THE DEVELOPMENT AND CHARACTERIZATION OF A NOVEL AUTOGENE BASED, DUAL PROMOTER CYTOPLASMIC EXPRESSION SYSTEM

by

Jonathan Douglas Finn

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ABSTRACT

The relatively low levels of transfection that can be achieved by current gene delivery systems have limited the therapeutic utility of gene transfer. This is especially true for non-viral gene delivery systems, where the levels of gene expression achieved are usually below the levels achieved by viral gene transfer systems. One strategy for increasing gene expression is to design a cytoplasmic expression system that does not require nuclear delivery for gene expression to occur. This can be achieved through the use of an autocatalytic cytoplasmic expression system using phage RNA polymerases.

In this work, the design, construction, and characterization of a novel dual promoter, autogene based cytoplasmic expression system is described. The purpose of this work was to develop new plasmids capable of giving rise to increased gene expression levels over standard plasmids currently used in the field of either non-viral gene therapy, or general gene expression. In Chapter 2 the system is introduced and characterized. Chapter 3 is concerned with attempting to optimize expression levels, and determining what factors are limiting gene expression. In Chapter 4, the differences between a high expressing cell line (BHK) and a low expressing cell line (COS-7) are investigated.

In Chapter 2, the ability of the autogene based expression system to give rise to exponential increases in gene expression is demonstrated, as well as the ability of the expression system to give rise to levels of gene expression in BHK cells that are 20 times higher per transfected cell than a standard CMV based nuclear expression plasmid. Further analysis determined that the mRNA transcripts made in the cytoplasm are not as stable as those made in the nucleus (most likely due to the lack of a 5' cap

structure on the cytoplasmic transcripts), and that the cytoplasmic expression system gives rise to much higher mRNA levels than the nuclear system.

In Chapter 3 an attempt is made to determine what factors are limiting the cytoplasmic expression system, in an effort to optimize the system to give rise to even higher levels of gene expression. After exploring various factors, such as the nuclear promoter, RNAP gene and IRES sequence, it was found that the critical factors responsible for limiting the cytoplasmic system is at the mRNA level, both the transcription and translation. It was found that the cytoplasmic transcripts are being translated 20 times less efficiently than their capped nuclear counterparts, and by measuring mRNA transcript levels, it was found that the autogene based cytoplasmic expression system appears to be saturating the cells ability to produce new mRNA, and that transgene transcript levels are reaching 30% of the total RNA in the cell, a level that is 10 times higher than the sum of every other mRNA transcript in the cell. In addition to this finding, insights into possible IRES function are gained.

In Chapter 4, the differences between a cell line that gives rise to high levels of cytoplasmic gene expression (BHK) are compared with a cell line that gives poor cytoplasmic expression (COS-7). After investigating the individual components of the cytoplasmic expression system, such as the IRES activity and RNAP function, it was found that the reason for the difference in the two cell lines is most likely at the level of the intracellular processing of the DNA:cationic lipid complexes, as the COS-7 cells appear to have less intact plasmid per cell over time than the BHK cells.

In summary, this work represents a substantial advance in the understanding of cytoplasmic expression systems, and demonstrates the potential of these systems to give rise to increased levels of gene expression. In addition, this study clarifies

potential areas that need to be addressed in order to further optimize cytoplasmic expression systems.

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ABBREVIATIONS

AAV = adeno-associated virus

Ad = adenovirus

BHK = baby hamster kidney

CAR = coxsackie and adenovirus receptor

CMV = cytomegalovirus

DEAE = diethylaminoethyl

DNA = deoxyribonucleic acid

DODAC = N,N-dioleyl-N,N-dimethylammonium chloride

DOPE = dioleyl phosphatidlyethanoamine

eIF4G = eukaryotic initiation factor 4G

EM = electron microscopy

EMCV = encephalopmyocarditis virus

FMDV = foot and mouth disease virus

HIV = human immuno-deficiency virus

HSV = herpes simplex virus

IF = immunofluorescence

IRES = internal ribosome entry sequences

LUV = large unilamellar vesicle

MLV= multilamellar vesicle

Mo-MLV = Moloney murine leukemia virus

mRNA = messenger RNA

PBS = phosphate buffered saline

PEG = poly(ethylene glycol)

PTB = pyrimidine tract binding protein

RES = reticuloendothelial system

RLU = relative light unit

RNA = ribonucleic acid

RNAP = RNA polymerase

RNAPII = RNA polymerase II

rRNA = ribosomal RNA

RSV = Rous sarcoma virus

SPLP = stabilized plasmid lipid particle

SUV = small unilamellar vesicle

SV40 = simian virus 40

TAFs = TBP-associated factors

TBP = TATA binding protein

TF = transcription factor

tRNA = transfer RNA

UAS = upstream activation sequences

ACKNOWLEDGEMENTS

There are so many people I would like to thank for helping me accomplish this goal. First and foremost, I would like to thank Pieter for taking on a young, naïve molecular biologist, giving him the freedom and the resources to make this project work, and beginning the labs foray into the wonderful world of plasmids, RNAPs, and other fun areas of molecular biology.

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I would like to also thank Ian MacLachlan, and the gang at Protiva (especially Amy Lee). Thanks for all of the support, both financial and intellectual. Ian, thanks for teaching me how to write, clone, and giving me insights into the world of industrial research.

On a personal note, I would like to thank my family, Mom, Dad, Michelle, Matthew, Patrick and Ashley. You have all been such a huge part of my life and I truly could not have done this without every ones support. You've all helped shape me into the person I am today. And another personal thanks to Steph, for being there throughout all the highs and lows. Sam, thanks for everything. You've been a real inspiration to me and have given me nothing but support for everything I've done and accomplished.

All in all, its been a great trip, mostly due to the people that I shared it with. I wish everyone the best of luck and success in whatever they choose to do.

It's all good baby.

*I dedicate this to my parents. You can never know what you've
given me and how much I truly appreciate it.
Thank you for all of the sacrifices
and the love you've given me.
I am most blessed.*

CHAPTER 1
INTRODUCTION

1.1 Gene Therapy

1.1.1 Overview

Ever since Avery, MacLeod and McCarthy determined that DNA was the genetic material of the cell (Avery, MacLeod et al. 1944), (later verified by Hershey and Chase (Hershey, Chase et al. 1952)), Watson and Crick elucidated the double helix structure of DNA (Watson and Crick 1953), and the genetic code was deciphered (Crick, Barnett et al. 1961), a new era of scientific research was ushered in. Since those times progression in the field of molecular biology has proceeded at an astounding rate. The discovery of restriction endonucleases (Meselson and Yuan 1968; Roulland-Dussoix and Boyer 1969) and T4 ligase (Fareed and Richardson 1967) gave researchers the tools they needed to start manipulating life on the genetic level. With recombinant DNA techniques developed and the first gene being cloned in the mid 70's (Cohen, Chang et al. 1973; Rougeon, Kourilsky et al. 1975; Maniatis, Kee et al. 1976), the idea of gene therapy, the treatment of disease at the genetic level, started to emerge. This was demonstrated by the first gene therapy publications that brought the idea of treating disease with genetic material to the attention of both the scientific and public community (Aposhian 1970; Osterman, Waddell et al. 1971; Rogers 1971; Freese, Friedmann et al. 1972; Friedmann and Roblin 1972).

In its most basic form, gene therapy can be defined as the insertion of a functioning gene into the cells of an organism to correct an error of metabolism or to provide a new function in a cell. The first reproducible *in vivo* gene therapy experiment

Vectors Used in Gene Therapy Clinical Trials
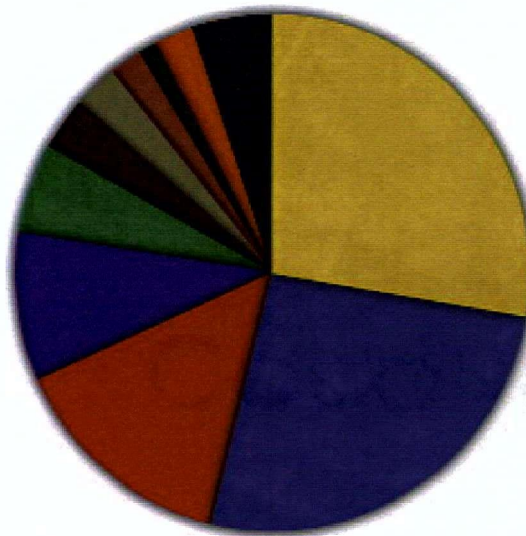
- Retrovirus 28% (n=254)
- Adenovirus 26% (n=240)
- Naked/Plasmid DNA 14% (n=132)
- Lipofection 9.3% (n=85)
- Pox virus 5.7% (n=52)
- Vaccinia virus 3.3% (n=30)
- Herpes simplex virus 2.8% (n=26)
- Adeno-associated virus 2.1% (n=19)
- RNA transfer 1.1 (n=10)
- Others 3% (n=23)
- N/C 5,1% (n=47)

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Indications Addressed by Gene Therapy Clinical Trials
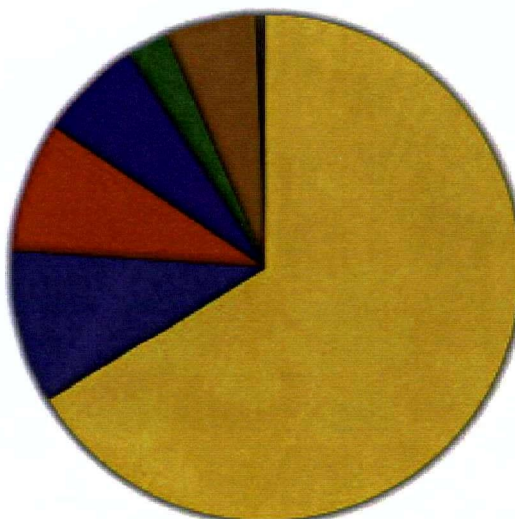
- Cancer diseases 66% (n=608)
- Monogeneic diseases 9.8% (n=90)
- Vascular diseases 8.3% (n=76)
- Infectious diseases 6.5% (n=60)
- Other diseases 2.6% (n=24)
- Gene marking 5.8% (n=53)
- Healthy volunteers 0.8% (n=7)

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Phases of Gene Therapy Clinical Trials
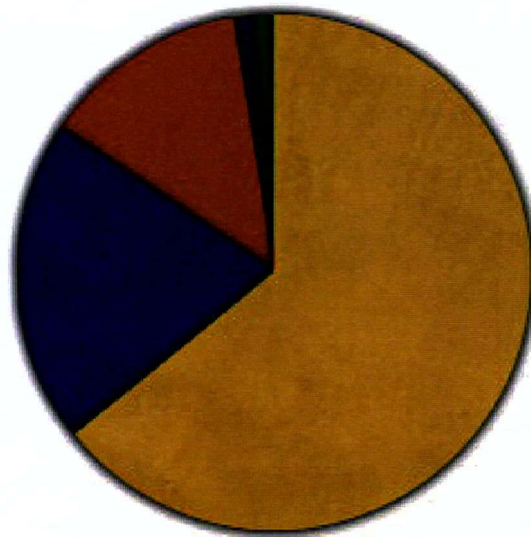
- Phase I 64% (n=589)
- Phase I/II 20% (n=185)
- Phase II 13% (n=120)
- Phase II/III 1% (n=9)
- Phase III 1.6% (n=15)

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Gene Types Transferred in Gene Therapy Clinical Trials
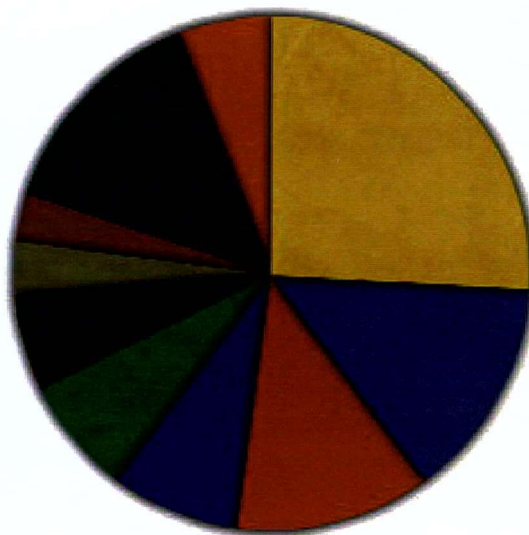
- Cytokine 26% (n=237)
- Antigen 14% (n=128)
- Tumor supressor 12% (n=113)
- Suicide 8.1% (n=74)
- Deficiency 7.8% (n=68)
- Drug resistance 6.1% (n=56)
- Receptor 3.4% (n=31)
- Replication inhibitor 2.9% (n=27)
- Others 14% (n=129)
- N/C 6% (n=55)

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Figure 1-1: A summary of gene therapy clinical trial information to date. Source:
http://www.wiley.co.uk/genmed/clinical/

was performed in Drosophila using transposable elements as carriers. Flies were 'cured' of a mutant eye color (Rubin and Spradling 1982).

Much research has been performed into ways of transmitting genetic material into cells. These vehicles for gene transfer have been termed 'vectors'. Gene therapy vectors can be divided into two major classes, viral and non-viral.

1.1.2 Viral Vectors

Ever since it was discovered that tumor viruses could cause cell transformation by inserting viral DNA sequences into host cells (Dulbecco, Sambrook et al. 1966; Dulbecco 1967; Sambrook, Westphal et al. 1968; Westphal and Dulbecco 1968; Dulbecco, Sambrook et al. 1969), viruses have been considered highly promising vectors for the transfer of genetic material into cells. In fact, the first human gene therapy experiment was performed using wild type Shope papillomavirus (Rogers, Lowenthal et al. 1973). In the early 70's while working with the Shope virus, Rogers discovered that cells infected with this virus, and in fact, even laboratory members working with the virus, had very low levels of arginine. He concluded that either the virus carried an arginase gene, or somehow activated a natural cellular arginase. When he was presented with two young German arginase-deficient girls, suffering from seizures due to extremely high systemic levels of arginine, he injected wild type virus in an effort to gain a therapeutic effect (Rogers, Lowenthal et al. 1973). There was no rigorous follow up of the two girls and no convincing evidence was found that the girls were either helped or harmed during the experiment (Terheggen, Lowenthal et al. 1975).

The use of recombinant DNA methods to produce recombinant virus vectors (Shimotohno and Temin 1981; Wei, Gibson et al. 1981; Tabin, Hoffmann et al. 1982)

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opened the door for a major advance in the area of viral gene therapy. Since that time, a number of different types of viruses have been used as gene transfer vectors (Figure 1-1).

1.1.2.1 Adenoviral Vectors

Adenovirus (Ad) is a non-enveloped, iscosahedral virus, 60-90 nm in diameter. It has a double stranded DNA (dsDNA) genome that is 30-40 kB in size (Epstein 1959; Horne 1962; Strohl, Rabson et al. 1967). Ad was first isolated from adenoid tissue in 1953, and since then, over 50 human serotypes have been identified. Ad has been shown to enter cells using the CAR (coxsackie and adenovirus receptor). Ad enters the cell via clathrin dependent, receptor mediated endocytosis (Wickham, Mathias et al. 1993; Li, Brown et al. 2001) (Figure 1-2). Some of the advantages of this vector are its high nuclear transfer efficiency, broad tissue tropism, and low pathogenicity (Buescher 1967; Meyer 1971). First generation Ad based vectors contained deletions that rendered the virus replication deficient; however they could only carry about 8 kB of genetic material (Fallaux, Bout et al. 1998; Danthinne and Imperiale 2000). Further research led to the development of high capacity, or 'gutless' Ad vectors. These vectors have no viral genes at all, and as a result, can carry up to 36 kB of foreign genetic material. However, they are entirely dependent on a helper virus for packaging (Kochanek 1999; Kochanek, Schiedner et al. 2001).

Other work using Ad based vectors include replicative, oncolytic adenovirus vectors. These are modified Ad viruses that are designed to only replicate in tumor cells (usually p53 deficient) (Alemany, Balague et al. 2000; Kirn, Hermiston et al. 2000; Kirn, Hermiston et al. 2001).

A disadvantage of Ad vectors is that while they are able to transduce non-
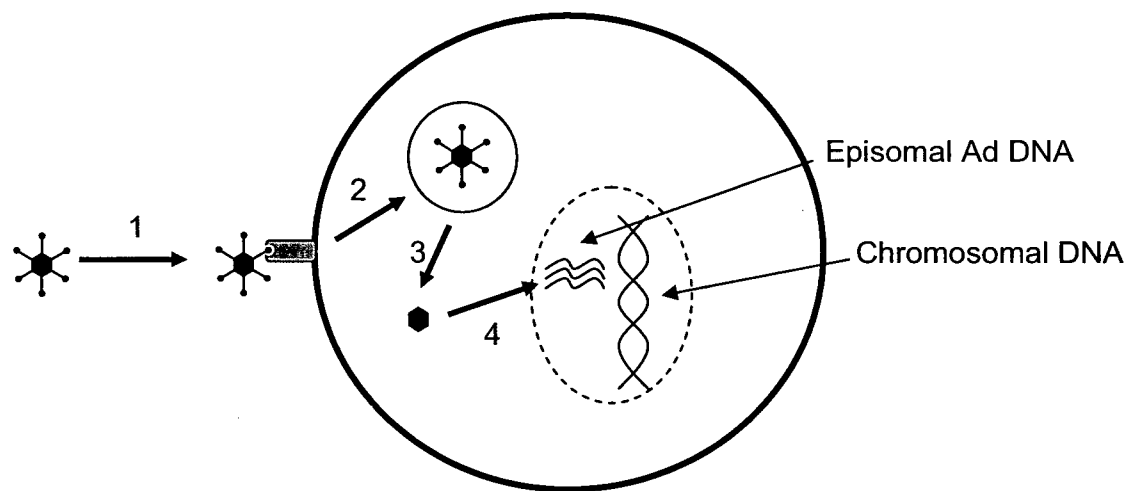
Figure 1-2: A diagram of adenovirus entry into a cell. The Ad virus binds to cell surface receptors (1), is internalized (2) and escapes from the endosome (3), and the DNA is imported into the nucleus where is remains episomal (4) (Modified from (Wolff 1994)).

dividing cells, the DNA stays in an episomal form, and therefore they are not efficient vectors for long-term expression. In addition, immune responses against the viral proteins limit re-application; however, use of different serotypes can help to overcome this problem. The first Ad clinical trial was in 1993, and to date, over 240 trials (26% of total gene therapy trials) (Figure 1-1) have been initiated using Ad vectors.

While Ad vectors have usually been well tolerated by patients in the clinic, it was an Ad vector clinical trial that led to the death of Jessie Gelsinger in 1999. This followed a very high dose of Ad vector, and his death was most likely caused by an Ad vector-induced shock syndrome due to a cytokine cascade that led to disseminated intravascular coagulation, acute pulmonary complications and multi-organ failure (Raper, Yudkoff et al. 2002). This adverse event cast a pall over the field of gene therapy (Somia and Verma 2000; Teichler Zallen 2000), and led to a reevaluation of gene therapy clinical trials, including vector dosage, safety, and toxicity.

1.1.2.2 Adeno-Associated Viral Vectors

Adeno-associated viral vectors (AAV) are based on the replication defective human "dependent' parvovirus. This virus requires co-infection with another virus (such as adenovirus) to result in a productive infection. The first recombinant adeno-associated viral vector (AAV) was made in 1989 by removing almost all of its genome and replacing it with a transgene. This virus was dependent on a helper cell line expressing the deleted AAV genes, as well as being infected with Ad virus in order to produce transduction competent particles (Samulski, Chang et al. 1989). It was not until 1992 that AAV viruses became commonly used tools for the transduction of mammalian cells (Muzyczka 1992). Some of the advantages of this vector system are

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that they demonstrate efficient gene transfer, are able to transduce non-dividing cells

(McCown, Xiao et al. 1996; Xiao, Li et al. 1996), are not associated with any known

human disease, and are able to integrate into a specific region on chromosome 19

(Kotin, Siniscalco et al. 1990). The disadvantages of this system include the difficulty of

production and purification from co-infected Ad virus, the small space for gene insertion

(~5 kB), and that some recombinant vectors have been shown to lose the nonrandom

integration property (Tenenbaum, Lehtonen et al. 2003). Currently, there have been 19

clinical trials (Figure 1-1) initiated using AAV vectors (2.1% of total gene therapy clinical

trials).

1.1.2.3 Retroviral Vectors

A retrovirus is a virus that uses RNA as its genetic material, and relies on a

reverse transcriptase to transcribe their RNA template into DNA, that can become

integrated into the host cells DNA. Retroviruses have long been used as gene therapy

vectors, with the first use of retroviral vectors in 1981 (Shimotohno and Temin 1981;

Wei, Gibson et al. 1981).

Moloney murine leukemia virus (Mo-MLV) is one of the most highly used

retrovirus vectors (Miller, Skotzko et al. 1992; Mulligan 1993; Crystal 1995). Mo-MLV is

an enveloped virus containing two single stranded RNAs (ssRNAs) in a nucleocapsid

structure. Both RNAs serve as a template to generate a dsDNA via reverse

transcriptase. The dsDNA then migrates into the nucleus and integrates into the host

genome (Figure 1-3). The advantages of this vector system are that the receptor for the

viral envelope protein is expressed on almost all mammalian cells, it is non-pathogenic,

able to integrate into the host genome, and the virus encodes its own strong promoter

and enhancer. A disadvantage of the system is that Mo-MLV based vectors are not

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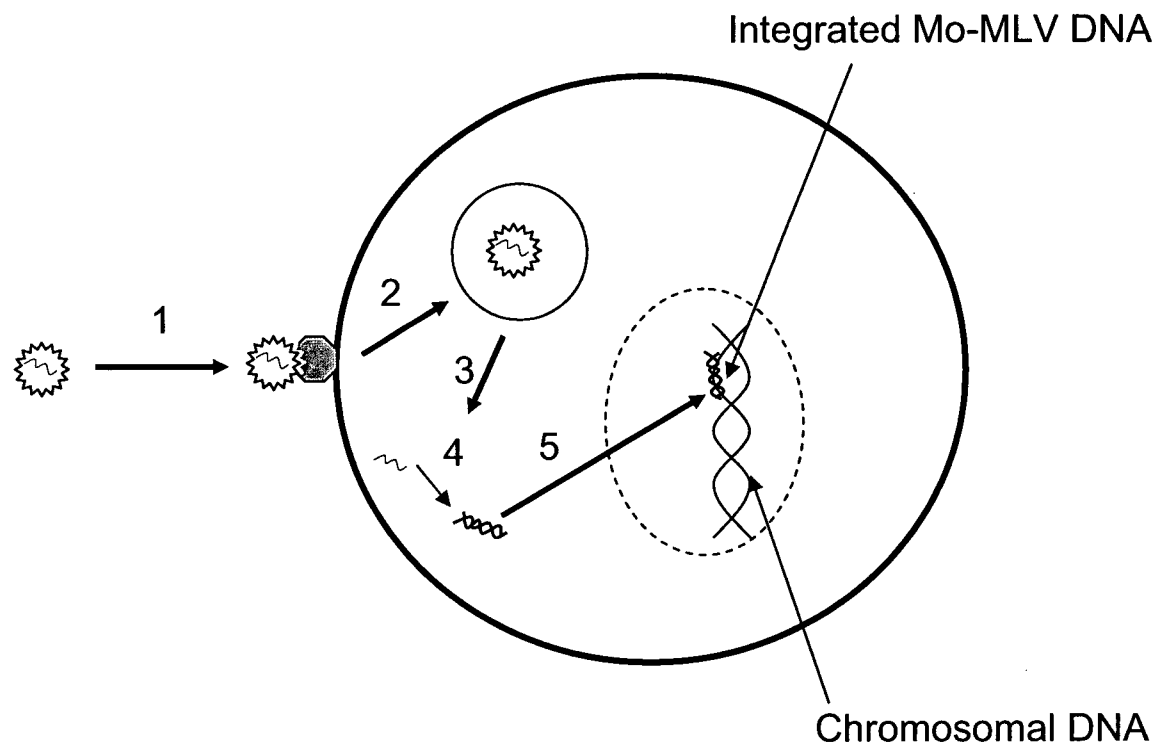
Figure 1-3: Molony murine leukemia virus vectors. These vectors bind to specific cell receptors (1), and are internalized (2). Once inside, the virus escapes from the endosome (3) and the viral RNA undergoes reverse transcription, converting it to DNA (4), where it then enters the nucleus and integrates into the host genome (4) (Modified from (Wolff 1994)).

able to transduce non-dividing cells (Roe, Reynolds et al. 1993). Retroviruses account for the highest percentage of clinical trial use at 28% of all clinical trials (over 250 trials) (Figure 1-1).

1.1.2.3.1 Lentiviruses

Lentiviruses are a special class of retroviruses that are able to transduce both dividing and non-dividing cells. This is due to the presence of three viral proteins, the matrix protein, Vpr, and integrase (Bukrinsky, Haggerty et al. 1993; Heinzinger, Bukinsky et al. 1994; Gallay, Hope et al. 1997). These proteins all contain a nuclear localization sequence that allows for import of the pre-integration complex into the nucleus. The most well studied lentiviral vector to date is the HIV-1 derived vector. By changing the envelope protein and removing genes essential for viral replication, this vector was first demonstrated by Naldini et al to transduce non-dividing cells both *in vitro* and *in vivo* (Naldini, Blomer et al. 1996). Lentiviral vectors also demonstrate long term, stable expression (Blomer, Naldini et al. 1997), making them very attractive vectors for viral gene delivery systems.

1.1.2.4 HSV Vectors

Herpes Simplex Virus (HSV) vectors are large, enveloped DNA viruses (Batterson 1985). They are the most complex of human viruses, carrying about 75 genes. The HSV genome is approximately 152 kB (McGeoch, Dolan et al. 1986), almost a third of which codes for non-essential viral replication proteins. By making specific deletions and using a packaging cell line, about 40 kB of the genome is available for carrying transgene cassettes.

1.2.2.5 Pox (vaccinia virus) Vectors

Pox viruses are double stranded DNA viruses that can infect both dividing and non-dividing cells. Similar to HSV, they have a large genome that can carry transgenes up to 25 kB in size (Smith and Moss 1983). The long history in the usage of these vectors as vaccines, their low toxicity, and high capacity for foreign DNA make them attractive candidates for gene delivery.

1.1.3 Non-viral Vectors

It was first discovered in the 1950' and 60's that cultured cells were able to take up radiolabeled DNA directly from the surrounding media (Gartler 1959; Sirotnak and Hutchinson 1959; Kraus 1961; Schimizu and Iwafuchi 1962; Rabotti 1963). These findings were further verified when it was demonstrated that adding purified viral genetic material onto cells in culture resulted in virus production from those cells (Klingler, Chapin et al. 1959; Dubes and Klingler 1961). These studies prompted interest in using non-viral methods of transferring genes into cells.

The first promising attempts at using a delivery system to deliver purified infectious viral nucleic acid *in vitro* used calcium phosphate (Graham and van der Eb 1973) or DEAE-dextran (Vaheri and Pagano 1965). These findings led to the second generation of non-viral gene delivery systems for plasmid delivery, such as liposomes (Fraley, Subramani et al. 1980), cationic lipids (Felgner, Gadek et al. 1987), polylysine/receptor mediated systems (Wu and Wu 1987), and the gene gun (Johnston, Anziano et al. 1988).

For *in vivo* applications, the most widely used non-viral gene delivery methods include injection of naked DNA (Wolff, Malone et al. 1990; Acsadi, Jiao et al. 1991; Jiao,

Acsadi et al. 1992; Jiao, Williams et al. 1992) and the use of DNA-cationic liposome complexes (Wu and Wu 1987; Curiel, Agarwal et al. 1991; Wagner, Zatloukal et al. 1992; Zatloukal, Schmidt et al. 1993; Zabner, Fasbender et al. 1995; Felgner 1997).

1.2 Lipids, Liposomes, and Lipid Polymorphism

Artificial lipid vesicles prepared from dispersions of phospholipids in aqueous media were first described in 1964 (Bangham and Horne 1964). Since this discovery, liposomes have been widely accepted as models of biological cell membranes and have important applications as drug (conventional or gene/protein based) carriers.

Membrane lipids are amphipathic, possessing hydrophobic and hydrophilic regions (see Figure 1-4). Within the bilayer, the hydrophilic head groups are oriented towards the aqueous environment, while the hydrophobic tails are facing each other.

Artificial lipid vesicles are generally designated by size and lamellarity (Figure 1-5). Multilamellar vesicles (MLVs) are generally large in size (>400 nm), while large and small unilamellar vesicles (LUVs and SUVs) are smaller in size (~100 nm or ~30 nm respectively) and contain only a single bilayer membrane. LUV's and SUV's can be prepared by a variety of techniques including sonication (Huang 1969), injection of lipids dissolved in ethanol or ether into aqueous buffer (Batzri and Korn 1973; Deamer 1978), and reverse phase evaporation (Szoka and Papahadjopoulos 1978). The most convenient and widely used method for producing LUVs today is via the extrusion technique. A comprehensive explanation of the extrusion technique is described in Hope et al, 1985 (Hope, Bally et al. 1985).

