could have resulted from multiple concatamers that are very similar in size. Since a regular agarose gel cannot resolve DNA fragments larger than approximately 20 kb, it was possible that the 23 kb signal was actually a mixture of several concatamers differing slightly in size.

The two major signals (2.7 and 3.9 kb) observed from digestion by a double cutter, Bsm I implied that the integrated concatamer(s) have multiple plasmids in a uniform orientation. However, the possibility that few plasmid units in the same concatamer might be present in the opposite orientation, producing non-uniform orientation of plasmids in the concatamer could not be completely excluded because of the presence of very weak third signal. The ends of the concatamers were not detected in the southern blots of Bsm I-digested DNA. It is possible that Bsm I site is near the linearization site of the plasmid. This would allow only a small portion of the plasmid DNA to be available for hybridization with the probe, making the detection difficult on the southern blot.

In addition to the concatamers, there seemed to be other independent single integration events as observed in a southern blot with EcoR I-digested DNA. These single integrations probably resulted in the integration of a partial plasmid as they were of smaller sizes than the intact plasmid. This was prominent with the stable cell line transformed with linear DNA as the intensity of the 3 kb signal was almost equivalent to that from the 23 kb concatameric insert. The 3 kb signal could not be detected when probe made from part of the LacZ was used for hybridization in another analysis (Fig 29B). In addition, the ‘linear’ stable cell line showed a very low level of stable B-gal activity when tested later (Fig 30). On account of these, the 3 kb insert could probably be ascribed to the GFP::Zeo fusion gene and the rest of the vector sequence.

Each weak intensity signal seen in the EcoR I-digested southern blot (Fig 27B) represents an independent integration event into a unique integration site. If the stable
cell lines are clonal, the signals from the different single integrations, such as the 3 kb signal from 'linear' line and 6.5 kb signal from B2 line, are expected to be the same in intensity. However, since the cell lines were established as polyclonal lines, the differences in the signal intensities are probably due to the difference in the proportion of the cells originating from such integration events. It is also possible that the concatameric integration and the single-plasmid integration(s) are not present within the same cell.

Many weaker signals (not 6.5 kb signal) detected in different stable cell lines in the Kpn I digestion are likely to be the ends of the concatamers. Kpn I digestion of a single concatamer should produce multiple copies of 6.5 kb DNA and two ends of the concatamer (Fig 28A). Hence, the presence of multiple signals other than the 6.5 kb signal implied that there may be more than one concatameric integrations and that they may be present in the different clonal populations of the same cell line, differing in their integration sites.

Figure 29 Southern blot of genomic DNA from various transformed stable cell lines hybridized with Sf9 actin promoter sequence or with transfected plasmid sequence

Transformed stable cell lines obtained from various transfection protocols have been analyzed for integration of the reporter plasmid. Refer to Fig 27 legend for the description of the cell lines. The genomic DNA was digested with either EcoR I, Kpn I, or Bsm I. Plasmids digested with either Kpn I (Kpn I pl.) or Bsm I (Bsm I pl.) were run as positive controls. Hind III-digested lambda DNA and untransformed Sf9 genomic DNA (Sf9) were run as markers and negative control, respectively. The blot (same blot as in Fig 27) was hybridized with probe made from 1.7 kb, BamH I/EcoR I fragment of previously cloned (Tara Colin, 1999) Sf9 actin promoter sequence (A). The same blot was stripped and hybridized with probe made from 1.9 kb, Kpn I/Sac I fragment (containing portion of the LacZ gene) of pBacTracer1-LacZ-smRS-GFP construct (B). The blot was washed in 2x SSC, 0.1% SDS at 60°C for both (A) and (B). Detection of radioactivity was performed using phosphoimager. The astericks, on the EcoR I- and Kpn I-digested DNA panels in (A), indicate the signal (same signal in every lane within the same panel) used to normalize DNA loading between lanes for quantitation. The astericks, on the EcoR I- and Kpn I-digested DNA panels in (B), indicate the signal (same signal in every lane within the same panel) used to quantitate the total amount of the plasmid DNA integrated into the genome.
Integration pattern of stable cell lines treated with Zeocin or Butyrate

Rationale
As introduced earlier in Section 3.1.6.1.1, zeocin and the topoisomerase inhibitor, VP-16 have been shown to increase random integration in mammalian cell lines. They cause DNA double-strand breaks in chromosomal DNA and as a consequence, induce DNA repair pathway during which the transfected DNA can become integrated into the genome as a repair substrate. Sodium butyrate is an inhibitor of histone deacetylase, thus, the treatment of cells with it was speculated to allow easier access of recombinant machinery to genomic DNA, allowing easier or higher frequency of integration events to occur in the chromosomal DNA. In theory then, cells treated with these chemicals would allow more frequent integration events than those not treated, in which such an event is less frequent. Higher frequency of random integration increases the probability of integration in transcriptionally active loci in the transformed cells. Therefore, the use of these chemicals were speculated to produce transformed stable cell lines, which would have a higher transgene copy number or higher reporter activity than those transformed using the regular transfection protocol.

Method
Because of the effect of chemicals on cell viability, these stable cell lines were harder to establish and had to be selected on a step-wise increment of zeocin selection for a more extended period of time than the cell lines transformed without the chemical treatment. Two stable cell lines were established after transfection with chemical treatment: ‘Zeo1’ and ‘But 2’ were treated with zeocin or sodium butyrate, respectively. The positive controls transformed without any chemical treatment were established into stable cell lines: ‘NoTreat 1’ and ‘NoTreat 2’. The VP-16-treated cells could not be established into stable cell lines.

Data
The major signal was 6.5 kb and 23 kb in the southern blot performed with Kpn I digested DNA (Fig 31B) and with EcoR I-digested DNA (Data not shown) for ‘Zeo1’,
‘But 2’, ‘NoTreat1’, and ‘NoTreat2’ cell lines. These data indicated that the integrated plasmids were also present in concatameric forms when the cells were treated with zeocin or sodium butyrate. *Bsm* I-digest southern blot analysis of these cell lines was not performed. Therefore, the orientation of the plasmids in the concatamers cannot be concluded here. Since the integrated plasmids were found in concatamers as in other transformed cell lines, the chemicals did not appear to have any apparent effect on the integration pattern.

