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Investigation of Potential Problems Associated with Gene Therapy

Thesis submitted for the degree of
Doctor of Philosophy at the University of Leicester

by

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Abstract

Investigation of Potential Problems Associated with Gene Therapy

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Safety requirements for gene therapy currently focus on preventing acute patient toxicity caused by the introduction of exogenous DNA. This project aimed to investigate the possibility that more subtle changes in cell regulation may occur as a result of transfection or transgene expression.

Mouse B16.F1 melanoma and CMT93 colorectal cell lines transfected with a plasmid (pNASSβ) or retroviral (pBabeNeo) construct were used as a model. Expression of the transgene was driven by a 2540bp (plasmid) or 769bp (retroviral) fragment of the mouse tyrosinase promoter. Results have shown that transfection *per se* can cause small changes in the proliferation rate of B16.F1 and CMT93 cells, but transgene expression did not produce this effect. However, expression of an IL-2 transgene in B16.F1 cells did decrease the rate of substrate attachment via an intracellular mechanism. These data suggest that cell regulation can be disrupted by transfection and transgene expression. Despite these changes, DDRT-PCR analysis of approximately 25% of the mRNA population indicated few changes in gene expression between parental and transfected cells.

Loss of transgene expression has proved an obstacle to success in clinical trials and was observed in both cell lines. In B16.F1 cells containing pNASSβ/IL-2, levels of IL-2 protein were reduced by more than 90% after 15 weeks in culture and this was mirrored by a decrease in IL-2 mRNA levels. The transgene was intact in high passage cells and restriction digest analysis did not indicate changes in methylation status. The continued presence of the construct was confirmed by treatment with 10^{-6} M melanocyte stimulating hormone, which induced IL-2 transgene mRNA and protein expression in cells that had lost expression from pNASSβ/IL-2. Induction was not observed in cells with the shorter promoter fragment in pBabeNeo/IL-2, indicating that the mechanism of downregulation can be overridden if the promoter contains suitable response elements.

For my parents

Thanks for giving me the opportunity

Don't forget, you've got to read every page

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Abbreviations

AAV	Adeno associated virus
ABTS	2,2′-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)
Ad	Adenovirus
ADA	Adenosine deaminase
Ag	Antigen
AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine 5′-triphosphate
CD	Cytosine deaminase
CTL	Cytotoxic T lymphocyte
DC-Chol	3-β-[N-(N′,N′-dimethylaminoethane)carbamoyl]cholesterol
dCTP	Deoxycytidine 5′-triphosphate
DEPC	Diethyl pyrocarbonate
DDRT-PCR	Differential display reverse transcription-polymerase chain reaction
DMEM	Dulbecco's minimal essential medium
DMRIE	1,2-dimyristyloxypropyl-3-N,N-dimethyl-hydroxyammonium chloride
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5′- triphosphate
DOPA	3,4-dihydroxphenylalanine
DOPE	Dioleoylphosphatidylethanolamine
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
G418	Geneticin
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GCV	Ganciclovir
GDEPT	Gene directed enzyme prodrug therapy
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
GTAC	Gene therapy advisory committee
HAC	Human artificial chromosome
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HSV-*tk*	Herpes simplex virus-*thymidine kinase*
ICAM	Intercellular adhesion molecule
ICRF	Imperial Cancer Research Fund
IFN	Interferon
IL-2	Interleukin-2
IPTG	Isopropyl β-D-thiogalactopyranoside
IRB	Institutional review board
IRES	Internal ribosomal entry site
ITR	Inverted terminal repeat
LAK	Lymphokine activated killer

LB	Luria-Bertani
LSD	Least significant difference
LTR	Long terminal repeat
MES	2-[N-Morpholino] ethanesulfonic acid
MHC	Major histocompatibilty complex
MMLV	Mouse Moloney Leukaemia virus
MOPS	(3-[N-Morpholino] propanesulfonic) acid
mRNA	messenger ribonucleic acid
Neo	Neomycin
NIH	National Institute of Health
NK	Natural killer
O.D.	Optical density
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RAC	Recombinant DNA advisory committee
RCV	Replication competent virus
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription-polymerase chain reaction
SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulphate
SSC	Saline-sodium citrate
TAA	Tumour associated antigen
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TE	Trypsin/EDTA
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TIL	Tumour infiltrating lymphocytes
TNF-α	Tumour necrosis factor-alpha
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

Chapter One

Introduction

INTRODUCTION

Gene therapy is an area of clinical research which eventually aims to treat a wide range of diseases. There are already strict guidelines in place governing the use of these techniques and patient safety and welfare is a primary concern. However, the safety requirements concentrate on preventing acute toxicity, and a short term view is generally taken of how gene therapy has affected a patient. There are undoubtedly aspects of the gene therapy process which warrant further investigation with regard to safety. It is the aim of this project to initiate an investigation, with the view to providing data, which would be useful in making general recommendations on longer term safety issues.

In the following introduction, the concept of gene therapy and the current safety and regulatory guide lines are outlined. An overview of the types of disease that can potentially be treated and the possible strategies available for use in developing gene therapy protocols is given. The current failings of gene therapy are discussed and the model system employed in this study is described.

1.1. The theory and history of gene therapy

The concept of treating a disease by transferring genetic material into a patient's cells is not new, but it remained an elusive technique until relatively recently. While the theory of gene therapy is easily understood, the methodology needed to execute it is very complicated, requiring the development of recombinant DNA technology to allow it to become a reality.

The advantage gene therapy has over more conventional treatments is that it aims to treat and cure the cause of the disease, rather than to alleviate the symptoms. The drawback is that in order to cure a disease a detailed basic understanding of the genetics and biology of that disease is needed. This may require the identification of a mutation in a gene encoding a receptor (Grossman et al, 1994), or a defective enzyme pivotal in a

metabolic pathway (Anderson et al, 1990) that is causing the condition. Alternatively, the situation may be more complicated as in the case of treating cancer (Culver and Blaese, 1994), where, to enable elimination of the transformed cells, a much more detailed knowledge of tumour biology and immunology is needed to understand what has caused the tumour to occur and to escape detection by the immune system.

In 1989, in the first gene therapy study to receive approval, patients were infused with their own tumour infiltrating lymphocytes (TILs) isolated from melanomas. These cells were modified to express the gene for neomycin phosphotransferase from a retroviral construct (Rosenberg et al, 1990). The aim of this trial was to provide information on the fate of the introduced cells and to study the safety of the vector system used. Since then further marker gene trials have been carried out to investigate aspects such as the efficiency of gene transfer and the effectiveness of bone marrow purging (reviewed by Brenner, 1996, Harris and Sikora, 1993a, Miller, 1992). These trials are unusual in that, while they provide useful information, the benefit to the patient is likely to be minimal at best and could potentially cause their condition to deteriorate. The initial trial with TILs could be justified because such treatment has been shown to provide therapeutic gain in combination with interleukin-2 (IL-2) (reviewed by Whittington and Faulds, 1993). However, approval for the initial gene marking trial was only received after much debate and then only patients that had failed all forms of therapy could be entered into the trial (Rosenberg et al, 1990).

The first clinical trial involving transfer of a therapeutic gene took place in 1990 (Anderson et al, 1990). The aim was to improve the condition of a patient suffering from the hereditary disorder adenosine deaminase (ADA) deficiency, a form of Severe Combined Immune Deficiency (SCID), which is caused by a mutation in the gene encoding adenosine deaminase. This enzyme is involved in purine catabolism in T lymphocytes. A retroviral vector carrying the cDNA for this enzyme was used to transduce the patient's T lymphocytes in the hope that they would express the functional protein and alleviate the symptoms of the disease. This disease is a good candidate for gene therapy because the corrected T-cell should have a selective advantage over untreated cells due to the disease causing defects in T-cell function. In

addition, it is possible to give the therapy in conjunction with conventional polyethylene glycol-ADA treatment so there is no ethical dilemma caused by cessation of conventional therapy to attempt an untried one. Finally, being a diffusible protein it is unlikely that all the patient's cells need to be transfected for the therapy to work. Subsequent phases of this trial have been encouraging with patients showing elevated levels of enzyme and increased peripheral blood T-cell counts (Blaese et al, 1995). Since this first trial many more protocols, designed to treat a variety of diseases, have been passed by review bodies in the United States and in Britain (Table 1.1).

1.1.1. The review process

In the United States the main body reviewing gene therapy protocols is the NIH Recombinant DNA Advisory Committee (RAC), which approves protocols submitted by groups supported by federal funding. Before being submitted to the RAC, approval from a local Institutional Review Board (IRB) is required. Following approval by the RAC, the protocol is submitted to the Food and Drug Administration (FDA). Protocols that are funded independently are not required to seek approval from RAC, but the local IRBs tend to insist on its approval anyway (Harris and Sikora, 1993b, Wivel, 1993). The aim of the RAC is to assess protocols for their safety to the patient and the general public and to determine the probable benefit to the patient or, in gene marking protocols, the probability of gaining useful information. The FDA concentrates on the safe production and use of biological agents used in gene transfer, including molecular details of the vector and insert, cell bank testing and consideration of any relevant safety data (Miller, 1992, Kessler et al, 1993). Both the RAC (NIH, 1990) and FDA (FDA, 1991) have issued guide lines to help researchers submitting protocols. Recently abolition of the RAC has been proposed and while this has been seen as premature, efforts are being made to streamline the regulatory process to avoid delays in developing new protocols (GTAC, 1997).

In Britain protocols are reviewed by the Gene Therapy Advisory Committee (GTAC) whose membership is both scientific and non-scientific (GTAC, 1995). The committee reviews proposals considering both the ethical issues and scientific risks and benefits

Table 1.1. Approved gene therapy research in Britain (1993-1996)

Disease	Vector type/Gene	Date of approval	No. of patients
SCID-ADA	retrovirus/ADA	1-93	1
Cystic fibrosis nasal trial	Liposome/CFTR	3-93	15
B-cell lymphoma	plasmid/anti idiotype immunoglobulin	7-93	6
Neuroblastoma	retrovirus/neo	2-94	_
Metastatic melanoma	plasmid/βGal, plasmid/IL-2	5-94	10
Metastatic melanoma	retrovirus/IL-2	2-94	12
Cystic fibrosis nasal trial	Liposome/CFTR	2-94	12
Cystic fibrosis nasal trial	Liposome/CFTR	5-94	16
Cystic fibrosis lung trial	Liposome/CFTR	9-94	_
Lymphoma	retrovirus/MDR	12-94	3
Breast cancer	plasmid/5-FC	10-95	4
Cervical cancer	vaccinia virus	6-95	8
Hurlers syndrome	retrovirus/α-L-iduronidase	12-95	_
Head and neck cancer	adenovirus/E1B attenuated	1-96	19
Cystic fibrosis nasal trial	Liposome/CFTR	5-96	11
Head and neck cancer	adenovirus/p53	9-96	16
Cystic fibrosis lung and nasal trial	liposome/CFTR	11-96	12
Glioblastoma	HSV	12-96	_

The symbol (-) in the patient column shows that, to date, patients have not been entered on the trial

data taken from GTAC, 1996,1997

raised by the proposal (Taylor and Lloyd, 1995) and publishes yearly reports on current progress (GTAC, 1995, 1996, 1997). Guidelines have been issued on preparing protocols for submission (GTAC, 1994) and researchers are encouraged to maintain a dialogue with the committee from an early stage. The committee works on the understanding that, at least for the present, gene therapy should not be considered as medical practice, but as research involving human subjects and so should be governed by the standards applied to other medical research. In addition, it has stated that no germ line therapy should be attempted and that gene therapy should only be applied to life threatening disorders or those causing serious handicap. In Britain, protocols addressing cystic fibrosis, breast cancer and metastatic melanoma are among those that have gained approval (Table 1.1.). Throughout the review process emphasis is placed on the clinical benefit to the patient, patient well being, public safety, fair patient selection, prevention of germ line effects, and informed patient consent. For a protocol to be approved there must either be no conventional therapy for the disease or this therapy must have failed. The patient must be made aware that the therapy is in its infancy and that they can withdraw at any point. Finally, the protocol must convince the board that there is enough background information to allow a reasonable prediction of the proposed outcome of the therapy and so outweigh the risks to the patient (Wivel, 1993). Thus, any trial must be backed up by animal studies, including studies in higher animals, provide data on the safety of the vector system and method of administration and estimate the benefit compared to existing treatment.

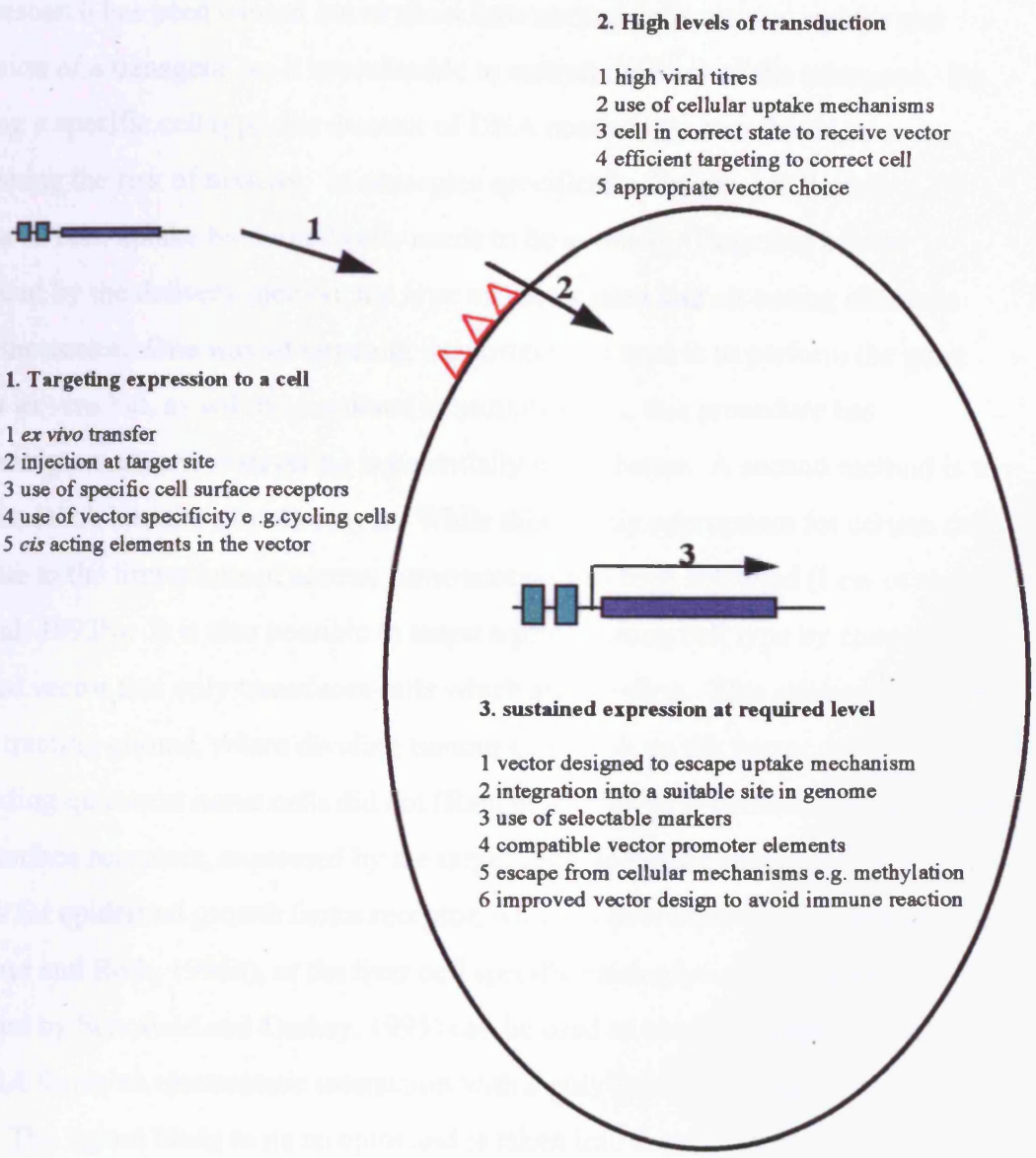
1.2. Requirements for success

For successful gene therapy there are several important requirements that the protocol must meet. These include the need for precise targeting of the transgene to the cell of interest, efficient uptake of the transgene by the cell and regulated expression of the transgene at an appropriate level.

1.2.1. Targeting

For expression of a transgene to be effective it has to be targeted to the correct cell type (Figure 1.1.). If the administered dose is spread throughout the body the transgene

Figure 1.1. Requirements for successful gene therapy



Scheme outlining the major obstacles to successful gene therapy and ways to overcome these obstacles.

product will be diluted. This may negate its effect or, more importantly, cause toxicity. Little research has been carried out to show how normal cells react to uptake and expression of a transgene, so it is preferable to restrict delivery of the transgene. By targeting a specific cell type, the amount of DNA needed can be reduced, so diminishing the risk of toxicity. In strategies specifically designed to destroy a transduced cell, uptake by normal cells needs to be avoided. Targeting can be influenced by the delivery method, the type of vector used and *cis*-acting elements within the vector. One way of targeting the correct cell type is to perform the gene transfer *ex vivo* but, as will be discussed in section 1.4.1., this procedure has disadvantages and *in vivo* targeting is potentially much better. A second method is to inject the DNA into the site of interest. While this is only appropriate for certain cell types due to the limitations of access, some success has been achieved (Lew et al, 1995, Vile et al, 1993b). It is also possible to target a proliferating cell type by choosing a retroviral vector that only transduces cells which are dividing. This strategy has been used in treating glioma, where dividing tumour cells took up the vector, while surrounding quiescent nerve cells did not (Ram et al, 1997). A different strategy is to utilise surface receptors, expressed by the target cells, to ensure specific uptake. Ligands for epidermal growth factor receptor, which is overexpressed in lung cancer (Cristiano and Roth, 1996a), or the liver cell specific asialoglycoprotein receptor (reviewed by Schofield and Caskey, 1995) can be used to obtain cell specific uptake. The DNA forms an electrostatic interaction with a polylysine/ligand conjugate of choice. The ligand binds to its receptor and is taken into the cell by receptor mediated endocytosis, used by the cell for uptake and transport of macromolecules. Other similar strategies have been proposed using antibodies to target cell receptors (Chen et al, 1994, Wickham et al, 1996) or employing other uptake systems such as potocytosis internalisation (reviewed by Cristiano and Curiel, 1996b), used by the cell to take up small molecular weight molecules.