Molecular shape theories have been used to rationalize and predict the behavior of various lipids upon dispersion in aqueous buffer (Gruner, Cullis et al. 1985). Lipids with a large head group area and a small hydrocarbon area (e.g. detergents such as

Hydrophilic

Hydrophobic

Biological
Membrane
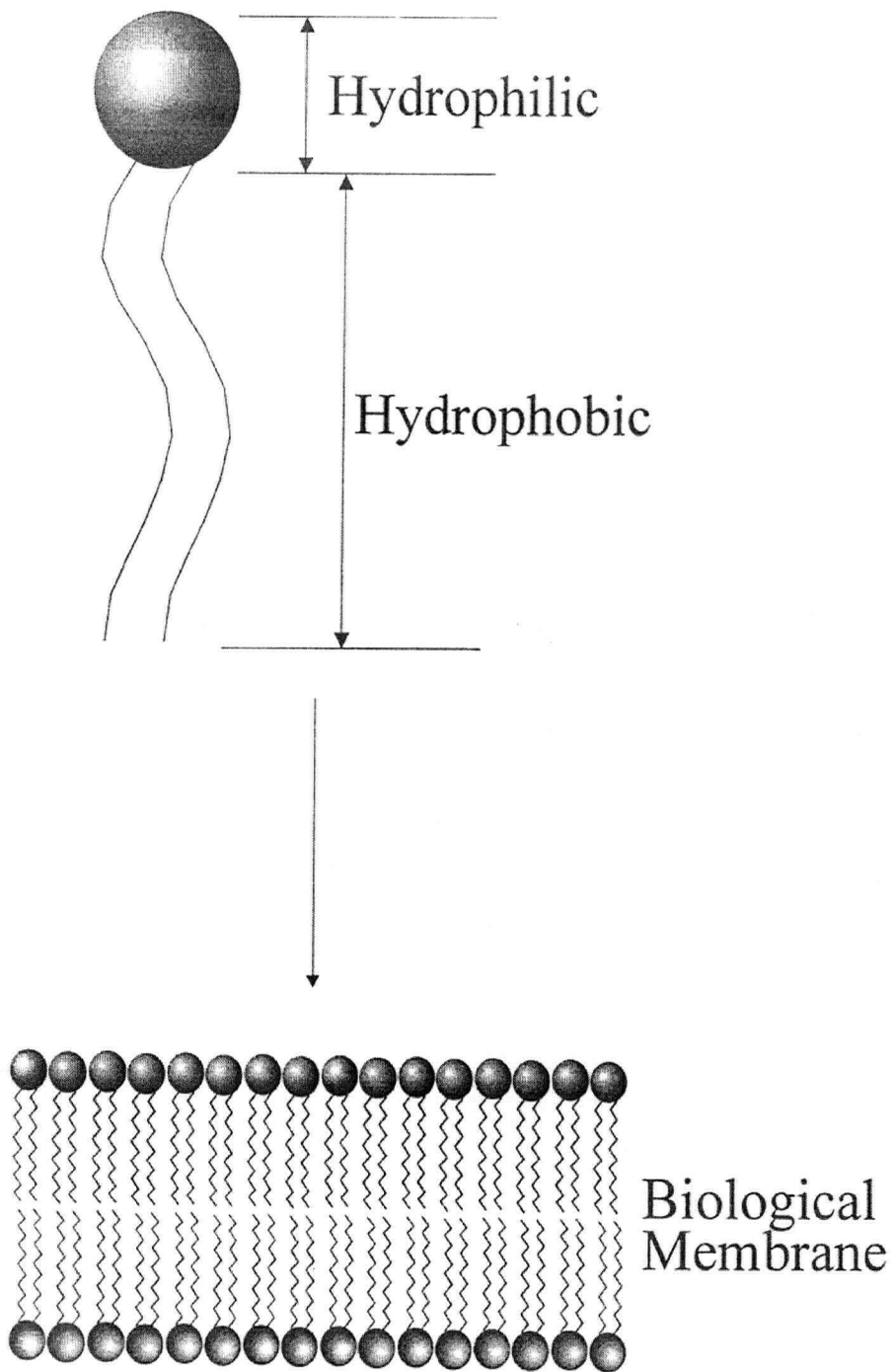
Figure 1-4: Schematic diagram of a typical lipid and how it self associates to form lipid bilayers.

Multilamellar vesicles (>400nm)

Large unilamellar vesicles (~100 nm)

Small unilamellar vesicles (~30 nm)
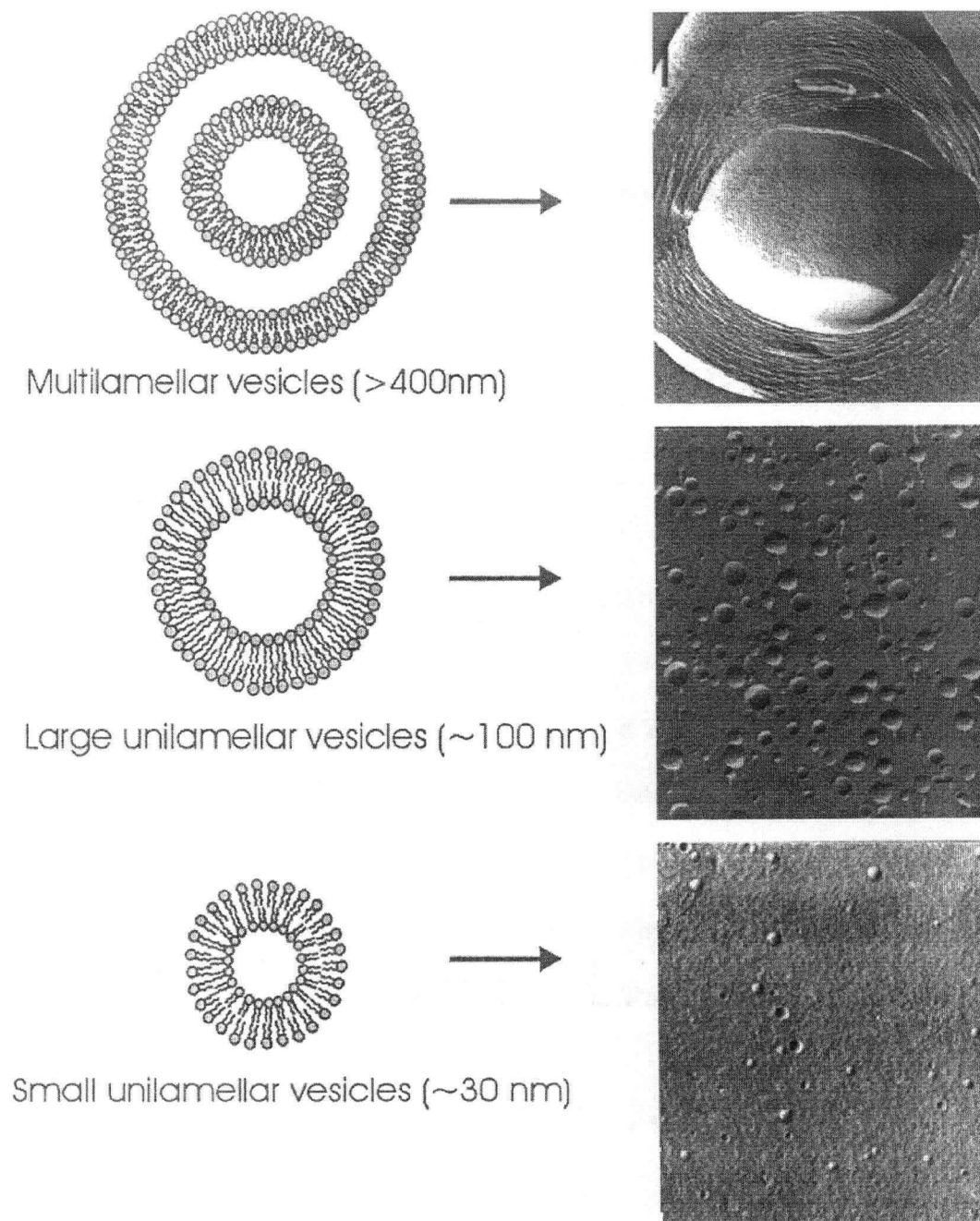
Figure 1-5: Classifications of liposomes. Schematic representation and freeze fracture electron micrographs of MLVs, LUVs and SUVs. The bar in the EM picture represents 200 nm.
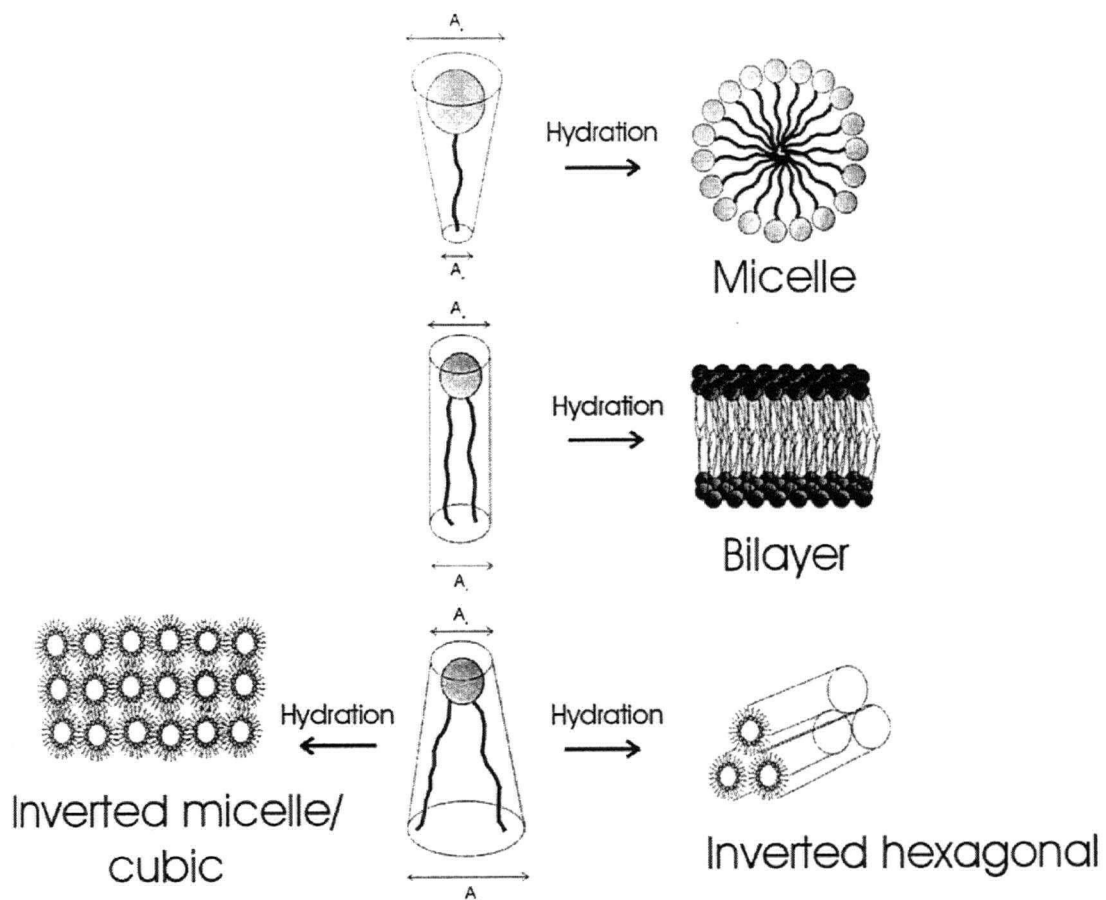
Figure 1-6: Molecular geometry of lipids and the predicted self-assembly of morphologically distinct structures.

Triton X-100), have a cone-like geometry and exhibit positive membrane curvature, self assembling into micelles (Figure 1-6). Lipids that are cylindrical in shape, having a ratio of the head group to hydrocarbon area nearly equal (e.g. DOPC), pack into lipid bilayers (Figure 1-6). Alternatively, lipids with a small head group area with a larger hydrocarbon area (e.g. DOPE) exhibit negative membrane curvature and adopt "inverted" lipid phases such as the cubic or inverted hexagonal (H_{II}) phase. These non-bilayer, H_{II} phase forming lipids are often known as "fusogenic" lipids as they help to promote membrane fusion, presumably by their ability to adopt structural intermediates involved in membrane fusion (reviewed in (Hafez and Cullis 2001)).

1.2.1 Cationic Lipids

Biologically, cationic lipids are very rare, appearing in nature only as sphingosine and stearylamine. Cationic lipids posses a positive charge in the head group. This allows for electrostatic interactions with polynucleotides. Since the first synthetic cationic lipids were prepared in the late 1970s (Kunitake 1977; Eibl 1979), there has been a large variety of synthetic cationic lipids prepared (see Figure 1-7). Cationic lipids are also thought to promote biological membrane destabilization through ion pairing with naturally occurring anionic lipids resulting in the formation of non-bilayer structures (Hafez and Cullis 2001). The inner endosomal membrane contains a high percentage of anionic lipids (18%) (Kobayashi, Stang et al. 1998), and formation of ion pairs with cationic lipids would be expected to exclude counter-ions and their associated water molecules, thus reducing hydration and resulting in formation of a cone-shaped zwitterion capable of adopting H_{II} phase structure, thereby promoting membrane fusion.

DODAC (N,N-dioleoyl-N,N-dimethylammonium chloride)

DOTAP (N-[2,3-(dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride)

DOTMA (N-[2,3-(dioleyloxy)propyl]-N,N,N-trimethylammonium chloride)

DDAB (N,N-distearyl-N,N-dimethylammonium bromide)

DMRIE (N-[2,3-(dimyristyloxy)propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide)

DC-CHOL (3-*B*-[N-(N`,N'-dimethylaminoethyl)carbamoyl] cholesterol)

DOSPA (2,3-(dioleyloxy)-N-[2-(spermine carboxamido)ethyl]-
N,N-dimethyl-1-propanaminium trifluoroacetate)

Figure 1-7: Structures of commonly used cationic lipids (Taken from (Palmer 2000))

1.3 DNA-cationic Liposome Complexes

The first use of a cationic liposome to deliver DNA was reported in 1987 (Felgner, Gadek et al. 1987). Since that time, the ability to use cationic liposomes as gene transfer vehicles has advanced significantly. The advantages of cationic liposomes as gene delivery vectors include their ease of manufacture via extrusion (Hope, Bally et al. 1985; Mayer, Hope et al. 1986), their spontaneous condensation with negatively charged DNA via electrostatic interactions, their interaction with negatively charged biological surfaces (e.g. glycoproteins and glycolipids containing negatively charged sialic acid residues on cell membranes), and their ability to aid in membrane disruption. This membrane disruption property is necessary to allow large molecules such as plasmid DNA to be delivered into the cytosol. (Felgner and Ringold 1989; Wattiaux, Jadot et al. 1997; Klemm, Young et al. 1998). It has been shown that higher charge ratios demonstrate more effective transfection, and that larger complexes generally give rise to higher transfection levels than smaller ones (Ross and Hui 1999).

DNA-cationic lipid complexes are heterogeneous in structure and size. There have been several structural models proposed (Figure 1-8). These include the "bead on a string" complexes (Felgner and Ringold 1989), lamellar complexes (Gustafsson, Arvidson et al. 1995), and the cylindrical complex model (Sternberg, Sorgi et al. 1994) . The 'bead on a string' complexes were first observed using metal shadowing EM (Gershon, Ghirlando et al. 1993). Evidence of lamellar complex formation came from cryo-TEM (Gustafsson, Arvidson et al. 1995), X-ray diffraction and optical microscopy (Radler, Koltover et al. 1997) (Spector and Schnur 1997). In this model, DNA is condensed by the cationic liposome and is trapped between fused lipid bilayers arranged in a typical multi-lamellar vesicle (MLV) pattern. Evidence for the cylindrical model arose from freeze fracture (Sternberg, Sorgi et al. 1994).

A   Bead on string complexes

Cationic liposomes

DNA

B   Lamellar complexes

Cationic lipid bilayers

DNA
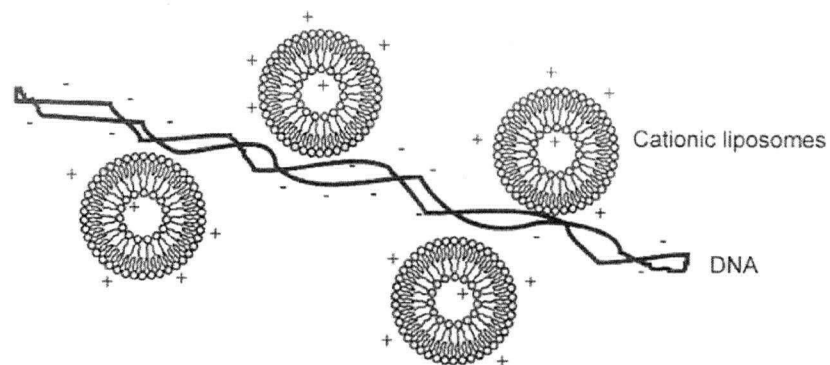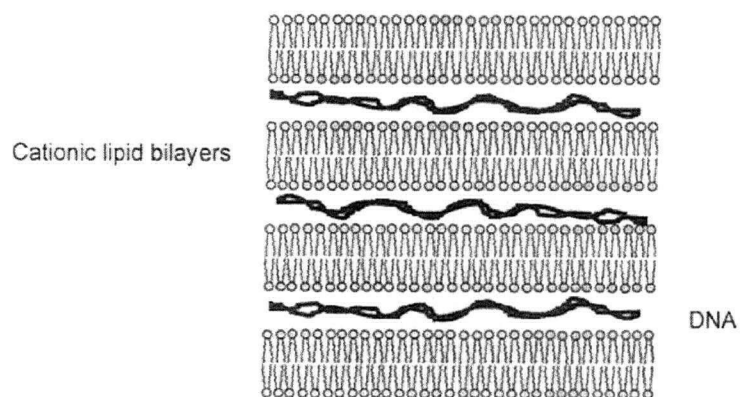
C   Cylindrical complexes
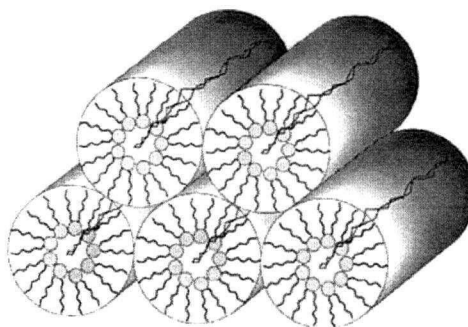
Figure 1-8: Diagrams of models of DNA-cationic lipid complexes.

There is currently no consensus as to which structure is responsible for mediating transfection.

While DNA-cationic liposome complexes demonstrate high levels of *in vitro* transfection (Felgner, Gadek et al. 1987; Felgner, Kumar et al. 1994), they also have a very large, heterogeneous size population, and a strong positive surface charge. Both of these features hamper their use for intravenous systemic gene delivery applications. DNA-cationic lipid complexes enter the cell via endocytosis. While it was once hypothesized that the complexes may fuse directly with the plasma membrane, studies have shown that this is not the case (Wrobel and Collins 1995). Further evidence for uptake via endocytosis has been generated employing protocols that modulate endocytosis pathways via ammonium chloride, hypertonic media, depleting cellular ATP levels, lowering temperature or changes in extracellular pH (Farhood, Serbina et al. 1995; Wrobel and Collins 1995; Stegmann and Legendre 1997). In addition, electron microscopy studies have given direct evidence for DNA-lipid complex uptake into endosomes (Zhou and Huang 1994; LabatMoleur, Steffan et al. 1996).

In order for genes to be expressed, the DNA must be released from its cationic lipid carrier (Zabner, Fasbender et al. 1995). Earlier studies have shown that free DNA is released into the cytoplasm from an early endosomal compartment (Zhou and Huang 1994; Friend, Papahadjopoulos et al. 1996). A likely hypothesis is that the free DNA is released into the cytosol when the cationic lipids dissociate from the DNA to associate with anionic lipids in the endosomal membrane. This results in the destabilization of the endosomal membrane and subsequent release of plasmid DNA into the cytosol.

Studies have also shown that the presence of the DNA carrier can affect the maturation of endosomes to lysosomes (Laurent, Wattiaux-De Coninck et al. 1999; Wattiaux, Laurent et al. 2000). In particular, endosomes containing cationic lipid

complexes are transported to and fuse with lysosomes much more slowly than normal endosomes (Wattiaux, Jadot et al. 1996). It has also been shown that not only the carrier system, but also the size of the complex affects the translocation process, with endosomes containing larger complexes being transported to, and fusing, with lysosomes more slowly than endosomes containing smaller complexes (Wattiaux, Laurent et al. 2000). This could be because larger complexes are taken up via phagocytosis. Phagosomes are known to mature and deliver their content to lysosomes more slowly than standard endosomes (Pitt, Mayorga et al. 1992; de Chastellier and Thilo 1997).

1.4 Systemic Gene Delivery Systems

Current gene delivery systems for systemic intravenous cancer gene therapy have met with limited success. A systemic gene delivery system showing extended circulation lifetimes, accumulation at distal tumor sites and significant target gene expression has not yet been developed. Upon systemic administration, viral gene transfer systems are rapidly cleared from the circulation (Huard, Lochmuller et al. 1995; Worgall, Wolff et al. 1997), resulting in accumulation in first pass organs such as the liver or lungs. In addition, the immunogenicity of viral systems results in an acute immune response that limits the effect of subsequent doses and can be hazardous to the patient (Worgall, Wolff et al. 1997). Non-viral systems, such as plasmid-DNA-cationic lipid complexes perform adequately *in vitro*, but due to their large size and positive surface charge lipoplexes are also rapidly cleared from circulation and are similarly limited to applications involving transfection of first pass organs (Hofland, Nagy et al. 1997; Templeton, Lasic et al. 1997). DNA-cationic lipid complexes have also been shown to exhibit significant toxicity (Li and Huang 1997).

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1.4.1 Liposomes as Carrier Systems

The most widely used clinical application of liposomes is for the delivery of anticancer drugs to solid tumor sites. These liposomes are small (~100 nm diameter), long circulating ($t_{1/2}$ >5 h), and these properties allow them to preferentially accumulate at tumor sites following intravenous (i.v.). injection (Gabizon, Price et al. 1990; Gabizon 1992). This tumor accumulation of liposomes is known as passive targeting, and is due to the increased permeability of the vasculature at these tumor sites (see Fig 1-9). Normal vasculature contains capillaries composed of a continuous lining of endothelial cells and have an uninterrupted sub-endothelial layer of basement membrane. Tumor vasculature is often described as hyper-permeable, due to large defects in the endothelial layer. These defects include fenestrations, widened inter-endothelial junctions, and in some cases, blood channels that have little or no endothelial lining at all (Dvorak, Nagy et al. 1988; Kohn, Nagy et al. 1992). In order for liposomes to target to tumor sites, they must be long circulating, either having a neutral surface charge, or containing a poly(ethylene glycol) (PEG) coating. The PEG coating acts as a shield, keeping plasma proteins from binding to the liposomes and preventing their rapid clearance (Allen, Hansen et al. 1991).

The promise of liposomes as conventional drug delivery systems has been demonstrated in the clinical use of various liposomal drug formulations, such as Doxil® or Calyx® (liposomal Doxirubicin for use in metastatic ovarian cancer and advanced Kaposi's sarcoma), or Vincristine®/Onco-TCS® (liposomal vincristine for use in non-Hodgkin's Lymphoma) (Maurer, Fenske et al. 2001).

From this information, it follows that DNA encapsulated within long circulating liposomes should lead in preferential accumulation at tumor sites due to passive targeting. It was this line of reasoning that lead to the formulation of Stabilized Plasmid

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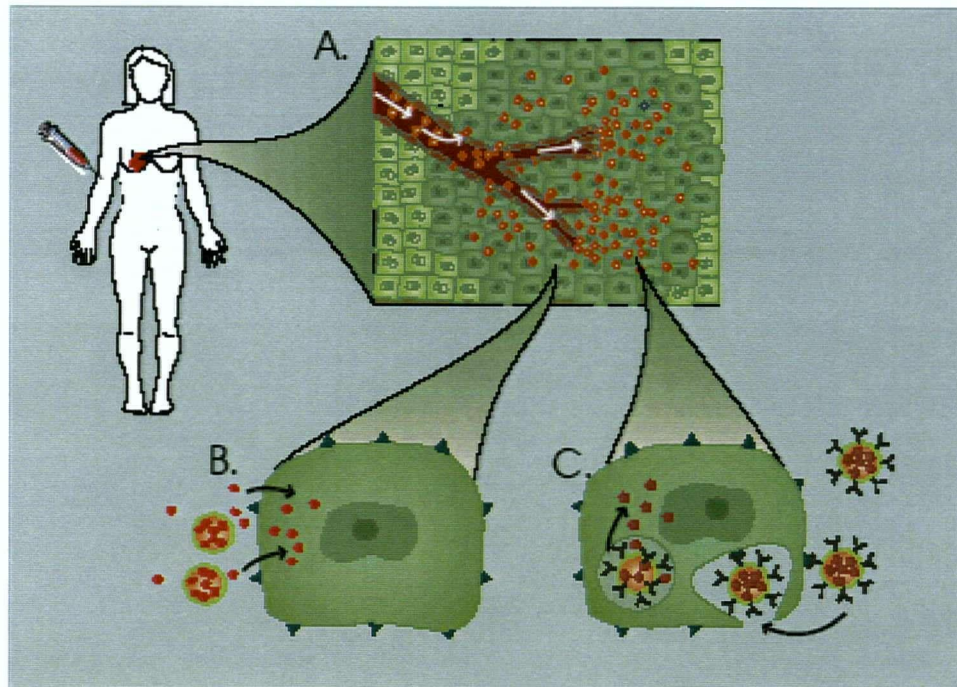
Figure 1-9: A schematic diagram depicting the passive accumulation of liposomal formulations at diseased tissues through the enhanced permeability and retention effect. **A.** Liposomes containing anticancer drug extravasate from the blood through gaps in vascular endothelial cells and accumulate in solid tumor tissue. **B.** Drug is released from the liposomes and taken up into cells. **C.** Liposomes containing nucleic acid-based therapeutics such as plasmid DNA or antisense oligonucleotides bind to the cell surface either through ligand-mediated binding to receptors or through charge-charge interactions, which triggers internalization of the liposomal formulation into endosomes. Some proportion of the encapsulated material escapes the endosome with subsequent intracellular release.

Lipid Particles, or SPLP (Wheeler, Palmer et al. 1999).

1.4.2 Stabilized Plasmid-Lipid Particles (SPLP's)

Previous work in this laboratory has resulted in a detergent dialysis procedure that allows complete encapsulation of plasmid DNA inside small (~70 nm), well-defined lipid vesicles (Wheeler, Palmer et al. 1999) (Figure 1-10). These vesicles contain fusogenic helper lipids (previously described in section 1.2) that are known to induce fusion with cellular membranes. Since fusogenic lipids do not readily form the bilayer structured needed for liposome formation they require stabilization by polyethylene glycol (PEG)-lipid conjugates.

1.4.2.1 Lipids used in SPLP Formulation

There are three primary lipids used in the composition of the SPLP particle (Figure 1-11). The most abundant of them is the fusogenic, non-bilayer forming phospholipid dioleyl phosphatidlyethanolamine (DOPE). DOPE is an important component of the SPLP particle as it helps to facilitate the fusion event required for escape from the endosome. A cationic lipid is also a very critical component of the SPLP particle. In our studies, we use the cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC). This cationic lipid was chosen due to its permanent positive charge at physiological pH (which simplifies the encapsulation process) and its ease of manufacture. Cationic lipids possess a positively charged head group, and it is this positive charge that allows the interaction and condensation of the

24
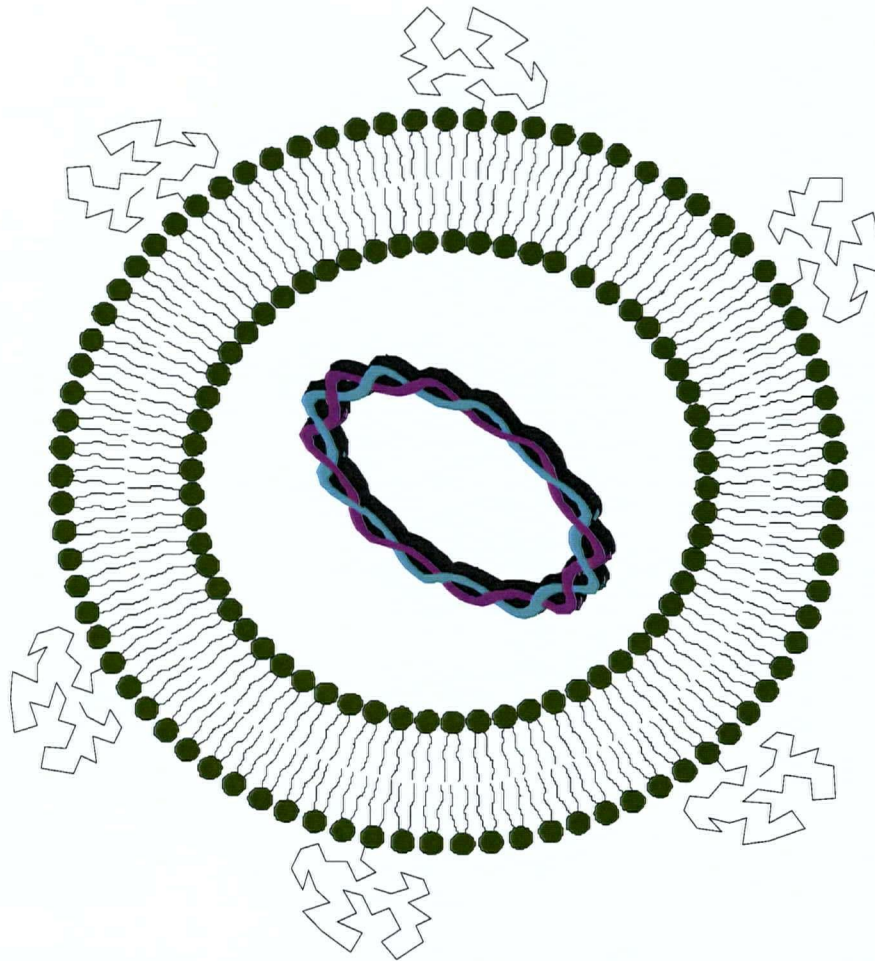
Figure 1-10: A schematic diagram of Stabilized Plasmid Lipid Particles.

plasmid DNA, driving the encapsulation process. In addition to encapsulating the DNA, the cationic lipids also play a role in helping to stabilize the DOPE, allowing a lipid bilayer to form. The third lipid component of the SPLP is the PEG-lipid. The PEG-lipids purpose is to stabilize the DOPE into a bilayer, as well as allow the liposome to avoid uptake and removal from circulation by the reticuloendothelial system (RES) (Klibanov, Maruyama et al. 1990; Allen, Hansen et al. 1991), which leads to dramatically increased circulation lifetimes. These PEG-lipids diffuse out of the membrane over time, leading to a more fusogenic particle (Wheeler, Palmer et al. 1999). The most commonly used lipid composition in our studies is 8:82:10 DODAC:DOPE:PEG-lipid, respectively.