![Graph](image)

**Fig 30 Comparison of reporter expression levels from transformed stable cell lines**

The *B-gal* reporter expression was measured from stable cell lines established from transformation using either optimized transfection protocol (A □ and B □ series), CellFECTIN protocol (C series □), or standard protocol (Circular, Linear □). ‘Circular’ and ‘Linear’ stable cell lines were transformed with untreated circular/supercoiled or linearized DNA, respectively. The background *B-gal* activity from untransformed Sf9 is shown (Sf9). The error bars represent 95% confidence intervals calculated from three *B-gal* assays.
Figure 31  Southern blot of genomic DNA from various transformed stable cell lines hybridized with \textit{Sf9} actin promoter sequence or with transfected plasmid sequence

Transformed stable cell lines obtained from various transfection protocols have been analyzed for integration of the reporter plasmid. Refer to Fig 27 legend for the description of the cell lines. \texttt{Zeol}, \texttt{But2}, \texttt{NoTreat1}, and \texttt{NoTreat2} cell lines were transfected with post-transfection chemical treatment with zeocin, sodium butyrate, and no chemical, respectively. The genomic DNA was digested with \textit{Kpn I} in both blots (A & B). Plasmid digested with \textit{Kpn I (Kpn I pl.)}, untransformed \textit{Sf9} genomic DNA (\textit{Sf9}), and \textit{Hind III}-digested lambda DNA were run as a positive control, negative control, and markers, respectively. In (A), the blot was hybridized with probe made from 1.7 kb, \textit{BamHI/EcoR I} fragment of previously cloned (Tara Colin, 1999) \textit{Sf9} actin promoter sequence. The same blot was stripped and hybridized with probe made from 1.3 kb, \textit{Bsm I/Pst I} fragment (containing the \textit{GFP::Zeo} gene) of pBacTracer1-LacZ-smRS-GFP construct (B). The blot was washed in 2x SSC, 0.1% SDS at 60°C for both (A) and (B). Detection of radioactivity was performed using phosphoimager. The asterick in (A) indicates the signal (same signal in every lane) used to normalize DNA loading between lanes for quantitation. The asterick in (B) indicates the signal (same signal in every lane) used to quantitate the total amount of the plasmid DNA integrated into the genome.
Summary

From the Southern blot analyses of *EcoR* I- and *Kpn* I-digested DNA, the concatameric form is the predominant form of the integrated plasmids. A few single-plasmid integrations of intact or partial plasmid were also detected in *EcoR* I digest. More than one concatamer were speculated to exist in some of the stable line as more than two signals representing the ends of the concatamer were observed in *Kpn* I digest. In addition, it could be inferred from the difference in the intensities and sizes of the weaker signals that the different concatamers could be present at different loci, in different sub-population of cells within the same cell line. This is characteristic of the polyclonal nature of the transformed cell lines. Southern blot analysis with pulsed field gel electrophoresis was attempted in order to determine if the 23 kb signal in the *EcoR* I digested DNA (Fig 27A) could be ascribed to more than one concatamer. However, it was not successful. The orientation of plasmids in the concatamer(s) was found to be uniform, however, the possibility of the presence of infrequent plasmids in the opposite orientation could not be excluded. There was no apparent difference in the pattern of integration between different cell lines transformed with circular/supercoiled DNA. Interestingly, transformation with linear DNA appeared to produce both concatameric and single integrations, but the latter was present more predominantly in the population than any other cell lines transformed with circular/supercoiled plasmids. Similar observations were made for the stable cell lines created with the post-transfection chemical treatment using zeocin or butyrate.

4.1.2 Analysis of integrated copy number

Rationale

The integrated transgene copy number is one measure of the efficiency of transformation. Higher copy number provides higher chance of obtaining high level of the transgene expression. However, the gene expression level may be determined by the genomic position of the integrated transgenes. Different genomic environments have a different level of transcription activity, thus controlling the level of transgene expression.
Consequently, the transgene expression level is not directly proportional to the number of integrated plasmids. In general, the presence of a larger number of transgene dispersed throughout the genome increases the likelihood of a high level of expression. Therefore, the copy number is one indicator of the transgene expression, but not an absolute one. To investigate the relationship between the copy number and the reporter expression level, I used a quantitative southern blot analysis and \textit{B-gal} assay, respectively. The positional effect of the reporter genes in the transformed stable cell lines was not investigated in this study.

\textit{Method}

In order to compare the relative number of the reporter gene copies between various transformed stable cell lines, the amount of DNA loaded in each lane of the southern blot was normalized by the amount of endogenous \textit{Sf9} actin gene. The blot (same blot as in Fig 27) was probed with 1.7 kb, \textit{Sf9}-specific actin promoter sequence (Fig 29A). Since the transformed stable cell lines will contain equal amount of actin promoter sequence in their genome, the amount of actin signal is a relative measure of the amount of total genomic DNA on the blot. Three and seven actin signals were detected from \textit{EcoRI}- and \textit{KpnI}-digested DNA, respectively. The largest molecular size signal from \textit{EcoRI} digest and the fourth largest molecular size signal from \textit{KpnI} digest were used to normalize the DNA loading (Asterisks are indicated in Fig 29A).

The same blot was stripped and hybridized with a 1.4 kb probe containing only part of the \textit{LacZ} sequence to detect the integrated reporter genes (Fig 29B). Transformed stable cell lines that were not included in the initial blot were studied in a separate southern blot analysis (Fig 31). Similar quantitation method was performed by hybridization with actin probe (Fig 31A) or with GFP::Zeo probe (Fig 31B) instead of \textit{LacZ}.

After the normalization of DNA loading, the relative amount of integrated reporter gene was determined for each stable transformant. The quantitation of the relative copy number was performed three times using i) \textit{EcoRI}-digested set of DNA probed with \textit{LacZ} (Fig 29B), ii) \textit{KpnI}-digested set probed with \textit{LacZ} (Fig 29B), and iii) \textit{KpnI}-digested set probed with GFP::Zeo in a different blot (Fig 31B).
Data

In general, similar results were obtained from three different quantitation sets (Fig 32). The average values of the three sets are summarized in Table 4. Some cell lines were quantitated only once (C5, C6, Zeo1, But1, NoTreat1, NoTreat2) or twice (C4, Cir, Lin). In comparison to the ‘Lin’ cell line, which contained the least amount of integrated plasmids (whose relative integrated copy number was set to one), the A series of transformants contained approximately 12 relative copies on average (Table 4) excluding A1 cell line. The copy number could not be determined for A6 because of incomplete digestion as evident from the high molecular weight signal in actin-probed blot (Fig 31 A). The B series contained 8 or fewer relative copies (Table 4). The C series, in general, contained more copies than any other series, ranging between average of 13 and 23 relative copies (Table 4).