The fact that viral vectors bind to receptors before infecting a cell can be used to target a particular cell type. There are a few examples of cell specific infection by a particular virus. Herpes Simplex Virus (HSV) has a natural tropism for neuronal tissue and murine parvovirus has been found to have specificity towards transformed cells (Perros

et al, 1995), but there is the potential for modification of other vector types to create such specificity (reviewed by Miller and Vile, 1995a). Work aimed at modifying the *env* protein and fibre and penton base proteins, which determine the cell types infected by retroviral and adenoviral vectors, is ongoing (reviewed by Harris and Lemoine, 1996).

In addition to the above methods, targeting expression to a particular cell type can be conferred by the expression vector itself. The expression of many proteins is restricted because the promoter regions of their genes contain *cis* acting elements that confer tissue specific transcription. By using such sequences in the vector, expression can be confined to cell types that would normally express the gene (reviewed by Miller and Whelan, 1997). Tyrosinase, which is only expressed in melanocytes and pigment epithelium, (Sakai et al, 1997) and α-fetoprotein, which is expressed in hepatocellular carcinoma, but not normal adult liver cells (Ido et al, 1995) are examples of genes with restricted expression. A variation is to use a promoter containing a stress responsive element. The GRP78 protein is induced under stress conditions such as chronic anoxia, which occur in tumours. This may be used to target expression preferentially to tumour cells (Gazit et al, 1995). The use of tumour specific targeting has a potential down side in that many tumour associated antigens are self antigens and are not exclusively expressed by the tumour, thus there is the potential for transgene expression in cells other than those of the tumour , with the possibility of causing a self immune reaction. A possible solution to this is to use a prokaryotic inducible promoter system such as the tetracycline-controlled transactivator-responsive promoter (Paulus et al, 1996).

The ultimate targeting strategy would be site specific recombination, whereby the mutated gene would be excised and directly replaced by a wild type copy. The technology to carry out this gene replacement strategy *in vivo* does not, as yet, exist.

It should be remembered that the target tissue does not necessarily have to be the tissue which is diseased. For example, by targeting expression to the epidermis, a protein whose action is systemic can be expressed. This tissue has the advantage of being easily accessible and its biology is reasonably well characterised (Greenhalgh et al, 1994,

Palmer et al, 1991). A protein such as factor IX could be expressed into the blood stream enabling the treatment of haemophilia B.

1.2.2. Achieving efficient transduction

Once a cell has been targeted the next hurdle is efficient uptake by that cell. This is a major consideration in vector choice and design (Figure 1.1.). For example, viral titre can differ dramatically with the design of a retroviral vector such that modifying it to contain more than one gene can lower the titre of the viral stock (Vile et al, 1994a). This can affect the level of transduction with transduction frequency being proportional to the number of viral particles to which the cell is exposed (Rettinger et al, 1994). Viral vectors have generally been found to give higher transfer efficiencies than non-viral vectors (Cristiano and Curiel, 1996b). This is understandable considering they have evolved to enter a cell in just such a fashion. Individual cell types may require particular growth factors for efficient uptake of a gene and parameters such as cell density (Pages et al, 1995) and proliferative state (Ram et al, 1997, reviewed by Schofield and Caskey, 1995) can affect gene uptake.

1.2.3. Achieving efficient expression

The transgene needs to be expressed at a level that will have a therapeutic effect (Figure 1.1.). This expression may also need to be long lived. The level of expression is influenced by the design of the vector and the promoter elements contained within it. Expression can also be influenced by the position of integration in the host genome and host regulatory mechanisms such as methylation or excision of the foreign DNA. These will be discussed in greater detail later (Section 1.7.2.).

1.3. Potential target diseases

Originally it was thought that diseases caused by inherited mutations would be the easiest to treat and early work focused on disorders such as ADA deficiency and the haemoglobinopathies. However, it has become clear that acquired diseases such as cancer are also good targets for gene therapy and may in fact be easier to treat. One of

the huge advantages of gene therapy is the ability of the basic technology to be applied to almost any type of disease situation.

1.3.1. Single gene disorders

These diseases are caused by a mutation in a gene which either prevents the final product being made or produces a non functional product. This product could be an enzyme such as adenosine deaminase, whose mutation causes a form of SCID (Anderson et al, 1990), a structural protein such as β globin whose mutation causes thalassaemia (Harris and Sikora, 1993a), or a receptor such as that for low density lipoprotein whose mutation causes familial hypercholesterolaemia (Grossman et al, 1994). The aim is to replace the mutated gene with one which will provide the wild type protein in sufficient quantities to cure the disorder. One problem that has been encountered is that strict regulation of these proteins may be required and this makes successful therapy more difficult.

1.3.2. Multifactorial disease

There are some conditions such as heart disease which are caused by a combination of different factors. However, there are aspects of such diseases which could be treated with gene therapy to improve a patient's condition. After heart surgery there is a chance of smooth muscle cell proliferation occurring (Foley et al, 1993). This is disadvantageous to the patient and could be prevented by inhibiting the expression of c-myb (Simons et al, 1992), a gene which is known to cause this proliferation. In this case, although the factors causing c-myb expression are likely to be multiple, one gene is affected and can be targeted.

1.3.3. Viral infections

There are several viral diseases which have a major impact on human health, for which there are inadequate treatments. These include infection with Hepatitis B and Human Immunedeficiency Virus (HIV) (reviewed by Chang and Wu, 1994, Gilboa and Smith, 1994). In both these diseases the virus particle shows a precise tropism for a cell type, thus delivery of the gene therapy vector can be targeted to the same cell using cell type specific receptors. In protocols developed to treat these infections, strategies include

inhibiting viral transcription, disrupting viral regulation and inducing an immune response to infected cells (reviewed by Sun et al, 1995).

1.3.4. Cancer

As gene therapy has progressed, it has become clear that cancer should be one of the major target areas. This is because, generally, the elimination of the tumour does not require precise regulation of the transgene. Two strategies are used in cancer treatment. The first aims to eliminate the tumour by killing the malignant cells. This can be achieved in a number of ways which will be outlined later and can be used to treat any type of cancer. A second approach is aimed at tumours with a mutation in a known oncogene or tumour suppressor gene, such as K*i*-ras in lung cancer or p53 in a number of different tumours (reviewed by Culver and Blaese, 1994, Harris and Sikora, 1993a). The therapy is designed to replace the lost function of the wild type protein, causing reversion of the transformed phenotype. A protocol delivering normal copies of p53 to human lung tumours has shown evidence of tumour regression (Roth et al, 1996).

1.3.5. Multidrug resistance

A clinical problem which can be treated with gene therapy is that of myelosuppression caused by chemotherapy, which occurs because bone marrow is more sensitive to the drug than the tumour and other body organs. This is particularly apparent at relapse when there may be reduced tolerance. Using gene therapy the normal bone marrow is transfected with the multidrug resistance gene, encoding the multidrug transporter, P-glycoprotein (reviewed by Licht et al, 1996). This confers an increased resistance to drugs such as Taxol, in the transfected progenitor cells. Higher doses of drug can then be delivered to the patient since modified bone marrow cells are able to resist the effects, due to the P-glycoprotein transmembrane protein pumping drug out, and so reducing toxicity. Similar approaches using enzymes such as glutathione S-transferase Yc, which confers resistance to anti-tumour, alkylating agents (Greenbaum et al, 1994), are also being developed. The transgene of choice is dependent on the type of drug that will be used because each enzyme or transporter is specific for a certain class of drug.

1.4. *Ex vivo or in vivo*

When preparing a gene therapy protocol the first decision relates to the location of the cells at the time of gene transfer.

1.4.1. Ex vivo

This strategy involves modifying cells in culture. These cells can be an established cell line (Arienti et al, 1996), or derived from the patient (Stingl et al, 1997). They are modified with the gene of choice and expanded further, before being infused into the patient. The advantage of this method is that the cells of interest can first be targeted and screened for expression of the transgene. The *ex vivo* approach was used in the first report of treatment of familial hypercholesterolaemia by gene therapy (Grossman et al,1995). The disadvantage is that extensive surgery is required to obtain and return the cells to the patient. There is a limit to how many cells can be returned to the patient safely which, in the case of hepatocytes, is approximately 2% of the liver mass (Rettinger et al, 1994). In addition, while the cells are in culture there is the possibility that they may succumb to infection, or their characteristics may alter, so they acquire a different antigenic profile. The *ex vivo* approach is not applicable to cells which are terminally differentiated and therefore unable to grow in culture.

1.4.2. In vivo

This approach aims to deliver the transgene to the target cells without removing them from the patient. Thus, surgery is minimised and the chance of any changes in the cells is reduced. However, this approach requires more technically advanced techniques, to allow precise targeting to the correct cell type. It has been used in animal studies (Dong et al, 1996, Kay et al, 1994, Lesoon-Wood et al, 1995) and clinical trials (Roth et al, 1996) with some success.

1.5. Methods of transgene insertion

Whether the *ex vivo* or *in vivo* approach is chosen, the range of methods for inserting the transgene into cells is the same. The gene can be carried into the cell by a viral or

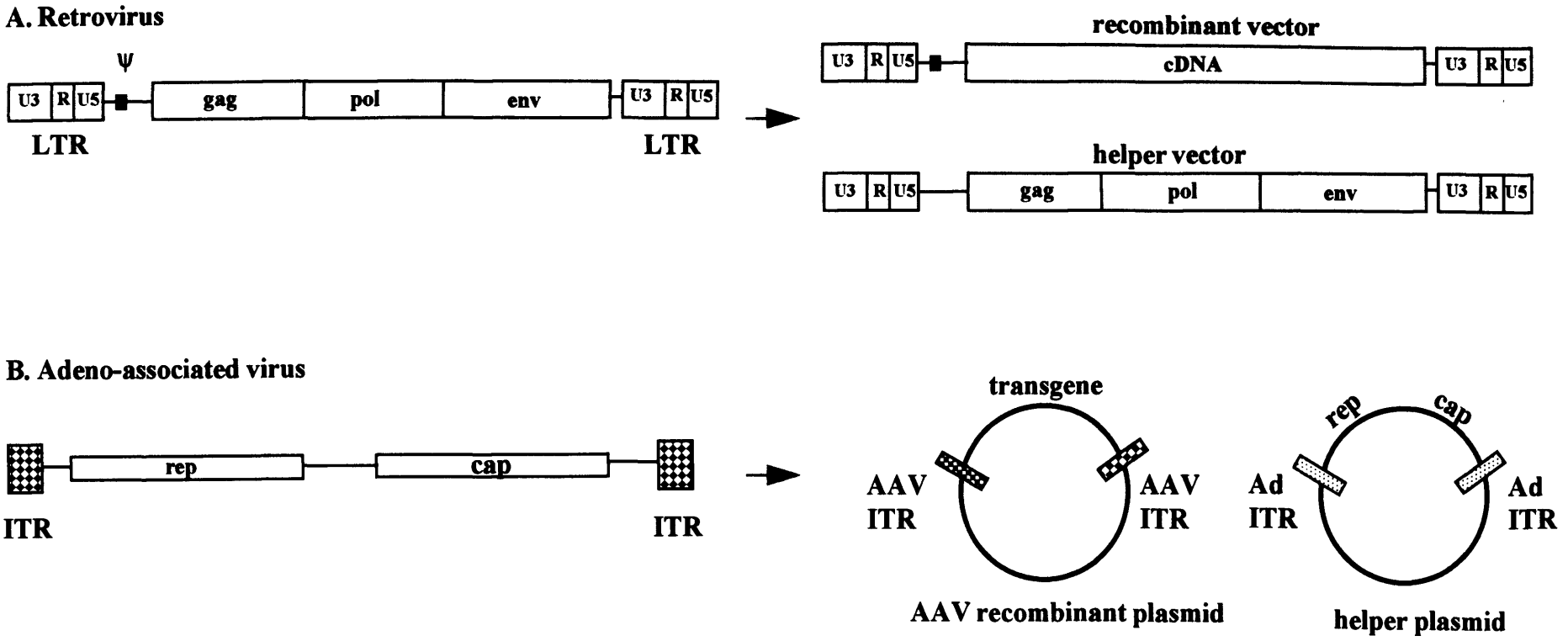
non viral method and several examples of each type of protocol are described below.

1.5.1. Retroviral vectors

These are envelope viruses which contain an RNA genome (reviewed by Gunzburg and Salmons, 1996). The virus envelope protein interacts with a receptor on the cell surface and the virus is internalised. The RNA is reverse transcribed by a viral protein and the resulting double stranded DNA can then integrate into the host genome where it is expressed as a host gene. This integration is stable, but random, except for some preferential integration into trancriptionally active areas (Mooslehner et al, 1990) and certain sequence specific sites (Shih et al, 1988). The retroviral constructs used to date are generally based on Murine Leukaemia Viruses, such as mouse Moloney Leukaemia virus (MMLV), which can infect human cells. The vector consists of a plasmid containing the essential provirus *cis* acting promoter and packaging signals in addition to the transgene. The viral protein coding genes *gag, pol* and *env*, are deleted from the vector, but it retains its reverse transcription and integration capabilities (Figure 1.2.). There are two reasons for removing the coding regions. First, the viral envelope can only package a limited amount of nucleic acid. Therefore, in order to accommodate the transgene some of the viral genes must be removed. Secondly, by removing these three viral genes the vector becomes replication defective. These functions are then provided *in trans* by a packaging cell line containing one or more plasmids expressing these proteins. The retroviral vector transcript, which cannot replicate on its own, is transfected into the packaging cell line where it is packaged into particles produced by the helper plasmid and released from the packaging cell. Such vectors can accommodate up to 8kb of nucleic acid (reviewed by Vile and Russell, 1995). These replication defective particles are then used to infect the target cell. Alternatively the packaging cell, producing the particles containing the transgene, can be used to treat the patient (Tamiya et al, 1995).

The vectors may contain several genes, one of which may be a selectable marker gene. These genes can be in either orientation and may be expressed from the same or different viral or non-viral promoters. Alternatively, an Internal Ribosome Entry Site (IRES) motif, which allows the production of polycistronic transcripts can be used.

Figure 1.2. Structure of viral genomes



Schematic diagram of the genome of two viruses used to construct gene therapy vectors.
A. Retroviral genome and two separate vectors which are used to infect the packaging line. The helper vector lacks a packaging signal (ψ), so makes empty virions. The transgene vector is unable to make virions, but can package itself into the empty virions.
B. Adeno-associated virus genome. The inverted terminal repeats (ITR) can be placed in a plasmid and used in conjunction with a second plasmid containing adenoviral (Ad) inverted repeats and the essential *cap* and *rep* genes.
(Berns et al, 1996, Kremer et al, 1995, Vile et al, 1995)

The advantage of this is that mRNA's can be expressed at equal levels from the same promoter (Gunzburg and Salmons, 1996, Vile and Russell, 1995). The main advantage of a retroviral vector is that it is stably integrated into the genome and therefore will be passed on to each new generation of cells, thus overcoming the need for repeated treatments. This is a very important consideration when treating diseases such as those caused by inborn genetic errors which will require life-long therapy. A disadvantage of retroviral vectors is that, because they are predominantly based on murine C-type virus, which cannot cross the nuclear membrane, they can only infect actively dividing cells. This means that terminally differentiated cells cannot be infected. However, this point can be used to advantage in targeting strategies (Section 1.2.1.). A further worry with the use of these vectors is the possibility that, when the virus integrates into the genome, it will disrupt the function of an important gene (Harris and Sikora, 1993b). Despite all their drawbacks retroviral vectors are the most widely used delivery system in gene therapy trials (Blaese et al, 1995, Grossman et al, 1995, Roth et al, 1996).

1.5.2. Adenovirus

These vectors are based on the family of adenoviruses and can take 7-8kb of inserted double-stranded DNA. They are capable of infecting a wide range of cell types and are maintained episomally, thus the possibility of insertional mutagenesis is removed (Kremer and Perricaudet, 1995). They also have a very high transduction efficiency and can infect non-proliferating cells. The adenovirus genome is divided into early and late regions. The early region contains 6 sets of genes, each of which is under the control of one promoter. Once the virus has entered the nucleus the E1A region is transcribed without the help of viral *trans*-activators. The E1A proteins then aid transcription of the other early genes. To construct the adenoviral vector the E1A region is deleted, making the vector replication deficient, and replaced with a cassette containing the gene of interest, its promoter region and a polyadenylation signal. The E3 region is also sometimes deleted. The deleted functions are provided *in trans*. One advantage these vectors have over retroviral vectors is that they are less pathogenic, causing only minor respiratory infections. They are also more robust than retroviral vectors and can be purified to much higher titres.

Adenoviral vectors do have some drawbacks. Despite having E1A deleted, there is the possibility that *trans*-activating sequences, already present in the patient, can promote replication. A further drawback is that expression from adenoviral vectors is often short-lived (Setoguchi et al, 1994, Yang et al, 1995). Despite these disadvantages these vectors have been used in many protocols, especially those proposing to treat cystic fibrosis (Dong et al, 1996, Goldman et al, 1995).

1.5.3. Adeno-associated virus

This vector system is based on the nonpathogenic human adeno-associated virus, which infects more than 90% of adults with no known side effects. The genome consists of a linear single stranded DNA molecule and there are five serotypes of which the AAV2 genome is best characterised (reviewed by Kotin, 1994, Kremer and Perricaudet, 1995). It has the advantage of efficiently and stably integrating into the host genome and will infect a wide range of host cells. These viruses require a helper virus such as adenovirus to replicate, without which they exist as a latent infection, during which they integrate stably into the host genome and are passed to daughter cells during cell division. Vectors preferentially transduce cells in S-phase but, in non dividing cells the virus persists until the cell divides, at which point it integrates into the host genome (Russell et al, 1994). Improvements in transduction efficiency of non dividing cells have been achieved by treating cells with DNA synthesis inhibitors (Russell et al, 1995). The wild type virus integrates preferentially into human chromosome 19q. To construct a vector the inverted terminal repeats (ITR), which act as a critical regulatory sequence and origin of replication in the AAV, are placed in a plasmid construct, with the promoter and transgene placed between the two repeats (Figure 1.2.). This is used to infect a cell in conjunction with a similar construct containing two essential AAV genes and a helper adenovirus ITR. The helper virus is unable to replicate, so the helper cell line produces recombinant AAV vectors, free of wild type virus, that can be used to carry out gene therapy (Berns and Giraud, 1996). An advantage of these vectors over retroviral vectors is that as DNA viruses, they are capable of processing introns, which is important in cases such as transfer of β-globin to thalassaemia patients. This gene requires the presence of the introns for production of full length transcripts (Einerhand et al, 1995). In addition, because the AAV termini are inverted,

rather than direct repeats, transcriptional activation of cellular oncogenes should not occur.