Most foreign particles (including liposomes) are cleared by the RES via a process known as opsonization. This process involves the coating of the particle with either components of the complement system (such as C3b) or with antibodies. These coatings are then recognized by macrophages such as Kupffer cells in the hepatic system, leading to their uptake (Gordon 1995). While it was first thought that the PEG coating shielded the liposomes from opsonization, recent studies have found that PEG-stabilized liposomes are efficiently bound by plasma proteins, such as opsonins and dysopsonins (for a more complete review, see (Moghimi and Szebeni 2003). It is hypothesized that either the binding of dysopsonins (proteins that degrade opsonins), or that the opsonins are buried within the PEG layer, being inaccessible to interacting with cells of the RES, is responsible for the long clearance lifetimes (Moghimi and Szebeni 2003).

In vivo studies have shown that systemic administration of SPLP resulted in not only a significant accumulation of plasmid at distal tumor sites, but tumor transfection levels that are over 1000 times higher than any other tissue (Monck, Mori et al. 2000)
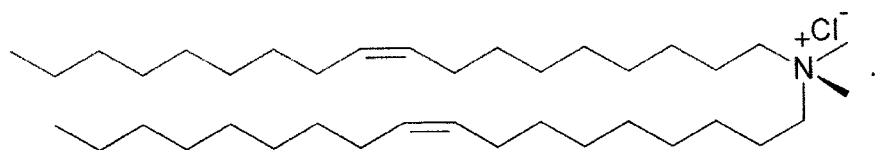
N,N-dioleyl-N,N-dimethylammonium chloride (DODAC)
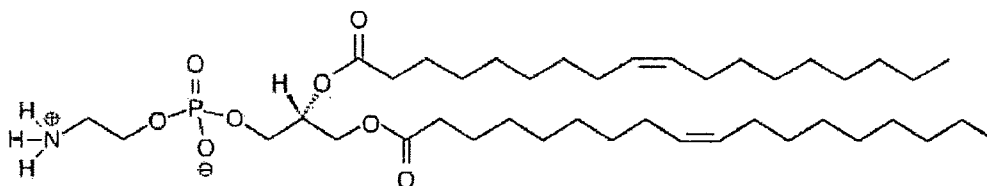
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)

C_8

C_{14}

C_{20}
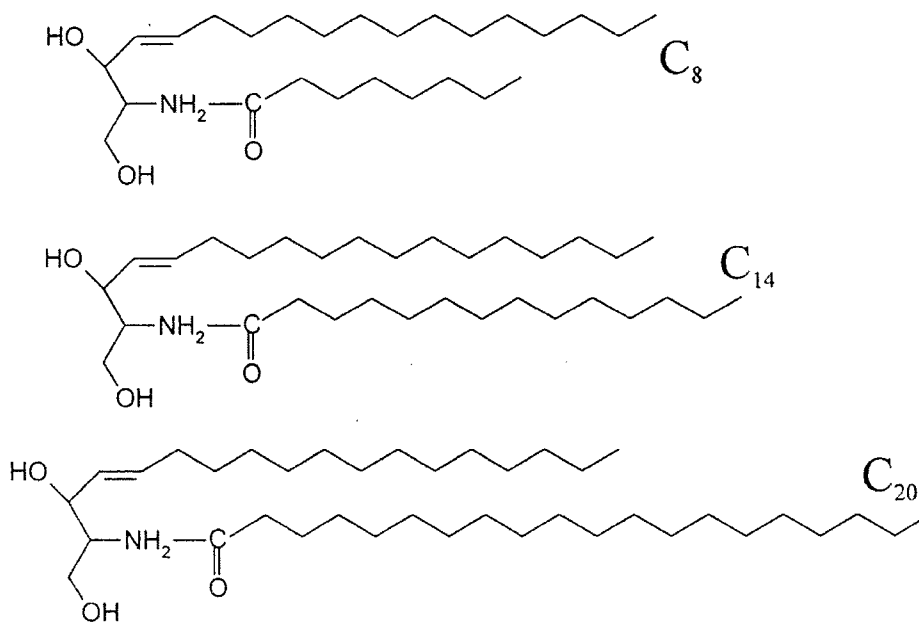
Ceramide structures

Figure 1-11: Structure of the major lipids used in the formulation of SPLP.
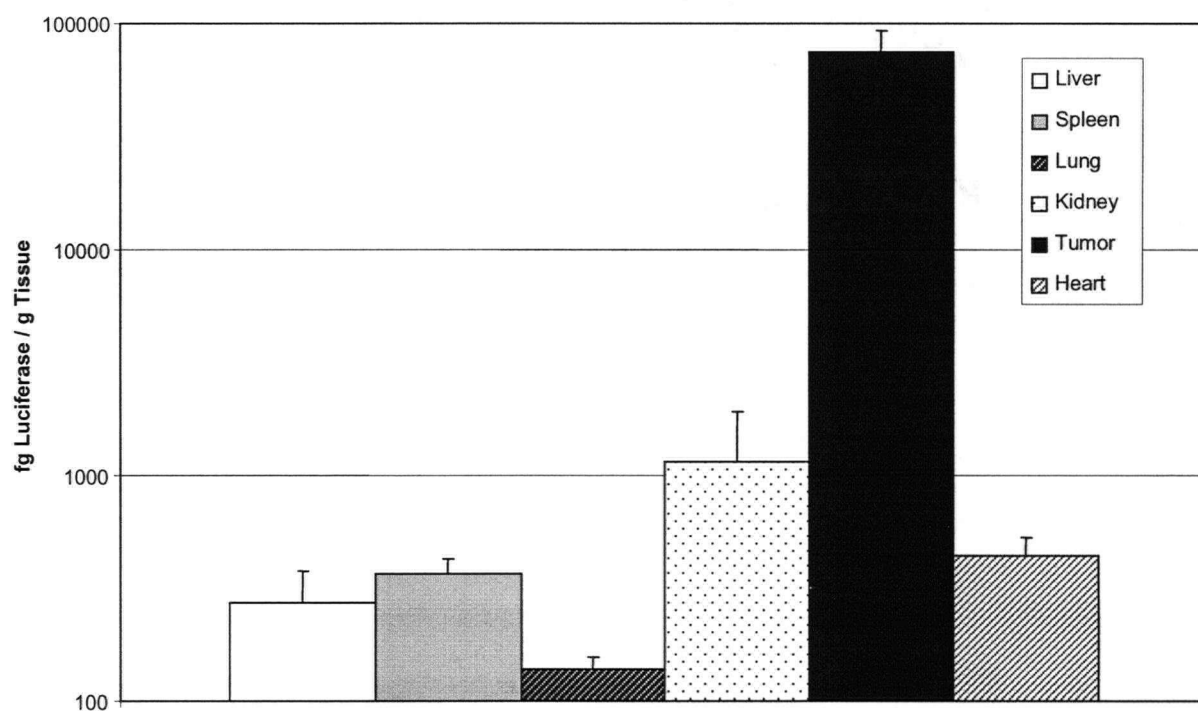
Figure 1-12: In vivo SPLP expression profile (at 24 h) following a single i.v. injection of 200 μg of SPLP containing a CMV-Luciferase plasmid in an A/J mouse, hind flank Neuro-2a tumor model.

(Figure 1-12). There are enough copies of plasmid delivered to the tumor site after a single intravenous dose of SPLP in tumor bearing mice to allow for more than 1000 plasmids per tumor cell. In spite of these encouraging results, a major limitation of these systems is that the gene expression levels obtained *in vivo* are quite low.

1.5 Transcription and Translation

1.5.1 Transcription

In eukaryotic cells, the majority of protein coding genes are transcribed by RNA polymerase II (RNAPII). RNAPII is unable to recognize gene promoters on its own, and relies on protein-protein interactions to recruit RNAPII to the promoter region. Specific transcription factors (TFs) are responsible for this recruitment. A number of TFs play a role in transcription. The major factors are TFIID (binds to TATA box), TFIIA (bound to TFIID as well as upstream DNA-binding proteins, such as those bound to enhancers), TFIIB, TFIIF and TFIIE, all of which contribute to stimulating transcription or to ensure correct initiation of transcription (Russell 1996).

1.5.2 Translation

Translation is the process by which the protein coded in the mRNA transcript is read and synthesized. The initiation of translation is complex, and involves the association and co-ordination of many factors. The association of the initiation factors eIF4A, eIF4E and eIF4G form the complex eIF4F, which is then able to bind to the 5' cap of the mRNA transcript along with eIF4B. This binding allows the recruitment of another complex, composed of the 40S ribosomal subunit, the eIF3, and eIF2 (bound to GTP and Met-tRNA) to the 5' cap. This complex is able to scan the transcript for the
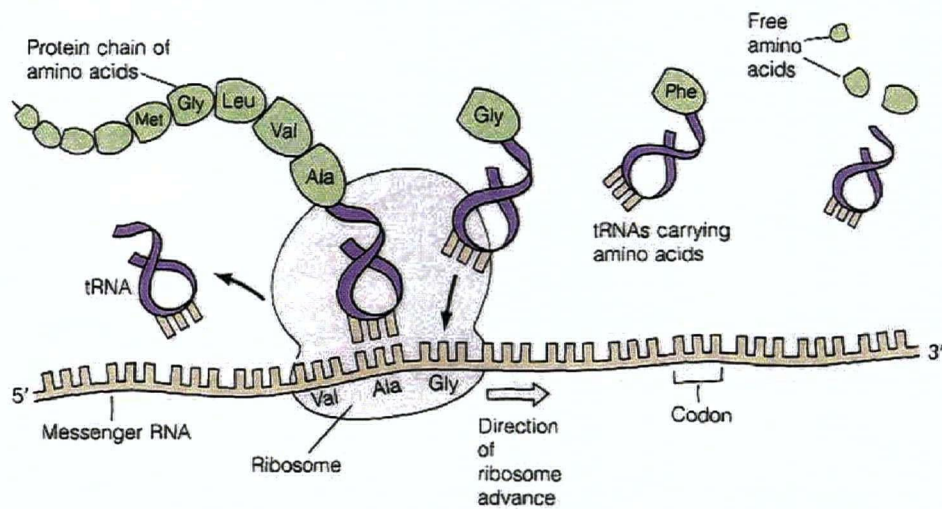
Figure 1-13: A diagram of translation. Incoming charged tRNAs with the appropriate anti-codons are brought into the ribosome and the amino acid they carry is added to the growing polypeptide chain (Russell 1996).

appropriate start codon (AUG), where the 60S subunit is recruited, forming a functional ribosome (80S) that is able to start synthesizing protein (Gallie 1998) (Figure 1-13).

1.6 Plasmids, DNA, mRNA

Plasmids are small, circular molecules made up of DNA. DNA is composed of a sequence of nucleotides joined together via phosphodiester linkages between the 5' hydroxyl group of the deoxyribose moiety on one nucleotide and the 3' hydroxyl group of another nucleotide. Nucleotides are composed of a purine (A or G) or pyrimidine (C or T) base attached to a deoxyribose sugar via a β-glycosidic bond and a phosphate group. The strands are linked together via hydrogen bonding between purine and pyrimidine bases. Double stranded DNA is stabilized by hydrophobic interactions resulting from base stacking within the helix (Figure 1-14). *In vivo*, a DNA molecule is usually found as a double helix possessing two nucleotide strands running anti-parallel.

Lederberg first coined the term plasmid in 1952 (Lederberg 1952) by studies on *E. coli* genetic factors that were not found on the chromosome. In more recent times, plasmids have become not only a workhorse of molecular biology, but also an integral part of non-viral gene therapy vectors. This can be attributed to their variable size (can carry from 1 ~ 30 kB of genetic material), their ease of isolation (Sambrook 1989), scale up, and manufacture.

Plasmids for gene therapy typically possess a number of essential discrete elements. They contain an origin of replication (such as the ColE1), that is responsible for plasmid replication in bacteria, as well as controlling the copy number, thereby having an impact on plasmid yield. Plasmids also contain an antibiotic resistance gene (such as kanamycin resistance) that allows selection for bacteria containing the desired plasmid. Plasmids for gene therapy also contain a promoter sequence, driving

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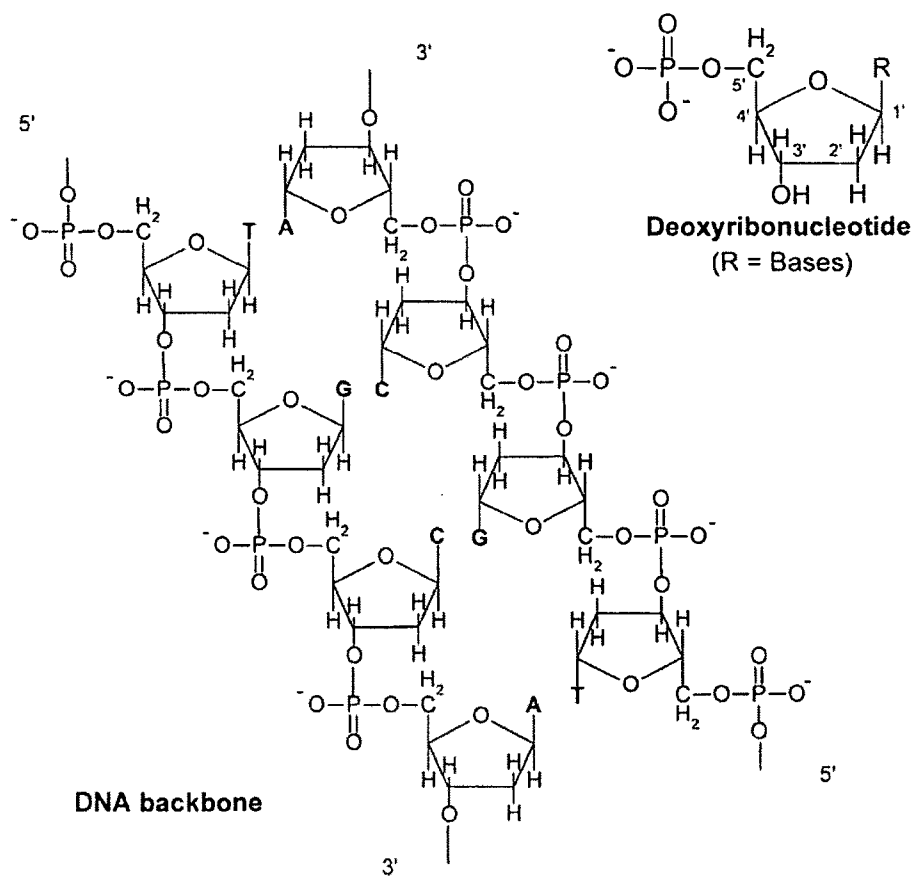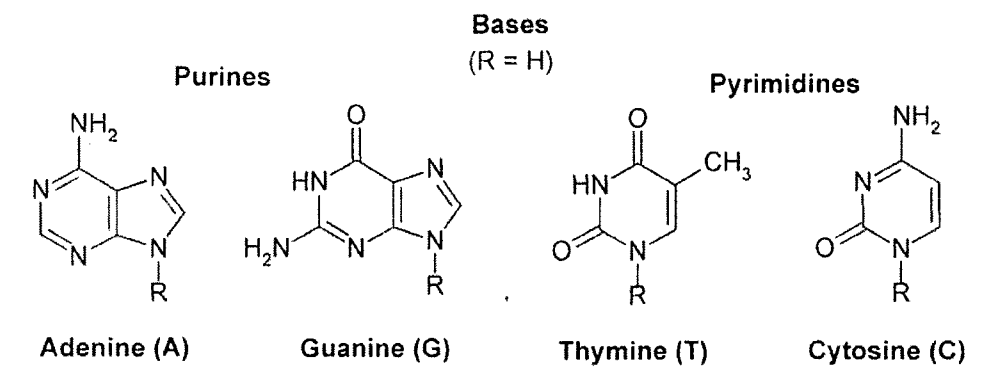
Figure 1-14: Structure of DNA (Taken from (Mok 1998))

expression of a protein coding gene, either a therapeutic gene (e.g. thymidine kinase), or a reporter gene (e.g. luciferase).

1.6.1 Eukaryotic Transcription Unit

The canonical transcription unit (encoding for a protein) is composed of many elements, such as the promoter, structural gene, enhancers, etc. The structural gene refers to the DNA unit that only encodes for a specific polypeptide chain (Jacob and Monod 1961). While this is an integral part of the transcription unit, a gene on its own will not be expressed without a promoter. The core promoter is usually 30-40 nt 5' to the start of transcription. This region contains the TATA box. This is the minimal element necessary for transcription. Proteins such as TBP (TATA-binding protein) bind to this region (Nikolov, Hu et al. 1992), and also interact with other proteins called TAFs (TBP-associated factors) (Greenblatt 1992). This complex eventually leads to the recruitment and positioning of the RNA polymerase, so that transcription can take place (Roeder 1991). Immediately upstream of the core promoter are located the Upstream Activation Sequences (UAS). This region can contain numerous binding sites for various transcription factors and activators.

Enhancers are required for maximal transcription of the gene to occur. They can be located quite far (over 1kB) away from the core promoter, can be found 5' or 3' to the promoter, and are able to act in either orientation (Khoury and Gruss 1983).

Silencers are control regions of DNA that, like enhancers, may be located very far from the core promoter, however, when transcription factors bind to them, expression of the gene they control is repressed, instead of enhanced (Brand, Breeden et al. 1985).

1.6.2 Promoters

The CMV promoter is one of the strongest promoters found in nature. It drives expression of the immediate-early genes of human cytomegalovirus (CMV) (Boshart, Weber et al. 1985; Foecking and Hofstetter 1986). This promoter/enhancer unit is the most commonly used promoter as very high expression levels are seen both *in vitro* and *in vivo*, and among different cell lines (Cheng, Ziegelhoffer et al. 1993; Wenger, Moreau et al. 1994; Tsan, White et al. 1995; Liu, Cashion et al. 1997) (Figure 1-15). It is for this reason that the CMV promoter was chosen as the primary nuclear promoter for the experiments in this thesis. The CMV promoter is down regulated by hyper-methylation (Scharfmann, Axelrod et al. 1991; Challita and Kohn 1994; Rettinger, Kennedy et al. 1994), and is sensitive to the levels of various transcription factors (e.g. increases in NFκβ leads to an increase in CMV expression (Lee, Sohn et al. 2004)).

In this thesis, the effect of two other promoters, the Rous Sarcoma Virus (RSV) promoter (Norton and Coffin, 1987), and the Simian Virus 40 (SV40) promoter (Bensimhon, Gabarro-Arpa et al. 1983; Luciw, Bishop et al. 1983) are examined. Studies comparing these promoters have found that there is often a dramatic difference in expression levels depending on the cell line used (Wenger, Moreau et al. 1994; Liu, Cashion et al. 1997; Zarrin, Malkin et al. 1999). In a recent study by Zarrin et al, it was found that for most cell types used, the CMV promoter gave much higher expression than either the RSV or the SV40 promoter. In fact, the SV40 promoter often demonstrated little if any expression in some cell lines.

```
-603                              RSRF
ATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTC


    CArG/SRF                                        GC-rich
ATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGA


SP1          SP1/AP2     CREB        CREB
CCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCC


     NFκB         CREB                                E - Box
AATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGG


      E - Box     E - Box         GC-rich       CREB     CREB
CAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAA


  GC-rich                                     NFκB
TGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTA


CATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATG


                               NFκB                    CREB
GGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAAT


                      NFκB                  Long SP1
GGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGC


(SP1)  CREB E - Box SP1                       TATA-Box
CCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTC


      +1  ---> mRNA Transcript
GTTTAGTGAACCG TCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAG
```

Figure 1-15: Sequence of CMV promoter. This shows 603 bp of the promoter and the first part of the transcribed message. Note the location of the TATA box and the numerous upstream activation elements that act as binding sites for numerous transcription factors (Taken from (Wolff 1994)).

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1.6.4 Capping

The 5' ends of eukaryotic mRNA transcripts are modified by the addition of a 5' cap. This capping procedure involves the addition of a 7-methyl guanosine nucleotide to the 5' terminal in an unusual 5'-5' linkage, and the addition of two methyl groups to the first two nucleotides of the RNA chain. Capping occurs in the nucleus during transcription when the transcript is around 30 nt long and is catalyzed by the capping enzyme (a guanylyltransferase) (Shatkin 1976). In addition to being the start of translation, the cap site also helps to stabilize the mRNA transcript (Coutts and Brawerman 1993; Ptushkina, von der Haar et al. 1999).

1.7 Cytoplasmic Expression Systems

1.7.1 Background

One strategy to increase levels of gene expression following non-viral cell transfection involves the use of a cytoplasmic expression system (Gao and Huang 1993). A cytoplasmic expression system is one that, as the name suggests, is able to mediate the expression of genes in the cytoplasm, as opposed to the nucleus. The advantage of such a system is that it bypasses the need for nuclear delivery of plasmid DNA, a major obstacle in present day gene therapy. The efficiency of nuclear delivery following intracellular delivery is very low and is dependent on the size of the plasmid DNA molecule (Hagstrom et al, 1997). Studies where plasmid DNA was microinjected into either the cytoplasm or the nucleus of cells demonstrated that less than 0.1% of the total cytoplasmic plasmid enters the nucleus (Lechardeur, Sohn et al. 1999). The addition of nuclear localization signals to plasmid DNA has been shown to enhance transfection, but with limited success (only a 3-fold increase in expression) (Aronsohn

and Hughes 1998). The primary barrier to nuclear delivery of plasmid DNA is thought to be the nuclear membrane, as plasmid DNA enters the nucleus more efficiently in mitotic or dividing cells, during the breakdown of the nuclear envelope (Coonrod, Li et al. 1997). As a result, gene expression following transfection is much higher in dividing versus non-dividing cells (Vitadello, Schiaffino et al. 1994; Mortimer, Tam et al. 1999). A further limitation of nuclear expression systems is the finite, saturable limit to the amount of DNA that can be taken up by the nucleus under any condition (Brisson, Tseng et al. 1999).

1.7.2 Phage RNA Polymerase

These limitations have led to the development of strategies that do not require nuclear localization of DNA. One of these involves the use of bacteriophage RNA polymerase proteins (phage RNAP). The phage RNAPs are single polypeptide enzymes that mediate transcription of phage genes in the cytosol of infected bacteria (Chamberlin, McGrath et al. 1970). Early on it was discovered that these phage RNAP proteins were able to also mediate transcription in the cytoplasm of eukaryotic cells with high promoter specificity and efficiency (Davanloo, Rosenberg et al. 1984). These properties have facilitated the development of a phage RNAP based cytoplasmic expression system. While most of the field has focused on the T7 RNAP (Davanloo, Rosenberg et al. 1984), this thesis also investigates the T3 as well as the SP6 RNAP proteins (Kupper, McAllister et al. 1972; Butler and Chamberlin 1982).

1.7.3 Initial Cytoplasmic Expression Systems

The T7 RNAP-based system requires cytoplasmic delivery of both a plasmid construct containing a gene of interest under transcriptional control of the T7 promoter and a source of T7 polymerase. Initial studies involved co-transfection of cells with plasmids carrying T7-controlled genes and purified T7 RNAP protein. These systems were able to bypass the need for the nuclear transcription machinery and yielded high levels of gene expression, however, due to the instability of the T7 RNAP protein, the resulting gene expression was short lived, and considerable T7 RNAP associated cytotoxicity was observed (Gao and Huang 1993). These studies led to the use of a T7 polymerase autogene, first described in 1991 by Dubendorff and Studier (Dubendorff and Studier 1991). This system consists of a T7 RNAP gene driven by its own T7 promoter, along with a reporter gene, also driven by the T7 promoter (Figure 1-16). When cells were co-transfected with these constructs and purified T7 RNAP protein, rapid and sustained levels of reporter protein were detected. The T7 autogene was able to replenish its supply of T7 RNAP, resulting in sustained gene expression (Chen, Li et al. 1994). While these autogenes are effective, the transfection cocktail is difficult to prepare and in practice has been shown to be cytotoxic. To overcome these problems, a dual promoter autogene was created in 1999 (Brisson, He et al. 1999). This construct contained a T7 RNAP gene under control of both T7 (cytoplasmic) and CMV (nuclear) promoters. The basis of this construct is that when plasmid was taken up into the nucleus it would result in some T7 RNAP protein being produced. This RNAP in turn would be able to transcribe the cytoplasmic plasmid (the major portion of plasmid in the cell), which would then lead to further expression of the T7 RNAP. Theoretically, one plasmid reaching and expressing from the nucleus
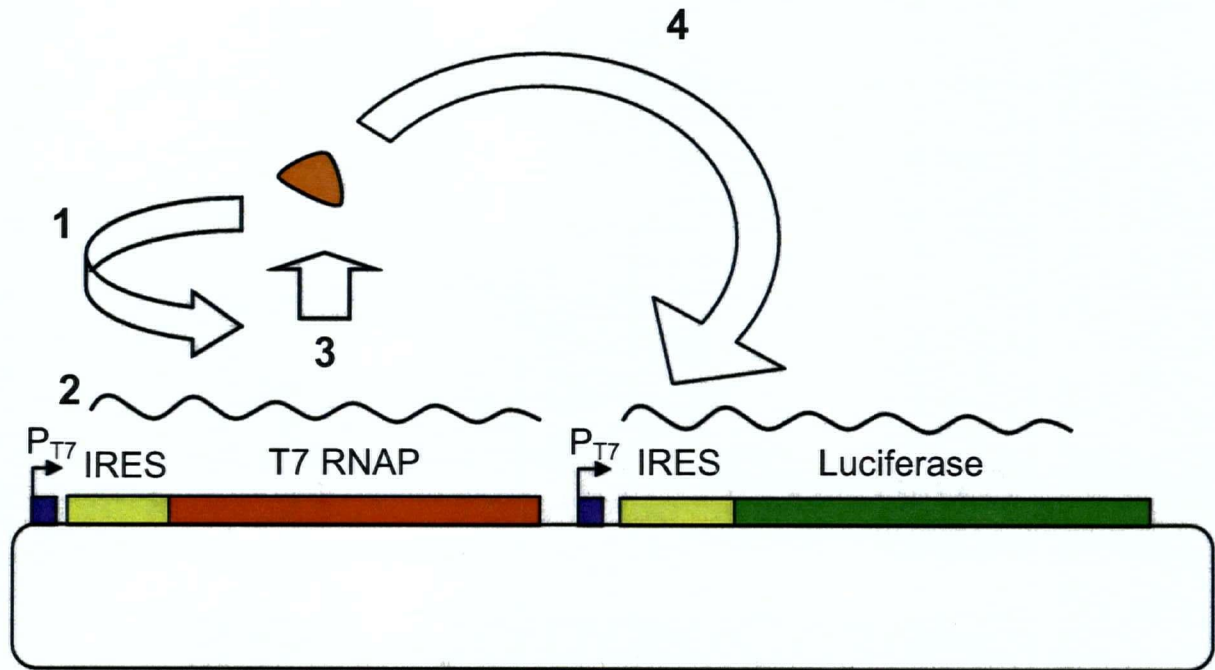
Figure 1-16: A diagram of the T7 RNAP autogene. An initial source of T7 RNAP in the cell binds to the T7 promoter (1) and transcribes some T7 RNAP mRNA (2). The mRNA is translated into T7 RNAP protein (3), which is then able to transcribe more T7 RNAP mRNA (1, 2), setting up an autocatalytic, exponentially increasing system. In addition, the T7 RNAP produced is able to transcribe mRNA of a reporter or therapeutic gene, either on the same plasmid or on a separate plasmid (4), allowing for high levels of transgene expression.
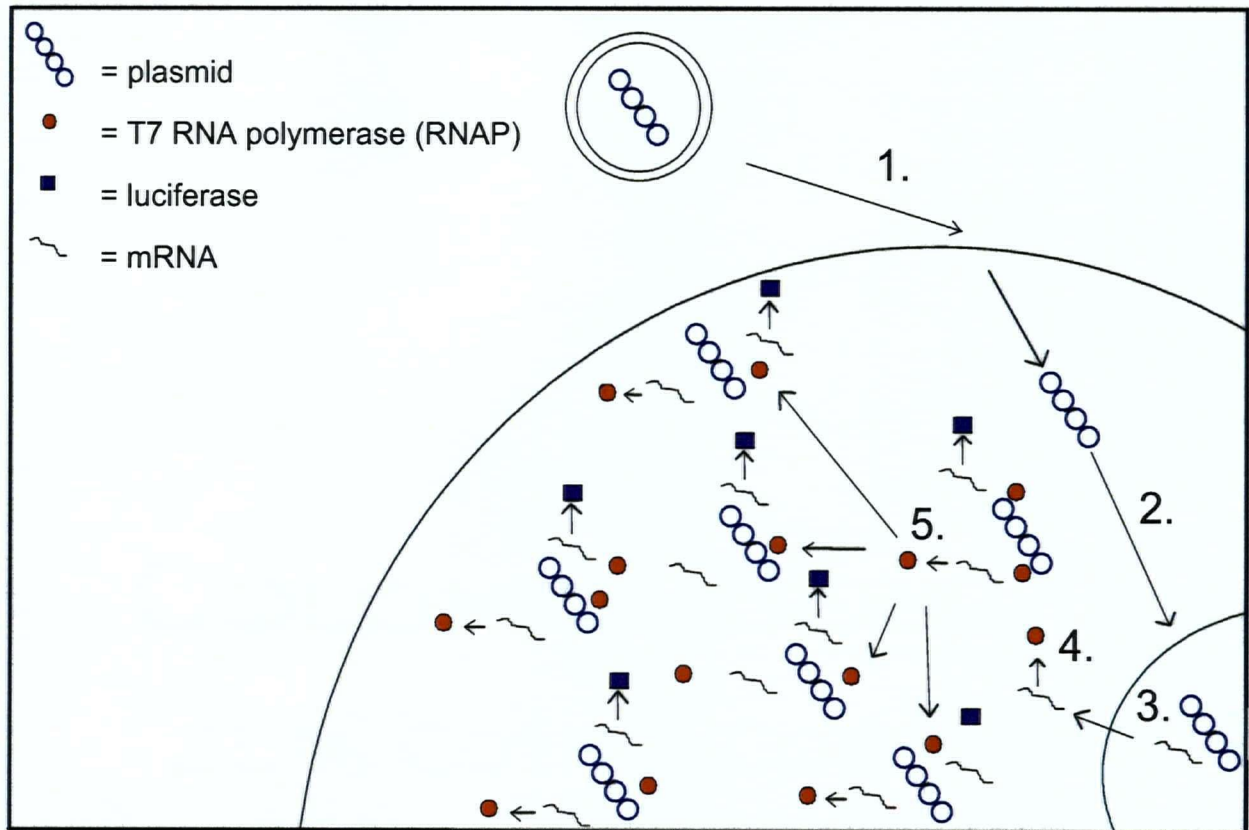
Figure 1-17: A schematic diagram of the cytoplasmic expression system. The plasmid DNA (circle) is delivered to the target cell via a carrier system (e.g. lipoplex, SPLP) (1). Upon delivery and release of plasmid, a small percentage of DNA will enter the nucleus (2), where the transcription machinery will make a T7 RNAP mRNA transcript (using the CMV promoter), which is then exported into the cytoplasm (3). In the cytoplasm the mRNA is translated into T7 RNAP protein (red circle) (4), where it can then transcribe T7 RNAP and target gene mRNA from cytoplasmic plasmids (via the T7 promoter). This in turn creates more T7 RNAP and more target protein (blue box) (5), which amplifies the cascade until all plasmids are being transcribed, or the cytoplasmic transcription machinery is being used to its full extent.

would be sufficient to activate and induce high levels of gene expression from thousands of cytoplasmic plasmids (Figure 1-17). While this system did represent an advance in the field, there were some problems that had not been addressed. First, they had the autogene and reporter gene cassette on separate plasmids, necessitating the co-delivery of two plasmids in order to observe gene expression. In addition, they only observed a 2-3 fold increase in gene expression. This small increase was most likely due to the fact that the autogene cassette that they were using did not contain an IRES element (described below), and therefore the cytoplasmic transcripts (lacking the 5' cap) were not efficiently translated.