The ‘But2’, ‘NoTreat1’, and ‘NoTreat2’ stable cell lines contained fewer than 7 relative copies. On the other hand, ‘Zeo1’ contained 27 relative copies of LacZ gene, containing the most copies among the examined transformants. ‘Cir’ and ‘Lin’ contained one to two relative copies of the LacZ reporter gene.

Conclusion

The stable cell lines transformed using CellFECTIN (C series) protocol had more integrations than those using the lab-generated liposomes (A and B series). Transformations performed using the optimized transfection protocol (A and B series, ‘Cir’, ‘Lin’, ‘NoTreat 1’ and ‘NoTreat2’) contained up to 13 relative copies whereas those performed using CellFECTIN protocol contained up to 23 relative copies.
### Table 4 Comparison of Relative Copy Number Between Transformed Stable Cell Lines

<table>
<thead>
<tr>
<th>Transformed Cell Lines</th>
<th>iv Average Copy Number Relative to 'Lin'</th>
<th>iii Stable B-gal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>3</td>
<td>3564 ± 56</td>
</tr>
<tr>
<td>A2</td>
<td>11.5 ± 1.3</td>
<td>1075 ± 84</td>
</tr>
<tr>
<td>A3</td>
<td>13.1 ± 3.8</td>
<td>520 ± 46</td>
</tr>
<tr>
<td>A4</td>
<td>12.0 ± 6.5</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>A6</td>
<td>N/D</td>
<td>652 ± 39</td>
</tr>
<tr>
<td>B2</td>
<td>2.3 ± 1.1</td>
<td>895 ± 106</td>
</tr>
<tr>
<td>B3</td>
<td>7.2 ± 0.7</td>
<td>558 ± 28</td>
</tr>
<tr>
<td>C1</td>
<td>17.9 ± 3.7</td>
<td>2908 ± 83</td>
</tr>
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<td>C2</td>
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<td>1269 ± 16</td>
</tr>
<tr>
<td>C3</td>
<td>22.8 ± 1.0</td>
<td>3321 ± 57</td>
</tr>
<tr>
<td>C4</td>
<td>18.4 ± 3.5</td>
<td>1255 ± 82</td>
</tr>
<tr>
<td>C5</td>
<td>12.8</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>C6</td>
<td>17.3</td>
<td>294 ± 13</td>
</tr>
<tr>
<td>Zeo 1</td>
<td>27.4</td>
<td>N/D</td>
</tr>
<tr>
<td>But 2</td>
<td>5.0</td>
<td>N/D</td>
</tr>
<tr>
<td>NoTreat 1</td>
<td>6.8</td>
<td>N/D</td>
</tr>
<tr>
<td>NoTreat 2</td>
<td>5.1</td>
<td>N/D</td>
</tr>
<tr>
<td>Cir</td>
<td>1.8 ± 1.1</td>
<td>147 ± 5</td>
</tr>
<tr>
<td>ii Lin</td>
<td>0.5 ± 0.7</td>
<td>149 ± 5</td>
</tr>
<tr>
<td>Sf9</td>
<td>0.3 ± 0.6</td>
<td>0</td>
</tr>
</tbody>
</table>

**Note:**

i. A1 transformant contained 3 copies of GFP::Zeo gene. This was determined by qualitative analysis of the southern blot probed with GFP::Zeo gene (Fig 31A & B).

ii. The copy number is relative to the integrated copy number determined for 'Lin' cell line which contained the least amount of integration.

iii. B-gal activities from stably transformed cell lines were obtained from 3 replica and the average with standard deviation is shown.

iv. The quantitation of relative copy number was performed three times using three different southern analyses. Results of three quantitations are averaged and shown with standard error. Some were performed once (C5, C6, Zeo1, But2, NoTreat1, NoTreat2) or twice (C4, Cir, Lin).

v. N/D denotes 'Not Determined.' The genomic digestion of A6 cell line was incomplete and therefore, could not be analyzed for quantitation.

vi. Stable B-gal activities could not be assessed for Zeo1, But2, NoTreat1, and NoTreat2 because the lines were lost shortly after being established into stable cell lines.
Figure 32  Relative number of integrated plasmids in transformed stable cell lines

The results of three different Southern blot quantitations using phosphoimager ImageQuant 5.2 are summarized. (■) series is quantitated from Kpn I-digested southern blot (Fig 3.2.2.2) hybridized with GFP::zeo probe. (□) and (■) series are quantitated from EcoRI- and Kpn I-digested southern blot (Fig 3.2.2.1), respectively, hybridized with LacZ probe. The values are relative to the intensity of signal from 'Lin' cell line. (■) indicates quantitation of integrated plasmids by qualitative analysis from a southern blot (Fig 31).
Sodium butyrate treatment did not result in a higher frequency of integration. The speculated property of sodium butyrate to increase the accessibility of transfected DNA or recombination machinery into the chromosomal DNA did not appear to improve the frequency of random integration. The VP-16 treated cells could not be established into the stable cell line.

On the other hand, the zeocin treatment resulted in the most frequent reporter integration. Although only one cell line was examined for this study, zeocin may, in fact, allow more frequent random integration. Since zeocin did not cause apparent change in the integration pattern of the transfected plasmid, it supports the argument that the mechanism of integration may be through DNA strand break and subsequent DNA repair pathway. Then by inference, one can speculate that the transfected DNA in an ordinary environment in untreated cells also takes a similar route of integration, that is, integration into sporadically occurring DNA breaks.

4.1.3 Analysis of integration pattern and copy number in A1 stable cell line

As the A1 transformant was established into a stable cell line later than the other A series cell lines, separate southern blot analyses were performed to determine the integration pattern and copy number. When its genomic DNA was digested with EcoRI and probed with the entire plasmid, one high molecular weight signal was detected (Data not shown). The size of the signal was slightly smaller than the high molecular weight signal detected from the rest of the stable cell lines in EcoRI southern blot. When a blot with KpnI-digested DNA was probed with GFP::Zeo fusion gene, three signals were detected (Fig 31 B). Two of the signals were larger and the other was smaller than 6.5 kb. The absence of 6.5 kb signal, on this blot, indicated that the integrated plasmids are not arranged in a head to tail concatamer. KpnI does not cleave within the GFP::Zeo gene, therefore, each signal detected should correspond to one copy GFP::Zeo gene. I speculated that the former two signals, larger than the size of a single plasmid unit, represent two full-length plasmids and that they express both the GFP::Zeo and LacZ reporter genes actively owing to its high B-gal activity (Table 4, Fig 30) and its resistance to high concentration of zeocin (selected on 1 mg/ml zeocin). The smaller
signal is likely to be a partial integration containing only the GFP::Zeo gene since it is smaller than a full length plasmid but large enough to be GFP::Zeo coding sequence, which is 1.1 kb.