A disadvantage of these vectors is that they can only accommodate about 5kb of transgene DNA, but this is sufficient to allow expression of small genes (Zhang et al, 1996). The AAV also tends to integrate in multiple copies, which is disadvantageous, due to the increased risk of insertional mutagenesis. Finally, in order to construct AAV vectors, the replication proteins are often deleted and it appears to be these that specify the site of integration in chromosome 19. Despite these drawbacks AAV vectors are currently being developed to treat diseases such as cystic fibrosis (Conrad et al,1996) and haemophilia B (Snyder et al, 1997).

1.5.4. Herpes simplex virus (HSV)

This is a family of nuclear DNA viruses with genomes of up to 250kbp. By establishing a latent infection as a stable episome, the viral genome is maintained with no apparent effect on the host cell. While some of the subtypes are able to replicate within dividing cells, so passing on their progeny to daughter cells, others such as herpes simplex virus are unable to do this. They rely on infecting long lived cells such as sensory neurones, although they are capable of infecting many human cell types.

There are several methods of constructing HSV vectors. The first is to delete genes in the wild type virus which are unnecessary for growth in cell culture to give vectors that are replication competent. These are likely to be unacceptable for use in gene therapy. The second is to delete the genes necessary for replication and replace them with a transgene and provide the necessary genes *in trans* in a packaging line. The third alternative employs a plasmid vector carrying the transgene , an HSV origin of replication and a packaging signal. This amplicon is transfected into a cell that is superinfected with defective helper virus. The necessary proteins are provided *in trans* and the amplicon is replicated and packaged into viral particles (Efstathiou and Minson, 1995). Their ability to maintain long term infections in differentiated cells makes them potentially useful in treating brain disorders (Neve and Geller, 1996).

1.5.5. Other viral vectors

Other viral genomes are also considered for development as vectors for gene therapy. The vaccinia virus can transduce dividing or quiescent human cells of all types. The virus does not integrate into the host cell genome, but replicates in the cytoplasm and is capable of taking inserts of up to 25kb without any adverse effect on growth (Moss, 1991, Wan et al, 1997).

A three plasmid vector system has been developed based on an HIV lentiviral retrovirus which produces a replication-defective retroviral particle able to integrate into non-proliferating cells (Naldini et al, 1996).

Plasmoviruses are plasmids developed to contain a defective recombinant proviral genome and a transcription unit coding for *env*. The aim was to produce a vector that had the benefits of viral vectors without the potential safety problems. The plasmid is transfected into a cell and transforms it into a packaging cell that will produce a recombinant defective retrovirus capable of transducing neighbouring cells (Noguiez-Hellin et al, 1996).

Another approach is to produce empty pseudocapsids of the mouse polyoma virus using a baculovirus expression system and package exogenous DNA into them. These can be used to transfer the transgene DNA into target cells, while avoiding the transfer of viral DNA, thus preventing any of the complications this can cause (Forstova et al, 1995).

1.5.6. Liposomes

Liposomes have been developed more recently as a non-viral alternative for gene transfer. They consist of a mono or polycationic lipid that forms a positively charged liposome, which interacts ionically with negatively charged DNA. The liposome/DNA complex fuses with the cell membrane, delivering the DNA into the cytoplasm. The advantages of liposomal vectors include the lack of size constraint on the amount of DNA incorporated, thus allowing large stretches of promoter sequence to be included, the ease and low cost of preparation and the low immunogenicity displayed by the lipids (Canonico et al, 1994, reviewed by Schofield

and Caskey, 1995). Additionally, being non-viral, they do not have the safety worries of expression of viral proteins and insertional mutagenesis associated with the viral vectors. The disadvantage of liposomes is that the DNA is maintained episomally so the vector is lost as cells replicate, requiring repeated administration.

Several factors are important in determining efficient uptake and expression of the liposome. Cell type seems to be important (Zabner et al, 1995) and proliferating cells show increased levels of expression compared to non-dividing cells (Schofield and Caskey, 1995). The optimal lipid to DNA ratio also varies, as does the amount of each type of liposome that can be administered before signs of toxicity are observed (Mahato et al, 1995).

Several combinations such as DC-Chol/DOPE (3-β-[N-(N′, N′-dimethylaminoethane)carbamoyl]cholesterol/dioleoylphosphatidylethanolamine) and DMRIE/DOPE (1,2-dimyristyloxypropyl-3-N,N-dimethyl-hydroxyammonium chloride/DOPE) have received approval for clinical trials, but work is still in progress to find combinations that give higher transfer efficiency with reduced toxicity. Despite the seeming inefficiency of this method, it has been used with some success. Mice bearing breast tumours with a p53 mutation have shown regression of tumours and decreased recurrence when injected with liposomes complexed with the transgene for wild type p53 (Lesoon-Wood et al, 1995).

1.5.7. Receptor mediated endocytosis

As discussed earlier (section 1.2.1.), this method uses an electrostatic complex to package DNA and target it to a specific cell surface receptor.

1.5.8. Direct injection

Direct injection of DNA has shown potential as a method of gene transfer. For instance, direct injection of a plasmid expressing human CD4 antigen into mouse muscle prevented development of tumours after a subsequent injection with a tumour cell line. This was thought to be due to the plasmid being taken up by cells which then expressed the transgene. This generated an immune response, which protected against

the tumour challenge (Wang et al, 1995). Direct injection has the advantage of being inexpensive and relatively non toxic. It is also capable of delivering large constructs (Schofield and Caskey, 1995), but persistence of expression may be poor (Lew et al, 1995, Nomura et al, 1997). This may be due to degradation of the DNA, or the inability of the DNA to replicate when the cell divides, a problem seen with all plasmid-based strategies. A possible solution would be the production of extrachromosomally replicating plasmids (Cooper et al, 1997). A variation of direct gene transfer has been described, whereby high frequency puncturing of the skin using fine needles had led to transfer of naked plasmid DNA (Ciernik et al, 1996)

1.5.9. Particle bombardment

This technology uses an electric field to accelerate gold particles, coated with plasmid DNA, into target cells. The particles can penetrate three to five epidermal cell layers and have been reported to efficiently transfer cytokines, giving high levels of expression (Sun et al, 1995).

All the vector systems are being improved to overcome the drawbacks mentioned and this often involves combining all or part of several different strategies. It seems likely that several different vector systems are required to meet the demands of different gene therapy strategies and that the vectors described above will provide the basis for a large amount of future development.

1.6. Gene therapy strategies

Several different gene therapy strategies have been developed to fulfill the requirements of treating various diseases. Each one has its merits and the most appropriate one will depend on the disease being treated.

1.6.1. Gene replacement

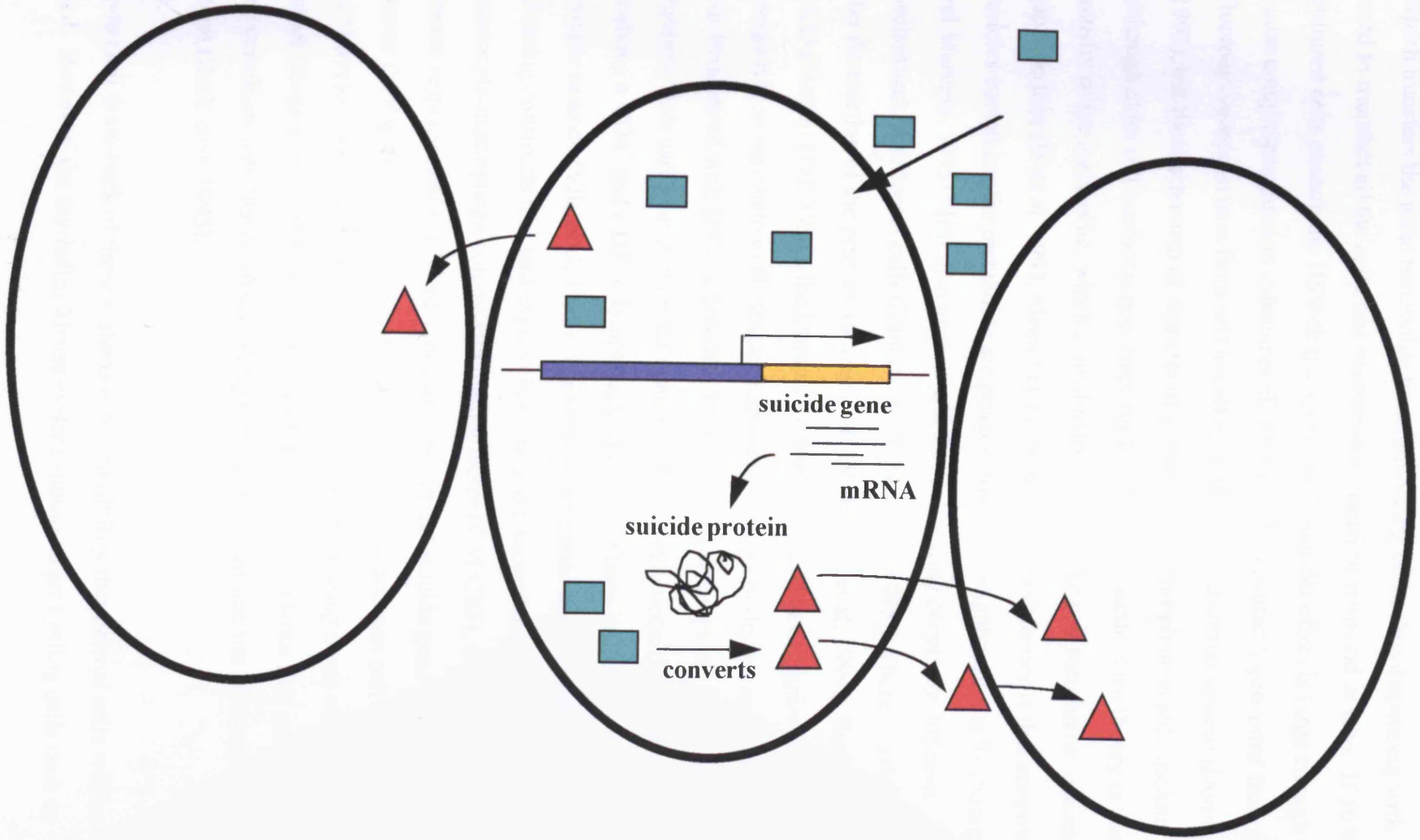
This strategy aims to replace a mutated or non-functional gene that is causing a disease state. As mentioned in sections 1.3.1. and 1.3.4. it can be used to treat inherited metabolic disorders such as ADA deficiency (Anderson et al, 1990) or cystic fibrosis

(Goldman et al, 1995) and certain cancers where a specific mutation has caused the cells to become transformed (Roth et al, 1996). Theoretically, it would appear that this approach is flawed because the probability of transfecting 100% of the affected cells is very small. However, for many of the metabolic disorders, the missing protein is normally secreted and the level of protein required is often low. This makes it is unnecessary for every cell to be transfected to produce a noticeable benefit to the patient (Anderson et al, 1990). The argument for treating tumours in this manner is more controversial, because it would be reasonable to assume that any cell that did not take up the transgene would continue to grow unchecked. Nevertheless, in tumours containing p53 mutations, tumour growth has been inhibited (Zhang et al, 1994) or has even regressed, due to some of the tumour cells arresting in G_1 or dying by apoptosis as a consequence of overexpression of wild type p53 (Lesoon-Wood et al, 1995, Roth et al, 1996), and a bystander effect appears to be present. There is the possibility that the dying cells induce an immune response to the tumour (Munshi et al, 1997). In addition, evidence suggests that when wild type p53 expression is restored there is a reversal in angiogenesis which can cause necrosis of the tumour (Nishizaki et al,1997).

1.6.2. Gene directed enzyme prodrug therapy (GDEPT)

Gene (or virus) directed enzyme prodrug therapy is being developed for use in diseases such as cancer where the target cells need to be destroyed. The aim is to incorporate a gene that encodes a non-mammalian enzyme into the vector construct (Huber et al, 1991). The construct is targeted to the cancer cells which take up the transgene and express the foreign protein. A nontoxic prodrug is given to the patient which is converted to a toxic compound by the enzyme encoded by the transgene so that cells that have taken up the transgene are killed (Figure 1.3.). Commonly used suicide genes include cytosine deaminase (CD), found in bacteria and fungi, which converts 5-fluorocytosine to highly toxic 5-fluorouracil, and HSV-*tk* which converts ganciclovir (GCV), an acyclic nucleoside, to a cytotoxic nucleoside analogue. Cellular kinases then complete the conversion to ganciclovir triphosphate (reviewed by Conners, 1995). This gene is employed in one of the constructs used in this project (Vile and Hart, 1993b).

Figure 1.3. The GDEPT strategy



A cell takes up the suicide transgene and begins to express the protein. If that cell then receives the non-toxic prodrug ▭ it will convert it into the toxic drug ▲ that will kill the cell. In addition, the toxic metabolite will be transferred to neighbouring cells that have not taken up the transgene, and these cells will also die.

A process which increases the efficiency of these 'suicide genes' is the mechanism known as the 'bystander effect' (Culver et al, 1992, Rowley et al, 1996). This process, which transfers the toxic metabolite to neighbouring cells, thus dispensing with the need to transfect all the cells, has been demonstrated *in vitro* and *in vivo*. If 10 % of cultured cells contain the HSV-tk transgene the bystander effect is large enough to cause complete regression (Mesnil et al, 1996). 5-Fluorouracil, generated from 5-fluorocytosine, can pass from cell to cell by diffusion or a carrier system (Domin et al, 1993), but the mechanism of transfer of ganciclovir triphosphate is still unclear, although close cell contact is necessary for the effect to occur. One theory is that transfer of the metabolite, which is unable to permeate the cell membrane, occurs via gap junctions (Bi et al, 1993, Mesnil et al, 1996). A second theory is that apoptotic vesicles containing the metabolite are phagocytosed by neighbouring cells (Samejima and Mervelo, 1995). It is also unclear whether cell killing occurs by different mechanisms in different cells (Kaneko and Tsukamoto, 1995). There is evidence that after destruction of the primary tumour by HSV-tk (Vile et al, 1994b, Barba et al, 1994) or CD (Mullen et al, 1994), the immune system is induced to recognise the tumour as foreign and so may help to eliminate the primary tumour or distant metastases. In B16 cells transfected with HSV-tk less than 1% of cells show apoptotic features after treatment with ganciclovir, with the majority of cell death occurring by necrosis and involving a $CD4^+$ and $CD8^+$ T lymphocyte and macrophage infiltrate with a Th1 type cytokine cascade (Vile et al, 1997). This type of response opens up the possibility of delivering constructs that will express HSV-tk and a second transgene, such as granulocyte-macrophage-colony-stimulating factor (GM-CSF), that will augment any immune response that is elicited. Choosing the correct suicide gene is of importance because the degree of cell death has been shown to vary between cell lines (Samejima and Mervelo, 1995), with one suicide gene system often being more efficient than another (Rogers et al, 1996, Trinh et al, 1995). There is evidence that sensitivity *in vitro* correlates with that *in vivo*, making it easier to determine the most appropriate system (Beck et al, 1995).

A potential draw-back of these systems is the possibility that normal cells will be killed. However, the thymidine kinase system should target cycling cells such as

proliferating tumour cells rather than differentiated normal cells. In addition, precise targeting such as could occur if the gene is linked to a tumour specific promoter should limit the effect on normal tissue. A further use for this type of cell killing is as a safety mechanism in conjunction with other genes. Any cell containing the construct will have the potential to be removed by treatment with the prodrug.

1.6.3. Suppression of gene expression

This method of gene therapy aims to disable an mRNA which is causing a disease condition.

Antisense

One method of doing this is to produce a short oligonucleotide of 15-30bp in length whose sequence corresponds to a portion of the non-coding strand of the gene of interest and is thus anti-sense to the mRNA molecule. The formation of the DNA/RNA duplex causes inhibition of protein expression (reviewed by Bennett, 1995). Antisense molecules have several advantages in that they are sequence specific, do not require cell replication, can enter cells by simple diffusion and any molecule that does not reach the target cell will be rapidly degraded. They are not infectious and do not integrate into the host genome. They do have disadvantages in that they have relatively short half lives and cannot be targeted to specific cell types. Uptake is concentration and length dependent with high concentrations and long contact times needed for cell uptake to be efficient (reviewed by Leonetti et al, 1993). Antisense technology is being developed to treat diseases such as HIV (Sun et al, 1995) and smooth muscle cell proliferation after heart surgery (Suzuki et al, 1997).

Triple Helix-forming Agents

These are antisense oligos designed to bind to the gene of interest, forming a triple helix structure (reviewed by Leonetti et al, 1993), so possibly blocking RNA polymerase progression or transcription factor binding. One study has shown their effectiveness at binding to promoter elements of HIV-1, so suppressing viral expression (McShan et al, 1992).

Aptamers

These are oligomers which bind to target sequences on a specific protein and alter its activity (reviewed by Bennett, 1995). This is potentially more specific than trying to target an mRNA. Suppression of protein activity has been seen *in vivo*, but it is not yet possible to predict which oligomer will bind to a particular protein.