1.7.4 Internal Ribosome Entry Sequence Elements

Internal Ribosome Entry Sequences (IRES) are elements that have been shown to drive expression of the second gene in a bi-cistronic mRNA transcript, as well as increase the translation of un-capped transcripts. IRES elements were first isolated from viral genomes, such as the encephalomyocarditis virus (EMCV), where they allow the translation of viral mRNA transcripts, made in the cytoplasm, and thus lacking the 5' cap structure essential for mRNA translation (Jang, Pestova et al. 1990). Various viral IRES elements, such as the EMCV, FMDV (foot and mouth disease virus), and other picornavirus based IRES elements share similar features. All are approx 450 bp long and share a conserved secondary structure, as well as a pyrimidine-rich tract that starts ~25 bp before the 3' end of the IRES (Agol 1991; Jackson, Hunt et al. 1994; Jackson and Kaminski 1995). The secondary structure is hypothesized to allow for proper alignment of ribosome subunits and other co-factors necessary for translation. Other IRES sequences (e.g. Gtx) are much shorter than picornaviral IRESs and contain

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sequences that are complementary to rRNA (Chappell, Edelman et al. 2000). These IRES work in a different fashion than the picornaviral IRES and the studies in this thesis (described in Chapter 3) show that these short rRNA complementary IRES sequences are not effective in driving expression from un-capped mRNA. Other mammalian IRES sequences (such as the one driving expression of mRNA encoding eIF4G) contain a polypyrimidine tract and some structural similarities to the EMCV IRES, however are shorter than picornaviral IRESs (~100 bp) (Gan, LaCelle et al. 1998). Similar to the rRNA complementary IRES elements, it is shown in this thesis that the eIF4G IRES is not effective in directing translation from un-capped mRNA. It is hypothesized that the picornavirus IRES possess a secondary structure that is important for cap-independent mRNA translation (Attal, Theron et al. 1999), an essential component of the autogene based cytoplasmic expression system.

1.8 Objectives of Thesis

As described previously, a systemic gene delivery system showing extended circulation lifetimes, accumulation at distal tumor sites and significant target gene expression has not been developed. While the SPLP technology developed in our lab allows for extended circulation lifetimes, accumulation at tumor sites, and preferential tumor gene expression, the levels of expression using this carrier system are quite low. It was the objective of this thesis to increase the levels of gene expression following non-viral, plasmid based gene delivery by increasing the effectiveness of the plasmid payload, as opposed to modifying the lipid-based carrier system. In Chapter 2, the novel dual promoter autogene based cytoplasmic expression system is introduced and characterized, demonstrating an exponential increase in gene expression levels, as well

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as a 20-fold increase in expression over a standard nuclear control plasmid. In Chapter 3, efforts are made to further enhance the expression levels of the cytoplasmic expression system and in the process, it is determined that the factors responsible for limiting cytoplasmic expression are the levels of mRNA transcription and translation. In Chapter 4, a comparison between a cell line that shows high cytoplasmic expression is compared with a cell line that shows relatively poor expression levels, and it is determined that the most likely cause for the difference is due to the differences in intra-cellular processing of the lipid:DNA complexes.

CHAPTER 2

CREATION AND CHARACTERIZATION OF A NOVEL AUTOGENE BASED CYTOPLASMIC EXPRESSION SYSTEM

2.1 Introduction

A major limitation of gene delivery systems is the relatively low level of gene expression in transfected tissues. One strategy to increase levels of gene expression following transfection employing a non-viral vector involves improving the plasmid design. The incorporation of a cytoplasmic expression system represents one such approach (Elroy-Stein and Moss 1990; Dubendorff and Studier 1991; Gao and Huang 1993). Cytoplasmic expression systems bypass the requirement for nuclear delivery of plasmid DNA, a major obstacle in present day gene therapy (Capecchi 1980),(Zabner, Fasbender et al. 1995; Wilke, Fortunati et al. 1996; Coonrod, Li et al. 1997). In addition, they take advantage of the large number of plasmids found in the cytoplasm of the cell following transfection with non-viral vectors (Lechardeur, Sohn et al. 1999).

Cytoplasmic expression systems can be designed to utilize the unique properties of the bacteriophage RNA polymerases (RNAPs). Phage RNAPs are moderately sized (~100 kD), single subunit proteins capable of synthesizing RNA from DNA templates. They require no additional co-factors and have demonstrated efficient cytoplasmic transcriptional activity (Chamberlin, McGrath et al. 1970; Dunn, Bautz et al. 1971). These features make phage RNAPs attractive candidates for the development of autocatalytic cytoplasmic expression systems using autogenes. Phage RNAP autogenes consist of an RNAP gene, driven by its own cognate promoter (Dubendorff and Studier 1991). In order to evade the requirement for exogenous RNAP to initiate the expression system, a nuclear promoter can be added upstream of the RNAP

promoter (Brisson, He et al. 1999). Although the first round of RNAP expression must occur via the nuclear promoter, the resulting RNAP in the cytoplasm drives the cytoplasmic expression system, producing RNA from plasmid DNA template in the cytoplasm.

RNA produced in the cytoplasm lacks the 5' cap that stabilizes nuclear transcripts and assists in ribosomal recruitment (Kaempfer, Rosen et al. 1978) (Drummond, Armstrong et al. 1985). Viral Internal Ribosome Entry Site (IRES) elements are sequences that have been shown to enhance the recruitment of the cytoplasmic translational machinery in the absence of 5' capping (Jang and Wimmer 1990). Early dual promoter cytoplasmic expression systems did not contain IRES elements, and as a result, the vast majority of the mRNA produced was not translated (Brisson, He et al. 1999). Although an autogene based on the T7 bacteriophage RNAP that contained an EMCV IRES has been previously described (Deng and Wolff 1994), it did not contain a eukaryotic promoter and required the co-transfection of RNAP protein or mRNA, thereby limiting its utility.

In this study, a novel autogene construct containing both the EMCV IRES element (to allow cap-independent translation), and the CMV promoter (to bypass the need for additional exogenous RNAP protein or mRNA during transfection) was characterized. In addition, the autogene and reporter gene cassettes were combined onto a single plasmid, whereas other systems have utilized separate plasmids. A cell-free system for the evaluation of RNAP based transfection reagents was developed and used to demonstrate the potential of the autogene system to elicit high levels of gene expression.

2.2 Materials and Methods

2.2.1 Plasmids and Primers

Plasmid R023 consists of a basic autogene cassette driven by a CMV promoter and intron. The autogene cassette was derived from the plasmid T7-G1, a gift of Dr. Jon Wolff (Waisman Center, Wisconsin). T7-G1 contains the basic autogene cassette, consisting of the T7 promoter, EMCV IRES, and T7 RNAP gene. The nuclear localization sequence was removed from the T7 RNAP via PCR prior to subcloning into R023. L059 consists of a pTRI-Amp (Ambion) backbone with EMCV IRES, *Photinus pyralis* luciferase and beta-globin poly-adenylation site derived from EMC-Luc (Dr. Jon Wolff). L053 consists of the CMV promoter (with intron) from NGVL3 and the *Photinus pyralis* luciferase gene. L069 and L070 consists of L053 containing one or two irrelevant 2.5 kb spacer fragments respectively. R037 consists of R023 without the T7 and T3 promoters. R011 is a bi-cistronic plasmid consisting of R023 with a downstream luciferase reporter gene cassette from L059 (bi-cistronic). PT7-Luc (Promega) consists of the *Photinus pyralis* luciferase gene driven by a T7 RNAP promoter. RPA-RNAP consists of a 350 bp *Kpn* I – *Afl* II T7 RNAP fragment blunted and ligated into the *Sma* I site of pTRI-Amp in reverse orientation. RPA-Luc consists of a 250 bp *Xcm*1 – *BsrG* 1 luciferase fragment blunted and ligated into the *Sma*1 site of pTRI-Amp in the reverse orientation.

The NVSC1 primer sequence is 5'-TCCTGCAGCCCGGGGGATCCTCTAG-3'.

2.2.2 Transcription and Translation Assay

A 25 µl reaction was set up using a Promega (Wisconsin) Coupled In Vitro Transcription and Translation kit as per the manufacturer's instructions. PT7-Luc (250

ng) was added to all reactions. R023 (250 ng) (containing a T7 RNAP gene driven by the T7, SP6 and T3 promoters) or R037 (containing a T7 RNAP gene driven only by the SP6 promoter) was then added to the reactions. SP6 RNAP (0.5 U) (Promega) was added and each reaction was incubated at 30°C. At the time points indicated, 2 µl of reaction mixture was removed and assayed for luciferase expression as described below. All reactions were performed in triplicate.

2.2.3 Transfections

Lipoplexes were formed by mixing plasmid DNA with large unilamellar vesicles (LUVs) composed of equimolar amounts of DOPE:DODAC (50:50) (prepared via extrusion) on ice and incubated for 20 min prior to use. All transfections were performed at a cationic lipid to plasmid DNA charge ratio of 3:1. Lipoplexes were diluted with serum-containing media before addition directly to cell media. BHK cells were plated at 25,000 cells per well in 24-well plates. Neuro-2a cells were plated at 30,000 cells per well in 24-well plates. The total mass of plasmid added was identical in all transfections. Equimolar transfections using plasmids of different sizes were achieved through the addition of an empty vector (pBlueScript) to normalize the total mass of DNA in each transfection. All transfections were performed in triplicate. Data is presented as mean values +/- standard error.

2.2.4 Luciferase and BCA Assays

Cells were washed twice with 1 mL PBS followed by the addition of 0.2 mL lysis buffer (PBS with 0.1 % Triton X-100) before being stored at –70 ° C. Cells were thawed and 5-20 µl of sample were assayed in duplicate on a 96-well plate. Samples were

assayed using a Berthhold Centro LB960 Microplate Luminometer and Luciferase Assay System (Promega). Standard luciferase assays were performed and transfection data is reported as mass quantities of luciferase protein using a standard curve obtained from serial 10-fold dilutions of a 20 mg/mL *Photinus pyralis* luciferase standard (Promega). Cell-free luciferase assays are reported in Relative Light Units (RLUs). Total protein was quantified using a Pierce BCA assay kit as per manufacturer's instructions.

2.2.5 Immunofluorescence

BHK cells were plated on glass coverslips in 6-well plates (150,000 cells per well) and transfected with 1.5 µg of plasmid DNA . At 24 h post-transfection, cells were washed once with 2 mL PBS-IF (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4) prior to fixation for 10 min with 2 mL 2% paraformaldehyde. Cells were subjected to three 30 s washes before permeabilization with 0.25% Triton X-100 in PBS-IF for 5 min. After washing three times for 30 s with PBS-IF, cells were incubated with blocking buffer (10% BSA in PBS-IF) for 1 h, shaking gently at room temperature. Cells were washed three times for 10 min with PBS-IF followed by addition of primary antibody solution consisting of a 1:1000 dilution of goat anti-T7 RNAP antibody (a gift from Dr. Paul Fisher at the Department of Pharmacological Sciences, State University of New York at Stony Brook) or 1:1000 dilution of mouse anti-luciferase monoclonal antibody (Abcam) in 2% BSA in PBS-IF. Cells were incubated with primary antibody solution for 2 h while shaking at room temperature. Cells were washed three times for 10 min in PBS-IF followed by the addition of secondary antibody (Rabbit anti-goat IgG, FITC labeled (QED Bioscience Inc) or Texas Red labeled Rabbit anti-mouse (Abcam),

1:200 dilution in 2% BSA-PBS-IF) and incubated for 2 h while shaking at room temperature. Cells were washed four times for 10 min with PBS-IF before being mounted and photographed on a Zeiss Axiovert S100 fluorescence microscope. The percentage of cells transfected was determined by counting transfected and non-transfected cells under the microscope. Data indicate the average of six separate counts from three different experiments.

2.2.6 RNase Protection Assay

RNAP and luciferase probes were prepared from *EcoR* 1 digested RPA-RNAP or RPA-Luc plasmid respectively. GAPDH probe was purchased from Pharmingen. Probes were labeled following the manufacturers protocol using ^{32}P-αUTP (3000 Ci/mmole, 10mCi/mL)(NEN).

BHK cells were plated on 6-well plates (150,000 cells per well) and transfected with 1.5 µg of R011 or L053 in triplicate. After 24 h cells were treated with 20 µg/mL Actinomycin D. At 0, 2, 4, 6 or 8 h after Actinomycin D treatments, cells were washed once with PBS and recovered by treatment with trypsin. Cells from triplicate wells were pooled before harvesting total RNA (RNeasy miniprep kit, Qiagen). Total RNA (10, 5 or 2.5 µg) was subjected to RNase protection analysis using the RiboQuant RPA system (Pharmingen) according to the manufacturer's protocol. All values shown are the average +/- standard deviation of two independent experiments. Data was collected using a Typhoon Phosphorimager (Amersham Biosciences) and analysis was performed using ImageQuant software (Amersham Biosciences).

2.2.7 Primer Extension

Primer extension analysis using ^{32}P-labeled primer NVSC1 and 100 μg of RNA isolated from R011-transfected BHK cells (24 h post transfection) was performed using a Primer Extension System (Promega). The ladder was prepared by end labeling ΦX174 *Hinf* I digested DNA markers with ^{32}P (from AT^{32}P. All values shown are the average +/- standard deviation of two independent experiments. Data was collected as described for RNase Protection assay above.

2.3 Results

2.3.1 Autocatalytic Gene Expression Results in an Exponential Time Dependent Increase in Gene Expression

A hallmark of an autocatalytic, self-amplifying system is an exponential, time-dependent increase in the product being amplified. This exponential relationship would be limited only by the amount of substrate available (i.e. charged tRNA, GTP, etc.), and would continue as long as the template plasmid is in excess. In order to verify the autocatalytic nature of the autogene, a cell-free transcription and translation assay was performed. R023 plasmid DNA (consisting of T7 RNAP driven by both SP6 and T7 RNAP promoters) was incubated with a PT7-Luc reporter gene plasmid (consisting of luciferase driven by only the T7-promoter) in the presence of rabbit reticulocyte lysate and SP6 RNAP. SP6 RNAP transcribes T7 RNAP RNA from the R023 plasmid, leading to the production of T7 RNAP protein that is then able to drive expression of both the T7 RNAP gene from R023 in an autocatalytic fashion, as well as expression of the luciferase gene from PT7-Luc. Figure 2-1 shows a dramatic increase in luciferase expression over time, indicating an exponential, autocatalytic increase in T7 RNAP protein. This increase is not observed when a control plasmid (R037, consisting of T7

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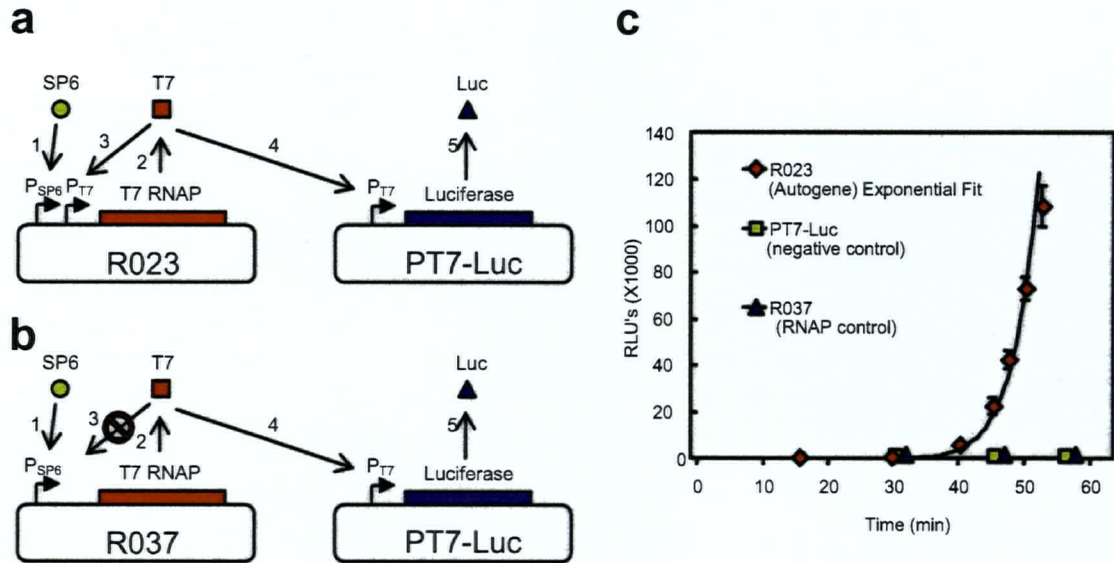
Figure 2-1: Transcription and Translation assay. a) Schematic diagram of the transcription and translation assay. SP6 RNAP binds to the SP6 promoter (PSP6) on R023 (T7 RNAP driven by SP6 and T7 promoters) (1) transcribing T7 RNAP mRNA, which is (2) translated into T7 RNAP protein. The T7 RNAP protein then binds the T7 promoter (PT7) on R023 (3) resulting in more T7 RNAP protein (2) and initiating the autocatalytic cycle and an exponential increase in T7 RNAP production. T7 RNAP also transcribes luciferase mRNA from PT7-Luc (4), resulting in an increase in luciferase expression proportional to the amount of T7 RNAP present.

b) In the control reaction, the lack of PT7 in R037 (T7 RNAP gene driven by only SP6 promoter) prevents any autocatalytic production of T7 RNAP (3).

c) An *in vitro* coupled transcription and translation (Promega) assay. 250 ng of PT7-Luc was combined with 250 ng of either R023 or R037 in a total reaction volume of 15 µl and 0.5 U of SP6 RNAP (Promega) was added and incubated at 30° C. 2 µl aliquots were removed at time points indicated and subjected to luciferase analysis as described in Materials and Methods. After an initial lag phase, the R023 reaction resulted in an exponential increase in luciferase expression, verifying the autocatalytic nature of the system.

RNAP driven only by the SP6 promoter) lacking the T7 promoter needed for autocatalytic amplification is used. The reason for the lack of expression from R037 is that without the autocatalytic amplification, the amount of T7 RNAP produced is not enough to give rise to detectable levels of luciferase expression.

2.3.2 A Bi-cistronic Construct Results in Higher Levels of Gene Expression Than a Dual Plasmid Transfection

Previously published work on cytoplasmic expression systems employed an autogene cassette and a reporter gene cassette on separate plasmids (Brisson, He et al. 1999). It was of interest to compare the expression resulting from a dual plasmid transfection system with a single plasmid bi-cistronic system in which the autogene and reporter gene were on one large plasmid. When equimolar amounts of autogene and reporter gene constructs were used to transfect BHK cells, it was found that the bi-cistronic construct yielded 2 to 4 fold higher levels of gene expression than the analogous dual plasmid transfection (Figure 2-2). This result was unexpected because previous results suggest that transfection (delivery to nucleus and subsequent expression) would be more efficient for the smaller autogene plasmid than the larger bi-cistronic construct (Kreiss, Cameron et al. 1999). For the dual plasmid transfection, this would result in a greater number of cells expressing RNAP via the CMV promoter in the nucleus, and accordingly greater levels of luciferase via the RNAP promoter in the cytoplasm. In order to understand this phenomenon, a series of luciferase plasmids of increasing size were prepared to determine the effect of plasmid size on transfection efficiency in BHK cells. It was found that L053 (5.8 kb) L069 (8.3 kb) and L070 (10.8 kb) yielded similar levels of gene expression when transfected in equimolar amounts (Figure 2-3). This suggests that for the system described here, larger plasmids are not at a disadvantage compared to

a

pR023
7940

CMV
KanR
Intron
TRI
EMC IRES
ColE1 Ori
T7 RNAP

pL059
5313

AmpR
Luciferase
PT7
EMC IRES

pR011
10769

KanR
CMV
Intron
PT7
EMC IRES
ColE1 Ori
T7 RNAP
Luciferase
PT7
EMC IRES
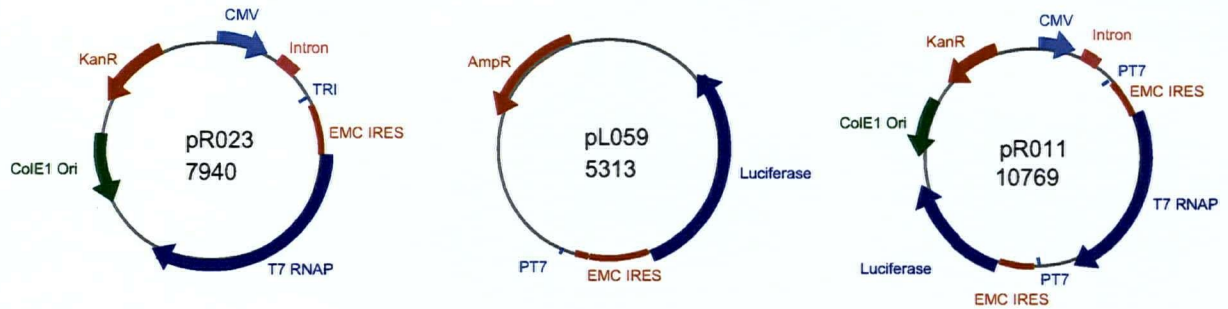
b
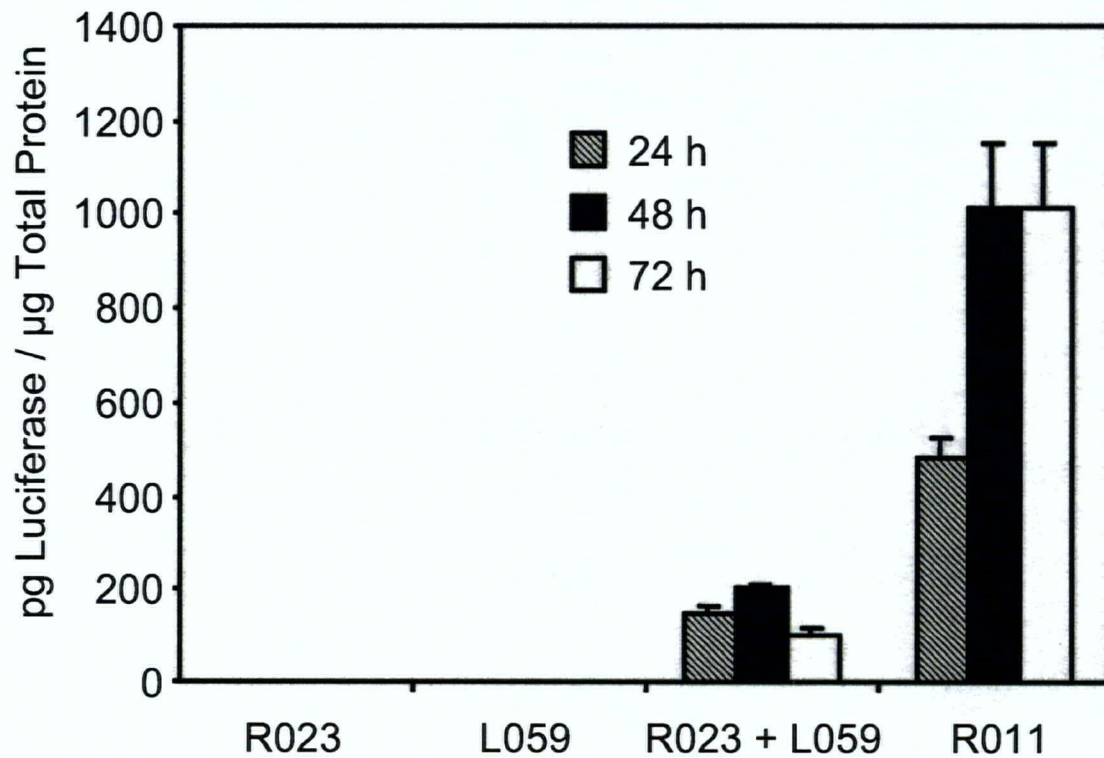
Figure 2-2: Comparison of bi-cistronic construct versus a dual plasmid transfection. a) Diagrams of plasmids used. b) BHK cells were transfected with 1 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pBlueScript). Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error.
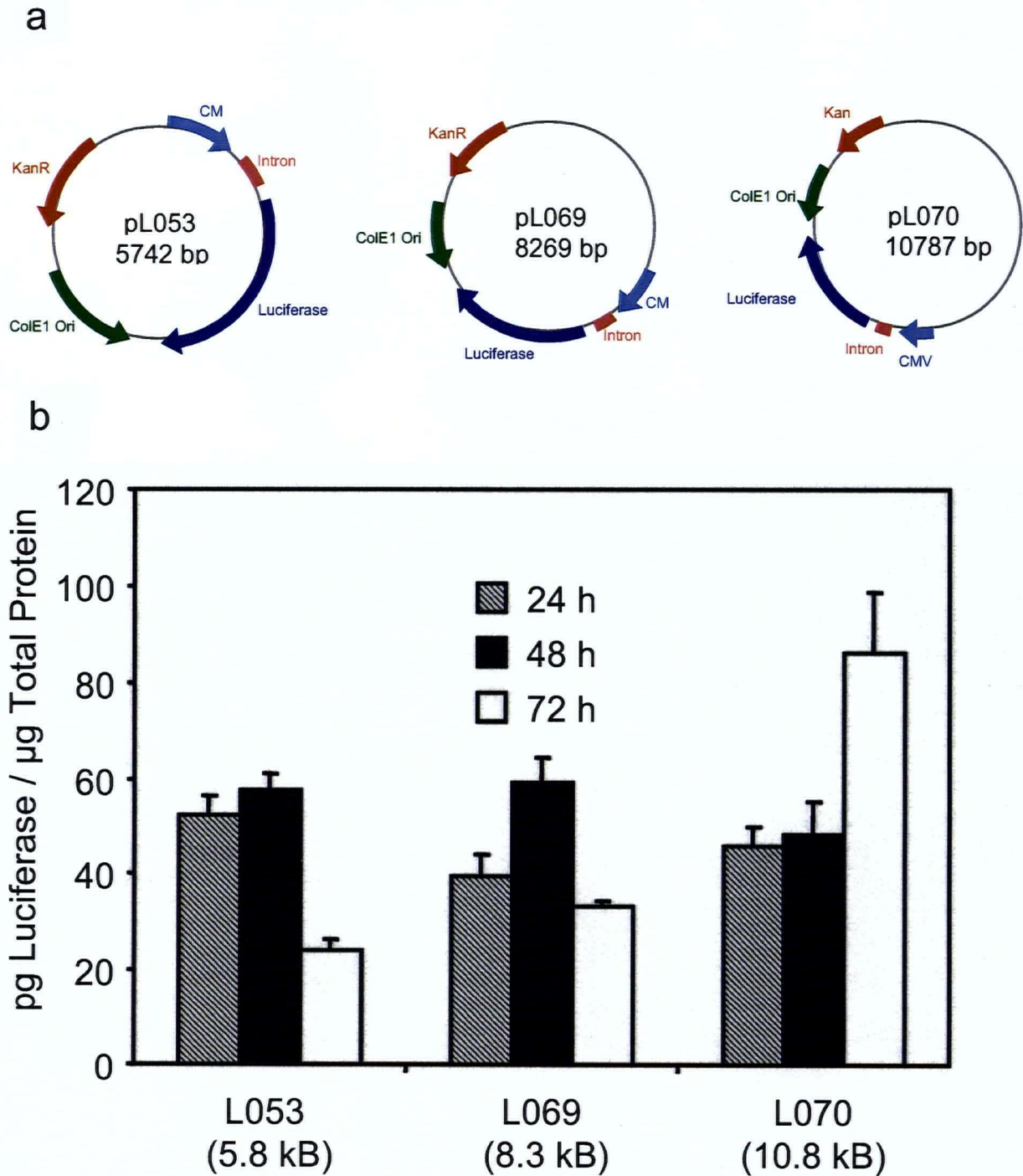
Figure 2-3: Plasmid size does not effect transfection of BHK cells. a) Diagrams of plasmids used. b) BHK cells were transfected with a total of 1 μg/well. Equimolar amounts of plasmid were added, and the total mass of DNA per transfection was normalized by adding an unrelated plasmid (pBlueScript).   Error bars indicate standard error.

the smaller plasmids. In addition, immunofluorescence studies using anti-T7 RNAP and anti-luciferase antibodies showed that the same percentage of cells are being transfected with the bi-cistronic construct as with the dual plasmid transfection (data not shown), further supporting this finding.