As only one signal was detected in EcoRI blot, the three integration events could have taken place in three different sites within approximately 20 kb of the genomic sequence with no EcoRI recognition sequence in between. It is also possible that two of the larger integrants are joined in a head-to-head or tail-to-tail orientation. The arrangement of the reporter genes shown in Fig 33 is a possible orientation of the two integrated plasmids. The partial plasmid might have been integrated in the vicinity (within ~ 20 kb) as a separate partial integrant (Fig 33). A southern blot analysis with BsmI-digested DNA was not performed.

In conclusion, the integration pattern of the transfected plasmids in cell line A1 was found to be different than that of other cell lines. All other transformants had concatameric integrations in a head-to-tail orientation. In this cell line, two full-length plasmids and a partial plasmid, containing GFP::Zeo gene, were present separately in 2 to 3 different loci within approximately 20 kb of genomic sequence.

4.1.4 Analysis of reporter expression level in transformed stable cell lines

**Rationale**

The decision as to which transformed stable cell lines are the most useful in expressing the transgene is made by comparing the stable expression level of the transgene regardless of the number of integrated transgenes. Therefore, the determination of the stable reporter expression is a necessary assessment. Since all the transformed stable cell lines were viable in a fairly high concentration of zeocin (1 mg/ml), the level of the LacZ reporter expression was expected to be equally high. The presence of full length plasmids in concatamers indicated that the expression of the reporter gene should be in a similar level as that of the GFP::Zeo gene in the same construct. In addition, comparison of the copy number and the reporter level will give an indication as to whether certain methods of transfection or conditions in transfection will characteristically produce
integrations into transcriptionally active genomic environment. To assess the reporter expression level of the stable cell lines, a B-gal assay was performed.

Data

The B-gal expression level was significantly variable between different stable cell lines within the same series of transformation (Fig 30). The low producers (A4, C5, C6, 'Circular', and 'Linear') were not confined to a certain series of transformation; however, the majority of the higher producers (at least 1000 B-gal units) were from C series, which were transformed using the CellFECTIN protocol. On the contrary, the highest producer was A1, which was transformed using the optimized transfection protocol with the lab-generated liposomes.

Because the B-gal activities obtained from these stable cell lines were as high as those of high transient expression, their counterpart transient B-gal activities were compared. The A series produced 963 transient B-gal activity (unit/min/mg) on average with standard deviation of 58, and the C series transformed on the same day produced 3310 ± 180 B-gal activity on average in a transient B-gal assay. Thus, a higher stable reporter activity was detected for A1 and A2 (Fig 30) than their transient expression level. On the other hand, the stable lines of C series produced lower or similar stable expression levels (Fig 30) than their transient levels, which varied from zero to 3400 B-gal units. The B series, transformed in a separate experiment, produced 824 ± 80 B-gal activity (unit/min/mg) in transient B-gal assay, which was similar to or lower than their stable expression levels (Fig 30). Transient B-gal assay was not performed on the 'Circular', 'Linear', 'Zeo1', and 'But2' stable cell lines.

In summary, although there was no direct relationship between transient and stable reporter activities, transformants with high transient reporter expression were more likely to be high producers of the reporter gene.
**Figure 33** Possible orientation of integrated plasmids in the genome of A1 stable cell line

The plasmid and genomic DNA are represented as and , respectively. The direction of the arrows and the orientation of the genes (GFP::Zeocin fusion and LacZ) on the plasmid represent head-to-head orientation. The $Kpn$ I cleavage sites are indicated on the plasmids.
4.1.5 Comparison of stable reporter activity with reporter gene copy number

The lower reporter activity generally corresponded to lower copy number. For example, A series cell lines except for A1 produced lower stable reporter activity than most of the C series cell lines (Fig 30). Similarly, the average relative copy number (~ 12 copies) of the A series cell lines was smaller than the relative copy numbers of C series cell lines (13 – 23 copies). The result is summarized in Table 4. Cell lines, B2, B3, Cir, and Lin, showed moderate to low reporter activities and low copy number between 1 and 7 copies. Therefore, cell lines with more copies of the transfected DNA seemed to produce higher reporter activity. This was not surprising since a large number of plasmids transferred into cells (a measure of high transient expression), more plasmids are likely to be integrated into transcriptionally active loci resulting in high expression level. This, however, was not a rule. Among cell lines within the same series, the reporter activity and copy number did not always show a correlation. For cell lines of series C, higher copy number correlated with higher reporter activity. However, with series B, such correlation was not seen. B2 cell line displayed higher reporter activity than B3, whereas the copy number was higher for B3 than B2. Similarly, the trend of reporter activity among A series cell lines did not correlate with that of the copy number. Interestingly, cell line A1, which appeared to contain only three copies of the plasmids, could produce the highest B-gal expression level. This high reporter expression level from, as few as three copies of template, could probably be attributed to their integration into a highly active site(s) of the genome for transcription.

In conclusion, there was a general correlation between high copy number and high level of transient expression.

4.1.6 Summary

The relative number of integrated plasmid DNA ranged from 1 to 27 copies. The relative copy number estimated for each cell line is relative to the number in ‘Lin’ stable cell line since it had the least number of plasmids integrated. Therefore, it meant that, for example, ‘Zeo1’ cell line had 27 times more integrants than the ‘Lin’ cell line.
The absolute number of integrated plasmids could not be determined in this study. The number of concatamers and their sizes can be used to determined the absolute number of integrated plasmids in each transformant, given the relative copy number. For example, if the ‘Lin’ cell line contained a single 20 kb concatamer, then fewer than 3 plasmids could be present in the concatamer since one plasmid unit is 6.5 kb. With this assumption, one can predict that the ‘Zeo1’ transformant, containing 27-fold more copies, contains 81 copies of the plasmid. I attempted separation of EcoRI-digested genomic DNA from stable cell lines by pulsed-field gel electrophoresis in order to determine the number of concatamers and their sizes; however, I could not obtain conclusive answers.

Interestingly, zeocin-treated cell line resulted in the highest copy number. Zeocin treatment did not alter the integration pattern but increased the integration frequency. This leads me to suggest that random integrations in zeocin-treated and untreated cells occur in a similar manner.