Ribozymes

Hammerhead ribozymes carry out sequence specific cleavage of mRNA and so are another method of suppressing target gene expression. They can be cloned into plasmid vectors and the construct transfected into the target cell. The ribozyme is expressed and cleaves its target RNA. They have been shown to effectively suppress expression of the H-ras oncogene, reversing the neoplastic phenotype (Kashani-Sabet et al, 1994) and are being used in HIV strategies (Sun et al, 1995). A further development is to use the ribozyme to repair a mutation in a defective gene. If the mutation is in the coding region, the gene will be regulated as normal and when expressed will be repaired by the ribozyme so restoring normal expression of the wild type protein. This approach is ideal for diseases where dominant mutant proteins are produced e.g. sickle cell anaemia and cancers caused by oncogene mutation (reviewed by Sullenger, 1996)

1.6.4. Production of a decoy

This type of gene therapy is particularly applicable to HIV like infections where a virus targets cells through a specific CD4 receptor. By producing large amounts of this receptor in the body, it is hoped to reduce the viral load by causing the virus to bind to the decoy rather than the cell receptor. Another possible use is to produce copies of an RNA binding site that will compete for binding of a regulatory protein such as Tat or Rev, both of which are involved in HIV replication (reviewed by Gilboa and Smith, 1994, Leonetti et al, 1993). The problem with these strategies is the unknown effects, caused by the release of these decoys, on the rest of the body.

1.6.5. Immunotherapy

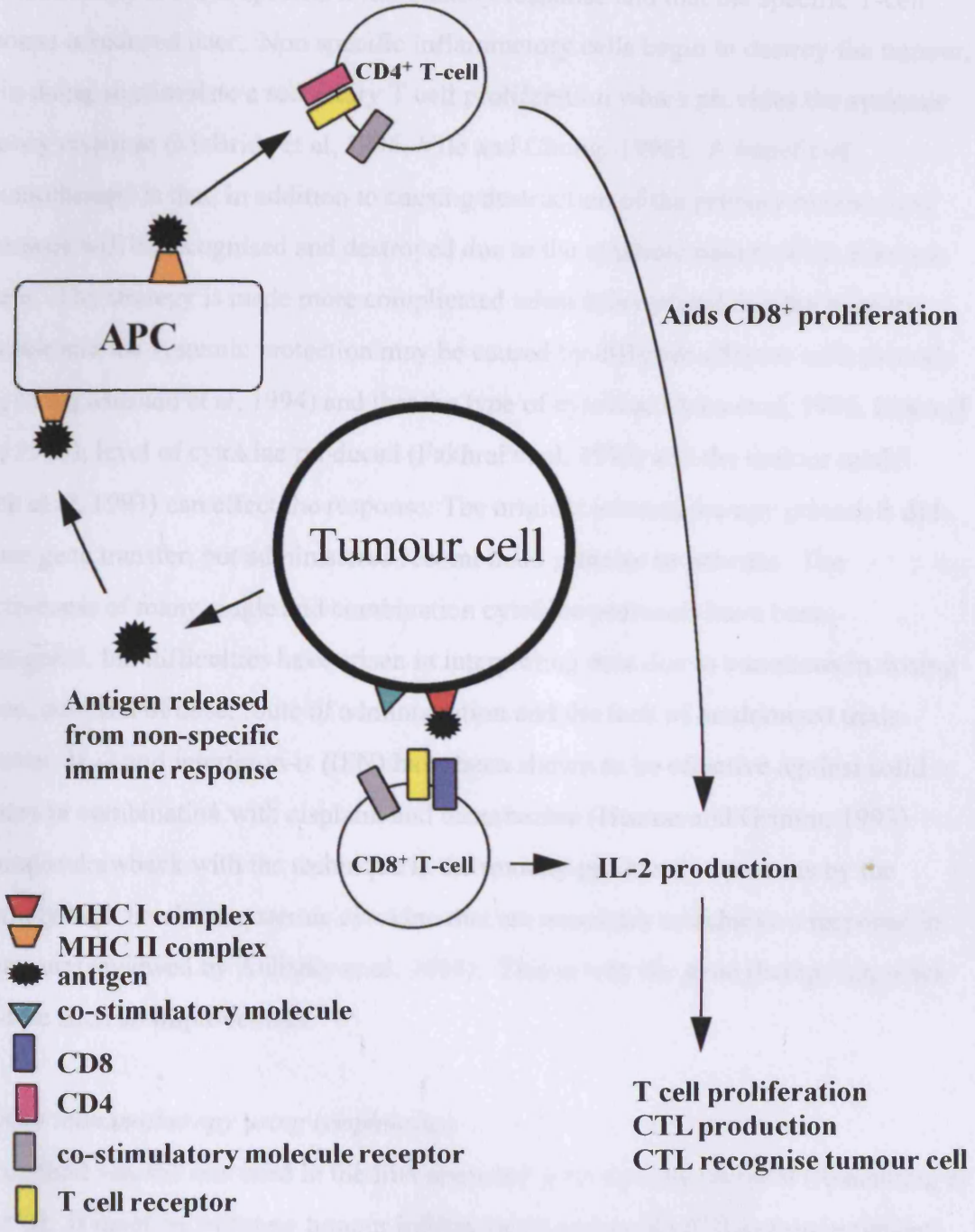
Immunotherapy has been developed to combat cancer. Once a cell has become transformed its ability to evade the immune system contributes to a large extent to its

survival and ability to proliferate (Fearon et al, 1990). The aim of immunotherapy is to overcome the blocks in the immune process. The strategies developed also provide another set of targets for the gene therapist.

During a normal murine immune response an antigen (Ag), presented by a major histocompatibility complex (MHC) protein expressed on the surface of an antigen presenting cell (APC), is recognised by a T lymphocyte. The T cell binds the MHC/Ag complex via its T cell receptor. This causes costimulatory molecules such as B7-2 to be expressed on the APC cell surface which in turn bind to CD28 on the T cell stimulating the production of interleukin-2 (IL-2). This cytokine induces T cell proliferation and differentiation. If the original T cell was a $CD4^+$ cell it forms Th1 (inflammatory) and Th2 (helper) cells. If the T cell was $CD8^+$ it can recognise the MHC/Ag complex expressed on a tumour cell and, in conjuction with IL-2 produced by itself or T_{helper} cells, a similar cascade follows, producing cytotoxic T lymphocytes specific to that tumour cell (reviewed by Galea-Lauri et al, 1996, Hollemweguer and Wormsley, Zier and Gansbacher, 1996).

This process appears to play an important part in tumour irradication by several cytokines (Hock et al, 1993) and there are stages in this process which, if impaired, could cause loss of tumour immunogenicity (Figure 1.4.). Tumours may express proteins on their cell surface which identify them as aberrant cells. However, if the tumour cell lacks MHC molecules the antigen will be inappropriately processed and presented and the immune system will be unable to distinguish the tumour cell from a normal one (reviewed by Browning and Bodmer, 1992, Vora et al, 1997). Alternatively, the tumour cell may present the Ag without the production of a co-stimulatory molecule. This will prevent the production of IL-2 and T cell proliferation will not occur, leading to T cell anergy (McBride et al, 1996, Zier and Gansbacher, 1996). Both immunotherapy and gene therapy strategies based on this approach aim to replace or augment the missing components in the cascade. This could be achieved by transferring tumour associated antigens (Kim and Cohen, 1994), MHC molecules (reviewed by Galea-Lauri et al, 1996), co-stimulatory molecules (Fenton et al, 1995) or activating cytokines (Dranoff et al, 1993, Vile et al, 1994c) to the tumour to overcome

Figure 1.4. Immunotherapy strategy



The tumour cell is recognised by $CD8^+$ T-cells. These release IL-2 which stimulates these cells to proliferate and produce CTL cells. In addition antigen released from the tumour cell by non-specific immune reactions is taken up by APCs, which stimulate $CD4^+$ T-cells. These T_{helper} cells aid the proliferation of CTLs by producing cytokines. (Chong and Vile, 1996)

T cell anergy and induce a specific immune response.

It is becoming clear that often the primary response caused by successful immunotherapy is a non specific inflammatory response and that the specific T-cell response is induced later. Non specific inflammatory cells begin to destroy the tumour, but in doing so stimulate a secondary T cell proliferation which provides the systemic memory response (McBride et al, 1996, Vile and Chong, 1996). A benefit of immunotherapy is that, in addition to causing destruction of the primary tumour, any metastasis will be recognised and destroyed due to the systemic nature of the immune system. The strategy is made more complicated when it is realised that the primary response and the systemic protection may be caused by different effector cells (Moody et al, 1995, Musiani et al, 1994) and that the type of cytokine (Arca et al, 1996, Dranoff et al, 1993), level of cytokine produced (Fakhrai et al, 1995) and the tumour model (Hock et al, 1993) can effect the response. The original immunotherapy protocols did not use gene transfer, but administered recombinant proteins to patients. The effectiveness of many single and combination cytokine protocols have been investigated, but difficulties have arisen in interpreting data due to variations in dosing regime, duration of dose, route of administration and the lack of randomised trials. However, IL-2 and interferon-α (IFN) have been shown to be effective against solid tumours in combination with cisplatin and dacarbazine (Heaton and Grimm, 1993). The major drawback with the technique is the toxicity produced in patients by the extremely high levels of systemic cytokine that are necessary to achieve a response in the tumour (reviewed by Aulitzky et al, 1994). This is why the gene therapy approach would be such an improvement.

Adoptive immunotherapy using lymphocytes

This method was the one used in the first approved gene therapy protocol (Rosenberg et al, 1990). It involves isolating tumour infiltrating lymphocytes (TILs) from a patient, modifying them to express a transgene, usually a cytokine, and re-injecting them into the patient. The hope is that the increased numbers of TILs and immune augmentation caused by the cytokine expression will cause tumour regression. Trials have been attempted to treat various tumour types including renal cell carcinoma, malignant

melanoma and colorectal cancer. Initial results were promising, although further experiments have proved less successful (reviewed by Whittington and Faulds, 1993).

Cancer Vaccines

Cancer vaccines can be prepared from a patient's own irradiated cells (Stingl et al, 1997), allogeneic tumour cell lines (Fenton et al, 1995, Melani et al, 1995), or fibroblasts (Fakhrai et al, 1995). The aim is to cause an immune response against the vaccine, that will also cause the tumour cells to be recognised and destroyed. An autologous cell line derived from a patient's tumour is often modified to express a cytokine to induce a T cell response. It may not be possible to culture a cell line from the tumour and so some strategies use irradiated allogeneic cell lines which express tumour associated antigens. These cell lines can be modified to express additional cytokines and are used to treat HLA matched patients (Dranoff et al, 1993, Melani et al, 1995). A third approach is to use an immunogenic cell line such as a fibroblast line and transfect it with a cytokine. This can be used, in a mixture with irradiated tumour cells, to cause an immune response (Fakhrai et al, 1995). A problem with all these approaches is that the tumour may have caused immunosuppression and these strategies will not overcome this unless MHC or co-stimulatory molecules are also transferred (Fenton et al, 1995), although it is possible that cytokines may have an autocrine effect on the tumour cell itself, upregulating MHC molecules, so overcoming the suppression (Cornetta et al, 1994). Cancer vaccines have been shown to cause less systemic toxicity than conventional methods due to the ability to produce the cytokine in a specific region of the body (Belli et al, 1997).

Immunotherapy based gene therapy

The final approach used in immunotherapy involves direct transfer of the genes to the tumour cell (Vile and Hart, 1994c). This has the advantage that no *ex vivo* methodology is required. Another advantage of expressing the gene at the site of the tumour, is that it allows more physiologically relevant expression of the protein. For example, cytokines can be expressed locally at high concentrations thus reducing systemic toxicity. Additionally, tumours are often heterogeneous and using an *in vivo* method, the immune system is presented with all the tumour associated antigens (TAA)

being expressed, improving the chances of the immune response killing a tumour. It has also been proposed that non irradiated cells are more effective at creating an immune response and it is more acceptable to do this by inducing the response *in situ*, rather than injecting a live cancer vaccine. As discussed earlier, this methodology does have the disadvantage of being very complex to control.

Animal studies have shown that tumour cell lines expressing a cytokine have a decreased ability to form tumours *in vivo* compared to parental cells (Arca et al, 1996, Armstong et al, 1994, Cao et al, 1995, Dranoff et al, 1993, Gerard et al, 1996, Gunji et al, 1996, Missol et al, 1995, Moody et al, 1994, Ohira et al, 1994,Vile and Hart, 1994c). Studies have shown that initial rejection is caused by a non specific immune mechanism, followed by a $CD8^+$ T cell response that confers specific immunity (Hock et al, 1993, McBride et al, 1996, Moody et al, 1994). It is unclear whether these studies mimic the conditions required to cause regression of an established tumour, as these studies are more akin to the cancer vaccine approach. More relevant studies are those which aim to treat established tumours by *in vivo* administration of the vector (Parker et al, 1996, Vile and Hart,1994c).

It has been much more difficult to show that established tumours will regress when treated, either directly or with a cancer vaccine, although some studies have shown such an effect (Parker et al, 1996, Sun et al, 1995, Toloza et al, 1996, Zhang et al, 1996). This may be because tumours of a certain size reach an equilibrium where the ability of the immune system to destroy them equals the tumour's ability to regenerate (Missol et al, 1995). In a clinical situation this may not be a problem. The main tumour could be removed by surgery and it would be the metastases which succumbed to immunotherapy. Proposals for combined cytokine or cytokine and co-stimulatory molecule therapy have been made and one possible advantage of these combinations is that different molecules may stimulate different arms of the immune system, giving a stronger overall response (Cao et al, 1995, McBride et al, 1996). For example, it has been shown that a mouse fibroblast vaccine expressing melanoma-associated antigens and a combination of IL-2 and IFN-γ is more effective at eliminating mouse melanoma cells than either of the two compounds expressed singly (Kim et al, 1995). However,

care must be exercised as evidence is emerging that combination therapy does not always produce improved efficacy (Cayeux, 1997).

A major complication is that the most effective cytokine appears to be different for each individual model or even that the same cytokine can be more or less effective depending on the site of the tumour (Dougherty et al, 1994). Thus each protocol must be developed individually.

1.7. Potential problems with gene therapy

1.7.1. *Level of transduction*

Low levels of transduction limit the effectiveness of gene therapy. Viral titres are often decreased as adaptations are made to vectors (Vile et al, 1994a) and liposomes generally have low levels of transduction efficiency (Missol et al, 1995, Schofield and Caskey, 1995). In addition, the efficacy of a vector may vary between species (Mazur et al, 1994), highlighting the need to find relevant animal models in which to test gene transfer systems. More research into the mechanisms of vector uptake will allow improved efficiency (Lee et al, 1997, Asante Appiah and Skalka, 1997). Greater specificity of targeting can also improve transduction efficiency by delivering the vector to the correct cell at increased concentrations, so increasing the likelihood of cellular uptake. Choosing the most appropriate vector for the conditions is important in this respect. A study to compare the uptake of HSV, RV and AV by brain tumour cells showed AV was not as specific at targeting the tumour cells as the other two vectors (Boviatsis et al, 1994).

1.7.2. Level of expression

Levels of initial transgene expression from a vector can vary enormously (Vile and Hart, 1994c). A major hurdle in gaining sustained expression at the required level is a lack of knowledge of how promoters function. Interactions between viral and tissue specific promoters leading to loss of tissue specificity, and position dependent expression leading to a loss of transgene expression are just some of the factors which can influence the efficiency of expression (Bonifer et al, 1996). Cellular promoter

elements have been found to lose their tissue restricted expression patterns when placed in close proximity to a viral promoter or in the context of multiple promoters (Paulus et al, 1996, Vile et al, 1994a). One reason for this may be that the strong transcriptional controls of the viral promoter override those of the cellular promoter (Miller and Whelan, 1997). Strategies to avoid this include placing the expression cassette in the reverse orientation to that of viral transcription and using cellular, tissue specific control sequences (Vile et al, 1994a). More recently, systems to replace LTR enhancer sequences with tissue specific sequences have been tried to avoid loss of expression (Diaz et al, 1998) The choice of internal promoter and the order in which the genes are placed has also been found to influence expression (McLachlin et al, 1993, Yan et al, 1994). It has also been shown that use of a viral promoter element that confers high expression *in vitro*, does not necessarily do so *in vivo* due to attenuation or inactivation, suggesting that cellular promoters are a preferable choice (Hoeben et al, 1991, Rettinger et al, 1994). One study has suggested a possible explanation for attenuation, reporting that expression is inhibited from some widely used viral promoters and enhancers by INF-γ and TNF-α (Qin et al, 1997). Viral promoters may also fail to promote expression at all in some cell types, and have been shown to differ in the ability to promote expression when placed in the same construct (Doll et al, 1996). However, it should be remembered that the requirement is for sustained expression at an appropriate level and promoters that give the highest expression do not necessarily improve results (Elshami et al, 1997).

The discovery of the β globin locus control region (Grosveld et al, 1987) has helped to shed light on some of these problems. This region is a 50-60kb region which confers high level, tissue specific, position independent expression of the gene it controls. It is chromatin dependent and will not function out of the context of normal chromatin structure. Experiments to reduce it in size to fit into current vector systems have led to loss of position independence (reviewed by Miller and Whelan, 1997). It is not surprising that by taking a much smaller amount of promoter region many functions are lost. To accommodate such a large controlling region, one type of vector being considered is that of the human artificial chromosome (HAC). These would carry one or more genes with all the regulatory sequences necessary and function as an extra

chromosome in the nucleus, passing to daughter cells with each round of replication. Some progress has been reported in the construction of HACs, although efforts have been hampered by the lack of data on the minimum requirements of a functional human chromosome (reviewed by Rosenfeld, 1997).

Many non-viral vectors have been found to give low levels of expression (Christiano and Curiel, 1996b). One of the stumbling blocks in improving levels of expression using liposomes or receptor mediated endocytosis is that this mechanism of gene transfer is still only partially understood. This has led to wide variations in protocol and formulation. A study using DMRIE/DOPE has shown that liposomes can form a very heterogeneous population (Zabner et al, 1995) which moves relatively slowly into the cell. It is thought they bind to the cell by charge interaction and are taken up by endocytosis. Much of the DNA fails to escape from the endosome and is then retained in the perinuclear area and not expressed. It has been suggested that once in the cell, the DNA needs to be released from the lipid for efficient expression in the nucleus. By understanding the mechanism of transfer it should be easier to increase transgene expression levels (Lee and Huang, 1997). Ways of increasing the efficiency of liposome transfer have been reported such as complexing liposomes with inactivated haemagglutinating virus of Japan (Sendai virus) or utilising mechanisms used by the adenovirus (Cristiano and Roth, 1996a, Cristiano and Curiel, 1996b, reviewed by Schofield and Caskey, 1995) to avoid endosome degradation. One study has shown pretreating SCID mice bearing ovarian carcinoma with cisplatin transiently sensitised the tumour to liposome uptake (Son and Huang, 1994).