2.3.4 The Cytoplasmic Expression System Results in a 20-fold Increase in Gene Expression per Cell Compared to a Nuclear Expression System

In order to compare the relative efficiency of nuclear versus cytoplasmic expression, BHK cells were transfected with equimolar amounts of a CMV-Luciferase (L053) and a bi-cistronic autogene plasmid containing both the autogene cassette, as well as the luciferase reporter gene cassette (R011). As shown in Figure 2-4, the autogene system yielded a 20-fold increase in luciferase expression when compared with the CMV-mediated nuclear expression system.

It was of interest to determine whether the increase in luciferase expression was the result of greater levels of luciferase production in each transfected cell or due to an increase in the total number of cells being transfected. The number of cells transfected with the autogene system was experimentally determined and compared with the number of cells transfected with the standard nuclear expression system. Transfected cells were quantified using immunofluorescence with both anti-T7-RNAP and anti-luciferase antibodies and BHK cells transfected with either the autogene or nuclear expression construct. As seen in Fig 2-5, the autogene and nuclear expression constructs both result in transfection of approximately the same number of cells (autogene 11.4% +/- 3.5, nuclear 10.7% +/- 2.9). The increase in reporter gene expression from the
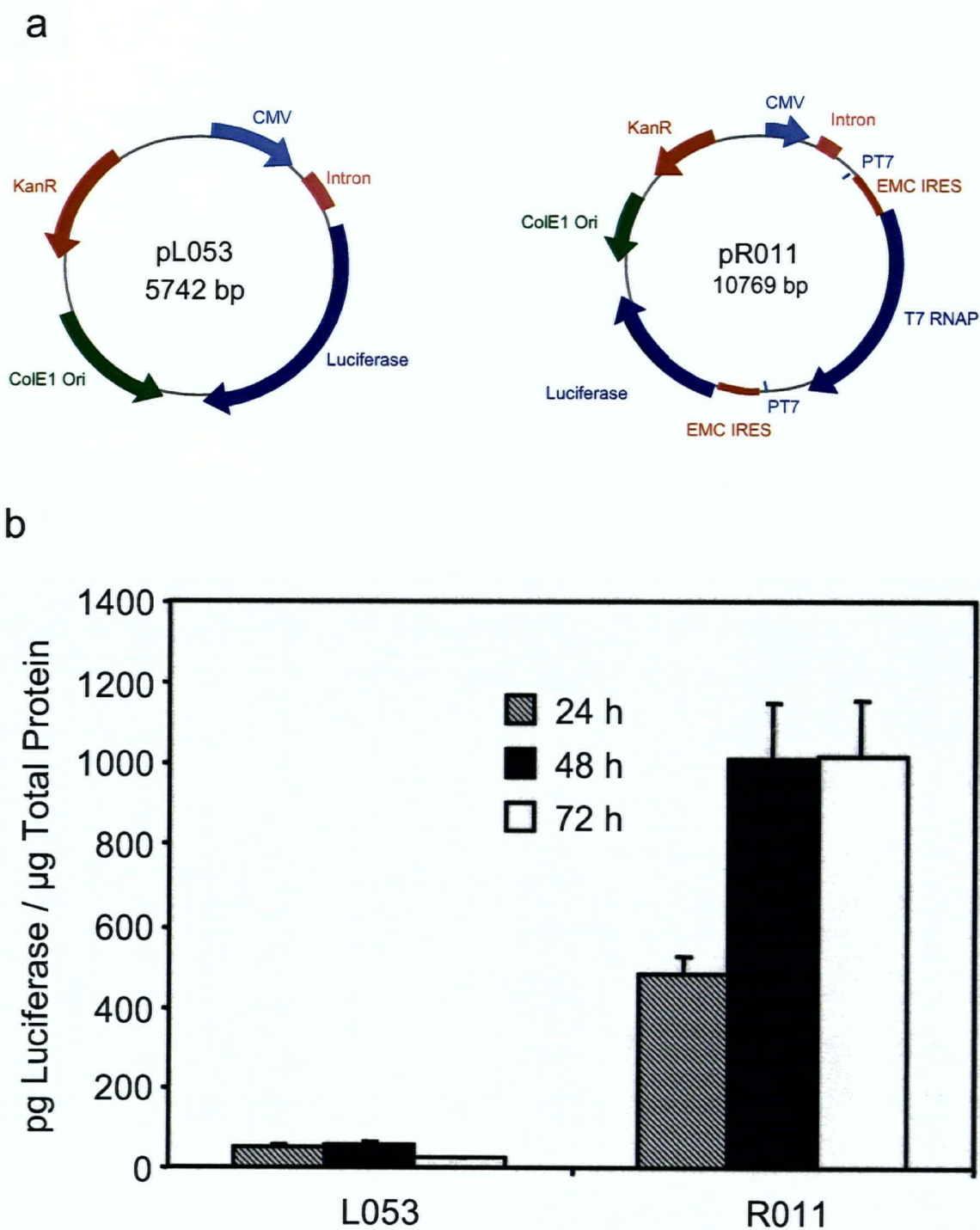
Figure 2-4: A comparison of autogene and nuclear expression. a) Diagrams of plasmids used (note, these plasmids were used for all following figures). b) BHK cells were transfected with a total of 1 µg/well. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pBlueScript). Error bars indicate standard error.
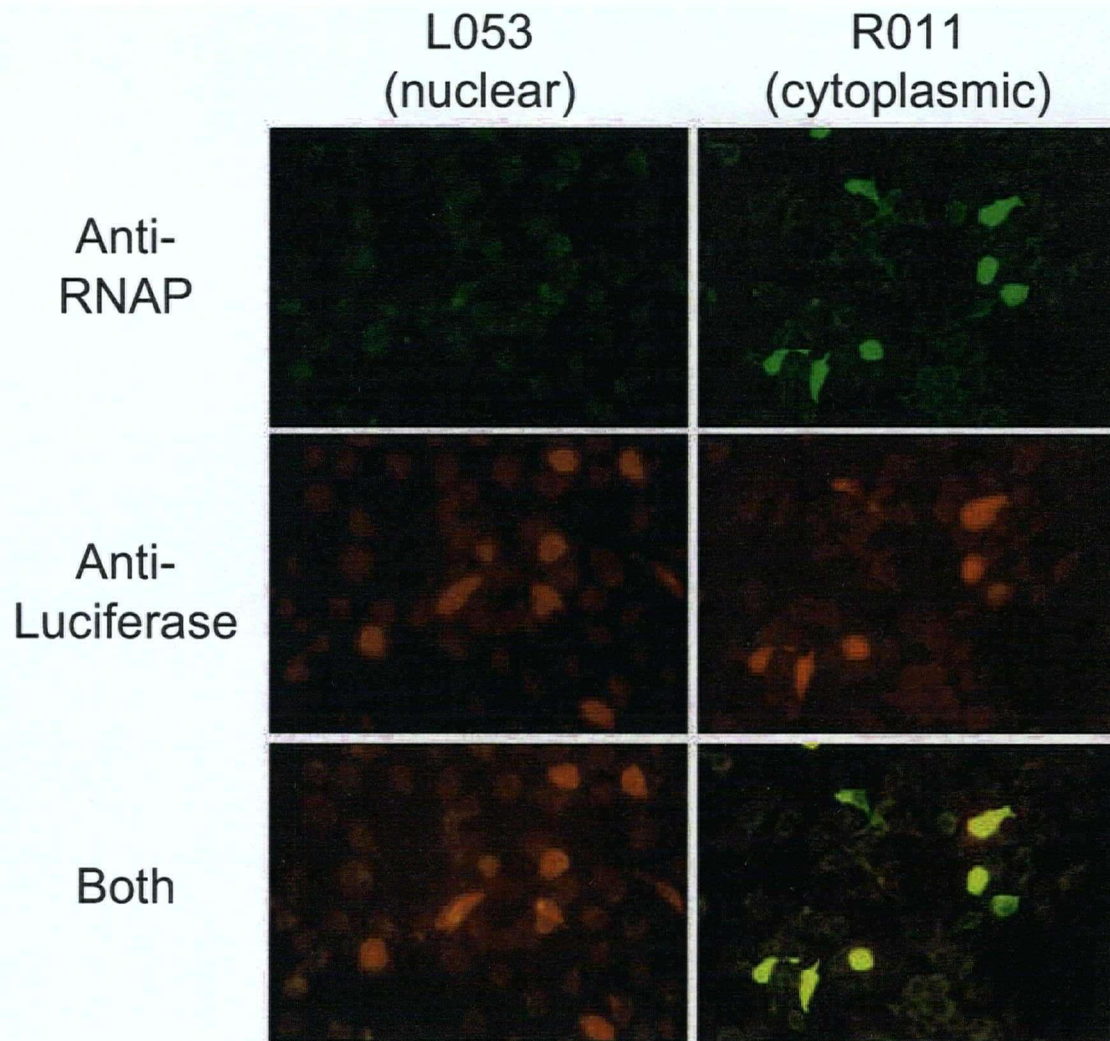
Figure 2-5: Immunofluorescence of BHK cells transfected with cytoplasmic or nuclear expression constructs. BHK cells were transfected with equimolar amounts of R011 (autogene) or L053 (nuclear) plasmids. Cells were fixed 24 h post transfection and subjected to immunofluorescence using anti-T7 RNAP antibodies (with a FITC conjugated secondary) and anti-luciferase (with a Texas Red conjugated secondary). A similar number of cells were transfected when using either the autogene or nuclear expression plasmids. In the case of the autogene transfection, every cell that is expressing RNAP is also expressing luciferase.

bi-cistronic autogene construct can therefore be attributed to an increase in the level of gene expression in transfected cells, as opposed to an increase in the number of cells being transfected.

It was important to make sure that the 20 fold increase in expression was not limited to BHK cells. As can be seen in Figure 2-6, a similar autogene-mediated improvement in gene expression was also observed in the Neuro-2a murine neuroblastoma cell line.

The system described here is initially dependent on the nuclear transcription of T7 RNAP. It was of interest to determine the proportion of nuclear transcripts derived from the CMV promoter versus cytoplasmic transcripts derived from the T7 promoter. As the two promoters have different transcription start sites, the two transcripts will have different length 5'-untranslated regions. Therefore, a primer extension assay was performed using a primer that binds downstream of the two promoters, 90 bp downstream from P_{T7} and 300 bp downstream of the P_{CMV}. Figure 2-7 shows that a much higher proportion of mRNA is transcribed from the T7 promoter than from the CMV promoter ($\sim$57 +/- 11 fold). This is consistent with previous work that found the large majority of transcripts in the cell were transcribed by the T7 RNAP in the cytoplasm (Brisson, He et al. 1999). This further demonstrates that only a catalytic amount of RNAP needs to be expressed in the nucleus for large amounts of cytoplasmic mRNA to be produced.

2.3.4 Cytoplasmic mRNA Transcripts have a Shorter Half-life than Nuclear Transcripts

It was of interest to determine the half-life of the cytoplasmic transcripts as the lack of 5' cap structure on the cytoplasmic transcripts may result in a decrease in mRNA

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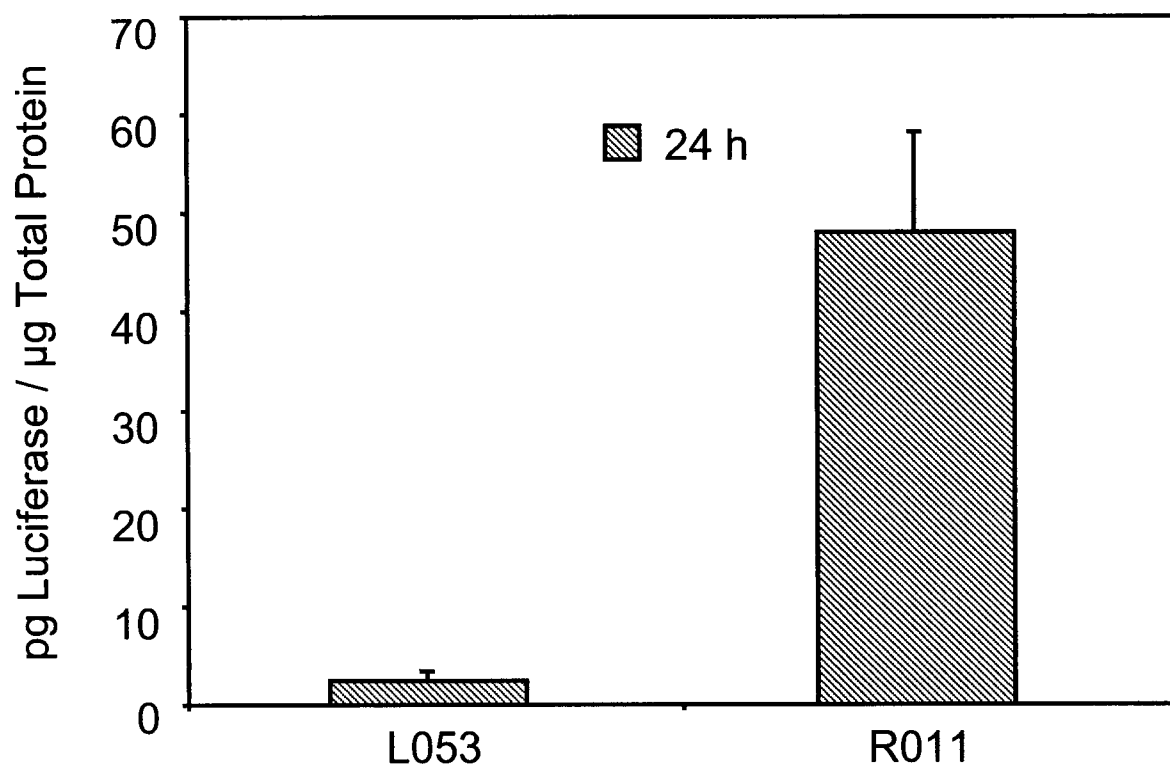
Figure 2-6: Increased autogene expression is also seen in Neuro-2a cells. Neuro-2a cells were transfected with a total of 2μg/well. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pBlueScript). Error bars indicate standard error. Transfection with the autogene (R011) yielded a 20-fold increase in expression over the standard nuclear expression plasmid (L053).
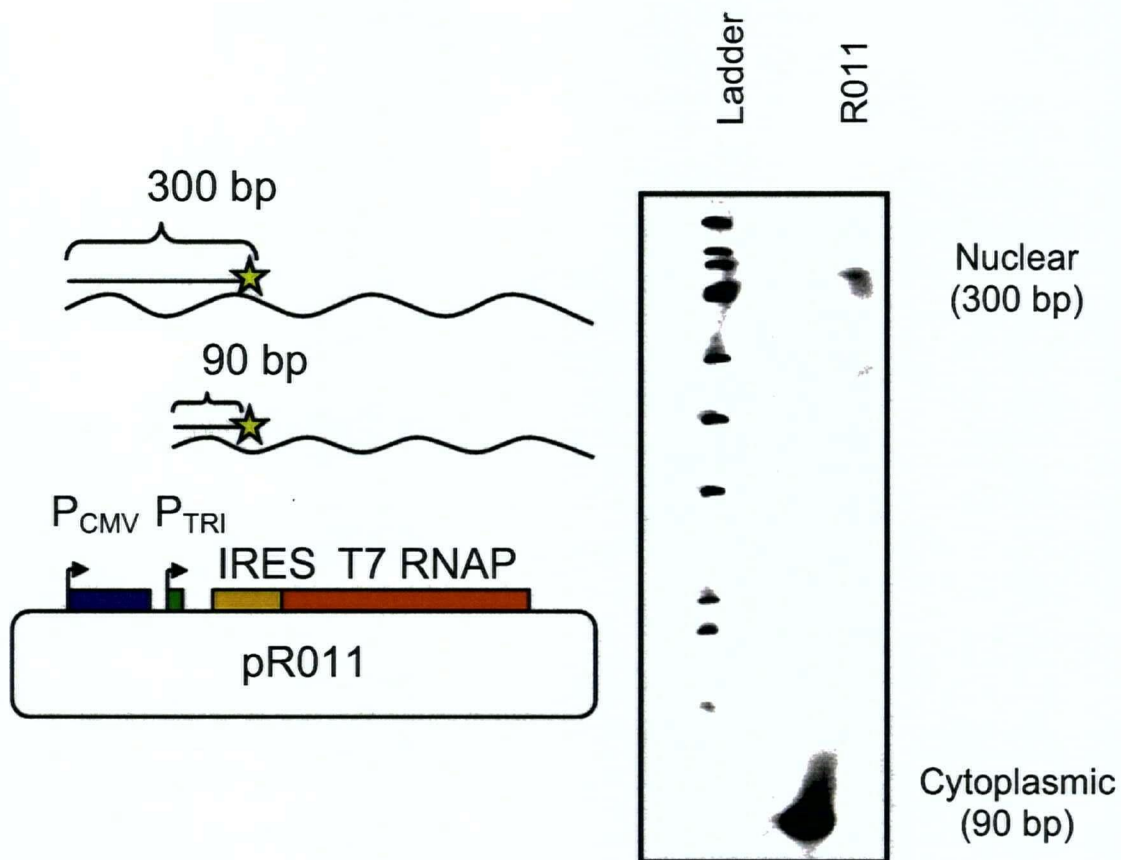
Figure 2-7: Primer extension assay performed on BHK cells transfected with the bi-cistronic autogene construct (R011). A) The transcripts initiated at the nuclear CMV promoter were predicted to have a longer 5' untranslated region resulting in larger fragments, ~300 bp in size, while transcripts initiated at the T7 promoter were predicted to have a shorter 5' untranslated region, ~90 bp in size. B) Transfection of BHK cells with the bi-cistronic autogene construct (R011) yielded approximately 57 +/- 11fold more short cytoplasmic transcripts as long nuclear transcripts, indicating that the majority of transcription is initiated by the T7 RNAP in the cytoplasm.

stability (Drummond, Armstrong et al. 1985; Bernstein and Ross 1989; Jackson and Standart 1990; Sachs 1990). An RNase Protection Assay (RPA) was used to measure both the half-life of the mRNA as well as the relative amounts of RNA present. BHK cells were transfected with equimolar amounts of R011 (autogene) and L053 (nuclear) plasmids. At 24 hours post-transfection, 20 µg/mL Actinomycin D was added to inhibit all de novo RNA synthesis. Previous work had demonstrated that this amount of Actinomycin D was sufficient to inhibit >99% of RNA synthesis (data not shown). Cells were harvested at 2 hour intervals and total RNA was isolated. As calculated from Figure 2-8, the half-life of the autogene transcripts average 103 +/- 6 min (88 +/- 3 min calculated using the RNAP probe, 115 +/- 5 min calculated using the Luciferase probe). The half-life of the nuclear transcripts was 317 +/- 6 min. By this analysis, it was determined that the cytoplasmic transcripts are not as stable as the nuclear transcripts. Densitometric analysis of the luciferase transcript band from the nuclear and cytoplasmic transfections, there are approximately 20-fold more autogene-derived luciferase transcripts as there are nuclear luciferase transcripts. Given that the half-life of the autogene transcripts is three times shorter than the nuclear transcripts; this suggests that the autogene produces at least 60 fold more mRNA than the standard nuclear expression system.
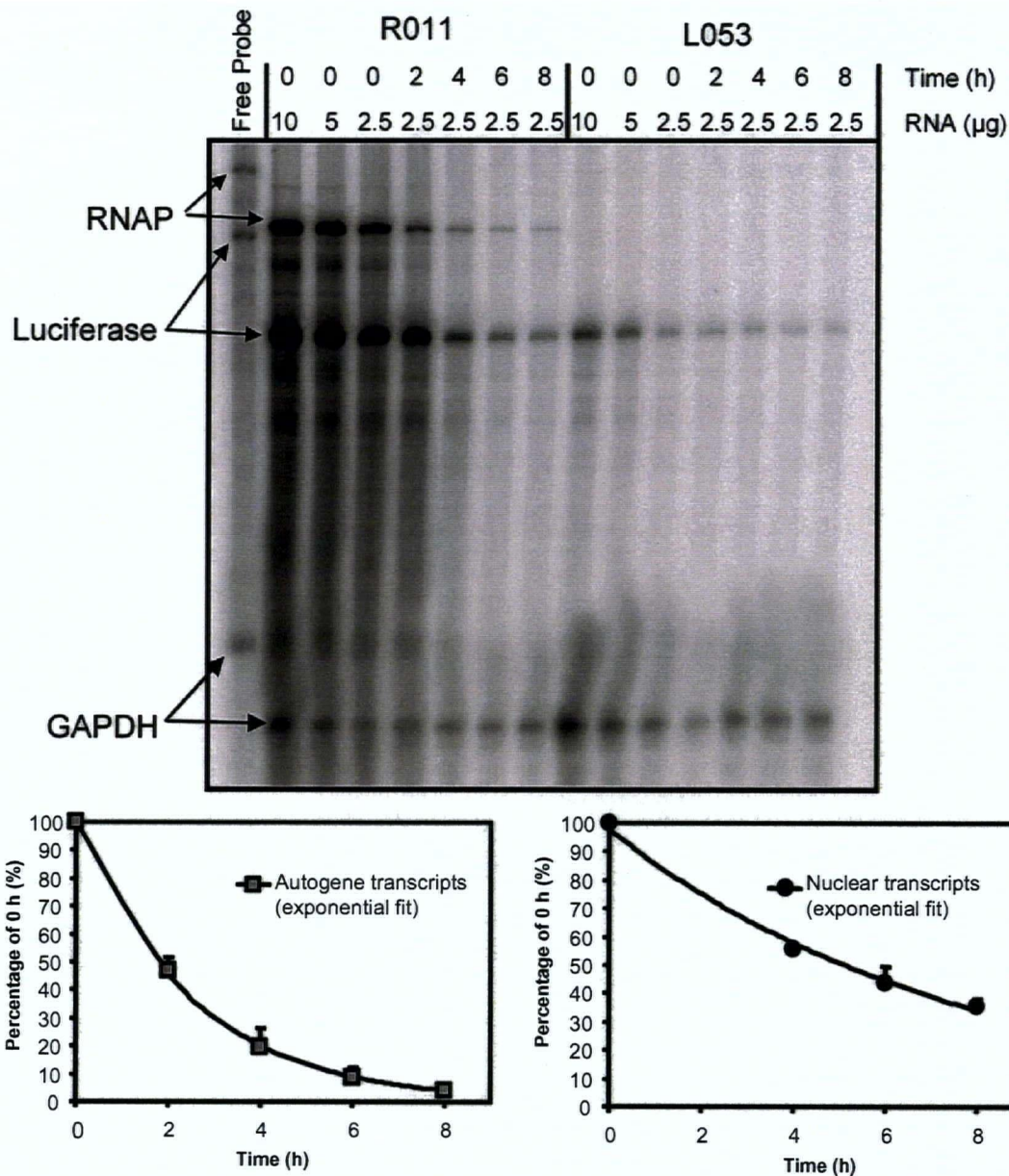
Figure 2-8: Ribonuclease Protection Assay of RNA derived from BHK cells transfected with bi-cistronic autogene construct (R011) or nuclear construct (L053). BHK cells were treated with Actinomycin D 24 h post transfection. Total RNA was harvested at 2-h intervals following treatment. 10, 5 or 2.5 µg of total RNA was subjected to an RNase Protection Assay using ^{32}P labeled probes against T7 RNAP (RNAP) and Luciferase (Luc) transcripts (see Materials and Methods). All values were standardized against the GAPDH control. Approximately 20 times as many luciferase transcripts were detected in the autogene transfected cells as the nuclear transfected cells. The half-life of the autogene transcripts is approx 103 min, approximately 3-fold shorter than the half-life of the nuclear transcripts, 317 min.

2.4 Discussion

A novel bi-cistronic autogene based cytoplasmic expression system is described that demonstrates a 20-fold increase in gene expression over a standard CMV-based nuclear expression system. The advantages of this system with respect to previous systems will be discussed, as well as the mechanism of action, and the potential utility of this system.

The system described here has a number of advantages over previously published systems. Previous work with an autogene based cytoplasmic expression system describes a dual promoter autogene, consisting of an autogene cassette driven by a CMV promoter (Brisson, He et al. 1999). In this system the initial supply of T7 RNAP was derived from nuclear transcripts via the CMV promoter, obviating the need to add exogenous RNAP protein or mRNA during transfection. Although this system showed increased expression when compared with a standard CMV-based nuclear expression system (approximately 2-3 fold increase), the authors found that the majority of the cytoplasmic transcripts were not being translated, likely due to the lack of 5' cap, required for efficient translation in eukaryotic cells. Other work described the creation of a T7 RNAP autogene containing an EMCV IRES sequence (Deng and Wolff 1994). This IRES element facilitated translation of the uncapped cytoplasmic transcripts. It was also noted that the inclusion of the IRES element attenuated the toxicity of the autogene plasmid in host *E.* coli cells, a major problem with earlier autogene plasmids (Dubendorff and Studier 1991). We observed some evidence of autogene toxicity in *E. coli*, but isolation of plasmid DNA was easily achieved from bacterial culture. The yield of autogene containing plasmids was approximately 0.5 mg of DNA per liter of bacterial culture. This is a marked decrease from the 5-10 mg/L yield normally obtained from

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plasmids that share the same vector backbone, but do not contain the autogene sequence. Methods by which the toxicity of autogene plasmids may be further alleviated are currently being investigated.

The bi-cistronic autogene system described here is distinguished from previous systems by two key properties. First, it contains both a CMV promoter, bypassing the need for addition of exogenous RNAP protein during transfection, as well as an autogene containing an EMCV IRES sequence, allowing for cap-independent translation of the autogene transcripts. In addition, our system has the autogene cassette and reporter gene cassette on the same plasmid, further simplifying the transfection process and resulting in increased transgene expression.

When the expression levels from the cytoplasmic expression system and a standard nuclear expression system were compared, the cytoplasmic system yielded 20-fold higher expression than the nuclear system. This is in contrast with previous systems that demonstrated a maximum of three-fold increase over a nuclear expression system control (Brisson, He et al. 1999). The improvement in performance is most likely due to increased translation of cytoplasmic transcripts generated from the modified expression system described here. The inclusion of an EMCV IRES element in the autogene cassette appears to enhance translation, overcoming the lack of a 5' cap on cytoplasmic transcripts and resulting in increased transgene expression levels.

The autogene system was also tested in Neuro-2a cells and a 20-fold increase in expression with the autogene as compared with the CMV-based system was observed. This indicates that the autogene system is not limited to BHK cells. However it should be noted that variable gene expression was seen when using other cell lines (see Chapter 4) and we are currently investigating this phenomenon.

The mechanism whereby the bi-cistronic autogene system results in increased gene expression is of obvious interest. It was important to verify that the T7 autogene, that has been the basis for all cytoplasmic expression systems to date, truly does exhibit an autocatalytic expression profile. For the results described in Figure 2-1, assuming that the amounts of substrates and PT7-Luc are not limiting, it is straightforward to show that if an autocatalytic process is occurring, then $N_L(t) = ce^{t/\tau}$, where $N_L(t)$ indicates the number of luciferase molecules at time t and c and τ are constants. The close fit ($R^2 = 0.94$) of an exponential profile to the luciferase expression observed in Figure 2-1 thus supports an autocatalytic mechanism. Any deviation from exponential characteristics at longer times can be attributed to either saturation effects as the amount of PT7-Luc becomes limiting, or the system running out of substrate (e.g. charged tRNA, GTP, etc).

The primer extension and RPA data in Figure 2-7 and Figure 2-8 provide further evidence of a cytoplasmic autocatalytic process. As demonstrated in the results presented in Figure 2-8, there is at least a 20-fold increase in transgene mRNA levels with the cytoplasmic expression system as compared to the standard nuclear expression system. These transcripts had a much shorter half-life than their nuclear counterparts, which is consistent with the lack of a 5' cap, an important determinant of mRNA stability. When combined with the primer extension data showing that the majority of the transcripts are being made by the T7 RNAP, this data suggests that the increase in gene expression is due to an increase in mRNA levels in the cytoplasm of transfected cells, consistent with the autocatalytic process.

There are many possible explanations for why the bi-cistronic construct is more effective than a dual plasmid transfection. One possible reason is that the T7 RNAP is able to transcribe RNA from either the first P_{T7}, driving T7 RNAP expression, or the

second P_{T7}, driving luciferase expression in the bi-cistronic construct. Due to the lack of a terminator sequence between these two genes, both transcripts will encode for the luciferase gene. Therefore, the cells transfected with the bi-cistronic plasmid should have more mRNA encoding luciferase than the cells in the dual transfection. Upon examination of the RPA data (Figure 2-9), it is clear that there are at least twice as many luciferase transcripts than RNAP transcripts following bi-cistronic transfection, lending support to this hypothesis. In addition, it was found that the luciferase transcripts had a slightly longer half-life than the T7 RNAP transcripts (115 min versus 88 min). This increased half-life may be attributed to the fact that the luciferase transcripts being made from the first P_{T7} in effect had a much longer 5' UTR. This would most likely add some stability to the transcript, therefore increasing its half-life and subsequent luciferase expression.

The potential applications of an autogene based cytoplasmic expression system are many. Aside from increasing the levels of gene expression in plasmid-based non-viral gene delivery systems, this system could also be used as a tool to express high levels of transgene *in vitro* for characterization or purification purposes.

In summary, the studies described here demonstrate a novel, bi-cistronic autogene based cytoplasmic expression system that shows 20-fold higher levels of gene expression compared with a nuclear expression system. This system has been shown to exhibit an exponential autocatalytic gene expression profile, and result in an increase in reporter gene expression per transfected cell, as opposed to an increase in the number of cells transfected. Furthermore, the bi-cistronic system is more effective than a cytoplasmic expression system carried on two plasmids. This system has a wide range of applications, not the least of which is increasing the therapeutic utility of plasmid based gene delivery systems.

CHAPTER 3

FACTORS LIMITING AUTOGENE BASED CYTOPLASMIC EXPRESSION

3.1 Introduction

In the previous chapter, a novel cytoplasmic expression system that resulted in a 20-fold increase in the levels of gene expression over a standard CMV-based nuclear expression system was described (Finn, Lee et al. 2004), comparing favorably with the 2-3 fold increase seen with previous similar systems (Brisson, He et al. 1999). The potential for this system to give even greater increases in expression prompted further examination of this system, specifically to determine the factors limiting the cytoplasmic expression system and ways in which the levels of gene expression can be increased.