Generally, cell lines with higher copy number produced higher B-gal expression. However, a noticeable exception was seen with the cell line A1, which was estimated to contain only two and a half copies of the plasmids, but produced the highest B-gal activity as stable cells. The position of the integration events within the genome is likely to be an important factor determining the expression of the reporter gene in stable cell lines. The positional effect could alter the transcriptional activity of the reporter genes in the chromosomal environment. Keeping the importance of the transgene’s position effect in mind, however, the correlation between high copy number and high expression cannot be ignored. I could not determine whether the difference in copy number between the transformants is caused by the difference in the number of concatamers or the difference in the number of plasmid units in a given number of concatamers. In the latter case, more frequent concatameric integrations into various loci of the genome would increase the likelihood of integration into a transcriptionally active chromosomal environment.

In conclusion, transformation of Sf9 insect cells using lipofection method resulted in varying degree of integration frequency of up to 27-fold difference between cell lines in the number of the integrated plasmid DNA. These plasmids were predominantly present in uniformly arranged concatamers. This was observed in the majority of the integrations regardless of the different liposomal formulation used. Interestingly, zeocin
appeared to improve integration frequency probably through its property to cause double-strand breaks into chromosomal DNA to allow more frequent integration.

4.2 Discussion

4.2.1 Common integration pattern but variable copy numbers observed in many transformed stable cell lines

The highly variable copy numbers suggest that there may be other factor(s) that control or influence the frequency of integration. These differences may be caused by the differences between cell lines in the amount of opportunities available for integration in the genomic environment. Although a variable degree of factor(s) that allow different stable cell lines to integrate plasmids in varying frequency may be present, a common pathway of integration appears to exist to allow such a uniform integration pattern between different stable cell lines.

Variable copy numbers and similar integration pattern have been reported in many studies from various organisms such as yeast, mammalian, and *Drosophila*. Highly repetitive head-to-tail organization of plasmids have been found integrated in *Drosophila melanogaster Schneider 2* cell culture after DNA-mediated cotransfection with 500 to 700 copies per cell genome (Vulsteke et al., 1993). In a *Drosophila hydei* cell line, stable transformants were found to have between 50 and 200 copies of plasmids in head-to-tail concatamers (Sinclair et al., 1985). In mammalian cells, 50 to 100 copies of transfected genes have been found in the concatameric pattern after microinjection of plasmid DNA (Folger et al., 1982). Stable transformants of yeast *Hansenula polymorpha* have also been shown to contain approximately 30-50 copies of plasmids integrated as a concatamer (Gatzke et al., 1995). In other studies, the copy number varied considerably, ranging up to several thousand copies per cell genome in *Drosophila Kc* cell line (Bourouis and Jarry, 1983). In contrast to varying degree of copy numbers, the integration pattern was commonly noted as head-to-tail concatamers. These observations again support the idea that a common pathway of integration can be triggered in different frequency, in different cells or transfection systems.
4.2.2 Correlation between transfection method and integration pattern

In addition to this well-recognized concatameric integration pattern, non-tandem, dispersed single integrations have also been observed in this study and in other studies. Single or concatameric integrations have been reported from different methods of DNA transfection and different amount of DNA used. For example, micro-injection of plasmids has been shown to result in either single-copy transformants when a few copies of plasmids were injected or to result in transformants with concatameric integrations when many copies were used (Folger et al., 1982). Liposome-mediated transfection results in concatameric integration as demonstrated in this study. A single-integration event per genome has been observed with transfection using retroviral infection method (Baer et al., 2000), whereas predominantly multiple dispersed single-integration events were seen with electroporation transfection (Baer et al., 2000). As evident from these studies, the methodology by which the transgene is introduced into cells, influence the type of integration pattern and the frequency of the transgene.

4.2.3 What causes different integration pattern in different transfection methods?

What mode of action in different transfection methods could bring about different integration patterns? Single integration events were observed with electroporation and viral infection methods. The lipofection and microinjection methods commonly produced concatameric integrations. These methods could be distinguished by the type of effect the transfection procedure has on the chromosomal DNA.

For example, electrical shock can result in single or double-stranded breaks in chromosomal DNA or in the interference of a normal interaction of chromosomal DNA with chromosomal proteins. This immediate damage to the genome, which creates opportunities for the transfected DNA to integrate, may allow prompt integration before the plasmid DNA can be eliminated by degradation or modified by cellular machinery in the cell (discussed in detail later), thus resulting in single integration events. In the case of viral infection, the use of viral integration mechanism could allow specific single
integrations of transgene. For instance, site-specific integration of adeno-associated virus DNA into a human cell genome has been shown to be dependent on viral-encoded Rep protein and the inverted terminal repeats of the viral sequence (Surosky et al., 1997; Weitzman et al., 1994).

On the other hand, the lipofection and microinjection of DNA do not cause direct damage to the target DNA or induce specific recombination pathway despite the apparent damage to cellular components, interruption of normal cellular processes, or toxicity of the transfection reagents. One possible opportunity for heterologous recombination is through spontaneous DNA double-strand breaks that occur during the regular cell cycle. Since these events seldomly occur, the transfected DNA may have to remain in cells longer than the DNA introduced by electroporation, before being integrated. As a consequence, it may be more prone to modifications such as degradation, homologous recombination, or ligations between plasmids (Folger et al., 1982). Such modifications to transfected DNA have been observed in many reports (Maryon and Carroll, 1989). Therefore, the effect of different transfection procedure on the target chromosomal DNA may cause different integration events to occur. It also seems logical that the number of plasmids could determine whether concatameric or single integration events would take place, under the assumption that transfected cells cannot generate more substrates from the plasmids they initially received. With higher concentration of plasmids in the nucleus during lipofection or microinjection, the aforementioned modifications may occur more easily or promptly by interacting with the relevant machinery.

Since this project examined the integration of transfected plasmid DNA in stable cell lines transformed using liposome-mediated transfection, which resulted in concatameric integration of the plasmids, the discussion will be focused on the mechanism of concatameric integration. Though the method of DNA transfer between lipofection and microinjection is different, the same integration pattern suggests that a common pathway for the transgene integration exists. As mentioned earlier briefly, the DNA double strand-repair model will be discussed to explain how different methods of transfection procedures could result in a common integration pattern.

4.2.4 Mechanism of concatameric integration
Double-strand breaks could offer opportunities for integration

The zeocin-treated stable cell line was found to have concatameric inserts like most other stable cell lines that were not treated with chemicals. This cell line had the largest amount of integrated plasmids (Table 4), which was approximately 27-fold more than the 'Lin' cell line. Since only one transformant treated with zeocin was analyzed, it is possible that this was an outlier among a large sample of zeocin treated stable cell lines that might have been obtained. The probability of obtaining a high-copy transfectant, defined as over 20 relative copies, is not very high, since only one out of 17 stable cell lines had such a high copy number (C3 contained 23 relative copies). Therefore, the probability of obtaining a transformant (zeocin-treated) with even higher copy number only by chance is much lower than the probability of obtaining a transformant which represents an average of a population of stable cell lines treated with zeocin. Thus, I believe that zeocin treatment is more likely to produce a higher number of integration on average than no treatment.