One approach for increasing expression levels is to use the *mdr1* gene as a selectable marker in conjunction with a second transgene. Increased expression of *mdr 1* has been found after treatment with taxol and this has been used to increase expression of the transgene by association. In addition by selecting the resistant cells and killing the non resistant cells the percentage of transduced cells in the population is increased, thus increasing the efficiency of the process (reviewed by Licht et al, 1996).

Expression of the transgene is often lost relatively rapidly. This could be due to the vector being maintained episomally, so that with each round of replication the copy number is reduced. Alternatively, the targeting of the virus infected cells by cytotoxic T lymphocytes due to low level expression of viral proteins may reduce expression (Sawchuk et al, 1996,Yang et al, 1995). Further modifications, such as eliminating expression of E2A genes, have been shown to improve the persistence of expression (Engelhardt et al, 1994). Retroviral vectors are susceptible to inactivation by complement (Gunzburg and Salmons, 1996, Rollins et al, 1996), and immune reactions against the transgene product, leading to loss of expression, have also been reported (Wells et al, 1997).

There are many cases where expression has been lost, despite persistence of the vector in the genome (Doll, et al, 1996, Dong et al, 1996). Various explanations have been proposed including viral promoter influence (Lund et al, 1996), methylation of the promoter (Szyf, 1996) or steric influences of the chromatin structure (Chen et al, 1997), all of which also influence levels of initial expression. Clearly, an increased understanding of promoter function will help to resolve this problem.

1.8. Potential safety concerns with gene therapy

As with all new technologies there are many safety concerns related to the clinical use of gene therapy.

1.8.1. Acute toxicity caused by the preparations

One cause for concern is the discovery that some of the components used in gene therapy could be potentially toxic to patients. For example, it has been found that many standard DNA isolation protocols do not separate DNA from lipopolysaccharide (endotoxin), a major component of the outer wall of gram-negative bacteria (Wicks et al, 1995). When contaminated DNA was injected into rats, it caused a significant increase in serum IgM and it is now accepted that DNA used in gene therapy should be endotoxin free.

A second worry is potential toxicity caused by the composition of the vector as in the case with liposomes (Mahato et al, 1995) or haemagglutinin spike protein (Mazur et al, 1994). In the latter study addition of this protein has induced toxicity, but differences in toxicity between species have been seen. Porcine cells showed similar levels of toxicity to human cells compared to much lower toxicity levels shown by canine cells, highlighting the problem of choosing a correct animal model. Specific DNA sequences encompassing viral promoters have been shown to cause toxicity (Holter et al, 1991), possibly due to these sequences binding cellular transcription factors more efficiently than cellular promoters, so depriving the cell of the necessary factors to regulate its genes. Another possible problem is that toxicity may not be related to any specific detail of the vector system, but the administration of a foreign particle may interact unexpectedly with the body causing side effects such as the production of a cytokine (McElvaney and Crystal, 1995). Additionally, transgene expression may cause unpredicted effects that could limit the efficacy of the treatment (Yan et al, 1994).

Finally, there is the potential for the administration procedure to cause toxicity and this has been seen in some of the clinical trials where large volumes of cells have been infused into the patient. In a trial treating low density lipoprotein deficiency a large number of cells were infused into the portal circulation causing fever (Grossman et al, 1995) and with the potential to cause more life threatening complications such as portal vein thrombosis.

1.8.2. Immune response

A potential problem with transferring foreign DNA into patients is the possibility of causing an autoimmune response. This can be to self antigens presented on the target cells or to DNA itself, although DNA has been found not to cause any such response when administered to melanoma patients (Nabel et al, 1993, Nabel et al, 1996). The transgene product itself may give rise to an immune response as has been seen in mice expressing the transgene β-galactosidase (Wells et al, 1997), or erythropoetin (Tripathy et al, 1996). Viruses have been found to express low levels of viral proteins which give rise to an immune response. This is especially seen with adenoviral vectors despite deletions in E1 and E3 (Dai et al, 1995, Dong et al, 1996, McDonald et al, 1997, Yang

et al, 1995). There is evidence of immune responses in macaques (Wilmott et al, 1996), mice (Yang et al, 1995) and humans (Zabner et al, 1996), but another study in cotton rats and macaques showed only a minor response (Zabner et al, 1994). One response was seen at higher doses, but on lowering the dose transfer of the gene did not occur (Wilmott et al, 1996).

The immune response may give rise to increased levels of neutralising antibodies or an inflammatory response caused by proliferation of inflammatory cells including lymphocytes, macrophages and neutrophils (Wilmott et al, 1996). The inflammatory cells target the infected cells, while the humoral antibody response is responsible for limiting the success of readministration of adenovirus by inhibiting transduction (Dong et al, 1996,Yang et al, 1995). Results from one study, however, have shown that the presence of these antibodies had no effect on the efficacy of the treatment (Zabner et al, 1994). The inflammatory response causes discomfort and can be potentially life threatening in situations such as treatment of cystic fibrosis, where patients already have a compromised immune system. In addition, the response targets cells expressing the proteins and destroys them, thus diminishing transgene expression. The inflammatory response has been shown to be increased after a second dose of adenovirus (Yang et al, 1995). This is a real problem with AV vectors because being maintained episomally they require repeated administration to treat diseases such as cystic fibrosis. Work is proceeding to produce vectors that do not cause an inflammatory response (Conrad et al, 1996, Goldman et al, 1995).

1.8.3. Replication competent virus (RCV) production

The possibility that contaminant replication competent viruses may be administered to patients has been one of the primary concerns with viral gene therapy. Replication competent MMLV has been shown to cause T cell neoplasm in non-human primates (Donahue et al, 1992). There has been rigorous improvement in screening to make certain that they are not present in packaging lines. The fear is that, if present, they could allow the spread of the transgene to non targeted areas of the body or to other individuals.

Despite the screening procedure, RCVs have been detected in retroviral packaging lines, probably due to recombination events. To reduce the possibility that recombination between the helper and transgene carrying vectors occurs, vectors are being developed whereby the necessary components are split into three vectors and the homology between the vectors is reduced to a minimum to decrease the likelihood of RCV formation (reviewed by Vile and Russell, 1995). One further possibility is that the vector can recombine with endogenous retroviral sequences in the host genome, although it is thought that the risk of this is very low (reviewed by Cornetta, 1992).

Adenoviral vectors also have the potential to recombine with endogenous sequences, but in addition they are susceptible to complementation. This occurs when the function of a gene such as E1A, which has been deliberately deleted, is restored by wild type virus. Again improved screening and vector design can reduce the possibility of this occurring (reviewed by Smith et al, 1996). Recombination with wild type viruses to create a replication competent virus is also possible. Development of packaging lines or vectors based on non human adenovirus may overcome these problems.

1.8.4. Insertional mutagenesis

Retroviral vectors insert into the host genome with some preference for transcriptionally active regions (Mooslehner et al, 1990). This means there is the potential to insert upstream of, or within, a gene. Either the insertion event or possibly the influence of the long terminal repeat (LTR) promoter may then disrupt the normal regulation of this gene. In many cases this will have no effect on cellular regulation or will cause the cell to die. However, there is the potential for the vector to disrupt an oncogene or a tumour suppressor gene resulting in transformation of the cell. The actual risk of this occurring is thought to be relatively low (reviewed by Cornetta, 1992, Moolten and Cupples, 1992). Retroviral vectors tend to integrate only once per cell (Harris and Sikora, 1993b) and it has been estimated that between 5 and 7 alterations may be needed for a cell to become transformed. The danger occurs if a cell, either by genetic predisposition or under the influence of environmental factors, has already acquired several mutations.

Other risks associated with retroviral vectors

The reverse transcription step of the RV life cycle has a high error rate and this could lead to the delivery of mutated copies of the transgene to the target cell causing cell transformation (Varela- Echavarria, 1993, reviewed by Vile and Russell, 1995). There is also a tiny risk that packaging of cellular mRNA, which has the potential to carry a mutation, or to transfer endogenous mouse retroviral sequences from packaging lines, could occur (reviewed by Cornetta, 1992).

1.8.5. Lack of toxicity data

Perhaps the most concerning safety issue in gene therapy is sheer lack of knowledge. Strict working practices and screening procedures have decreased the dangers of contamination of cell lines and viral vector preparations and improved reproducibility (reviewed by Smith et al, 1996). Extensive protocol development *in vitro* and in animal models and gene marking studies (Brenner, 1996) have highlighted the possible acute toxicity problems. What is required now is investigation into the longer term effects of transferring genetic material into cells.

In the course of developing a clinical trial proposal, the safety of the procedure must be determined. Here extensive investigation in animal models, including toxicological data, is required (Anderson et al, 1990, Klatzmann et al, 1996, Parker et al, 1995, Walker et al, 1996) and *in vivo* data on distribution of the vector and transgene expression and its effects on non target organs are often presented. These studies use physical examination, levels of blood haemoglobin, white blood cell counts, levels of serum electrolytes, biochemical markers and arterial blood gases, antibody levels and histological examination to monitor toxicity of the procedure (Canonico et al, 1994, Goldman et al,1995, Halbert et al, 1997, Lesoon-Wood et al, 1995, McDonald et al, 1997, Nabel et al, 1994, Simon et al, 1993, Stewart et al, 1992, Xing et al, 1997). Immunohistochemical or histochemical analysis, polymerase chain reaction (PCR), radioactive labelling, reverse transcription-PCR (RT-PCR) or *in situ* hybridisation are used to look at tissue distribution of the inserted DNA and distribution and efficacy of transgene expression (Goldman et al, 1995, Grossman et al, 1995, Lew et al, 1995, Osaka et al, 1996, Oshiro et al, 1995, Ram et al, 1997, Roth et al, 1996,) or of the

carrier (Parker et al, 1997). The inadvertent transfer of DNA to healthy tissue is an important issue, which has again been highlighted very recently in the literature (Boyce, 1998). Some studies have looked at a wide range of tissues including reproductive tissue to examine the possibility of DNA uptake here (Goldman et al, 1995, Nabel et al, 1992, Winegar et al, 1996). While not all studies use all of these methods, they will use those needed to obtain enough data to satisfy the regulatory boards.

1.9. Malignant melanoma

The work described in this thesis used a rodent model for metastatic malignant melanoma.

The incidence of skin cancer is increasing yearly. Malignant melanoma is a fatal form of skin cancer and in the United States 32 000 cases are diagnosed each year with 25% proving fatal (Sakai et al, 1997). The five year survival rate has increased dramatically over the last 40 years due, at least partially, to increased awareness and early diagnosis. The increasing incidence is thought to be associated with the increased exposure to sunlight and increased use of sun beds. The progress of the malignant disease is categorised such that local disease is considered to be stage I and II, stage III represents disease occurring in the lymph nodes and stage IV is distant metastatic disease (reviewed by Stratigos and Sober, 1996).

Melanocytes are specialised dendritic cells, mainly derived from the neural crest (Hearing and Tsukamoto, 1991, Sakai et al, 1997). They are found in skin, hairbulbs and the eyes and contain specialised organelles called melanosomes. These structures produce melanin and are transferred to keratinocytes and the hair shaft to provide protective colouration . Melanins have a crucial role in protecting the host from ionising radiation and absorbing free radicals in the cytoplasm. Melanomas form from melanocytes if they become transformed.

1.9.1. Conventional therapy

If a patient has a suspected lesion, a biopsy is taken to confirm diagnosis and prognosis. The diameter of the lesion gives a fair indication of prognosis, but other factors such as site of the lesion are also important. Further tests are carried out, the details of which vary depending on the stage of the disease, but the further the disease has progressed, the more stringent the search for metastatic disease. Techniques such as X-rays, computed tomographic imaging and PCR are all used to determine the extent of the disease and possibility of relapse. The primary lesion is excised by surgery and in some cases elective lymph node dissection is carried out, although there is still debate on the efficacy of this procedure (reviewed by Stratigos and Sober, 1996). Patients with larger primary lesions are given adjuvant therapy to increase their chance of remaining disease free by preventing recurrence and destroying metastases. Chemotherapy and immune stimulants have often proved ineffective, but a randomised trial using interferon α-2b has produced an increase in relapse free intervals and there is evidence that radiotherapy may be beneficial in this respect. Treatment of metastatic disease have been less effective to date. Small increases in response rates have been achieved with chemotherapy. Combination therapy using chemical and immune components has proved more successful (reviewed by Falkson, 1995). Radiotherapy has proved to be effective, especially when combined with other treatment strategies, with a complete response being seen in 25% of cases.

1.9.2. Immunotherapy

While malignant melanoma can be successfully treated when diagnosed at an early stage, the poor record of treatment of metastatic disease prompted research into other treatment regimes. Melanoma has always been regarded as an immunogenic tumour, with cases of spontaneous regression being reported. Immunotherapy protocols have been developed to treat melanoma patients. The administration of cytokines such as IL-2 or IFN, either on their own or in combination with chemicals, lymphokine activated killer (LAK) or TIL cells isolated from the patient has shown some success (Legha, 1997), but side effects such as capillary leak syndrome seen with high dose IL-2 have proved a problem (Philip and Flaherty, 1997, Stratigos and Sober, 1996).

1.9.3. Cancer Vaccines

As described in section 1.6.5. the aim of a cancer vaccine is to present tumour specific antigens in a form that the immune system will recognise and thus make the tumour, in this case a melanoma, susceptible to this same immune response. Most of the approved cancer vaccine trials have used autologous cells isolated from the patient and modified *ex vivo* to increase their immunogenicity (reviewed by Dalgliesh, 1996). A phase I study is in progress, assessing the efficacy of this approach, using IL-2 transfected autologous tumour cells (Stingl et al, 1997).

Cancer vaccines have also been prepared from allogeneic melanoma cell lines modified to express cytokines. The cell lines are chosen so that they express a range of antigens likely to be expressed by the melanoma cell. Several melanoma antigens have been described and these include the MAGE family of antigens (reviewed by Itoh et al, 1996), gp 100, MART-1 and tyrosinase (Sakai et al, 1997). Studies have been reported using allogeneic melanoma cell lines, positive for several melanosome specific antigens such as Melan-A/MART-1, tyrosinase and gp 100, and modified to produce IL-2. These were injected into HLA matched patients and some patients responded to the treatment (Arienti et al, 1996, Belli et al, 1997). Alterations in HLA and co-stimulatory molecule expression can also alter the ability of tumour cells to be recognised and positive results using this strategy have been reported (Fenton et al, 1995, Stopeck et al, 1997). Another approach is to create a tumour vaccine using cells, such as fibroblasts, that are immunogenic. In one study murine LM cells have been transfected with the gene for IL-2 followed by DNA from B16F1 or B16F10 cells. The hope was that the tumour associated antigens would be expressed on the surface of the cells and the IL-2 aid an immune reaction against small established B16F1 or B16F10 melanomas when injected into the animal. Indeed, the treated mice survived significantly longer than controls and an antimelanoma response mediated by cytotoxic T lymphocytes (CTLs) and natural killer/lymphokine activated killer (NK/LAK) cells was observed (Kim et al, 1995). Fibroblasts have also been modified to express a cytokine and have been injected in conjunction with irradiated tumour cells as a cancer vaccine (Fakhrai et al, 1995). An alternative is to modify fibroblasts to produce retroviral particles expressing

a suicide gene at the site of the tumour. The melanoma can then be eradicated by treating the patient with the appropriate prodrug (Klatzmann et al, 1996).

1.9.4. Gene therapy for metastatic malignant melanoma

As described in section 1.6.5., there are many advantages to carrying out gene therapy *in vivo*, including minimised surgical procedures and the occurrence of transgene expression in the context of the body rather than in culture. Gene therapy protocols are being developed in animals for treating metastatic malignant melanoma. These include transferring constructs expressing cytokines (Parker et al, 1996, Vile and Hart, 1994c) and suicide genes (Vile and Hart, 1993b) directly into the tumour. A clinical trial is in progress, which transfers HLA molecules to the tumour (Nabel et al, 1996). Some of these have shown some success in causing growth arrest or regression of established tumour.

1.9.5. Interleukin-2 gene therapy

One of the treatments for malignant melanoma which has shown some benefit is IL-2 therapy (reviewed by Aulitzky et al, 1994, Whittington and Faulds, 1993). Under normal circumstances IL-2 is localised to areas of immune response and inflammation and is not found systemically at high levels. Immunotherapy has proved only partially successful and the ability of gene therapy approaches to express IL-2 at the tumour site, thus mimicking the body's natural pattern of cytokine expression and decreasing the potential for toxicity, has renewed interest in this technique.

There is growing evidence from animal studies to suggest that tumourigenic cell lines secreting IL-2 have greatly reduced tumorigenicity when injected into animals (Hock et al, 1993, Moody et al, 1994, Musiani et al, 1994, Vile and Hart, 1994c, Wan et al, 1997). Studies using cancer vaccines expressing IL-2 have shown reduced tumour growth or remission in animal models (Fakhrai et al, 1995) and human tumour patients (Arienti et al, 1996, Belli et al, 1997, Sobol et al, 1995) and direct transfer of an IL-2 expressing construct has also caused retarded growth of a tumour (Toloza et al, 1996).