Specifically, the effects of changing the nuclear promoter, RNAP gene, and IRES sequence on autogene expression are examined. We find that mRNA production, as well as translation, are most likely the limiting factors for cytoplasmic autogene expression.

3.2 Materials and Methods

3.2.1 Plasmids and Primers

Plasmid R011 is a bi-cistronic plasmid consisting of a basic autogene cassette (driven by the T7, T3 and SP6 RNAP promoters) driven by a CMV promoter and intron, as well as a downstream *Photinus pyralis* luciferase reporter gene cassette (for construction details see Finn et al, 2003). L053 consists of the CMV promoter (with intron) from NGVL3 and the *Photinus pyralis* luciferase gene. R031 and L071 consist of R011 or L053, respectively, with the CMV promoter replaced with the SV40 promoter.

R032 and L072 consist of R011 or L053, respectively, with the CMV promoter replaced with the RSV promoter. R063 and R068 consists of R011 with the T7 RNAP gene replaced with the SP6 RNAP gene from pSR3, or the T3 RNAP gene from pTG100, both of which are a gift from Dr. W.T. McAllister (Department of Microbiology and Immunology, State University of New York Health Science Center at Brooklyn). R017 consists of the T7 RNAP gene driven by the CMV promoter (and intron) from NGVL3. L059 consists of a pTRI-Amp (Ambion) backbone with EMCV IRES, *Photinus pyralis* luciferase and beta-globin poly-adenylation site derived from EMC-Luc, a gift from Dr. Jon Wolff (Waisman Center, Wisconsin). L076 consists of L059 with the EMCV IRES replaced with the eIF4G IRES from pCatEIF4GGal, a gift from Dr. Caroline Lee (Faculty of Medicine, National University of Singapore). L059+GTX consists of L059 with the EMCV IRES replaced with the 10 tandem GTX sequences from p(Gtx133-141)10(SI)9BRPh, a gift from Dr. Vincent Mauro (Department of Neurobiology, Scripps Research Institute). PT7-Luc (Promega) consists of the *Photinus pyralis* luciferase gene driven by a T7 RNAP promoter. L080 consists of PT7-Luc with the Luciferase gene driven by both the T7 and the SP6 promoter. RPA-Luc consists of a 250 bp *Xcm*1 – *BsrG* 1 luciferase fragment blunted and ligated into the *Sma*1 site of pTRI-Amp in the reverse orientation.

3.2.2 mRNA Synthesis

mRNA synthesis was performed using a MEGAscript high yield transcription kit (Ambion). For all transcripts used for *in vitro* transfection, plasmid template (L059 for IRES-Luc and Cap-IRES-Luc mRNA; L080 for Cap-Luc mRNA; PT7-Luc for Luc mRNA) was linearized using *EcoR* 1 and 1 µg of each plasmid was used as per manufacturers

protocols. mRNA was recovered by the LiCl procedure as outlined in manufacturer's

protocol. For the synthesis of R011 mRNA (used for RPA standard curve), undigested

R011 was used as template for mRNA synthesis as the undigested plasmid more

closely resembles that present in the cytoplasm and would serve as a better control

than digested plasmid. After LiCl precipitation, the RNA pellet was washed 3 x with

70% ethanol to completely remove any unincorporated NTPs.

3.2.3 Transfections

Lipoplexes were formed by mixing plasmid DNA with large unilamellar vesicles

(LUVs) composed of equimolar amounts of DOPE:DODAC (50:50) on ice and

incubated for 20 min prior to use. All transfections were performed at a cationic lipid to

plasmid DNA charge ratio of 3:1. Lipoplexes were diluted with heat inactivated serum-

containing media before addition directly to cell media. BHK cells were plated at 30,000

cells per well in 24-well plates. The total mass of plasmid added was identical in all

transfections. Equimolar transfections using plasmids of different sizes were achieved

through the addition of an empty vector (pPUC19) to normalize the total mass of DNA in

each transfection. For mRNA transfections, TransMessenger reagent (Qiagen) was

used to transfect 0.6 µg of various mRNA transcripts into BHK cells (30,000 cells per

well in 24-well plates). An RNA to TransMessenger reagent ratio of 1:4 was used for all

transfections. Transfections were performed as per manufacturer's instructions. Three

hours post transfection, the medium was removed and replaced with fresh serum

containing medium. Cells were harvested and subjected to luciferase assays at time

points indicated. All transfections were performed in triplicate. Data is presented as

mean values +/- standard error.

3.2.4 Luciferase and BCA Assays

Cells were washed twice with 1 mL PBS followed by the addition of 0.2 mL lysis buffer (PBS with 0.1 % Triton X-100) before being stored at −70 ° C. Cells were thawed and 5-20 µl of sample were assayed in duplicate on a 96-well plate. Samples were assayed using a Berthhold Centro LB960 Microplate Luminometer and Luciferase Assay System (Promega). Standard luciferase assays were performed and transfection data is reported as mass quantities of luciferase protein using a standard curve obtained from serial 10-fold dilutions of a 20 mg/mL *Photinus pyralis* luciferase standard (Promega). Total protein was quantified using a Pierce BCA assay kit as per manufacturer's instructions.

3.2.5 Immunofluorescence

BHK cells were plated in 6-well plates (150,000 cells per well) and transfected with various amounts of plasmid DNA. At 24 h post-transfection, cells were washed once with 2 mL PBS-IF (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4) prior to fixation for 10 min with 2 mL 2% paraformaldehyde. Cells were subjected to three 30 s washes before being permeabilized with 0.25% Triton X-100 in PBS-IF for 5 min. After washing three times for 30 s with PBS-IF, cells were incubated with blocking buffer (10% BSA in PBS-IF) for 1 h, shaking gently at room temperature. Cells were washed three times for 10 min with PBS-IF followed by addition of primary antibody solution consisting of a 1:700 dilution of goat anti-T7 RNAP antibody, a gift from Dr. Paul Fisher (Department of Pharmacological Sciences, State University of New York at Stony Brook) in 2% BSA in PBS-IF. Cells were incubated with primary antibody solution for 2 h while shaking at room temperature. Cells were washed three times for

10 min in PBS-IF followed by the addition of secondary antibody (Rabbit anti-goat IgG, FITC labeled (QED Bioscience Inc), 1:200 dilution in 2% BSA-PBS-IF) and incubation for 2 h while shaking at room temperature. Cells were washed four times for 10 min with PBS-IF before being mounted and photographed on a Zeiss Axiovert S100 fluorescence microscope. Percentage of cells transfected was determined by counting transfected and non-transfected cells under the microscope. Data indicate the average of six separate counts from at least three different experiments.

3.2.6 RNase Protection Assay

Luciferase probe was prepared from *EcoR* 1 digested RPA-Luc. GAPDH probe was purchased from Pharmingen. Probes were labeled following the manufacturers protocol using ^{32}P-αUTP (3000 Ci/mmole, 10mCi/mL)(NEN).

BHK cells were plated on 6-well plates (150,000 cells per well) and transfected with various amounts of R011. At various time points, cells were washed once with PBS and recovered by treatment with trypsin. Total RNA was harvested from cells using an RNeasy miniprep kit (Qiagen). Various amounts of sample RNA was subjected to RNase protection analysis using the RiboQuant RPA system (Pharmingen) according to the manufacturer's protocol. In all experiments, the total amount of RNA was brought up to 2 µg using untransfected BHK total RNA. Standard curves were prepared from *in vitro* synthesized mRNA from supercoiled R011 as described above,). These mRNA transcripts are similar to those that will be generated from the cytoplasmic system. Serial dilutions of the R011 mRNA were made and various amounts were added to 2 µg untransfected BHK total RNA and subjected to RPA analysis. All values are standardized to the GAPDH control, and are the average +/- standard deviation of at

least three independent experiments. Data were collected using a Typhoon Phosphorimager (Amersham Biosciences) and analysis was performed using ImageQuant software (Amersham Biosciences). Note that when quantitating the GAPDH control, the blots were overexposed to give a sufficient value for GAPDH mRNA levels.

3.2.7 Calculation of Amount of Transgene mRNA per Transfected Cell

Using the standard curve from the quantitative RNase protection assay, the amount of luciferase mRNA present in each mRNA sample was calculated. Knowing the total amount of RNA loaded on the gel, the amount of luciferase mRNA per μg of total RNA was determined. Using immunofluorescence to determine the percentage of cells transfected, the amount of luciferase mRNA produced per transfected cell was able to be determined. For example: for 24 h time point on Figure3-5, 1.7 ng of luciferase mRNA was present in the sample (value derived from standard curve in Figure 3-5c). 0.5 μg of total RNA was used for that sample, so the total amount of luciferase mRNA per μg of total RNA is 1.7 ng/0.5 μg = 3.4 ng luciferase mRNA/μg total RNA. By examining the immunofluorescence data, it was found that ~3% of the cells were transfected with the autogene. Therefore, 3.4 ng luciferase mRNA/μg total RNA X 100/3 (dilution factor) = 113 ng luciferase mRNA/μg total RNA/transfected cell. Therefore, 0.113 μg luciferase mRNA/μg total RNA/transfected cell.

3.3 Results

3.3.1 Replacing the CMV Promoter with either the RSV or SV40 Nuclear Promoter has Little Effect on Autogene Expression

The enhanced dual promoter autogene system described previously (Finn, Lee et al. 2004) relied on the CMV promoter for the first round of nuclear transcription, effectively "triggering" the cytoplasmic expression system. It was of interest to explore the effect of other commonly used promoters. To this end, autogenes and their nuclear controls containing either the RSV or the SV40 promoters were constructed. As can be seen in Figure 3-1b, the different promoters had very little effect on the maximum levels of autogene activity. In contrast, the different promoters had a dramatic effect on the levels of nuclear gene expression. In the case of the SV40 promoter, the autogene system demonstrated over a 200-fold increase in expression over the nuclear system. It is important to note that even though the different promoters resulted in dramatically different amounts of nuclear expression (consistent with previously published data (Zarrin, Malkin et al. 1999), they had very little effect on the maximum levels of cytoplasmic expression. This is consistent with only a small, catalytic amount of nuclear expression being required to drive the cytoplasmic expression system, and that the cytoplasmic expression was reaching an apparent saturation level.

3.3.2 Autogene Expression is not Sensitive to the Type of Phage RNAP used.

It was of interest to compare the T7 RNAP protein with the two other RNAP proteins commonly used for *in vitro* transcription purposes, the SP6 and T3 RNAPs (Krieg and Melton 1987; Leary, Baum et al. 1991; Meador, McElroy et al. 1995). To this

73
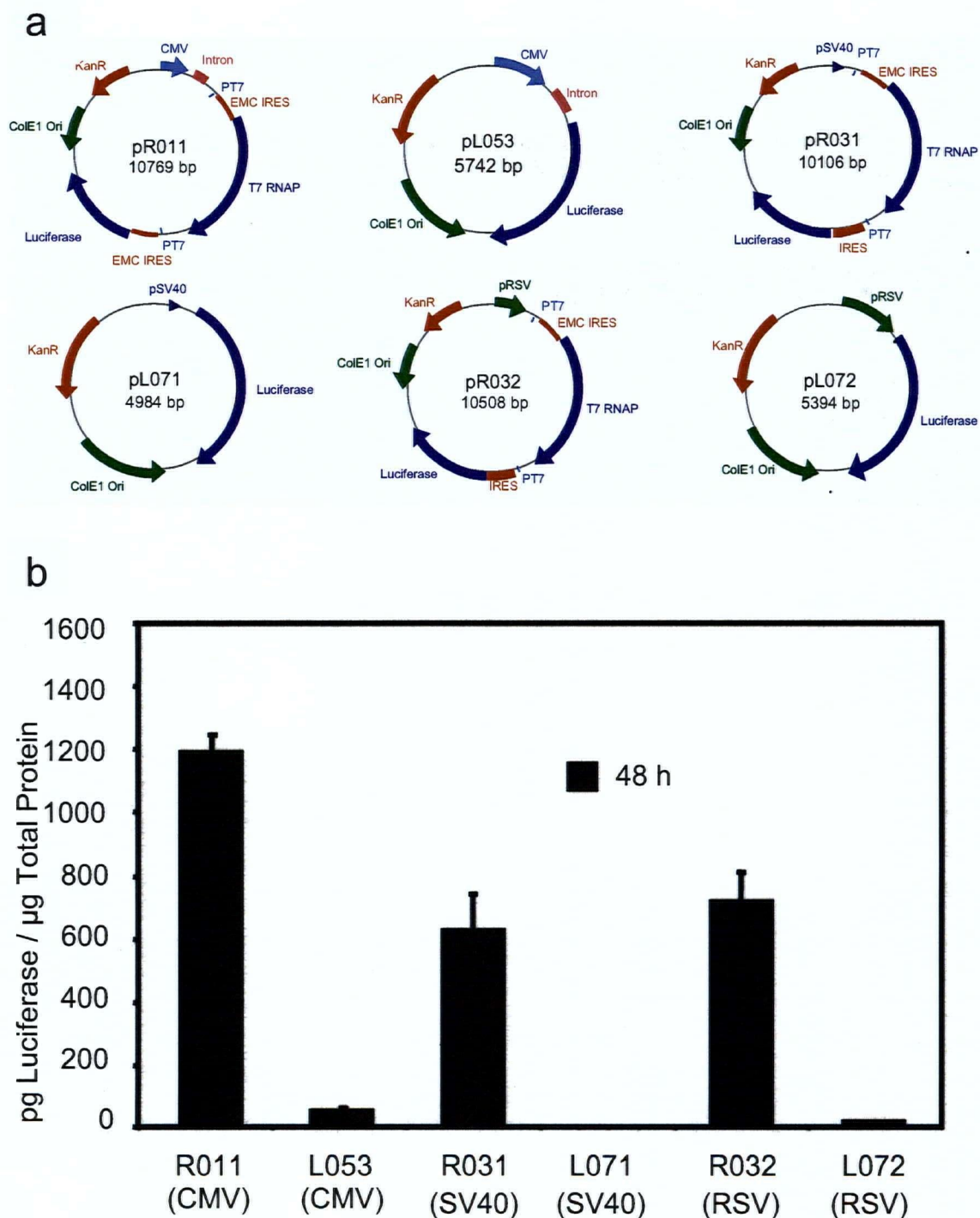
Figure 3-1: Nuclear promoter comparison a) Diagrams of plasmids used. b) Effect of various nuclear promoters on cytoplasmic versus nuclear expression. BHK cells were transfected with .75 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pPUC19). Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error.

end, the T7 RNAP gene in R011 was replaced with either the SP6 or the T3 RNAP gene. The R011 vector plasmid already had all three of the RNAP promoters in tandem, so there was no need to "add" in the promoter fragments. As can be seen in Figure 3-2b, using either the T3 or the SP6 RNAP in place of the T7 RNAP did not give a substantial increase in gene expression. It is noted that the T3 RNAP did show almost a 2 fold increase in gene expression, however, this increase was not found to be significant ($p > 0.05$).

3.2.3 Substitution of the EMCV IRES by Either the Gtx or eIF4G IRES Elements Reduced Autogene Expression

Higher expression levels have been observed employing IRES elements other than the EMCV used in the expression system employed here (Chappell, Edelman et al. 2000; Wong, Ngoi et al. 2002). Therefore, luciferase reporter plasmids were constructed containing either the EMCV, eIF4G, Gtx, or no IRES element at all, and these plasmids were tested in an *in vitro* transfection system, co-transfecting with a nuclear plasmid encoding for the T7 RNAP. As can be seen in Figure 3-3b, only the EMCV IRES was efficient at driving cap-independent translation of cytoplasmic transcripts. The eIF4G and Gtx IRES sequences appeared to have little if any effect on translation over the control that contains no IRES (which demonstrated background levels of gene expression). Similar results were seen using a cell free transcription and translation assay (data not shown). These results indicate that the EMCV IRES was the most effective IRES in the cytoplasmic expression system and that no advantage was to be gained by using either of the other two classes of IRES sequences.
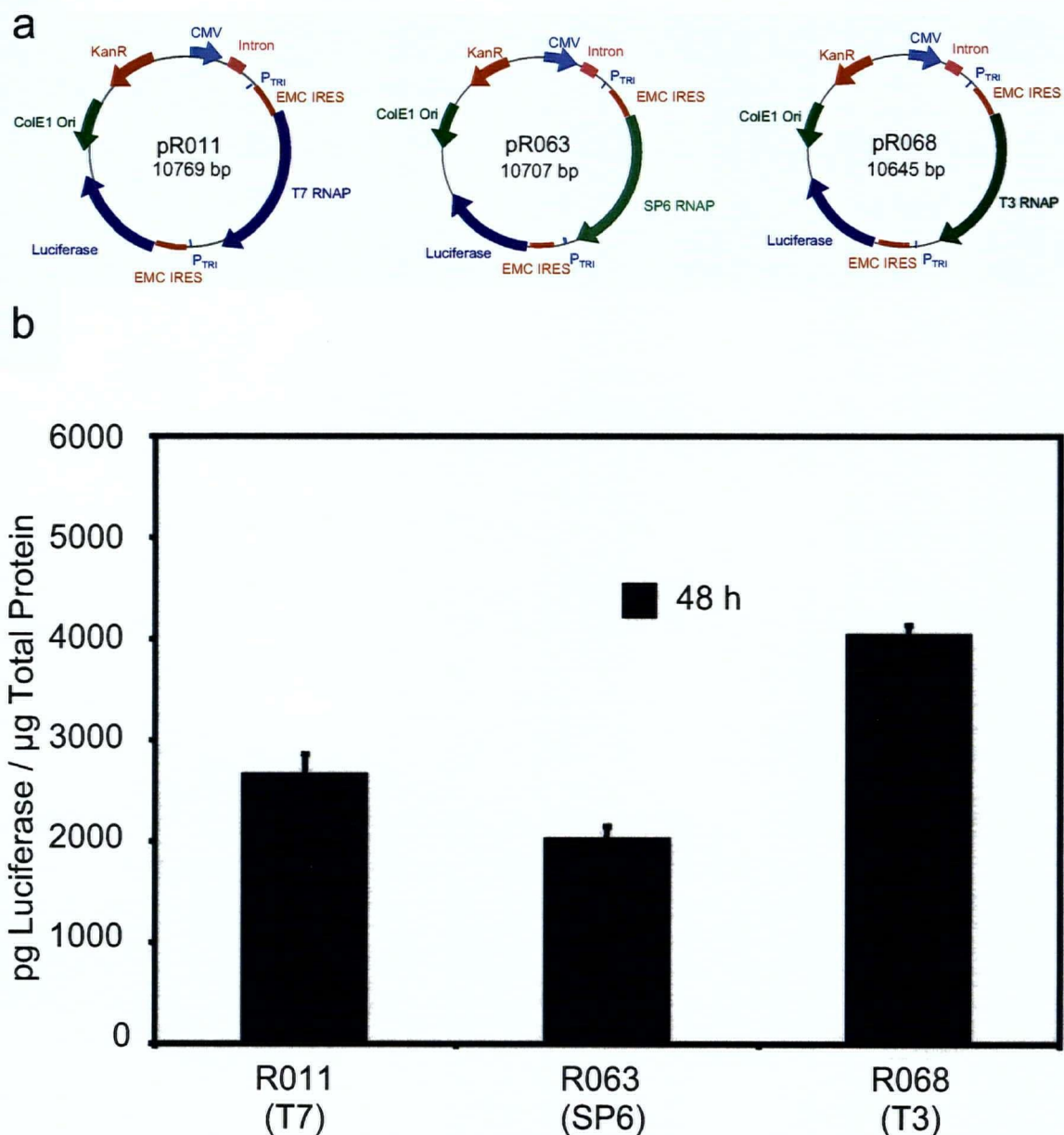
Figure 3-2: RNAP comparison a) Diagrams of plasmid constructs used. b) Comparison of T7, SP6 and T3 RNAP in autogene based cytoplasmic expressions system. BHK cells were transfected with 0.75 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmids (pPUC19). Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error. Transfection with either of the bi-cistronic autogene constructs (R011, R063 or R068) resulted in similar expression levels after 48 h. The T3 RNAP based autogene (R068) resulted in slightly (less than 2 fold) higher expression than the T7 RNAP based autogene (R011).
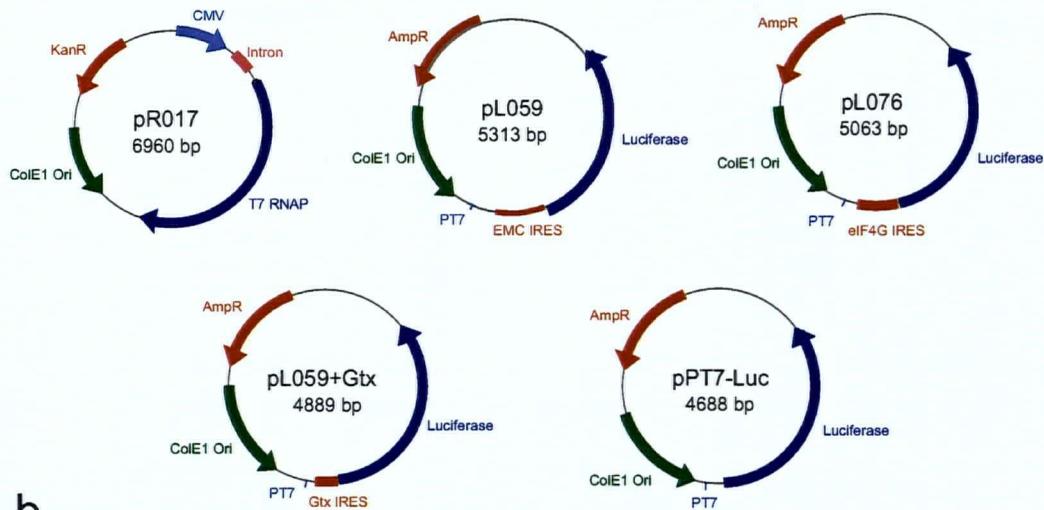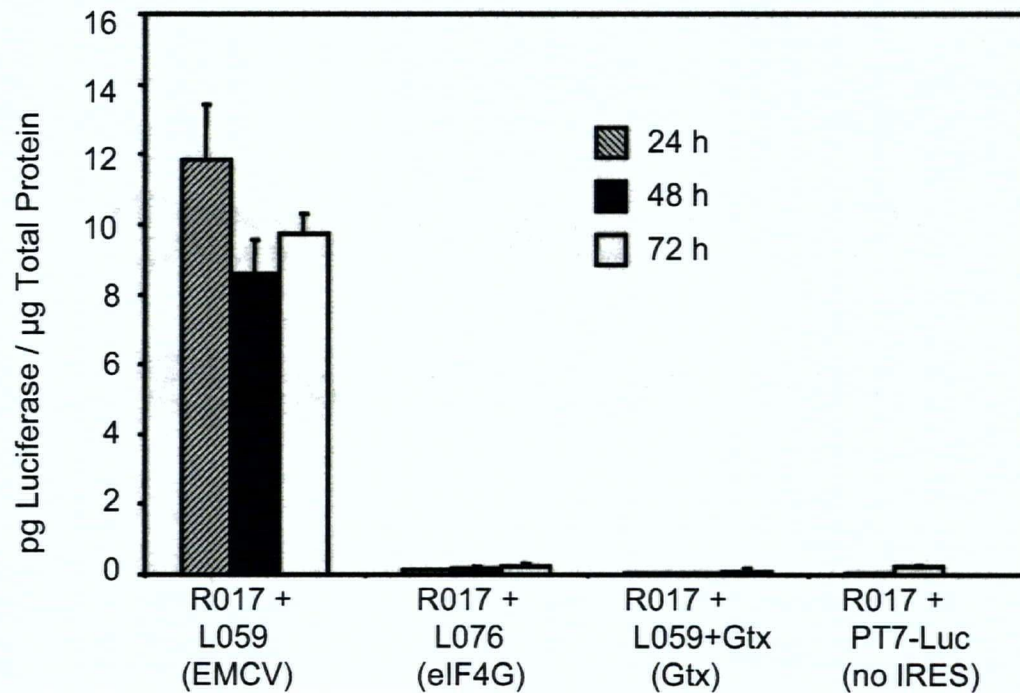
Figure 3-3: IRES comparison a) Diagrams of plasmid constructs used. b) Effect of IRES on cytoplasmic expression. BHK cells were transfected with a total of 0.75 μg/well of plasmid DNA. Half of the amount of DNA was R017 (CMV-T7-RNAP), and the other half was the T7 RNAP based luciferase expression cassette. Equimolar amounts of experimental plasmid were added, and the total mass of DNA per transfection was normalized by adding an unrelated plasmid (pPUC19). Error bars indicate standard error.

3.2.4 Nuclear mRNA Transcripts are Translated 20 Times More Efficiently Than Cytoplasmic Autogene Transcripts

In an effort to understand what other factors may be limiting the expression levels, the translation efficiency of the cytoplasmic transcripts (IRES-Luciferase) was compared with the nuclear transcripts (5' cap-Luciferase). mRNA was synthesized *in vitro* and the translation efficiency of these transcripts was determined via *in vitro* mRNA transfection. As can be seen in Figure 3-4, while the inclusion of the EMCV IRES does result in an increase in expression over no IRES at all, the cytoplasmic transcripts are translated ~20 times less efficiently than the capped nuclear transcripts. It is also interesting to note that the inclusion of the EMCV IRES into a capped transcript inhibited the expression ~5 fold. This is consistent with previously published data (Attal, Theron et al. 1999). While it has long been known that IRES elements are not as efficient at recruiting ribosomes as the 5' cap of mRNA transcripts, it is clear that the low translation of the cytoplasmic transcripts (as compared with a capped mRNA) is playing a major role in lower levels of gene expression than were expected. However, the low translation of the cytoplasmic transcripts may not be the primary factor limiting autogene expression. The observation that a 20 fold increase in luciferase protein expression is obtained with the cytoplasmic expression system, while the mRNA transcripts are being translated 20 times less efficiently than the capped nuclear transcripts, suggested the possibility that one of the factors responsible for reaching limiting levels of gene expression was at the level of mRNA production.
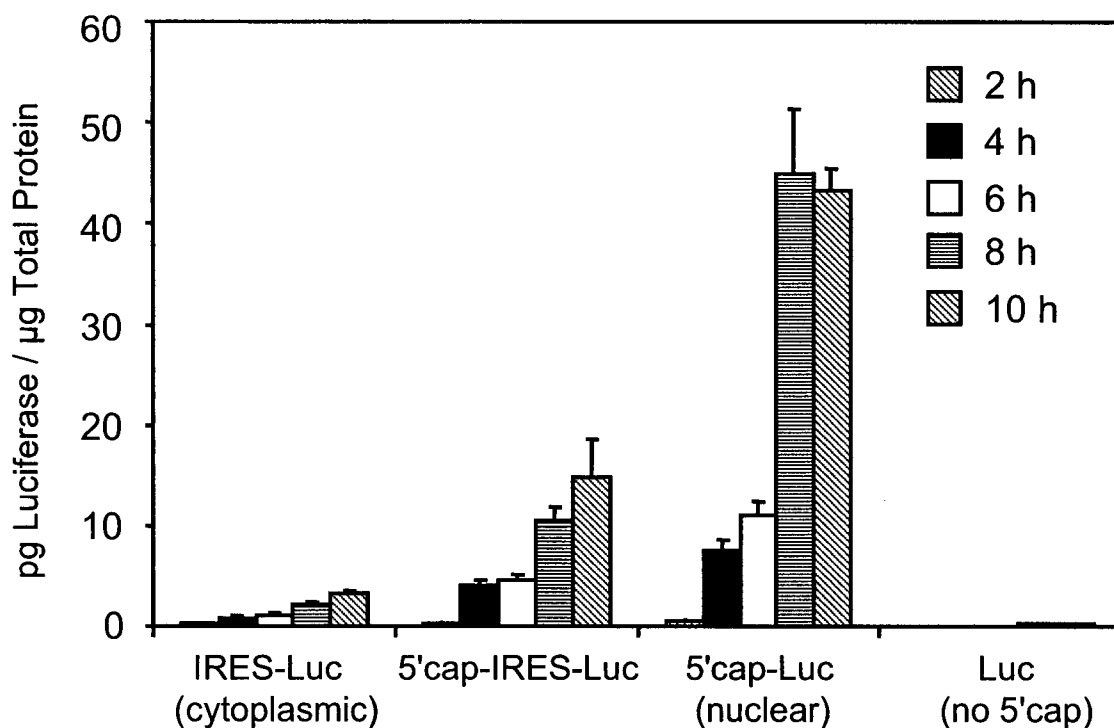
Figure 3-4: Comparison of translation efficiency of nuclear versus cytoplasmic mRNA transcripts. BHK cells were transfected with a total of 1 μg/well of *in vitro* synthesized mRNA using Transmessenger reagent as described in Materials and Methods. All transcripts were of a similar size. Error bars indicate standard error. Capped transcripts (nuclear) were translated 20 times more efficiently than uncapped transcripts containing the EMCV IRES (cytoplasmic). The insertion of the IRES into a capped transcript decreased the translation 5 fold. The lack of any IRES sequence or cap resulted in background levels of expression.

3.2.5 mRNA Production is a Limiting Factor in Autogene Expression

Given that the cytoplasmic transcripts are translated much less efficiently than the nuclear transcripts, it was clear that the RNA output of the cytoplasmic system must be much higher than that of the nuclear system in order to achieve the marked increase in luciferase expression. Therefore, a quantitative RNase Protection Assay (RPA) was performed to determine the amount of luciferase mRNA being produced. As can be see in Figure 3-5c, the amount of luciferase mRNA in the autogene transfection peaks at around 24 h post transfection. Using the standard curve and determining the percentage of cells transfected using immunofluorescence, it was determined that at 30 h, ~12 (+/- 0.4)% of the total RNA in each transfected cell was luciferase mRNA. Noting that in BHK cells only ~3% of the total RNA is mRNA (data not shown), this indicates that ~ 3.5 times more luciferase transcript was being produced than the sum of all other mRNA transcripts in the cell. Since this represents a significant allocation of cellular resources (such as NTPs (including ATP, the major energy source of the cell)), it appeared likely that mRNA production was a major factor limiting autogene expression.