From the observation that the same integration pattern was recovered in the zeocin stable cell line, one might infer that the same mode or mechanism of the integration process had occurred in zeocin-treated and the non-treated cell lines. On the other hand, the increased copy number indicates that the presence of zeocin could enhance the integration process, without changing its quality of mechanism. Judging from its chemical property to introduce double-stranded breaks (DSBs) into DNA, I speculate that the route of the integration of foreign DNA could be through DSB repair pathway. By introducing DSBs, more target sites for integration of the plasmids can be created, thus this could result in increased integration sites and increased copy number in the transformed cell lines.

It is estimated that a human cell encounters approximately 10 DSBs in a normal cell cycle (Haber, 1999). These occurrences of DSB could be induced in higher frequency by zeocin treatment, therefore, providing more potential integration target sites.

Zeocin could induce DSBs on the ectopic plasmid DNA, as equally as it would introduce DSBs into the chromosomal DNA. DSBs would result in the linearization of
some of the transfected plasmids. Depending on the efficiency of zeocin to induce DSBs on ectopic DNA molecules, some portion of the plasmids will be linearized with the remaining still in the circular/supercoiled form. Linearization would enable the plasmids to be more recombinogenic than the circular form of DNA, and stimulate extrachromosomal homologous recombinations between the linear and circular plasmids to produce uniformly arranged concatameric arrays containing multiple units of plasmids (discussed in detail below). This, together with the zeocin-induced chromosomal DSBs, could then result in illegitimate recombinations of the concatameric arrays into the chromosomal DSB sites during the DSB repair pathway. Therefore, the effect of zeocin on increased copy number is likely to be through the introduction of DSBs on both the ectopic plasmid DNA and the chromosomal DNA.

Both homologous and illegitimate recombinations are probably used for concatameric integrations

It is recognized that integrations of transfected DNA into a host genomic DNA occur by non-homologous recombination since the transfected plasmids usually do not have a homology to the host genome. Analyses of the integration sites in stably transformed cells show no homology to the integrated DNA despite some association with palindromes and A + T-rich DNA segments (Stary and Sarasin, 1992). However, the concatenation process may involve homologous recombination between the transfected plasmids as suggested by Folger et al. (1982).

During the residency of the transfected plasmids in the cell prior to integration after liposome-mediated transfection, they may go through modifications such as the linearization of plasmids, the exonucleolytic processing of linear plasmid ends, and homologous recombination between plasmids. Stimulated extrachromosomal homologous recombination has been demonstrated by transfection of plasmid DNA, which contained the I-Sce I endonuclease recognition sequence (18 bp-long recognition site) into a mammalian cells stably expressing I-Sce I enzyme (Rouet et al., 1994). Since linearization made the transfected plasmid DNA recombinogenic in the presence of homologous sequence, the linear form of DNA might be a necessary intermediate substrate in an integration process. The linearization of the transfected plasmids in the
insect cells was probably not as efficient as in above *I-Sce I*-expressing mammalian system since the process would depend on the natural ability of insect cells to cause DSBs. However, only one linear DNA molecule among many circular plasmids is needed to produce a linearized concatameric array of plasmids by homologous recombination. The product of a single homologous recombination event between a linear plasmid and a circular plasmid would result in a linear piece of DNA with two tandemly arranged plasmids. Subsequent recombinations with other circular plasmid molecules would result in a linear form of concatameric DNA. Homologous recombinations between only the circular forms of plasmids, though may be less recombinogenic, would lead to a circular form of concatameric DNA. On the other hand, homologous recombinations between two linear forms of plasmids would be futile, resulting in the same two pieces of linear DNA. Much fewer concatameric integration events observed in the stable cell line transfected with linear plasmids than in other cell lines transfected with circular plasmids could be explained by inefficient homologous recombinations between the linear plasmids. The few concatameric inserts could have resulted from recombination between the linear plasmids and the few circular plasmids, which would have been generated by the cellular religation activity.

In summary, a few linear plasmids occasionally generated by cellular modification, among the rest of the circular plasmids, could stimulate these extrachromosomal homologous recombination events. The products of these pre-integration processes would be a linear piece of DNA with head-to-tail concatameric array of plasmids. The integration of these modified DNA molecules into a site of chromosomal DSB by illegitimate recombination would result in the integration of a concatamer containing a head-to-tail array of plasmids.

A slightly different order of events could also lead to the same head-to-tail concatameric integrations (Folger et al., 1982). Following an integration of a plasmid, modified by linearization, into chromosomal DNA by non-homologous recombination, subsequent homologous recombination events between the integrated plasmid and ectopic circular plasmids, in multiple occurrences, could result in the same concatameric integration pattern.
The third mechanism would involve amplifications of the single, integrated plasmid into a concatamer through a mechanism similar to the rolling-circle replication of viral DNAs in the host genome. This possibility could be excluded since transfection by microinjection of two identical plasmids that differed only in a portion, by inversion, resulted in head-to-tail concatamers with both types of plasmids, indicating the origin of the plasmids in the concatamer to be different. Folger et al. (1982), however, could not determine whether the homologous recombination happened before (first mechanism) or after (second mechanism) integration into the chromosomal DNA.

Other studies, however, provide some insights as to which mechanism might be actually occurring to produce the final concatameric insert in transformed cell lines. A stably transformed insect trypanosomatid *Leptomonas seymouri* cell line has been shown to maintain its transfected DNA as extrachromosomal concatamerically arranged multimeric element that replicates autonomously (Bellofatto et al., 1991). That the transfected plasmids are put together into a concatameric unit rather than being maintained as single units of autonomously replicating plasmids indicates that plasmid DNAs sharing homology may be preferentially put together through homologous recombination. A herpes simplex virus thymidine kinase gene when transfected into mouse fibroblast cells by microinjection or calcium phosphate-mediated transfection is modified shortly after its introduction into cells, forms regularly arranged concatameric structure, and eventually integrates into the genome (Czernilofsky et al., 1985). Extrachromosomal homologous recombination between two strains of baculovirus during viral replication has also been reported to be a frequent event (Hajos et al., 2000).