1.10. Interleukin-2

Interleukin-2 is a 15.5 kDa glycoprotein which acts as a cytokine. It is expressed, in conjunction with its receptor, by T_{helper} lymphocytes in response to presentation of an antigen on the surface of an antigen presenting cell in the presense of interleukin-1 or interleukin-6 and is released from the cell. It is generally involved in a cell mediated or Th1 response (Dalgliesh, 1994). Here it acts in an autocrine and paracrine fashion to stimulate the division and differentiation of T_{helper} cells that are expressing an interleukin-2 receptor on their cell surface. In addition to T_{helper} cells, other T cell sub types, B lymphocytes, natural killer cells and cytotoxic macrophages are also induced to divide and differentiate by the binding of IL-2 to its receptor. Peripheral blood mononuclear cells can be stimulated to carry out lymphokine activated killing. Once activated by IL-2 the T cells go on to produce a cascade of cytokines including tumour necrosis factor (TNF), IFN and other interleukins such as IL-1 and IL-3 and GM-CSF (granulocyte macrophage colony-stimulating factor), which cause changes in other immune cells. The range of cells activated by IL-2 at any one time seems to be dependent on several criteria, including concentration. Natural killer cells are only activated by high concentrations of IL-2, while low levels activate T cells. It is also thought that the proportions of different cell types can change with time (reviewed by Whittington and Faulds, 1993). There is evidence that recombinant IL-2 can reverse suppression of the CTL response (Inge et al, 1992) and prevent T cell anergy (Chen et al, 1997), making it an obvious candidate for immunotherapy.

When IL-2 is expressed in a tumour cell as a transgene a non-specific inflammatory response is elicited, which may include natural killer cells (Hock et al, 1993), neutrophils (Musiani et al, 1994) and LAKs (Wan et al, 1997), causing suppression and possibly destruction of the tumour. In addition the cytokine producing tumour cell is recognised by T cells, which are induced to divide to produce $CD8^+$ CTL cells. This mechanism is thought to bypass the $CD4^+$ T helper response (Fearon et al, 1990). Antigens released from the destroyed cells cause other branches of the immune system to become involved. The antigens can be presented by APC's attracted to the area by the IL-2 (Figure 1.4.). It is thought to be the CTL cells which cause total tumour

destruction and go on to give protection against subsequent challenge (Arienti et al, 1996, Hock et al, 1993, Kim et al, 1995, Moody et al, 1994, reviewed by Vile and Chong, 1996). It is this response which is likely to aid in the rejection of metastases. Additionally, it has been shown that IL-2 expression can cause increased expression of intercellular adhesion molecule (ICAM) and major histocompatibilty complex (MHC) molecules on the tumour cell surface, possibly aiding adhesion of effector cells to the tumour cell and facilitating antigen presentation (Wan et al, 1997). It seems likely that the final tumour response does not depend entirely on the transgene produced, but the induction of a range of other cytokines and immune cells, which are attracted to the area is also relevant.

An interesting observation to come from these studies is that the amount of cytokine being secreted could be important. In a tumour vaccine animal model, if large amounts of cytokine are excreted the cell is immediately destroyed, producing small amounts of antigen. However, if a smaller amount is produced the cell may form a small tumour before the IL-2 levels are sufficient for immune upregulation, at which point the therapy takes effect. The amount of tumour antigen produced will be far larger in this case and is more likely to create a specific T-lymphocyte response (Musiani et al, 1994). It has been shown in one animal model that low levels of IL-2 are more effective at creating a systemic response, whereas high levels of IL-2 production elicit no such 'memory' (Fakhrai et al, 1995). This may relate to the fact that T-cells are stimulated by IL-2 binding to high affinity receptors while NK cells and LAK precursors are stimulated by binding to intermediate receptors which require much higher levels of protein. However, the situation is far from straightforward, as there is evidence to show that, in some cases, high local concentrations of IL-2 are necessary for tumour cell eradication (Moody et al, 1994) and in an M3 murine melanoma model systemic immunity was only obtained from high expressing cells (Zatloukal et al, 1993).

So to summarise, in animal models, in response to IL-2 secretion, the tumour is initially infiltrated by non T cell specific immune effector cells, while the long lasting systemic response is caused by $CD8^+$ CTLs. Human trials have also shown evidence for the

presence of $CD4^+$ cells when IL-2 is used (Arienti et al, 1996, Belli et al, 1997). When other cytokines are used, $CD4^+$ cells have also been implicated (Dranoff et al, 1993).

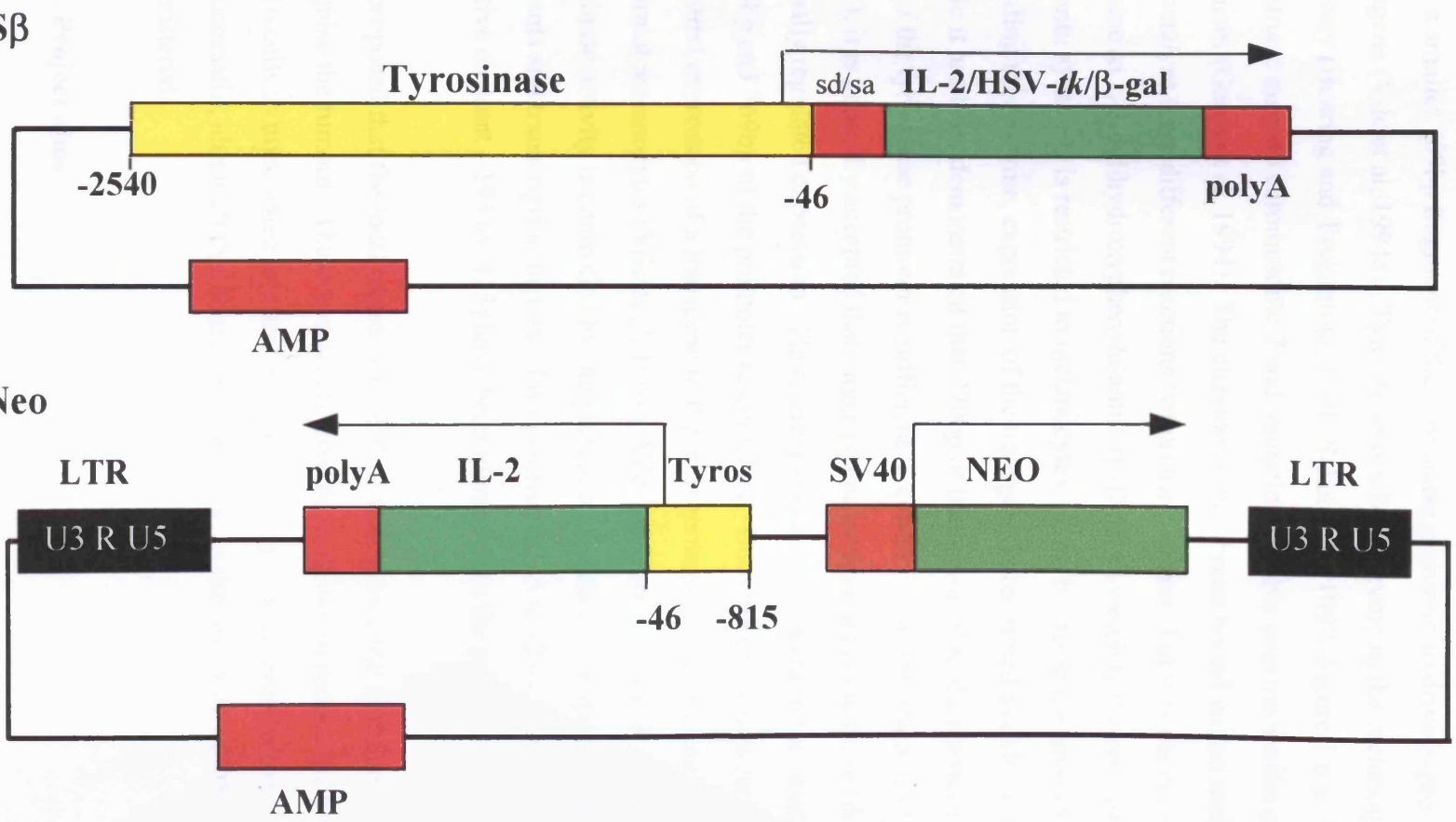
1.11. Model system

Dr. R. Vile's group (ICRF) is developing several gene therapy approaches for treating malignant melanoma (Vile and Hart,1993b, Vile and Hart, 1994c). This project focuses on an immunotherapy model they have developed to explore treatment of malignant melanoma. In this strategy a vector expressing IL-2 is transferred directly into the tumour (Vile and Hart, 1994c), with the aim of overcoming T cell anergy (Chen et al, 1997) or suppression of CTL responses (Inge et al, 1992), causing the tumour to be destroyed by the immune system. I have used the B16.F1. mouse melanoma line obtained from Dr. Vile's group. This is a highly malignant cell line derived from a spontaneous melanoma that occurred in a C57BL/6 mouse (Fidler, 1975). When injected intravenously into a mouse tail vein, the animal develops pulmonary tumour nodules. These are equivalent to pulmonary metastases from a primary melanoma. Well encapsulated tumours, lacking invasive ability, are formed. The B16.F1 sub clone has a lower metastatic potential compared to B16.F6 or B16.F10 sub clones. A mouse colorectal cell line CMT93 (Franks and Hemmings, 1977) was also used in order to test a second tumour system. This is an epithelial line derived from a methyl azoxymethanol acetate induced mouse carcinoma.

1.11.1. Constructs

The plasmid construct (Figure 1.5.) is based on the commercially available plasmid pNASSβ (Macgregor and Caskey, 1989). A 2494bp fragment of the mouse tyrosinase promoter was inserted into the vector to drive expression of β-galactosidase (Vile and Hart,1993a). In the other two plasmid constructs used, the coding region was excised and replaced with either the Herpes Simplex Virus-*thymidine kinase* gene (Vile and Hart, 1993b) or a 561bp fragment of the coding region of murine IL-2 (Vile and Hart, 1994c). The retroviral MMLV based vector, pBabeNeo, was designed (Morgenstern et al, 1990) to transduce and express the inserted gene with the same efficiency as endogenous genes expressed by the virus. Two versions were used in these

Figure 1.5. Constructs



A. shows the plasmid vector pNASSβ which contains the coding region of the β-galactosidase, HSV-*tk* or mouse IL-2 gene under the control of 2494bp of the mouse tyrosinase gene.
B. shows the retroviral vector pBabeNeo which contains the coding region of mouse IL-2 gene under the control of a 769bp fragment of the mouse tyrosinase gene. A second version of this vector (pBabeNeo/IL-2LTR), which promoted expression of IL-2 from the LTR, was present in CMT93 cells (Morgenstern et al, 1990, Vile and Hart, 1993a, Vile and Hart, 1993b, Vile et al, 1994a, Vile and Hart, 1994c).

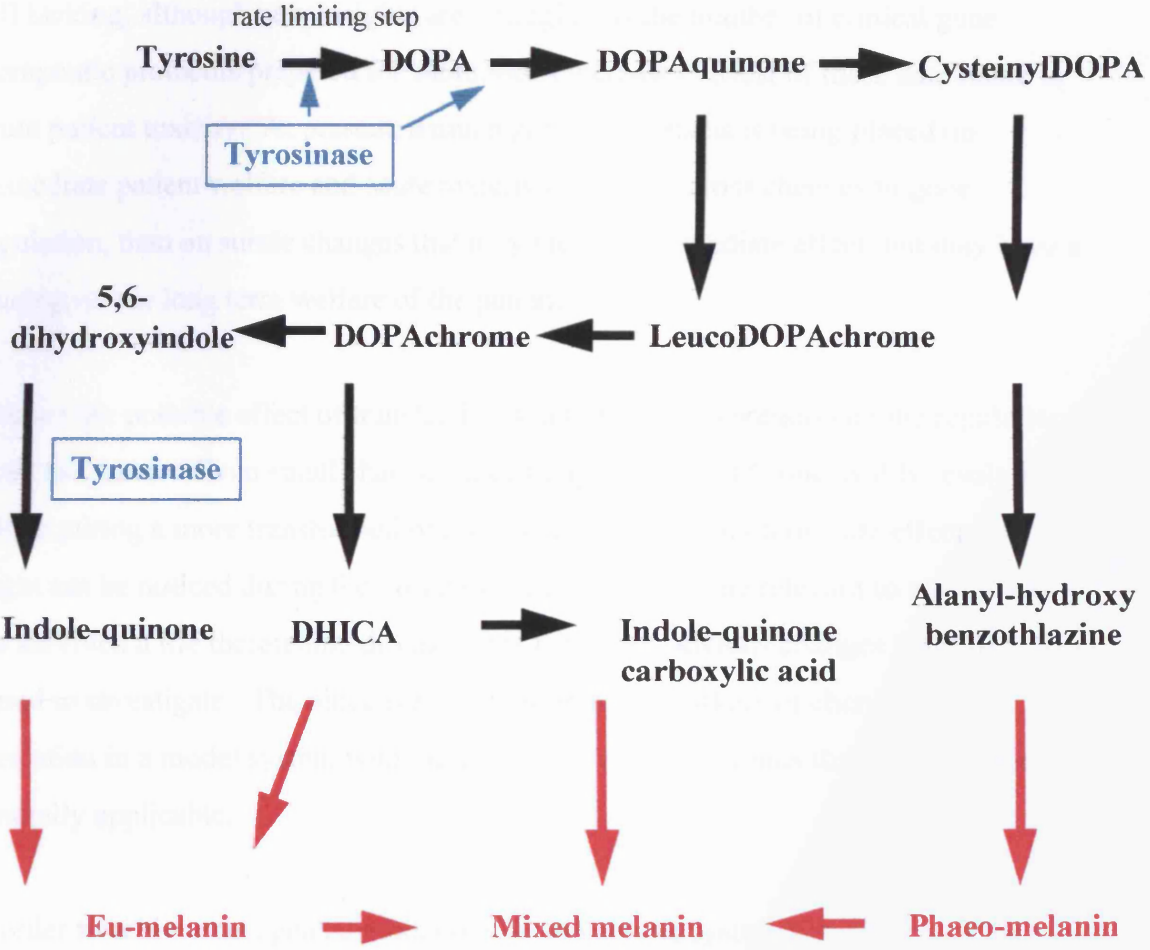
experiments and both carried the 561bp fragment of IL-2. The version found in CMT93 cells promoted IL-2 expression from the LTR, while that found in the B16 cells used a smaller 769bp fragment of the tyrosinase promoter to drive expression of the transgene (Vile et al, 1994a). Tyrosinase is a key enzyme in the melanogenesis pathway (Hearing and Tsukamoto, 1991, Sakai et al, 1997, Figure 1.6.). Mouse tyrosinase maps to chromosome 7 and mutations in this enzyme are the cause of classic albinism (Ganss et al, 1994). The enzyme is membrane bound within melanosomes and can catalyse three different reactions within the pathway, but it is the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) which is the rate limiting step. Melanin synthesis is restricted to melanocytes. Thus by using the promoter of the gene encoding this enzyme, expression of the transgene is restricted to cells of this lineage. While it has been demonstrated that 270bp of the region 5′ to the transcriptional start site of the tyrosinase promoter is sufficient to confer tissue specificity (Kluppel et al, 1991), it is generally accepted that larger promoter fragments are more likely to give normally regulated expression. Therefore, the constructs used in this study contained 2494bp and 769bp of the promoter respectively. Both the constructs have been shown to restrict expression of a transgene to the melanoma-derived cell line, B16, *in vivo* and to normal melanocytes (Vile et al, 1993a, Vile and Hart, 1994c). It is thought that tyrosinase activity is controlled by the presence of melanocyte specific promoter elements and transcription factors. Two positive (-245 to -230, -104 to -93) and one negative element (-195 to -125) have been identified in the promoter (Ganss et al, 1994)

It is proposed that the local expression of IL-2 will induce the immune system to recognise the tumour. These constructs have been shown to reduce the tumorigenicity of B16 cells in mice when injected *in vivo*. A study to treat established tumours was not successful, although this could have been due to the low dose of DNA administered.

1.12. Project aims

In the 137 patients who have received gene therapeutics in this country (to Jan 97) no life threatening side effects have been seen (GTAC, 1997). The most common side

Figure 1.6. Melanin synthetic pathway



Synthesis pathway of melanin showing the role of tyrosinase as the enzyme that controls the rate limiting conversion of tyrosine to DOPA. Tyrosinase additionally catalyses two other steps in the pathway.

DOPA, 3,4-dihydroxyphenylalanine; DHICA, 5,6-dihydroxyindole-2-carboxylic acid

(Hearing and Tsukamoto, 1991)

effect observed in animal models is the unwanted immune response mentioned above (1.8.2.). Uptake of DNA and expression of the transgene in non-target tissues does not appear to be associated with any significant toxicity, and there has been no evidence of vector uptake by reproductive tissues. Nevertheless, data useful in safety evaluation is still lacking, although new insights are emerging as the number of clinical gene therapeutic protocols prepared for submission increases. Most of these data relate to acute patient toxicity. At present, a much greater emphasis is being placed on immediate patient welfare and acute toxicity caused by gross changes in gene regulation, than on subtle changes that may yield no immediate effect, but may have a bearing on the long term welfare of the patient.

Data on the possible effect of transfection and transgene expression on the regulation of a cell are scarce. Even small changes in cell regulation could conceivably result in the cell acquiring a more transformed phenotype or in other long term side effects. These might not be noticed during the initial treatment, but become relevant to a patient who has survived a life threatening disease. It is these less obvious changes that this project aimed to investigate. The objective was to search for markers of change in cell regulation in a model system with the view to discovering trends that might be more generally applicable.

In order to achieve this goal, a model mouse melanoma system was chosen. This model system is part of our collaborator's ongoing attempts to design gene therapeutic approaches for malignant melanoma. Thus the project used a system that has clinical relevance and contacts were maintained with Dr. Vile's group throughout the project to ensure that questions applicable to the field of gene therapy were being addressed. While it may be considered a drawback to have chosen a cancer therapy model in which the aim of the treatment is to destroy cells, our goal was to find generic changes, applicable to other systems. A model based on a long term strategy such as treatment of CFTR may have been considered preferable, but the abundance of gene therapy trials aimed at cancer treatment seemed to render the approach taken here pertinent.

Effects which transfection and transgene expression exerted on the model system were determined by assessment of changes in growth parameters (Chapter 3) and cellular mRNA population (Chapter 4).

Whilst many clinical protocols are now in progress, successful gene therapy has proved to be difficult to achieve. One of the factors contributing to the lack of success is downregulation of the transgene, which causes protein levels to decrease, often within days of the procedure. The model used in this study displayed loss of transgene expression. This time dependent loss of transgene expression was investigated to gain information on the mechanism of loss of transgene expression, which may ultimately improve vector design (Chapter 5 and 6).