To test this hypothesis, a dose response study was performed and RPA and immunofluorescence were used to determine the amount of luciferase transcript per transfected cell. If mRNA production was a limiting factor, we would expect to see a point at which the addition of additional plasmid has no effect on mRNA production. Cells were harvested at 24 h post transfection to reduce any cytotoxic effects that may be seen due to the large amount of foreign mRNA production. As can be seen in Figure 3-6, the amount of luciferase transcript produced reaches a saturation point at approximately 30 (+/- 5) % of the total RNA. This observation is supported by the presence of large molecular weight mRNA species when total RNA from transfected

a

8 h 24 h 33 h

48 h 72 h

b

	Free probe	R011 (total RNA (µg))					Standard Curve (R011 mRNA (ng))				
Time (h)		8	24	33	48	72						
RNA (µg or ng)		2.0	0.5	0.5	0.5	0.5		0.2	1.0	2.5	5.0	

Luciferase

GAPDH

$R^2 = 0.9752$

Luciferase/GAPDH (intensity)

ng R011 mRNA
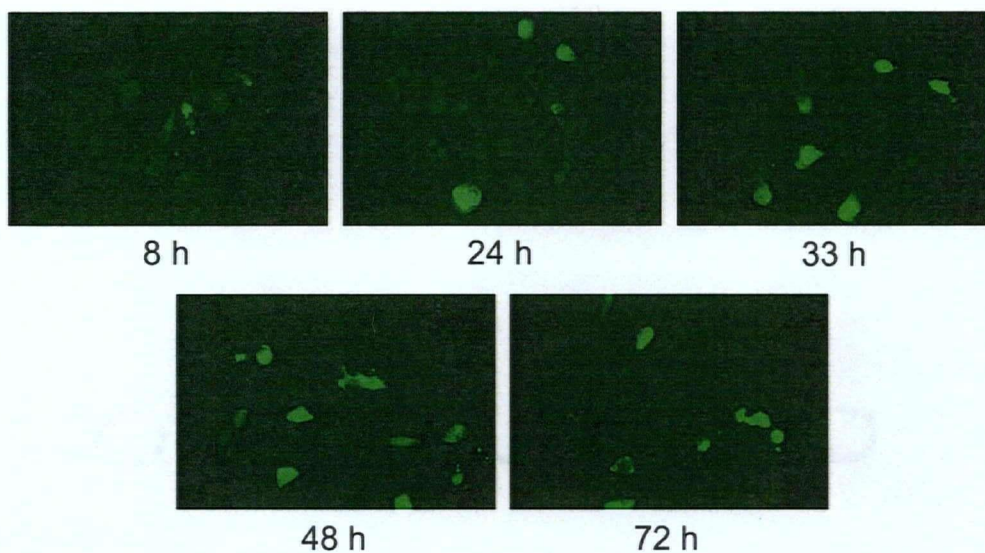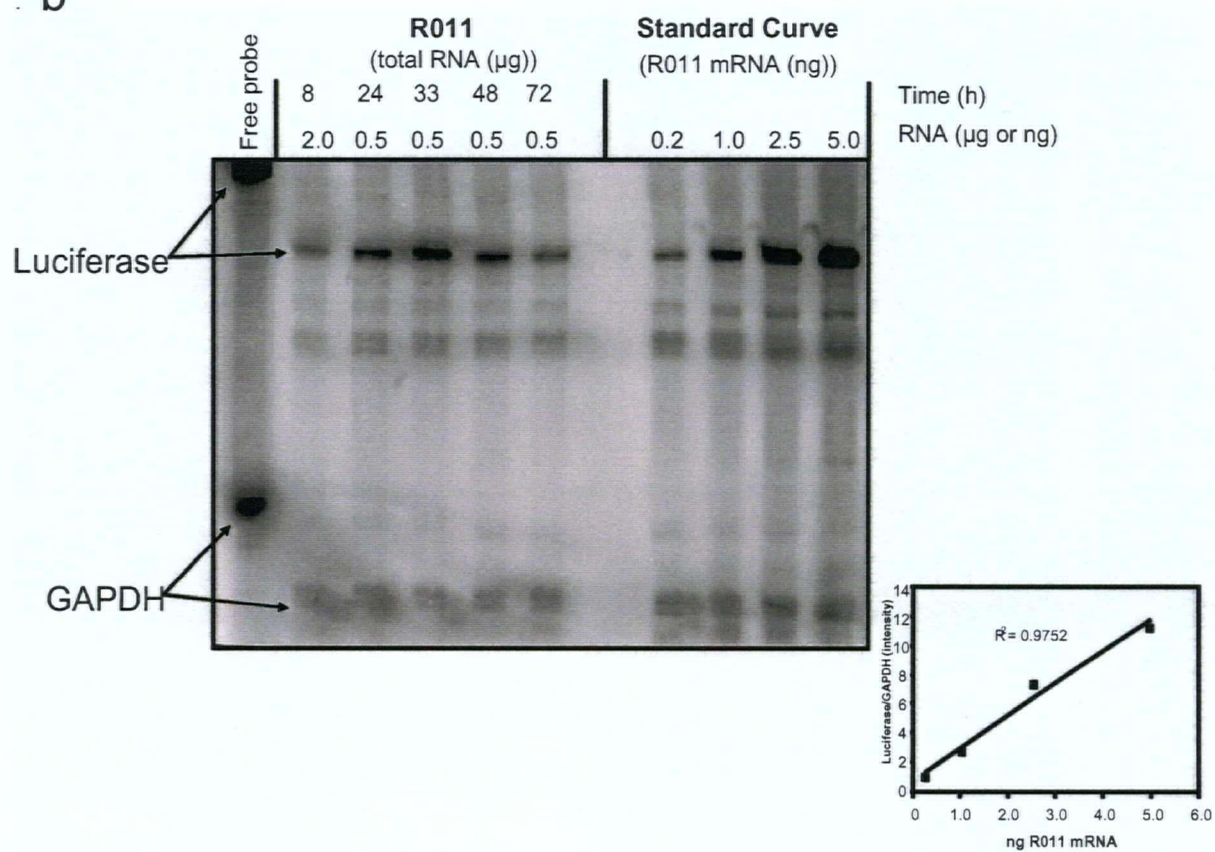
C
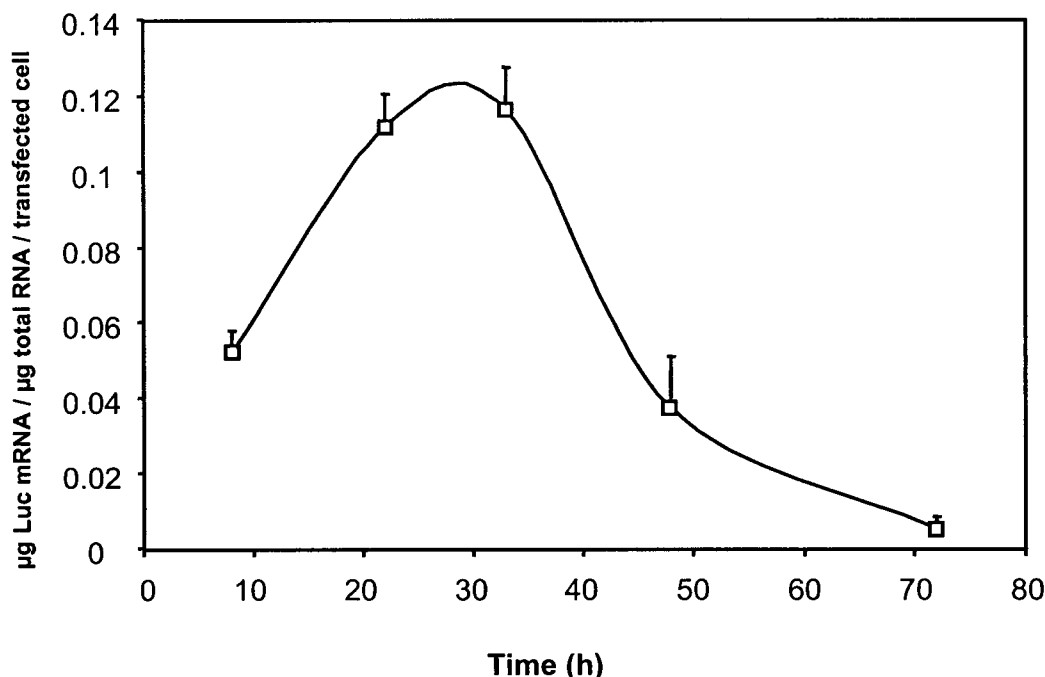
Figure 3-5: Amount of transgene mRNA per transfected cell a) Immunofluorescence of BHK cells transfected with R011. BHK cells were transfected with 0.75 µg of R011. Cells were fixed at time points indicated and subjected to immunofluorescence using anti-T7 RNAP antibodies and a FITC conjugated secondary antibody. The percentage of cells transfected was used to calculate the amount of transgene mRNA expressed per transfected cell.

b) RNase Protection Assay (RPA) of BHK cells transfected with R011. BHK cells were transfected with 0.75 µg of R011 and total RNA was harvested at time points indicated as described in Materials and Methods. Various amounts of total RNA (ranging from 0.5 to 2 µg) was subjected to RPA analysis using a ^{32}P labeled probe against luciferase mRNA as well as GAPDH control mRNA. An R011 standard curve was prepared by spiking 2 µg of untransfected BHK total RNA with various amounts (0.25 to 20 ng) of *in vitro* synthesized R011 mRNA. The inset is a graph of the standard curve of R011 mRNA. Intensity values were obtained using ImageQuant and luciferase mRNA values were standardized against GAPDH values (to control for lane loading differences). Blots were overexposed to get sufficient values for GAPDH controls. Standardized intensities were plotted against amount of R011 mRNA and this standard curve was used to determine the amount of luciferase mRNA in experimental samples.

c) Amount of luciferase mRNA produced per transfected cell over time. BHK cells were transfected with 0.75 µg of R011 as seen in Figure 5. The amount of luciferase mRNA per transfected cell was determined as described in Materials and Methods. The amount of luciferase mRNA peaks at around 30 h at ~12% of the total RNA, and then rapidly decreases after 72 h.
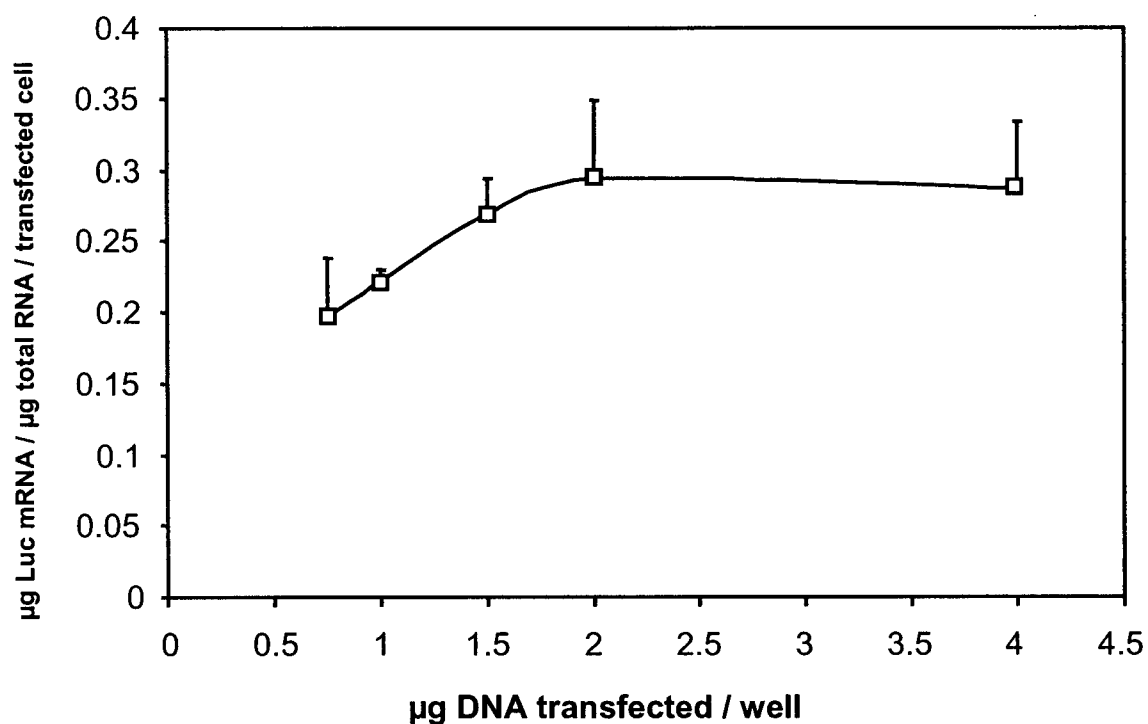
Figure 3-6: R011 dose response study in BHK cells. Values for mRNA levels and percentage of cells transfected were obtained as described in Materials and Methods and shown in Figure 5. The amount of luciferase mRNA produced per transfected cell increases with increasing amount of plasmid transfected up to a point, at which it then appears to reach a saturation level (~30 % of total RNA levels) at which point the addition of more plasmid has no subsequent increase on luciferase mRNA levels.

BHK cells was run on a denaturing agarose gel and subjected to ethidium bromide staining (data not shown). These large mRNA species were not present in RNA from non-transfected cells. This is consistent with the cells only being able to produce a finite amount of luciferase mRNA, and adding more plasmid cannot increase this level. This indicates that the amount of transgene mRNA production, in addition to its poor translation, is the major factor limiting autogene based cytoplasmic expression.

3.4 Discussion

Previous work described a dual promoter autogene based cytoplasmic expression system that demonstrated a 20-fold increase in gene expression over a standard CMV-based nuclear control. In the present work, attempts were made to increase the levels of cytoplasmic gene expression further, revealing some of the factors limiting gene expression in this system. Here we discuss the changes made to the autogene system, the finding that mRNA production is a limiting factor in autogene expression, and the implications of this result for future studies.

In an effort to further increase gene expression levels, the effect of changing either the nuclear promoter (CMV vs. RSV vs. SV40), the RNAP gene (T7 vs. SP6 vs. T3), or the IRES (EMCV vs. eIF4G vs. Gtx) element was examined. It was found that altering any of these three parameters did not significantly increase autogene expression.

For example, replacing the T7 RNA polymerase with either the SP6 or the T3 RNAP (both commonly used in *in vitro* transcription systems) resulted in very minimal (maximum 2-fold) increase in autogene expression. In fact, the SP6 RNAP appeared to be slightly less effective at driving autogene expression than either the T7 or the T3

RNAP. It should be noted that the T7 and T3 RNAP share almost 80% amino acid identity, while the SP6 RNAP shares about 25% identity with both the T7 and the T3 RNAP. It is known that the SP6 RNAP does not effectively transcribe linear DNA (Promega). It may be that cytosolic nucleases convert the supercoiled plasmid into a linear form (Pollard, Toumaniantz et al. 2001), therefore accounting for the decreased efficiency of the SP6 RNAP when compared with the T7 or T3 RNAP. Regardless of the mechanisms behind the differences, using either the SP6 or the T3 RNAP did not give any appreciable increase in autogene expression.

While other laboratories have reported increased expression when using other classes of IRES elements (60 fold increase with Gtx IRES (Chappell, Edelman et al. 2000), 100 fold increase with eIF4G IRES (Wong, Ngoi et al. 2002)) in a bi-cistronic context, no such enhancement was observed here. The most likely explanation for this is that the eIF4G and Gtx IRES sequences have thus far only been used to drive expression of the second gene in a bi-cistronic transcript. These transcripts are made in the nucleus and therefore contain a 5' cap structure. It is most likely that these small sequences do not recruit ribosomes *de novo*, but facilitate the re-initialization of translation of the ribosome after the first gene has been translated. The type of bi-cistronic system that these small IRES elements were studied in previously is not the same as the system tested in this study. Here the ability of the IRES elements ability to directly recruit ribosomes to the 5' end of an uncapped mRNA transcript is assessed.

These studies show that the eIF4G and GTX IRES elements are not able to recruit ribosomes to an uncapped mRNA transcript. The larger viral EMCV IRES has a complex secondary structure (Jang, Krausslich et al. 1988; Jang and Wimmer 1990; Evstafieva, Ugarova et al. 1991; Le and Maizel 1997) that may be able to recruit

ribosomes *de novo*, thus accounting for its ability to increase the translation of uncapped cytoplasmic transcripts. This hypothesis is consistent with certain controversial aspects of IRES sequences and how they function. There is convincing evidence that IRES elements act as true internal ribosome entry sites (indicated by the ability for the EMCV IRES to allow translation of circular transcripts (Chen and Sarnow 1998)), as well as evidence suggesting that the IRES element only act as rescue translation stimulators (Attal, Theron et al. 1999), allowing the ribosome to re-initiate translation when inserted after the stop codon of the first cistron in a bi-cistronic construct. The evidence presented here suggests that while the Gtx and eIF4G IRES elements may be effective in driving second gene expression in a bi-cistronic construct, they are ineffective for recruiting ribosomes *de novo* in the absence of a 5' cap structure.

The studies performed to determine factors that may be limiting the autogene expression indicate that the major factors limiting the autogene expression were both at the level of mRNA production and translation. It is clear that the cytoplasmic expression system is producing a large amount of mRNA (corresponding to transgene mRNA levels that are 10 times the sum of every other transcript in the cell). This significant allocation of cellular resources (such as NTPs) would be expected to eventually have a detrimental effect on cell growth. This is consistent with the results summarized in Figure 3-6, where it is demonstrated that the autogene expression rapidly decreases after 24 h. This could arise because of the short half-life of the cytoplasmic transcripts, the high levels of foreign mRNA production having a cytotoxic effect, or because of the loss of cytoplasmic plasmid (due to cytosolic nuclease activity).

While it cannot be stated conclusively that transgene mRNA saturation is responsible for limiting the autogene expression, it cannot be ruled out as a potential factor. Alternately, the cytoplasmic translation machinery may be saturated, meaning that the translation of the mRNA is the limiting factor. There most likely exists an equilibrium between mRNA levels and the translation of the cytoplasmic transcripts, and increasing either the levels of mRNA, or increasing the translation of the cytoplasmic transcripts would lead to a further increase in expression.

The cytoplasmic expression system described here has many potential applications. An example is suicide cancer gene therapy where a large amount of a suicide gene (e.g. Thymidine Kinase) is required to be expressed, leading to the death of that cell, and a corresponding large bystander effect. For researchers using siRNA or ribozyme expression, this system could be used for producing high levels of RNA in transfected cells. In addition, future work aimed at increasing the translation efficiency of the cytoplasmic transcripts could lead to a dramatic increase in gene expression, which would improve the utility of non-viral gene therapy based systems.

In conclusion, the results presented here demonstrate that modifications to the dual promoter autogene based cytoplasmic expression system, including changes in the nuclear promoter, RNAP gene, or IRES element, had a minimal effect on autogene expression. The factors responsible for limiting autogene expression are most likely at both the level of transgene mRNA saturation, and the poor translation of the cytoplasmic transcripts. This work demonstrates the ability of autocatalytic expression systems to produce extremely large amounts of transgene mRNA, and that advances in improving the translation efficiency of un-capped, cytoplasmic transcripts could lead to a significant increase in the utility of such systems.

CHAPTER 4

INVESTIGATION OF FACTORS RESPONSIBLE FOR CELL LINE CYTOPLASMIC EXPRESSION DIFFERENCES

4.1 Introduction

Previous chapters have described a novel cytoplasmic expression system that results in a 20-fold increase in the levels of gene expression over a standard CMV-based nuclear expression system (Finn, Lee et al. 2004), as compared with a 2-3 fold increase seen with previous similar systems (Brisson, He et al. 1999). While this increase was seen with BHK and Neuro-2a cells, further studies revealed that some cell lines, such as COS-7, demonstrated relatively poor levels of cytoplasmic expression.

The objective of this chapter was to determine what factors were responsible for the different expression levels between BHK (a high expressing cell line) and COS-7 (a low expressing cell line). After investigating numerous factors, including the Internal Ribosome Entry Sequence (IRES) element, T7 RNAP activity, cytosolic nuclease levels and intra-cellular processing, it was found that differences in the intra-cellular processing of the DNA-cationic lipid complex was the most probable factor responsible for the difference in gene expression.

4.2 Materials and Methods

4.2.1 Plasmids:

Plasmid R011 is a bi-cistronic plasmid consisting of a basic autogene cassette (containing the T7, T3 and SP6 RNAP promoters) driven by a CMV promoter and intron, as well as a downstream *Photinus pyralis* luciferase reporter gene cassette (for construction details see Chapter 2). L053 consists of the CMV promoter (with intron)

from NGVL3 and the *Photinus pyralis* luciferase gene. L059 consists of a pTRI-Amp

(Ambion) backbone with EMCV IRES, *Photinus pyralis* luciferase and beta-globin poly-

adenylation site derived from EMC-Luc, a gift from Dr. Jon Wolff (Waisman Center,

Wisconsin).   PT7-Luc (Promega) consists of the *Photinus pyralis* luciferase gene

driven by a T7 RNAP promoter. L080 consists of PT7-Luc with the Luciferase gene

driven by both the T7 and the SP6 promoter.

4.2.2 mRNA Synthesis:

mRNA synthesis was performed using a MEGAscript high yield transcription kit

(Ambion). For all transcripts used for *in vitro* transfection, plasmid template (L059 for

IRES-Luc and Cap-IRES-Luc mRNA; L080 for Cap-Luc mRNA; PT7-Luc for Luc mRNA)

was linearized using *EcoR* 1 and 1 µg of each plasmid was used as per manufacturers

protocols. mRNA was recovered by the LiCl procedure as outlined in the

manufacturer's protocol.   After LiCl precipitation, the RNA pellet was washed 3 x with

70% ethanol to completely remove any unincorporated NTPs.

4.2.3 Transfections:

Lipoplexes were formed by mixing plasmid DNA with large unilamellar vesicles

(LUVs) composed of equimolar amounts of DOPE:DODAC (50:50) on ice and

incubated for 20 min prior to use. All transfections were performed at a cationic lipid to

plasmid DNA charge ratio of 3:1. Lipoplexes were diluted with serum-containing media

before addition directly to cell medium. BHK or COS-7 cells were plated at 30,000 cells

per well in 24-well plates.   The total mass of plasmid added was identical in all

transfections. Equimolar transfections using experimental plasmids of different sizes

were achieved through the addition of an empty vector (pPUC19) to normalize the total mass of DNA in each transfection. For mRNA transfections, TransMessenger reagent (Qiagen) was used to transfect differing amounts of various mRNA transcripts into BHK cells (30,000 cells per well in 24-well plates). An RNA to TransMessenger reagent ratio of 1:4 was used for all transfections. Transfections were performed as per manufacturer's instructions. Three hours post transfection the medium was removed and replaced with fresh serum containing medium. Cells were harvested and subjected to luciferase assays at time points indicated. For mRNA pretreatment studies, cells were transfected with mRNA as described above, followed by a DNA transfection 24 h later. All transfections were performed in triplicate. Data is presented as mean values +/- standard error.

4.2.4 Luciferase and BCA Assays:

Cells were washed twice with 1 mL PBS followed by the addition of 0.2 mL lysis buffer (PBS with 0.1 % Triton X-100) before being stored at –70 º C. Cells were thawed and 5-20 µl of sample were assayed in duplicate on a 96-well plate. Samples were assayed using a Berthhold Centro LB960 Microplate Luminometer and Luciferase Assay System (Promega). Standard luciferase assays were performed and transfection data is reported as mass quantities of luciferase protein using a standard curve obtained from serial 10-fold dilutions of a 20 mg/mL *Photinus pyralis* luciferase standard (Promega). Total protein was quantified using a Pierce BCA assay kit as per manufacturer's instructions.

4.2.5 Cytoplasmic Extract Preparation:

Cytoplasmic extracts were prepared using a hypotonic buffer solution. Cells (2 million) were washed with PBS (2 x 1mL) before a 15 min incubation on ice in 500 μl hypotonic extraction buffer (10 mM Hepes-KOH pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.6% NP-40). The samples were centrifuged at 13,000 x g for 3 min at 4 °C and the supernatant was collected and saved as the cytoplasmic fraction. Protein levels were quantitated using a Pierce BCA assay (as described above) after exchanging the hypotonic extraction buffer with PBS using a centrifugal filter device with a MW cut off of 3000.

4.2.7 Nuclease Assay Procedure:

Various amounts of cytoplasmic extract were incubated with plasmid DNA (50-300 ng extract/μg plasmid DNA) at 37°C. At time points indicated, aliquots were removed and flash frozen in liquid nitrogen and stored at -70°C until all assays were completed. Samples were then thawed and run on a 0.8% agarose gel and bands were visualized using ethidium bromide. Nuclease activity defined by the proportion of intact supercoiled plasmid left over time.

4.2.8 Plasmid Labeling:

R011 (cytoplasmic expression plasmid) was labeled using a *Label* IT Tracker Fluorescein Kit from Mirus Corporation following manufacturers instructions. A ratio of 1 μl *Label* IT Tracker Reagent per μg of plasmid DNA was used. Transfections were performed as previously described.

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4.2.9 Fluorescence Microscopy:

At time points indicated, cells were washed 3 x with room temperature PBS before fixing for 5 min with 2% paraformaldeyde. Cells were further washed with 2 x 1mL PBS before being treated with Prolong Antifade (Molecular Probes) and mounted. Pictures were taken using a Zeiss Axiovert S100 fluorescence microscope. In all cases, exposure times were kept constant to allow for ease of comparison of samples.

4.3 Results

4.3.1 Autogene Transfection in COS-7 Cells Results in Lower Transgene Expression than in BHK Cells

In previous chapters, it was found that the enhanced dual promoter autogene system demonstrated transgene expression levels that were 15-20 times higher than the equivalent nuclear control. This was demonstrated for both BHK and Neuro-2a cells. However, when the system was tested in the COS-7 cell line, the autogene system resulted in levels of gene expression that were lower than the nuclear control (Figure 4-1). It had been thought that the autogene system should produce high levels of gene expression in any transfected cell. Possible factors that would affect autogene expression levels including IRES function, T7 RNAP activity, cytosolic nuclease levels, and intra-cellular processing differences were therefore examined.

4.3.2 EMCV IRES is Functional in COS-7 Cells

The first parameter that was tested was whether or not the EMCV IRES was functional in COS-7 cells. In order to determine this, mRNA was synthesized *in vitro* and transfected directly into either BHK or COS-7 cells. As is observed in Figure 4-2,

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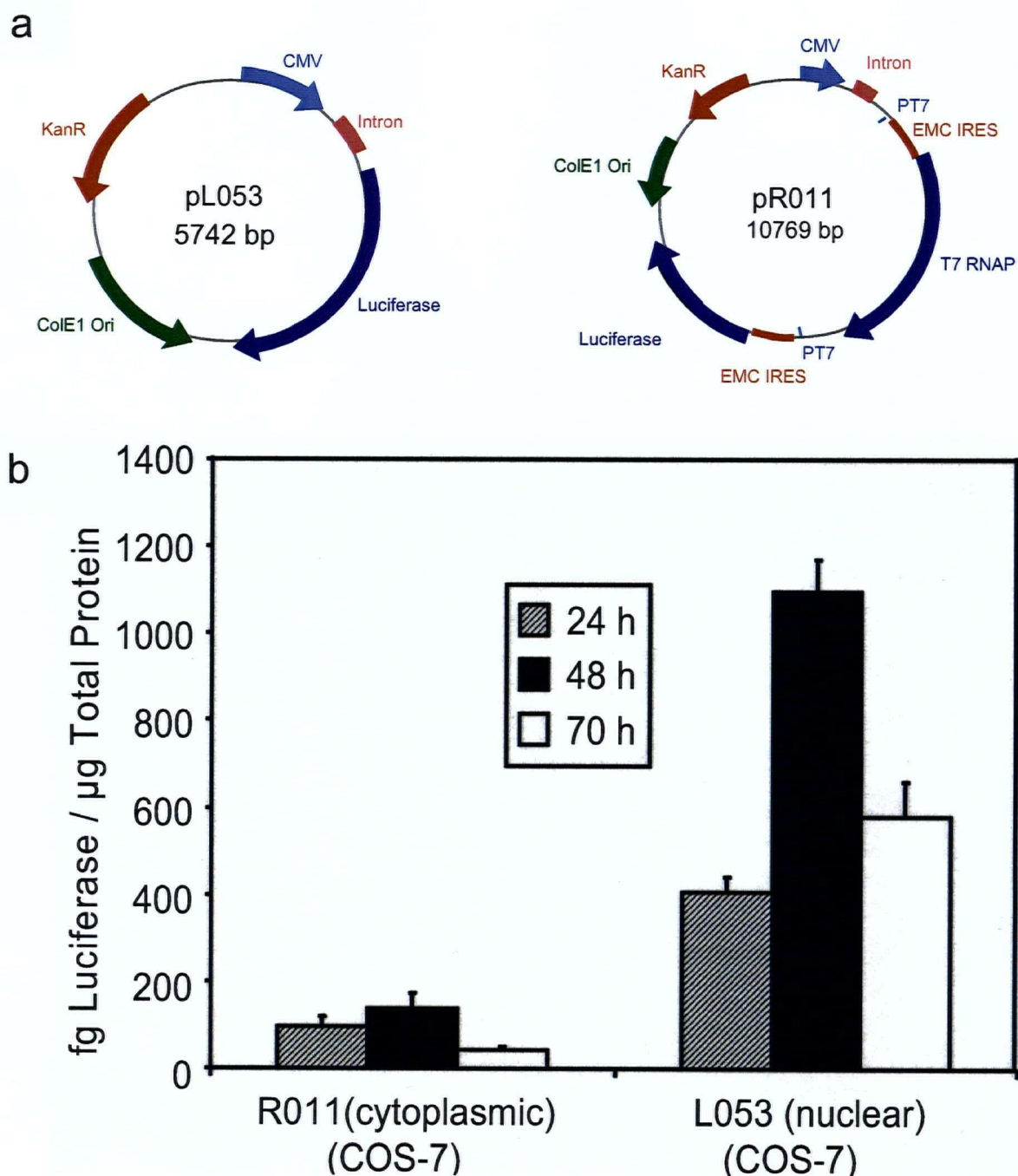
Figure 4-1: Autogene expression in COS-7 cells A) Diagrams of plasmids used. B) Comparison of cytoplasmic (R011) and nuclear (L053) expression plasmids in COS-7 cells. COS-7 cells were transfected with 0.75 µg/well of plasmid. Equimolar amounts of plasmids were added, and the total mass of DNA per transfection was kept equal by adding an unrelated plasmid (pPUC19). Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error.

the pattern of gene expression is similar for both cell lines. This indicates that while the IRES containing transcripts are not translated as efficiently as capped transcripts (previously described in Chapter 3), the EMCV IRES has the same relative expression pattern in both of the cell types, indicating that differences in IRES function are not the reason for the poor expression levels in COS-7 cells. It is interesting to note that the while the pattern of gene expression is similar, the absolute levels of gene expression are much lower in the COS-7 cells than the BHK cells. While it is known that cell division is an important factor for plasmid transfection, the BHK and COS-7 cells were found to have similar doubling times (data not shown), ruling this out as a factor responsible for the low expression levels in the COS-7 cells. Another reason could be because COS-7 cells are transfected (nucleic acid taken up and delivered into the cytoplasm or nucleus and subsequently expressed) less efficiently than BHK cells, or the intracellular processing of the nucleic acid-lipid complexes are different in the two cell lines, with the BHK cells having a greater amount of intact nucleic acid being released into the cytoplasm than the COS-7 cells. This possible difference in nucleic acid:cationic lipid complex processing could be responsible for the low levels of cytoplasmic autogene expression.