As seen in above examples, homologous recombination does occur extrachromosomally in higher eukaryotic cell cultures, and it is possible that there may be a cellular machinery which modifies the transfected plasmids, facilitating homologous recombination. Furthermore, the extrachromosomal homologous recombination between transfected plasmids would be viewed as conceptually simpler and physiologically easier process than the homologous recombination between a extrachromosomal plasmid and a plasmid integrated into the chromosomal DNA. The complexity of the chromatin structure could hinder the search of the homologous partner for recombination, thus making such event less frequent than recombination between unhidden, easily accessible
ectopic plasmids. Unless the initially integrated plasmid and its neighboring chromosomal environment stay open or the subsequent homologous recombination events happen rapidly before the alteration of that chromosomal location, search for the same site for the second, third, or more recombination events would be very rare in higher eukaryotes. The complexity and the large genome size of higher eukaryotic organisms are reflected upon the phenomenon of inability or the difficulty of gene targeting in higher eukaryotes in comparison to yeast and bacteria. Therefore, the extrachromosomal homologous recombinations forming a multimeric concatamer followed by a subsequent illegitimate integration of the concatamer into genome seems more plausible than multiple homologous recombination events into a chromosomal site, where a prior illegitimate recombination occurred for the first plasmid.

What induces homologous recombination between extrachromosomal plasmids?
Specific components of homologous recombination machinery such as \textit{RAD} family of genes (Baumann and West, 1998), \textit{XRCC} (Pierce et al., 1999) and \textit{Brca} genes (Callebaut and Mornon, 1997) from various eukaryotic organisms have been identified in recent years. The recombination machinery is implicated in repairing the chromosomal damage such as single-stranded break (SSB), DSB, and nucleotide mismatch, which occur in moderate frequency during replication (Haber, 1999). Replication is held back until such a repair is complete. A similar pathway may be taken by the transfected plasmid DNA through spontaneously occurring SSB or DSB, or through cellular defense mechanism such as nuclease or DNase activities. The recombination machinery could also recognize the modified (linear or nicked) and unmodified (circular) plasmids, but perhaps in less efficient manner than the chromosomal DNA, and carry on homologous recombination events to repair the damage. Although such repair, after the cell’s effort to rid of the foreign DNA, seems inefficient, some marginal leeway may be tolerated, thus resulting in acquisition of new genes or DNA sequences.
Illegitimate recombination is preferentially used to introduce foreign DNA into genome

The repair by homologous recombination is limited by the presence of homologous sequences. Moreover, the mechanism of homologous recombination involving cross over is mechanistically more complicated than that of non-homologous recombination. In fact, illegitimate recombination events are stimulated 10-fold more frequently than homologous recombination by DSB in mammalian cells (Roth and Wilson, 1985; Sargent et al., 1997). This observation suggests that cells may utilize illegitimate recombination either optionally or preferentially when homologous sequence is not available or accessible (Kucherlapati et al., 1984).

4.2.5 Relationship between copy number and stable transgene expression

Correlation between high copy number and high transgene expression and the effect of genomic location

Each of the transformed stable cell lines contained varying number of transgene copies, which are in general correlated with their stable reporter expression levels. A higher reporter activity generally corresponded to a higher copy number. For example, the stable cell lines of series C, which had the most copy numbers, generally produced higher β-gal reporter activities than other cell lines (Table 4). One exceptional cell line A1 was found to have only 3 copies of integrated plasmids and to have the highest stable reporter expression.

A single copy of transgene integrated into an actively transcribed chromosomal site may produce higher expression than multiple copies arranged as a concatamer in transcriptionally inactive site. The high stable expression of A1, despite a low copy number, may be explained by the integration of those two copies into a transcriptionally active site(s) of the S99 genome. As evident from the analysis of A1, a high copy number does not provide an absolute benefit for a high expression of the transgene in a system such as here, where the site of integration cannot be controlled.

The genomic location appears to play an important part in the gene expression, but having high copy number could also be very advantageous. A higher copy number in
the transformed stable cell lines in this study could have been due to either more frequent concatameric integrations or integration of larger concatamers. If the former is true, then this would increase the probability of the integration of the concatamers into transcriptionally active sites, thereby increasing the probability of obtaining a higher transgene expression than cell lines with lower copy number. On the other hand, if a higher copy number were obtained as a result of the integration of the same number of concatamers of larger sizes, then having more copy would not increase the probability of integration into transcriptionally active sites of the chromosome.

The lack of an absolute correlation between copy number and stable gene expression could be attributed to the transcriptional activity of the neighbouring chromosomal DNA of the integrated concatamers. However, some degree of correlation between the copy number and expression level cannot be ignored.

Silencing or repression of concatameric transgenes in transgenic organisms
A number of documents also report concatameric integrations of transgenes in transgenic animal models. A concatameric form of integration seems to be a more efficient way to introduce many copies of the transgene than multiple single integration events to produce high transgene expression. Logically, more copies would mean more templates for transcription and thus more gene expression. However, this is the opposite of what has been observed in many transgenic studies. Concatameric arrays of transgene copies in transgenic animals such as mouse (Garrick et al., 1998), Drosophila (Dorer and Henikoff, 1994), and Arabidopsis (Assaad et al., 1993) show silencing or repression of gene expression, which results in lower expression of the transgene per copy number. Silencing or cosuppression of repeated transgenes appears to be a prevalent phenomenon in higher eukaryotes (Dorer and Henikoff, 1994).

On the contrary, a lower eukaryotic organism, yeast, does not appear to follow the same rule. Multiple tandem integrations have been correlated with a high transgene expression level in many studies (Clare et al., 1991; Fogel and Welch, 1982; Gatzke et al., 1995; Gjuracic and Zgaga, 1996; Orr-Weaver and Szostak, 1983; Plessis and Dujon, 1993). Since yeast maintains autonomously replicating elements, the copy number of a gene and its expression level may not be regulated in the same manner as in higher
eukaryotes such as insects, plants, and mammals, which do not have naturally known autonomously replicating elements.

Although the Lepidopteran stable cell lines utilized in this study is a higher eukaryotes, comparable to *Drosophila*, they resemble the yeast system rather than the transgenic animals in that correlation exits between copy number and gene expression. Other similar integration studies of transfected transgenes done in cell cultures show a correlation between high copy number and high transgene expression (Lycett and Crampton, 1993; Merrihew et al., 2001; Vulsteke et al., 1993). The inconsistency between cell culture (insect cell culture and yeast) and transgenic organism studies, however, can be explained.