Chapter Two

Materials and Methods

MATERIALS

2.1. Source of materials

2.1.1. Chemicals and equipment

40% Acylamide:bis acrylamide	Scotlab
Ampicillin	Sigma
Avidin peroxidase	Sigma
2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)	Sigma
DMEM medium	Gibco BRL
deoxynucleotide triphosphate (dNTPs)	Pharmacia
Ganciclovir (GCV)	Leicester Royal Infirmary
Geneticin (G418)	Gibco BRL
Glutamax I	Gibco BRL
Hybond N^+	Amersham
Interleukin-2 (cell culture supplement)	Cetus
IPTG	Melford Laboratories
MES buffer	Sigma
Melanocyte stimulating hormone	Sigma
Nap 5 columns	Pharmacia
Phalloidin	Sigma
Phenol pH 4.3	Sigma
RPMI medium	Gibco BRL
Ultima gold scintillation fluid	Canbera Packard
X-gal	Melford Laboratories
X-ray film (Hyperfilm MP ^{TM})	Amersham

2.1.2. Enzymes

AMV-reverse transcriptase	Promega
T4 DNA ligase	New England Biolabs
Restriction enzymes	New England Biolabs
	Boehringer Mannheim

Rnase A	Pharmacia
Rnasin	Promega
Sequenase kit	Amersham
Superscript II reverse transcriptase	Gibco BRL
Taq DNA polymerase	Perkin Elmer, Gibco BRL
T4 polynucleotide kinase	Promega

2.1.3. Antibodies

Biotinylated anti-mouse IL-2 antibody	Pharmingen
FITC conjugated CD25 (IL-2 receptor α) antibody	Pharmingen
Mouse IgG negative control antibody	DAKO
Rat anti-mouse IL-2 antibody	Pharmingen

2.1.4. Radioisotopes

[γ ^{32}P] Adenosine 5′-triphosphate (ATP)	Amersham
[α ^{32}P] dATP	Amersham
[α ^{33}P] dATP	NEN
[α ^{35}S] dATP	NEN
[α ^{32}P] deoxycytidine 5′-triphosphate (dCTP)	Amersham, NEN
5-[methyl ^{3}H]-thymidine	Amersham

2.1.5. Dyes, molecular weight markers , DNA and standards

100bp ladder	New England Biolabs
Bromophenol blue	USB
Interleukin-2 standard	Pharmingen
Oligonucleotide primers	In house
RNA ladder	Gibco BRL
Salmon sperm DNA	Sigma
Xylene cyanol	BDH

2.1.6. Bacterial cells

INVαF′ cells	InVitrogen

LigATor kit competent cells	R and D systems

2.2. Solutions and buffers

Unless otherwise stated all chemicals were made up in autoclaved water distilled to 18.5 Ohms (ultrapure). To prepare RNase free water, 0.5mls of diethyl pyrocarbonate (DEPC) was added to a litre of ultrapure water which was shaken and left overnight. The water was autoclaved twice before use, in order to degrade the DEPC.

Acid fixative	*final concentration*
glacial acetic acid	10% (v/v)
methanol	50% (v/v)

The fixative was prepared in water and stored at 4°C.

Acrylamide gel	*final concentration*
11.25mls 40% acrylamide: bis acrylamide (19:1)	6% (v/v)
15mls 5 x TBE	1 x TBE

To prepare a non-denaturing gel the components were combined and the volume adjusted to 75mls with water. To this was added 450µl ammonium persulphate (10%) and 75µl of TEMED. The gel was swirled and immediately used to prepare a 0.4µm thick gel.

To prepare a denaturing gel, 31.5g urea was dissolved by heating in the TBE buffer. The acrylamide was added and the volume adjusted to 75mls with water. The gel was cooled before proceeding as for the non-denaturing gel.

Agarose gel

To prepare a 1% gel, 1g of ultrapure electrophoresis grade agarose was dissolved in 100ml of 1 x TAE buffer by heating in a microwave. Once the agarose was completely dissolved the solution was allowed to cool to approximately 50°C before pouring into the casting tray.

Ampicillin stock solution

Ampicillin was dissolved in water to a concentration of 50 mg/ml, sterilised by passing through a 0.22µl filter and stored in aliquots at -20°C.

Avidin peroxidase

Avidin peroxidase was dissolved in phosphate buffered saline (PBS)/10% fetal calf serum (FCS) to give a stock of 1mg/ml which was stored at -20°C.

β–galactosidase substrate solution	*final concentration*
13µl magnesium chloride	1.3mM
600µl 50mM potassium ferricyanide	3mM
600µl 50mM potassium ferrocyanide	3mM
500µl X-gal (20mg/ml)	1mg/ml
5mls 200mM sodium phosphate buffer	100mM

The components were dissolved in 10mls of water and filtered through a 0.45µm filter before use.

Coating buffer	*final concentration*
Sodium hydrogen carbonate, pH8.2	0.1M

Column wash (2 x)	*final concentration*
2.941g sodium citrate	0.1M
5.844g sodium chloride	1M
0.4mls 0.5M EDTA	2mM

The components were dissolved in 100mls of water and treated with DEPC before being autoclaved. After autoclaving, 1ml of 20% sodium lauryl sarcosinate was added.

Denhardts (50 x)	*final concentration*
1g ficoll	1% (w/v)
1g polyvinyl pyrrolidone	1% (w/v)
1g bovine serum albumin	1% (w/v)

The components were dissolved in 100mls of water previously treated with DEPC and autoclaved. The solution was stored in aliquots at -20°C.

Dulbecco's minimal essential medium (DMEM)

One litre of DMEM (containing Glutamax I, sodium pyruvate (110mg/l), glucose (1000mg/l) and pyridoxine) was supplemented with 10% heat inactivated fetal calf serum, 5 units/ml penicillin and 50µg/ml streptomycin

ELISA substrate solution (preparation of)

To prepare the substrate 150mg of 2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) was dissolved in 500ml of 0.1M citric acid. The pH was adjusted to 4.35 with NaOH pellets and stored at -20°C in 11ml aliquots. The substrate is very sensitive to temperature so should be defrosted at the last possible moment before use.

Elution buffer	*final concentration*
10mls Tris.HCl pH 7.6	10mM
1ml 0.5M EDTA	1mM
2.5mls 10% sodium dodecyl sulphate (SDS)	0.05%

The Tris.HCl and EDTA were combined and the volume adjusted to 497.5mls with water. The solution was treated with DEPC and autoclaved before adding the SDS.

Ethidium bromide solution

A solution of 5µg/ml in water was used to stain agarose gels. Gels were generally stained for 10 minutes and destained in water for a further 10 minutes before viewing under UV light. The stock solution was stored at room temperature and protected from the light.

Formaldehyde gel

To prepare the gel 0.8g of agarose was dissolved in 57.6mls of RNase free water. After cooling slightly, 8mls of 10x MOPS and 14.4mls of formaldehyde was added. The gel was mixed gently by swirling and poured into the casting tray.

Freezing medium	*final concentration*
10mls heat inactivated fetal calf serum	50%
1.5mls dimethyl sulphoxide	30%
0.5mls 200mM Glutamax I	5mM
8mls appropriate medium	

The components were mixed and incubated at 37°C until needed. This medium was freshly made when required.

G418 stock solution

Being a mammalian cell selection agent, G418 is toxic, so the powder is used in a fume hood. The stock solution was prepared by adding phosphate buffered saline (PBS) to the chemical container (thus avoiding weighing out the powder), replacing the lid and shaking gently. Once in solution G418 can be handled with care in a flow hood. The stock was sterilised through a 0.22μm filter and stored in aliquots at -20°C.

Ganciclovir stock

Ganciclovir (Cymevene) was obtained from Leicester Royal Infirmary pharmacy. The contents of the vial were dissolved in sterile PBS by injecting the solution through the rubber seal of the vial. This gave a solution of 100mgs/ml which was further diluted to 1mg/ml and stored at -20°C.

Hybridisation buffer

This buffer contains the same components as the prehybridisation buffer, but also contains 10% dextran sulphate. This should be added before the Denhardts and the formamide and incubated at 65°C as this helps the dextran sulphate to dissolve. Once the dextran sulphate has dissolved the other components can be added.

Interleukin-2 standards

The purchased stock of IL-2 (75 000 units) was dissolved in 40μl of PBS/10% FCS and diluted 1 in 10 in PBS/FCS to give a stock of 187.5 units/μl. This was stored at -20°C. To generate the standard curve, 2μl of the stock (187.5 units/μl) was added to 198μl of

PBS/10% FCS to give a working solution of 1.875 units/μl. This was diluted as set out below:-

		picograms of IL-2/well
1:25	40μl working stock plus 960μl PBS/FCS	200.00
1:50	500μl 1:25 plus 500μl PBS/FCS	100.00
1:100	500μl 1:50 plus 500μl PBS/FCS	50.00
1:150	500μl 1:100 plus 250μl PBS/FCS	33.75
1:200	350μl 1:150 plus 115μl PBS/FCS	25.00

Further dilutions were made as required up to 1:800. Fresh dilutions were prepared from the stock (187.5units/μl) for each experiment.

LB agar

Agar (15g) was added to 1 litre of LB medium before autoclaving.

LB medium	*final concentration*
10g bactotryptone	1% (w/v)
5g bactoyeast	0.5% (w/v)
10g sodium chloride	1% (w/v)

The chemicals were dissolved in 1 litre of water and the pH adjusted to 7.0 with sodium hydroxide before autoclaving.

LB-agar plates

Sterile L-agar was melted and allowed to cool to 55°C before ampicillin (50μg/ml) was added. The agar was poured into 9cm bacterial petri dishes under sterile conditions and allowed to set before the plates were inverted and placed at 37°C for 30 minutes to dry. Plates were stored at 4°C until required.

LB plates containing X-gal for blue/white colony selection

A stock of X-gal was prepared by dissolving in dimethyl formamide to a concentration of 25mg/ml. This was stored in the dark at -20°C in aliquots in glass or polypropylene tubes. Isopropyl β-D-thiogalactopyranoside (IPTG) (60mg) was dissolved in 2.5mls of water to make a 0.1M solution.

An aliquot of 2mls of X-Gal (25mg/ml) and 2.5mls of IPTG (0.1M) was added to 500mls of L-agar, containing ampicillin.

Loading buffer I	*final concentration*
1ml 0.5M EDTA	0.1M
2.5g sucrose	50% (w/v)
few grains bromophenol blue	approx. 0.2% (w/v)

The components were dissolved in 5ml of water, sterilised through a 0.22μm filter and stored at 4°C.

Loading buffer II

100μl deionised formamide
30μl formaldehyde
20μl 10x MOPS
0.9μl ethidium bromide

The components were combined and 3 x the sample volume added to each sample before loading.

Loading buffer III	*final concentration*
2.5ml 1M Tris-HCl	0.1M
4ml glycerol	40%(v/v)
20mg bromophenol blue	0.2%
20mg xylene cyanol	0.2%

The components were combined and the final volume adjusted to 10ml with water. The solution was sterilised by passing through a 0.22μm filter.

Loading buffer IV	*final concentration*
9.5ml formamide	95%
9mg bromophenol blue	0.09%
9 mg xylene cyanol	0.09%

The components were combined and the volume adjusted to 10ml with water. The solution was sterilised by passing through a 0.22μm filter.

MES buffer	*final concentration*
2.172g MES	50mM
0.228g EGTA	3mM
0.203g $MgCl_2$	5mM
1ml Triton-X-100	0.5%

The first three components were dissolved in 200ml water and the pH adjusted to 6.0 with sodium hydroxide. The Triton-X-100 was added and the solution sterilised through a 0.22μm filter.

MOPS buffer (10 x)	*final concentration*
20.93g MOPS	0.2M
20.51g sodium acetate	0.5M
1.86g EDTA	10mM

The components were dissolved in 500ml of RNase free water in a bottle that had been treated with DEPC and autoclaved. The pH was adjusted to 7.0 with sodium hydroxide and stored in the dark at 4°C.

Paraformaldehyde fixative

Using a fume hood 8g of paraformaldehyde was weighed out and dissolved in 150ml of 0.1M sodium phosphate buffer, pH 7.3. To this 10M sodium hydroxide was added at a rate of 1 drop per minute until the solution cleared. The volume was adjusted to 200ml with sodium phosphate buffer. To prepare the working solution, 50ml of 4% paraformaldehyde was added to 49.2ml of 0.1M sodium phosphate buffer, pH 7.3 and 0.8mls of 25% glutaraldehyde.

Phalloidin stock solution

Phalloidin is extremely hazardous and should be handled with caution. Working in a fume hood at all times, the stock chemical (0.1mg) was dissolved in 1.5ml of methanol by injecting the solvent into the bottle. The stock was stored at -20°C. A working solution was made by diluting 10μl of the stock in 1ml of PBS.

Phosphate buffered saline (PBS)

One PBS tablet (Oxoid) was dissolved in 100ml of distilled water and autoclaved.

Poly-L-lysine

The stock solution was made up in PBS to a concentration of 1mg/ml and stored at -20°C. This was diluted to 0.1mg/ml in PBS and used to coat coverslips by incubating the sterile coverslips in this solution for 30 minutes. The coverslips were rinsed in PBS before use.

Potassium ferricyanide/ferrocyanide

A 50mM solution of each chemical was prepared in autoclaved bottles with sterile water and stored at 4°C in the dark where it is stable for at least 3 months. These solutions should not be autoclaved.

Prehybridisation buffer	*final concentration*
5ml deionised formamide	50%(v/v)
1ml 50 x Denhardts	5 x
3ml 20 X SSC	6 x
1M sodium phosphate buffer, pH6.8	100mM
100µl sonicated salmon sperm DNA	100µg/ml

The components were combined and the volume adjusted to 10 mls with water.

Rnase A stock

A stock of 5mg/ml was prepared in water and stored at -20°C. The stock was diluted to 1mg/ml in 10mM Tris HCl, pH 7.5, 15mM NaCl before use.

RPMI 1640 medium

One litre of RPMI 1640 medium was supplemented with 2mM Glutamax I, 15mM hepes and 2mM sodium pyruvate. Once CTLL/2 cells were resuspended in the medium, IL-2 (20 units/ml) was added, to promote cell growth.

Sodium phosphate buffer (0.1M), pH 7.3

Solutions of 1M disodium hydrogen phosphate and sodium dihydrogen phosphate were prepared.

	final concentration
11.55ml of 1M disodium hydrogen phosphate	77mM
3.45ml of 1M sodium dihydrogen phosphate	23mM

To prepare the buffer the above volumes of each 1M solution were combined and the volume adjusted to 150mls with water. The pH was adjusted to 7.3 if necessary.

Solution D	*final concentration*
250g guanidine thiocyanate	6.25M
sodium citrate pH 7.0	40mM
sarcosyl	0.78%

The components were combined and stored at -20°C. Prior to use 36µl of β-mercaptoethanol was added to 5 ml of solution D.

Sonicated salmon sperm DNA

The DNA was prepared in water to give a final concentration of 10mg/ml. The solution was allowed to stand until the DNA had dissolved and once in solution was sheared using a sonicator (Branson Sonifier 250). Ten to twenty pulses were used until a change in the viscosity could be seen. Aliquots were stored at -20°C.

SSC buffer (20 x)	*final concentration*
175.3g sodium chloride	3M
88.2g sodium citrate	0.3M

The chemicals were dissolved in 800ml of water, the pH adjusted to 7.2 using HCl and the volume adjusted to 1000ml before autoclaving. Dilutions were made in water.

TAE buffer (50 x)	*final concentration*
242g Tris base	2M
57.1ml glacial acetic acid	5.71% (v/v)
100ml 0.5M EDTA (pH 8.0)	50mM

The components were dissolved in 1 litre of water and autoclaved. Working solutions were diluted in water.

TBE buffer (5 x)	*final concentration*
54g Tris base	0.45M
27.5g boric acid	0.45M
20ml 0.5M EDTA (pH 8.0)	0.01M

The chemicals were dissolved in water, the volume adjusted to 1 litre and the solution autoclaved. Working solutions were diluted in water.

Trypsin/EDTA Solution (10 x)	*final concentration*
5g Trypsin	0.05% (w/v)
2g EDTA	5.4mM
8.5g NaCl	145mM

The chemicals were dissolved in 1 litre of sterile phosphate buffered saline (PBS), aliquoted and stored at -20°C. A working solution of 1 x Trypsin/EDTA (TE) was prepared using PBS.

X-Gal Solution

X-Gal was dissolved in N,N-dimethyl formamide to the required concentration and stored in a glass container in the dark at -20°C.

METHODS

2.3. Maintenance of cell lines

2.3.1. Routine cell maintenance

Cell culture was carried out in a class II laminar flow cabinet under aseptic conditions. Cells were maintained in a Sanyo incubator at 37°C in 10% CO_2. The adherent B16.F1 mouse melanoma cell line (Fidler, 1975) and mouse colorectal cell line, CMT93 (Franks and Hemmings, 1978), were obtained from Dr. R. Vile (ICRF, Royal

Postgraduate Medical School, London). The non-adherent mouse CTLL/2 cytotoxic T lymphocyte line obtained from Dr. C. Hewitt (University of Leicester) was also used in this work. The adherent cells were maintained in DMEM and the T cell line was maintained in RPMI 1640. After revival from liquid nitrogen, cells were maintained through 3 passages, before being used in growth experiments and through one passage, before determination of levels of protein expression. Adherent cells were routinely passaged twice a week. B16 cells were washed with phosphate buffered saline (PBS), released from the substrate using 1 x trypsin/EDTA and seeded at $1x\ 10^5$ cells per $75cm^2$ flask to maintain the cells in logarithmic phase. The colorectal cells were detached using 5 x TE and seeded at 2×10^5 cells per $75cm^2$ flask. All the transfected variants of the adherent cell lines (see Table 3.1.) were maintained in a similar fashion. The T cell line was passaged three times a week and seeded at 5×10^3 cells/ml. Cell counts for the B16 lines were obtained using a Coulter counter (model ZM, settings:current 100, attenuation 32, Tl 14, Tu 99.9, Kd 15.26). All the other cell lines were counted using a haemocytometer. The passage number quoted throughout the text is relative to time of transfection (passage 1), where known. All the cell lines used were tested for mycoplasma contamination and found to be negative (Mycoplasma Experience).