4.3.3 T7 RNAP is Functional in COS-7 Cells

The next parameter that was examined was whether the T7 RNA polymerase (that is the basis of the cytoplasmic expression system) is active in COS-7 cells. Therefore, *in vitro* synthesized T7 RNAP mRNA was transfected directly into BHK or COS-7 cells. Twenty-four hours later cells were transfected with a luciferase reporter plasmid that would only express luciferase if functional T7 RNAP was being produced in those cells. As can be seen in Figure 4-3, while the levels of luciferase expression were
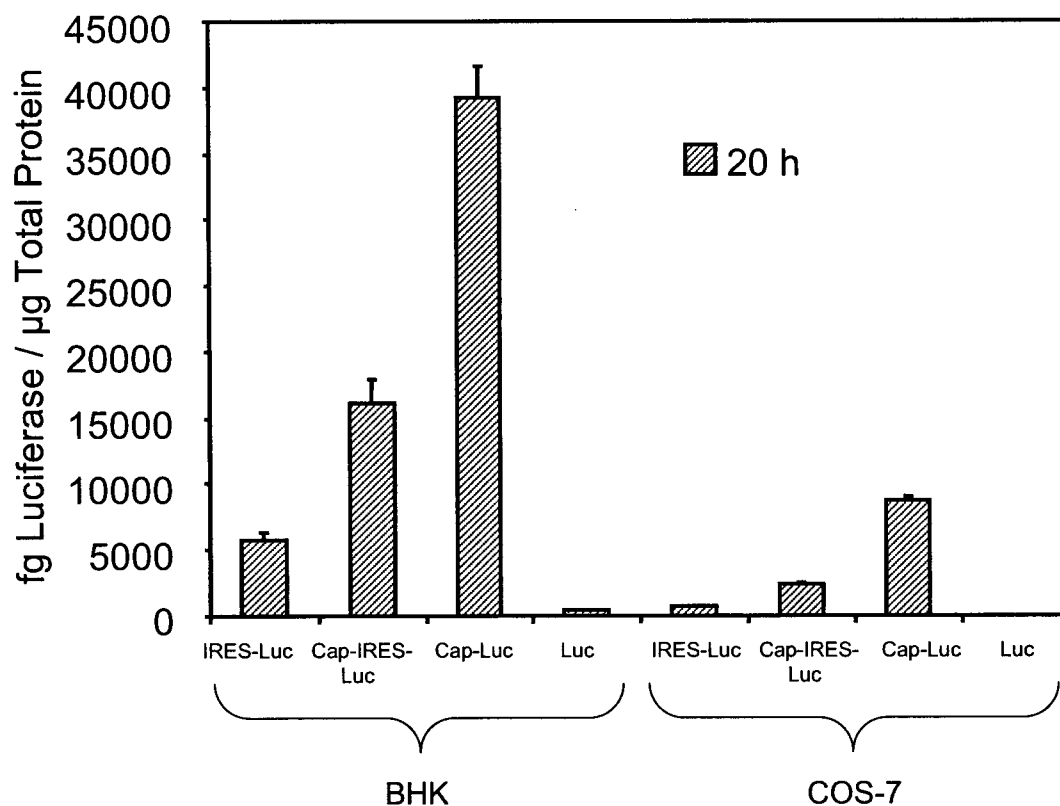
Figure 4-2: Transfection of BHK or COS-7 cells with various mRNA species. Cells were transfected with 0.75 μg/well of mRNA. Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error.

lower in the COS-7 cells, these lower levels are consistent with the overall lower expression levels also seen in Figure 4-2. Note that in the absence of T7 RNAP pretreatment, no luciferase was detected. This data demonstrates that the T7 RNAP was indeed functional in the COS-7 cells. Therefore, these results indicate that the activity of the T7 RNAP gene was not the reason for the poor expression levels.

4.3.4 Effect of T7 RNAP mRNA Pretreatment on Autogene Expression

Since it appeared as if all of the individual components of the autogene expression system were functioning in the COS-7 cells, it was suggested that perhaps the poor expression levels were due to temporal constraints. The cytoplasmic expression system is dependent on the initial source of T7 RNAP coming from the nucleus via the CMV promoter, which is then able to 'trigger' the cytoplasmic system. Perhaps by the time the first T7 RNAP proteins were produced via the nuclear transcripts (at least 8-12 hours post transfection), cytosolic nucleases had degraded the cytoplasmic plasmid, therefore leaving no substrate for the T7 RNAP. In order to test this hypothesis, BHK or COS-7 cells were transfected with T7 RNAP mRNA. Twenty-four hours post mRNA transfection cells were transfected with a plasmid encoding either the cytoplasmic expression system (R011) or the nuclear control (L053). As is apparent in Figure 4-4, the pretreatment of the COS-7 cells led to a 10 fold increase in autogene based expression that was not seen with the nuclear control. It was also observed that only about a 2-3 fold increase in expression was seen with the BHK cells (data not shown). This result appeared to support the hypothesis that timing was a major factor. If the cells were already primed with T7 RNAP protein prior to autogene expression, a substantial increase in gene expression was seen. These results suggest that certain factors affecting the amount of free cytoplasmic plasmid (such as

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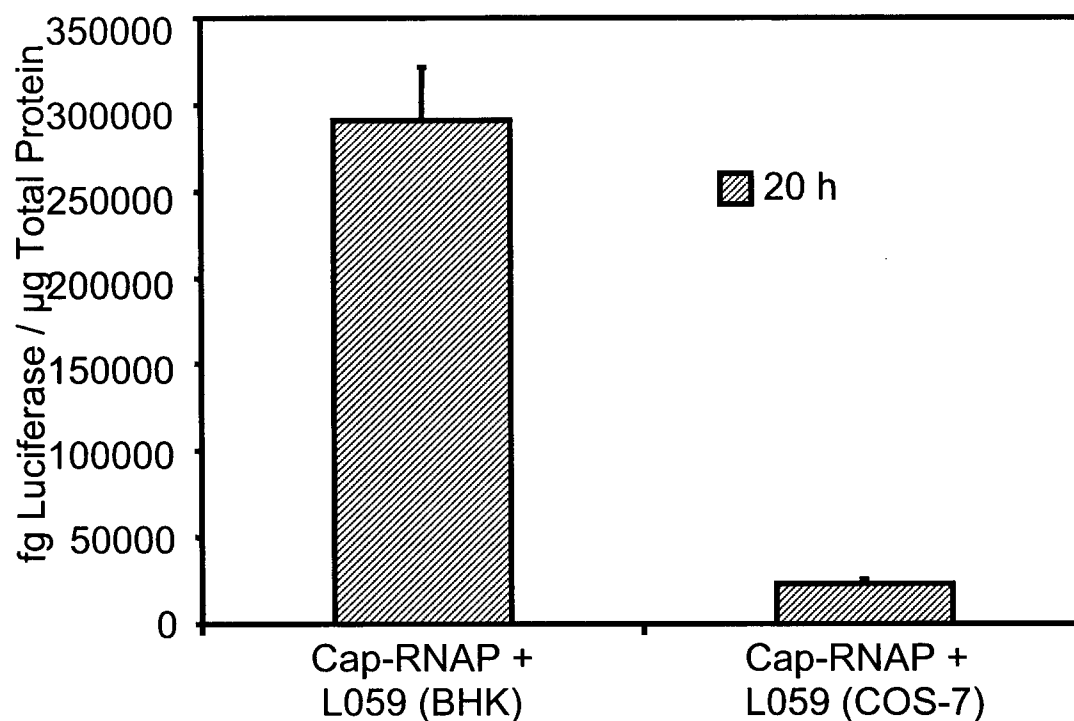
Figure 4-3: T7 RNAP is functional in COS-7 cells. Transfection of BHK or COS-7 cells with 0.75 µg of capped T7-RNAP mRNA for 24 hours, followed by a transfection with 0.75 µg of a luciferase reporter construct (L059) mRNA species. Cells were harvested 20 h post plasmid transfection. Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error. Note that in the absence of T7 RNAP (no pre-transfection) no luciferase was detected.

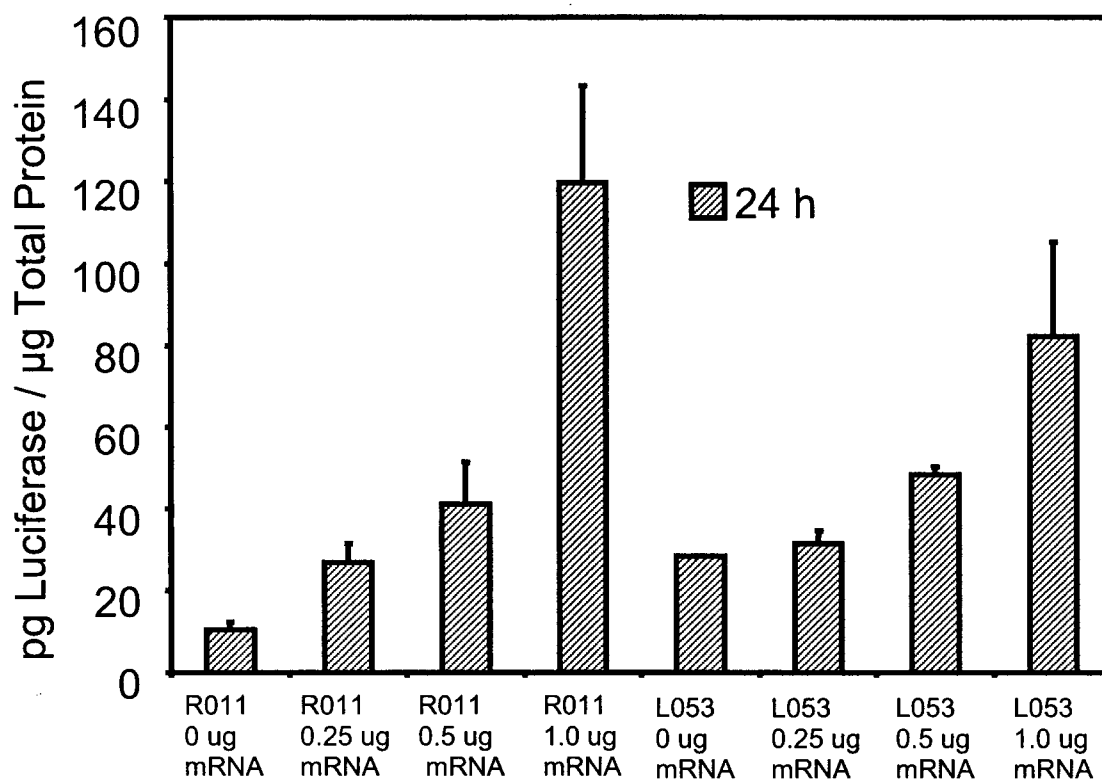**Figure 4-4:** Pretreatment of COS-7 cells with T7 RNAP mRNA. Transfection of COS-7 cells with 0.75 μg of capped T7-RNAP mRNA for 24 hours, followed by a transfection with 0.75 μg of R011 (cytoplasmic) or L053 (nuclear) expression plasmids. Cells were harvested 24 h post plasmid transfection. Transfections and luciferase assays were performed as described in Materials and Methods. Error bars indicate standard error

cytoplasmic nuclease levels or the intracellular processing of complexes) are playing an important role in autogene expression differences between the BHK and COS-7 cells.

4.3.5 BHK and COS-7 Cells have Similar Levels of Cytosolic Nucleases

In order to understand the difference between the high expressing (BHK) and low expressing (COS-7) cell lines, it was hypothesized that perhaps the BHK cells had lower levels of cytosolic nuclease, therefore more plasmid survived in the cytoplasm during the 8-12 h lag period before T7 RNAP was produced from the nuclear mRNA. Therefore, cytoplasmic extracts of both cell lines were obtained and subjected to nuclease assays. As can be seen in Figure 4-5, it appears as if the BHK cells did not have lower levels of cytoplasmic nuclease activity than the COS-7 cells, and may even appear to have slightly higher nuclease activity. Therefore, it appears as if the levels of cytosolic nuclease are not a major determining factor with respect to autogene based cytoplasmic expression.

4.3.5 Intracellular Processing of DNA is Different in BHK and COS-7 Cells

While it had been determined that the low levels of autogene expression in COS-7 cells was not due to increased levels of cytosolic nucleases, it could occur due to differences in the intracellular processing of DNA-lipid complexes between the two cell lines. In order to investigate this, plasmid DNA was labeled with a fluorescent label, transfected into both BHK or COS-7 cells, and then florescence microscopy was performed at various time points. As can be seen in Figure 4-6, while there appears to be a similar level of plasmid taken up into both cell lines at early time points (24 h), at later time points (48 h), the BHK cells seem to have accumulated a significant amount
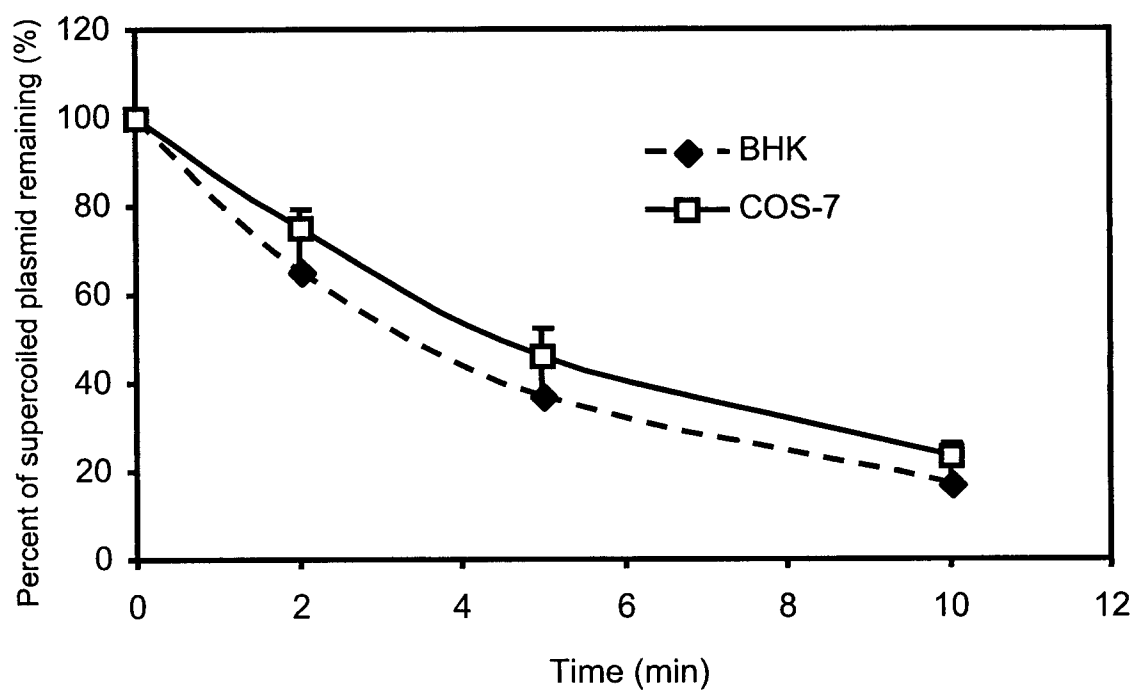
Figure 4-5: Cytosolic nuclease assays of BHK and COS-7 cytoplasmic extracts. 200 ng/μg plasmid DNA was incubated and aliquots were taken at time points indicated. No significant difference in nuclease activity between the BHK and COS-7 cytoplasmic extracts was observed.
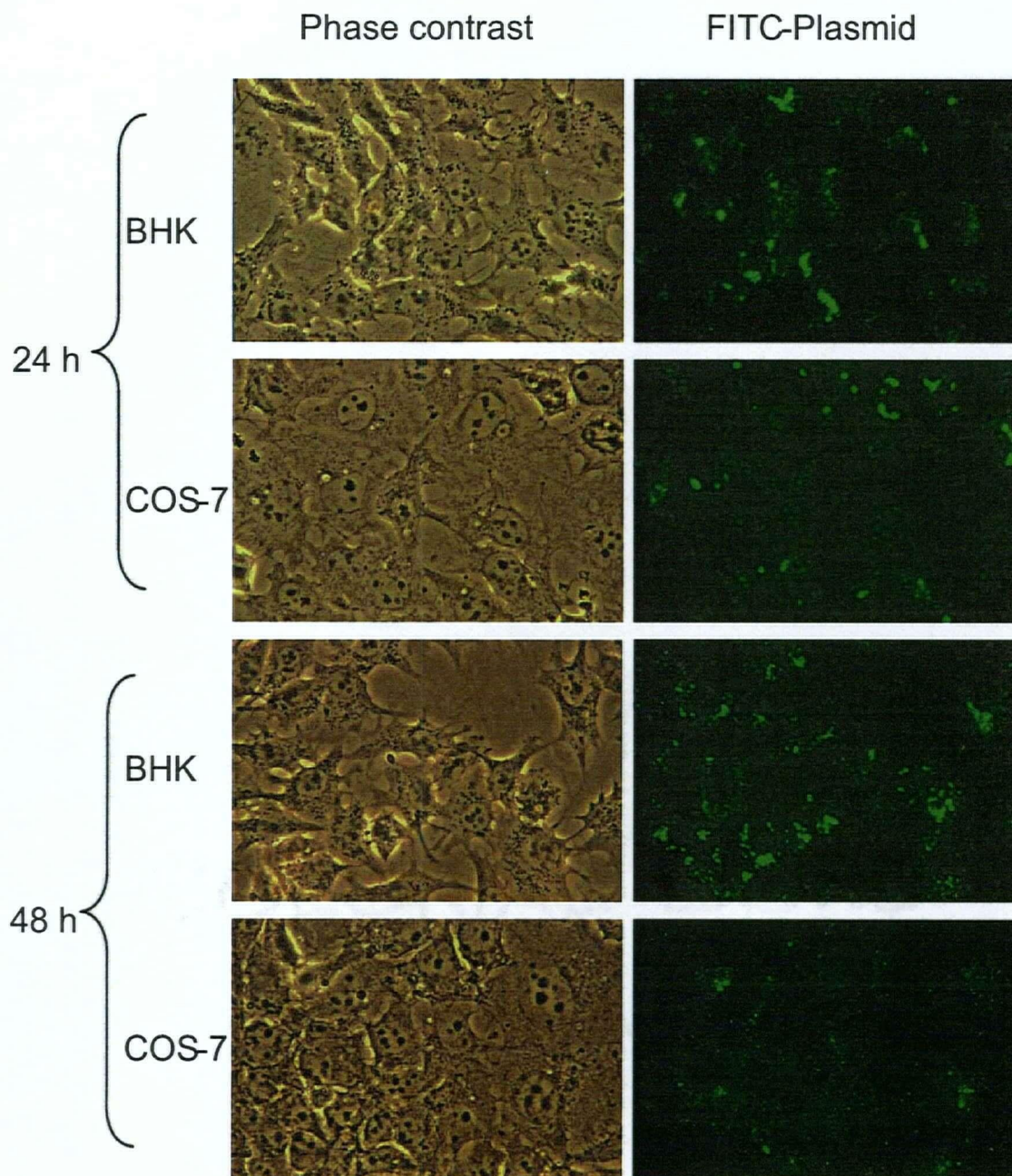
Figure 4-6: Intracellular processing of complexes. Transfection of BHK or COS-7 cells with FITC labeled R011. Cells were transfected with 0.75 µg of FITC-R011. Cells were fixed at time points indicated and subjected to phase contrast and fluorescence microscopy.

of plasmid in punctate, perinuclear structures, while the COS-7 cells have lost a significant amount of their fluorescence. Thus, it appears as if the BHK cells contain a pool of plasmid DNA for a longer period than the COS-7 cells. This perinuclear pattern seen with the BHK cell is characteristic of DNA-lipid complexes, and previous work (Zabner, Fasbender et al. 1995) has demonstrated that these structures are not associated with lysosomes, and appear to be large structures, presumably formed when multiple endosomes have fused into larger vesicles. These studies clearly show that there is a difference in the intracellular processing of the DNA-lipid complexes between the BHK and COS-7 cells, and this is most likely the reason behind the lower levels of cytoplasmic gene expression seen with the COS-7 cells.

4.4 Discussion

While previous work (Chapter 2) demonstrated a 15-20 fold increase in levels of gene expression using an autogene based, cytoplasmic expression system (Finn, Lee et al. 2004) in BHK and Neuro-2a cell lines, recent studies showed that the levels of cytoplasmic expression were significantly lower in some other cell lines, such as COS-7. In the present work, an attempt is made to determine what the reason for the difference was. Here various factors are discussed, including the activity of the various components of the autogene system in BHK and COS-7 cells, the finding that pretreating the COS-7 cells with T7 RNAP led to an increase in expression, and that the differences in expression between the two cell lines is most likely due to differences in intracellular processing of the DNA-lipid complexes.

Previous studies have shown that IRES function is dependent on trans-acting cellular factors (Belsham and Sonenberg 1996). The pyrimidine tract binding protein (PTB) binds to the EMCV IRES (Kolupaeva, Hellen et al. 1996), and functions by maintaining the IRES secondary structure, thereby facilitating translation through the IRES (Kaminski and Jackson 1998). The La antigen has also been demonstrated to bind to the IRES (Meerovitch, Svitkin et al. 1993; Belsham and Sonenberg 1996). There is also evidence for other proteins binding to various regions of IRES elements (Hoffman and Palmenberg 1996) that have been reported to have an effect on the IRES function. Therefore, it was thought that perhaps one of the differences between the BHK and COS-7 cells was the presence and/or abundance of various trans-acting cellular factors. However, transfection with various mRNA transcripts showed an identical pattern to that of the BHK cells, indicating that the EMCV IRES was functioning similarly in both of the cell lines, ruling out the IRES as the reason for the expression level differences.

It was then suggested that perhaps the T7 RNAP protein was not active in the COS-7 cells. This would also lead to poor expression levels. Transfecting the cells with capped mRNA encoding T7 RNAP, and then following 24 h later with a plasmid that will only express luciferase in the presence of T7 RNAP demonstrated that this was not the case. Even though the levels of expression were much lower than in BHK cells, a substantial amount of protein was expressed. It appears as if the COS-7 cells in general may not be very amenable to mRNA transfection, as both the transfections with the luciferase encoding mRNA transcripts (Figure 4-2) as well as the T7 RNAP encoding transcripts (Figure 4-3) gave much lower expression levels in the COS-7 cells than the BHK cells. This may be indicative of some difference in intracellular

processing of the nucleic acid-lipid complex, allowing for less cytoplasmic delivery of mRNA.

As it appeared that the individual components of the autogene system were functioning, it was thought that perhaps the difference was due to a matter of timing. It is known that it takes at least 8-12 h post transfection in order to detect any expression from the nucleus, with expression usually peaking at around 24-48 h post-transfection. Perhaps in this time the majority of cytoplasmic plasmid in the COS-7 cells was degraded by cytosolic nucleases. By priming the cells with T7 RNAP (via pre-transfection of cells with T7 RNAP mRNA) prior to autogene transfection, a 10 fold increase in autogene expression was observed, as opposed to about a 2 fold increase with the nuclear control (most likely due to non-specific effects associated with increased lipid or nucleic acid delivery). Thus it was clear that the lag time between transfection and T7 RNAP expression from the nucleus was playing a role in the poor expression levels in COS-7 cells.

In order to further understand this phenomenon, it was proposed that the COS-7 cells had higher levels of a cytosolic nuclease, and this was degrading the cytoplasmic plasmid, decreasing the amount of intact plasmid left for cytoplasmic expression. However, nuclease assays on cytoplasmic extracts from BHK or COS-7 cells showed that in fact, the COS-7 cytoplasmic extracts had less nuclease activity than the BHK cells. Thus, increased levels of cytosolic nucleases in the COS-7 cells was not the reason for the decreased expression.

The observation that the mRNA transfections gave much lower levels of expression in COS-7 cells than BHK cells hinted at the fact that perhaps there were

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differences in the intracellular processing of the DNA-lipid complexes, with the COS-7 cells allowing for less free plasmid to be released into the cytoplasm.

By labeling the plasmid with a fluorescent label (FITC) and looking at the internal distribution of plasmid in both BHK and COS-7 cells over time, it was determined that there was a qualitative difference in not only the localization of the labeled plasmid, but also in the amount of plasmid. It appears as if in the COS-7 cells, the plasmids are degraded more quickly, shown by the less intense overall fluorescence as well as the diffuse green staining throughout the cell, indicative of plasmid degradation. This could be due to either a greater number of complexes being transported to lysosomes in COS-7 cells, or that the complexes are being released from the endosomes faster, and therefore being degraded by cytosolic nucleases before the cytoplasmic expression system can be triggered. In contrast, the BHK cells contained larger pools of plasmid DNA in discrete, most likely non-lysosomal, perinuclear structures, even after 48 h. It is hypothesized that perhaps plasmid is being released into the cytoplasm over time, thereby allowing more free cytoplasmic plasmid to be available for cytoplasmic transcription at later time points.

In conclusion, it has been demonstrated that cell line differences in cytoplasmic expression (when compared to nuclear expression) are most likely due to differences in the intra-cellular processing of the nucleic acid-cationic lipid complexes, as opposed to differences in how the individual components (IRES, RNAP, etc.) function.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

In this work the design, construction, and characterization of a novel dual promoter, autogene based cytoplasmic expression system is described. In Chapter 2 the system is introduced and characterized. Chapter 3 is concerned with attempting to optimize expression levels, and determining what factors are limiting gene expression. In Chapter 4, the differences between a high expressing cell line (BHK) and a low expressing cell line (COS-7) are investigated.

In Chapter 2, the ability of the autogene based expression system to give rise to exponential increases in gene expression is demonstrated, as well as the ability of the expression system to give rise to levels of gene expression in BHK cells that are 20 times higher per transfected cell than a standard CMV based nuclear expression plasmid. Further analysis determined that the mRNA transcripts made in the cytoplasm are not as stable as those made in the nucleus (most likely due to the lack of a 5' cap structure on the cytoplasmic transcripts), and that the cytoplasmic expression system gives rise to much higher mRNA levels than the nuclear system.

In Chapter 3 an attempt is made to determine what factors are limiting the cytoplasmic expression system, in an effort to optimize the system to give rise to even higher levels of gene expression. After exploring various factors, such as the nuclear promoter, RNAP gene and IRES sequence, it was found that the critical factors responsible for limiting the cytoplasmic system is at the mRNA level, both the transcription and translation. It was found that the cytoplasmic transcripts are being translated 20 times less efficiently than their capped nuclear counterparts, and by measuring mRNA transcript levels, it was found that the autogene based cytoplasmic

expression system appears to be saturating the cells ability to produce new mRNA, and that transgene transcript levels are reaching 30% of the total RNA in the cell, a level that is 10 times than the sum of every other mRNA transcript in the cell. In addition to this finding, insights into possible IRES function are gained.

In Chapter 4, the differences between a cell line that gives rise to high levels of cytoplasmic gene expression (BHK) are compared with a cell line that gives poor cytoplasmic expression (COS-7). After investigating the individual components of the cytoplasmic expression system, such as the IRES activity and RNAP function, it was found that the reason for the difference in the two cell lines is most likely at the level of the intracellular processing of the DNA:cationic lipid complexes, as the COS-7 cells appear to have less intact plasmid per cell over time than the BHK cells.

In summary, this work represents a substantial advance in the understanding of cytoplasmic expression systems, and demonstrates the potential of these systems to give rise to increased levels of gene expression. In addition, this study clarifies potential areas that need to be addressed in order to further optimize cytoplasmic expression systems.

Future work in this field should focus on not only increasing the amount of gene expression per transfected cell, but also on how to increase the percentage of cells transfected with this system. With respect to increasing the translation of cytoplasmic transcripts, improvement can be expected either through the use of more potent IRES elements, or by co-transfecting with a capping enzyme, allowing for the production of capped, cytoplasmic transcripts. Either of these approaches should give rise to increased expression by increasing the translation of the transcripts. With respect to increasing the proportion of cells that are transfected, approaches that bypass the need

for nuclear delivery of the plasmid (in order to trigger the cytoplasmic system) should be explored. These could include the co-transfection of the autogene plasmid with mRNA encoding for the RNAP gene, allowing for production of RNAP in the cell from the mRNA, without the need for nuclear delivery. Alternatively, purified RNAP protein could be co-transfected along with the autogene plasmid, again bypassing the need for nuclear delivery, and resulting in a greater percentage of cells transfected. An extension of this would be to engineer a secretable RNAP protein that would be able to exit an expressing cell and enter into a neighboring cell, thereby serving as the initial source of RNAP in that cell, leading to an increase in the percentage of cells transfected, without the need for any co-transfection.

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