Although the transgenic animals are confirmed by resistance to antibiotics or by selectable markers, cells or tissues in the transgenic animals can display variegating expression. On the other hand, cultured cells when selected for stable transformants are selected for the presence of the transgene expression whether low or high level. The non-expressors become selected out during the establishment of the stable cell lines, unlike those cells with lowered or silenced expression in transgenic organisms, which remain in the organism and are detected as variegating phenotypes. Therefore, the absence of silencing of the integrated concatameric transgenes in cell cultures are likely due to the limit of detection in the experimental design.

4.2.6 Relationship between transient and stable expression level

High transient reporter activity was not directly correlated with high stable reporter activity, indicating that high transient expression, and thus a presumable large amount of DNA uptake, did not secure a high stable expression level or high number of integrated copies. The transient reporter activities of the cell lines, that were established into stable cell lines, were similar between replicates (3 to 6 replicates were performed for each transfection experiment) with small confidence intervals (Note the error bars in Fig 24). On the contrary, the stable reporter activities of each replicate when established into stable cell lines showed large variations (Fig 30, Note large differences between cell lines within a series, i.e. Series A, B, and C). This variation was observed regardless of the
different transfection protocols. For example, a large variation in stable reporter activity was seen within the cell lines transfected with the optimized protocol (Series A) and the cell lines transfected with the CellFECTIN protocol (Series C) (Fig 30).

With more careful observations, the CellFECTIN protocol, which yielded a higher mean transient reporter activity, appeared to have higher chance of producing high-producer cell lines (C1 and C3 among C series (2 out of 6)) than the optimized protocol, which produced one high-producer cell line (A1) among 7 cell lines (A and B Series). Although each of the best-producer cell lines from the optimized and the CellFECTIN protocol may be similar in the stable expression level, there seems to be higher chance of obtaining the best producer by using the protocol that produces higher transient expression.

One could speculate that a higher level of the transient reporter, as seen with the CellFECTIN protocol (Fig 24) is attributable to the large amount of DNA taken up by cells during transfection. This would mean that the cell lines C1 to C6 would have retained a large amount of vector DNA molecules transiently. However, the large quantity of vector DNA molecules was not sufficient to bring about a large amount of integration, in other words, high reporter activity in all of the C series cell lines. This suggests that not only the large amount of integration substrate, but also other condition(s) may have to be suitable, or other factor(s) may be necessary.

For instance, the transfected cells may have to have coincident DNA damage such as DSBs for the transfected DNA to be integrated. The formation of DSBs activates cell-cycle checkpoints, and this allows cells to pause replication and repair DNA damage before segregation of the replicated genome (Kanaar et al., 1998). During this checkpoint, cells may be in different stages of DSB repair when they are presented with transfected plasmids. Cells that had just started the DSB repair may allow most DSBs to be available for integration. Conversely, cells that had completed the repair of most DSBs may not allow much opportunity for the transfected DNA for integration. The increased copy number in the zeocin-treated cell line may be a consequence of elevated amount of DSBs artificially induced by zeocin.

A certain cell cycle-specific factors may allow the cells to be more prone to accepting foreign DNA for integration. The components of the DSB repair machinery
may be regulated by the cell cycle. In fact, NBS1 gene, a potential cell-cycle regulator, has been found to be one of the components of the Rad50-Mre11 complex, the DSB repair proteins, suggesting a cell-cycle dependence of DSB repair (Carney et al., 1998).

Since the cellular state of individual cells in a culture cannot be controlled, few cells that fortuitously meets the favorable conditions could be established into a high-producer cell line. Therefore, the high transient expression, that is a consequence of a large amount of DNA uptake, may be necessary to provide the substrate for potential integration events, but is not sufficient to generate stably transformed cell lines with high copy number or high transgene expression. The intrinsic state of the transfected cells, such as cell cycle phase or the state of DSB repair process may determine the frequency of the integration of the transfected plasmids and subsequently the stable expression of the transgene.

4.2.7 Application for production of stable cell lines with high transgene expression

The production of a stable cell line expressing a large quantity of recombinant protein is a goal in many transfection experiments. This involves the examination of many cell lines for high expression level of the transgene of interest or for the reporter gene. Though the copy number is a comparable measure of transgene expression between different stable cell lines, having a high copy number does not guarantee a high expression level due to the genomic position effect. Since the integration is not targeted to a specific sequence of the chromosome or to a region with constant transcriptional activity, every concatamer integrated into different genomic loci would produce different level of expression per copy of the transgene. Therefore, screening the stable cell lines for their transgene expression is a necessary step for selection of a high-producing cell line.

Although evidence was not formally presented in this study, transfected cells with high transient expression levels appeared to have higher probability of becoming a stable cell line expressing high transgene expression. The optimization of the transfection efficiency to increase the transient expression would be useful, since fewer clones may have to be examined to select a high producer cell line. The most notable increase in transfection efficiency was made by optimizing the liposome formulation and the
liposome to DNA ratio. Therefore, the examination of these transfection variables, though less than complete, would provide rapid optimization.
Chapter 5  Conclusion

The objectives of this study were to optimize the efficiency of liposome-mediated transfection in Sf9 insect cells and to analyze the integration pattern and the copy number in transformed stable cell lines. Upon the optimization of the transfection variables, the transfection efficiency could be improved by 7.8-fold, most of which could be attributed to the suitable choice of liposome formulation and liposome-to-DNA ratio. The individual improvements made for each variable were not additive suggesting a complexity of the interactions between the transfection variables. Examination of a number of stable transformants established by selection in zeocin revealed that integrations were predominantly in uniformly arranged multiple concatamers. It was speculated that the mechanism of concatameric integrations would involve extrachromosomal homologous recombination between transfected plasmid molecules and subsequent illegitimate recombination of the concatamer into genomic locations during the DSB repair pathway. Highly variable numbers of integrated plasmid, up to 27-fold difference, resembled the variable expression level of the reporter gene in the stable cell lines. The reporter expression levels generally correlated with the integrated copy number. One exceptional cell line, which produced the highest reporter expression level from only three copies of the plasmid, demonstrated the importance of the chromosomal sequence neighbouring the integrated plasmids in enhancing the transcription of the transgene. The comparison of the transient and stable reporter gene expressions from each stable cell line demonstrated that a large amount of DNA uptake reflected upon the high transient expression is necessary but not sufficient for high copy number reflected upon the high stable expression. However, having a high transient expression level appeared to increase the probability of obtaining a cell line with high stable expression level. In production of a cell line having high transgene expression, use of an optimized transfection protocol, which produces high transient expression level, could increase the chance of obtaining a high-producer cell line with a high copy number and, hence, could also reduce the number of stable cell line to be screened.
Chapter 6  References


Yang, J. P., and Huang, L. (1997). Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther 4, 950-60.