2.3.2. Preparation of a single cell suspension

The medium was removed from a flask of cells growing in log phase and the cells were washed with PBS. The cells were released from the substrate by overlaying them with 1ml of 1 x TE and incubating at 37°C for 3 minutes. Once the cells were detached they were resuspended in fresh medium and transferred to a universal tube. The cells were taken up into a 1 ml Gilson pipette tip 20 times to create a single cell suspension. They were counted and diluted to the appropriate density per ml of medium.

2.3.3. Cell storage in liquid nitrogen

Cells were detached from the substrate using 1 x TE, resuspended in medium and pelleted at 300g, 20°C using a Jouan centrifuge to remove the trypsin. The pellet was resuspended in freezing medium and aliqoted into cryovials. The samples were frozen

slowly overnight at -80°C before being transferred to liquid nitrogen for long term storage.

2.4. Preparation of the empty pBabeNeo vector

2.4.1. Transformation of bacteria

Competent INVαF′ cells contained in the TA cloning® kit from InVitrogen were transformed with the plasmid pBabeNeo/IL-2 (Morgenstern et al, 1990). One aliquot of cells was thawed and 2µl of β-mercaptoethanol added, followed by 25ng or 50ng of the plasmid. The sample was incubated on ice for 30 minutes and then heat shocked for 30 seconds at 42°C, before being incubated for a further 2 minutes at 4°C. To this, 450µl of SOC medium was added and the cells were shaken at 37°C, 250rpm (Gallenkamp orbital shaker) for 1 hour. The bacteria were plated neat and at appropriate dilutions onto LB agar plates containing ampicillin and the plates incubated at 37°C overnight.

2.4.2. Preparation of plasmid and plasmid stocks

Individual colonies were picked using aseptic technique and used to inoculate 10mls of LB medium containing 50µg/ml of ampicillin. These cultures were shaken overnight at 37°C. To make stocks of each clone, 0.85ml of stock was mixed with 0.15 ml of autoclaved glycerol and stored at -80°C. A further 3 mls of the culture was used to prepare plasmid using the Wizard™ plasmid preparation kit from Promega. This kit uses a silica resin to bind plasmid DNA in a bacterial lysate. The resin is isolated on a spin column and DNA retained by the resin is washed, before being eluted in 50µl of water. A 1 in 100 dilution of plasmid was prepared and the optical density of the sample at 260, 280 and 330nm was read (Perkin Elmer Lambda 2). The DNA concentration was then calculated using an $O.D._{260}$ reading of 1 = 50µg/ml.

2.4.3. Gel analysis of DNA

Using the O.D. reading as a guide, 100ng of plasmid was diluted to a final volume of 9µl with water and 1µl of loading buffer I was added. The sample was run on a 1% agarose gel containing 1 x TAE to verify the plasmid was the correct size. The DNA was visualised by staining the gel with ethidium bromide and viewing under UV light.

2.4.4. Large scale restriction digest of plasmid DNA

Plasmid (9μg) was digested in 1 x restriction buffer (supplied with the enzyme) using 13.6 units of *Eco*R1 per μg of plasmid DNA at 37°C for 2 hours. A sample (125ng) of the digest was diluted to a volume of 9μl with water and 1μl of loading buffer I was added. This sample was run on a 1% agarose gel containing 1x TAE to verify that the digest was complete.

2.4.5. Purification of DNA from agarose

The restricted DNA was run on a 1% low melting point agarose gel containing 1x TAE and stained with ethidium bromide. The DNA was viewed under ultraviolet (UV) light and the fragments were excised with a clean razor blade. The DNA was extracted using the Wizard™ PCR purification system from Promega, which uses a resin to bind the DNA from the melted agarose sample and then isolates the resin on a spin column. The sample was washed with 80% isopropanol and then DNA eluted in water, or in the case of a restriction fragment, in TE pH 8.0. An aliquot of 200ng was run on a 1% agarose gel containing 1 x TAE to verify the purification.

2.4.6. Religation of a plasmid

Linear plasmid (50ng) was incubated at 16°C overnight in 1 x buffer (50mM Tris-HCl pH7.5, 10mM $MgCl_2$,10mM dithiothreitol, 1mM ATP, 25μg/ml bovine serum albumin) with 400 units of T4 DNA ligase in a final volume of 10μl. The religated plasmid was transformed into INVαF′ as above, using 5μl of the ligation reaction and glycerol stocks were prepared.

2.5. Preparation of B16 and CMT93 cells containing pBabeNeo

2.5.1. Electroporation

A cell suspension was prepared as described in 2.3.2. and the cells spun down and resuspended in 1ml of serum free medium, such that each ml contained 0.5-1 x 10^7 cells. From this suspension 900μl was placed in a 4mm electroporation cuvette, together with 4.3μg of the plasmid pBabeNeo, and incubated on ice for 10 mins. The cells were resuspended using a gilson pipette and electroporated (Biorad Gene

Pulsar™) at 250V, 950μF, giving a time constant of 17.1 for B16 cells and 13.1 for CMT93 cells. Each cell line was incubated for 5 minutes before being diluted 1 in 50 and 1 in 250 with complete medium and seeded in 24 well plates.

2.5.2. Selection of plasmid-containing cells using G418 sulphate

To select cells that contained pBabeNeo, the aminoglycoside Geneticin (G418) was added to the medium on the day after transfection. This antibiotic inhibits protein synthesis and works more efficiently in dividing cells. It can take several days to work, therefore it is necessary to seed the cells at a low enough density such that they will not have overgrown by the time the antibiotic starts to take effect. To select B16 cells, G418 was added to the growth medium at a concentration of 5mg/ml, while to select CMT93 cells, a concentration of 0.5mg/ml was used. It is important to include a well of non-transfected, control cells in the selection.

2.6. Determination of cell growth characteristics

2.6.1. Growth studies to determine doubling time

A single cell suspension of 2 x 10^4 cells/ml of medium was created from cells growing in log phase and used to seed 35mm diameter wells. Each well contained 1ml of cell suspension plus 2ml of medium. The plates were grown under normal conditions and the medium changed after 2 days. Duplicate wells were counted at each time point using 1ml of TE to detach the cells from the substrate and a further 1ml of medium to resuspend the cells. Cell numbers were then obtained using a Coulter counter for B16 cells, or in the case of the CMT93 cells, using a haemocytometer. To obtain the doubling time of each cell line, the $\log_{10}$ of the cell number was plotted against time. The gradient of the curve was used to estimate the doubling time.

2.6.2. Statistical analysis

Statistical analysis of doubling time data was carried out using an analysis of variance (ANOVA) contained in the MiniTab computer package. The significance of these results was calculated using Fisher's least significant difference (LSD) test according to

the formula $LSD = t_{.05} \times \sqrt{\frac{2s^2}{n}}$

where n = no. of events

s^2 = error mean standard

t at the 5% level was obtained from a t probability table

2.6.3. Tritiated thymidine incorporation

Cells were seeded in triplicate 35mm wells at a density of 2×10^4 cells/well from a suspension prepared from a culture in log phase. At each time point the medium was removed by aspiration and replaced by 1ml of fresh medium containing 5-[methyl ^{3}H]-thymidine (1μCi/ml). The plate was incubated for 1 hour at 37°C, 5% CO_2 (Dale and Gescher et al, 1989). Each well was washed three times with ice-cold PBS and then fixed at 4°C for at least 1 hour, using 1ml of acid fixative. Each well was again washed twice with ice -cold PBS and the cells were lysed using 1ml of SDS (1% w/v). Each sample was added to 10mls of Ultima Gold scintillant before being vortexed and counted (2 x 1 minute) in a scintillation counter (Packard Tri-carb 500).

2.7. Analysis of cell cycle

A single cell suspension of 2×10^6 cells/ml was prepared and a 1ml aliquot spun at 300g, 20°C for 3 minutes and resuspended in 200μl ice-cold PBS. The cells were fixed by adding 2mls of ice-cold 70% ethanol/30% PBS while vortexing. The sample was then kept at 4°C for 30 minutes. After centrifugation at 1075g and 4°C for 10 minutes, the cells were washed in ice-cold PBS, spun as before, and resuspended in 800μl of PBS. A 100μl aliquot each of RNase A stock I (1 mg/ml) and propidium iodide (500μg/ml) was added to the cell suspension and the sample incubated at 37°C for 30 minutes (Ormerod, 1990). The cells were stored at 4°C until analysis by flow cytometry, using an argon-ion laser tuned to 488nm (FACScan, Becton Dickinson).

2.8. Investigation of IL-2 receptor status

2.8.1. Flow cytometric analysis

Cells growing in log phase were spun down at 200g, for 3 mins and resuspended in PBS. The cells were counted and 1 x 10^6 cells aliquoted into a round-bottom tube (Falcon, 12 x 75mm). Each sample was spun again at 200g for 3 mins and the cells were resuspended in 90µl of PBS, to which was added 10µl of FITC-conjugated anti-mouse IL-2 receptor α chain antibody (0.5mg/ml). A mouse IgG antibody (Dako) was also used to control for non specific binding. The samples were incubated in the dark on ice for 1 hour before 1ml of PBS was added. The cells were spun down once more and resuspended in 1 ml PBS and analysed using a FACScan (Becton Dickenson). If cells expressed the receptor on their cell surface they should bind the antibody, increasing their level of fluorescence. The FACScan detects the increased fluorescence and a graph of fluorescence against frequency can be plotted.

2.8.2. Preparation of total RNA

Total RNA was prepared using a modified version of the method developed by Chomczynski et al (1987). Cells growing in culture were detached from the substrate using TE and spun at 300g, 22°C for 5 minutes. The cells were washed with PBS and respun before being homogenised for 2 minutes in solution D, using 1ml per 10^7 cells. Each ml of sample was split in half and to this 33µl sodium acetate, pH 5.2, 500µl aqueous phenol, pH 4.3 and 100µl of chloroform/isoamyl alcohol (ratio 24:1) were added. The sample was mixed well and left on ice for 15 minutes before being spun at (15 300g), 4°C for 15 minutes. The top aqueous layer was transferred to a fresh tube and precipitated with an equal volume of ice-cold isopropanol for 15 minutes at -20°C. The sample was spun again at 15 300g , 4°C for 15 minutes and the resulting pellet resuspended in 100µl each of solution D and cold isopropanol. The sample was again mixed and incubated at -20°C for 15 minutes before being spun as before. The supernatant was removed and the pellet resuspended in 800µl of 75% ethanol/RNase free water and incubated at -20°C for 15 minutes or longer before being spun for a final time as above. The ethanol was removed and the pellet air dried for 5-10 minutes at room temperature, before being resuspended in 50µl of RNase free water. If necessary

the pellet was heated to 65°C in a water bath for 10 minutes to aid resuspension. The total RNA was then stored at -80°C.

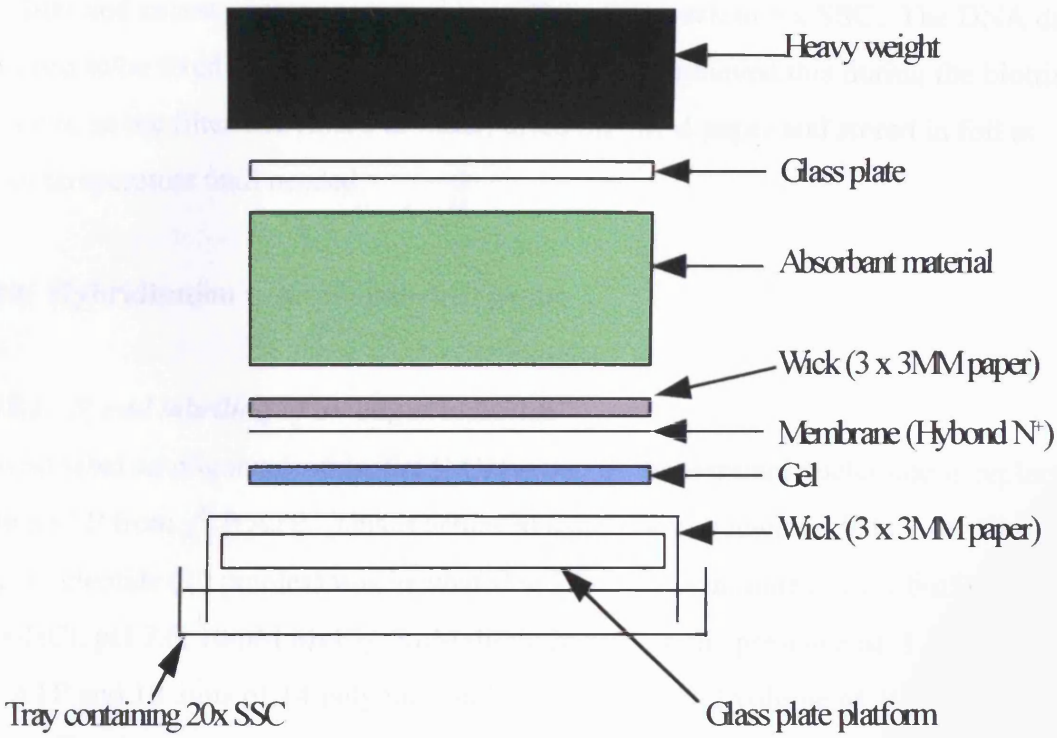
2.8.3. Continuous RT-PCR

This method was carried out according to Mallet et al (1995). A 1μg aliquot of total RNA was reverse transcribed and then amplified in the same reaction tube in the presence of 310nM of 5′ and 3′ primers, 250mM dNTPs, 10 units of AMV-RT, 2.5 units AmpliTaq DNA polymerase,10mM Tris-HCl pH 8.3, 50mM KCl and 0.01% gelatin in a final volume of 100μl. A Biometra thermal cycler was used with the cycling conditions of 10 minutes at 65°C for RNA denaturation and primer hybridization, 8 minutes at 50°C for reverse transcription, 5 minutes at 95°C to denature nucleic acid and inactivate the reverse transcriptase, followed by 35 cycles of 1 min at 95°C, 2 minutes at 55°C and 2 minutes at 72°C. A final extension of 7 minutes at 72°C was carried out and the reactions were then cooled to 4°C. An internal control reaction was run in parallel to each primer set using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is a house keeping gene which should be expressed at very similar levels in each cell type (Fort et al, 1985). The signal obtained from each sample can be used to control for different amounts of RNA being placed in each reaction, due to errors in spectrophotometer readings and sampling error.

2.9. Agarose electrophoresis and Southern blotting of PCR products

A sample of 10μl of each of the RT-PCR reactions was mixed with 1μl of loading buffer I and electrophoresed at 70V on a 1% agarose gel, prepared with 1 x TAE. The gel was stained with ethidium bromide and photographed under UV light before being blotted onto Hybond N+ according to the method described by Southern (1975). The gel was not pretreated in any way, as the small size of the PCR products made it unnecessary. The DNA was transferred to Hybond N+ by capillary blotting overnight, using 0.4M NaOH as the transfer buffer (Figure 2.1.). Three layers of 3MM paper acted as the wick. The gel was placed on the wick with wells facing down and the membrane placed on top of the gel with care being taken to remove all the air bubbles. Once in place, the membrane cannot be moved as nucleic acid will begin to be

Figure 2.1. Capillary blot apparatus



Capillary blot apparatus used for Southern and northern blot analysis

transferred immediately. Clingfilm was laid along the edges of the gel to avoid buffer moving through the wicks and bypassing the gel. The rest of the apparatus was placed on top of the membrane and the gel left to blot for 18 hours. The wells were marked on the filter and excess agarose removed by a 30 second wash in 5 x SSC.. The DNA did not need to be fixed on the filter as the transfer buffer achieved this during the blotting process, so the filter was rinsed in water, dried on 3MM paper and stored in foil at room temperature until needed.

2.10. Hybridisation to a radiolabelled probe

2.10.1. 5' end labelling of an oligonucleotide

To end label an oligonucleotide, the 5' OH group of the terminal nucleotide is replaced with a γ^{32}P from γ^{32} P ATP. This is achieved using T4 polynucleotide kinase. The oligonucleotide (10 pmoles) was incubated at 37°C for 10 minutes in 1 x buffer (70mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 5mM dithiothreitol in the presence of 1.11MBq of ^{32}P ATP and 10 units of T4 polynucleotide kinase in a final volume of 10µl.

2.10.2. Purification of a radiolabelled oligonucleotide

The labelled oligonucleotide was diluted by the addition of 170µl of water and denatured by heating to 95°C for 2 minutes, before being placed in ice. A Nap-5 column was prepared by running 2 volumes of water through it. The sample was applied to the column and the eluate collected in an eppendorf tube. A further 9 fractions were collected in fresh tubes by the addition of 180µl of water for each fraction. The fractions were counted for 1 minute using a bench count (Scotlab Easicount 2000) to obtain the dpm value for each fraction. A characteristic graph should be obtained with the count values rising sharply at approximately fraction 5 and then decreasing again. This peak represents the labelled oligonucleotide. When the counts start to increase again towards the later fractions, this indicates the unlabelled nucleotide is eluting from the column.

2.10.3. Hybridisation using Amersham Rapid Hybridisation buffer

The end-labelled oligonucleotide was hybridised to the blot using Rapid-hyb buffer (Amersham). This buffer contains all the necessary components for the hybridization reaction, requiring only addition of the oligonucleotide. Although the protocol states that ^{32}P labelled oligos do not need to be purified to remove unlabelled nucleotides this procedure was carried out. The protocol was followed exactly using prehybridisation times in excess of 1 hour and hybridisation times of 2 or more hours. All the hybridisations were carried out at 42°C. The probe was added to the buffer to a specific activity of 10^6dpm/ml. The blots were washed in 2 x SSC, 0.1% SDS, 42°C for 30 minutes and then further if necessary with solutions containing 1 x SSC and then 0.1 x SSC. The concentration of SDS remained constant. The blot was wrapped in Saran wrap and exposed to Hyperfilm™ MP film overnight at -80°C. The film was developed using an automatic developer (X-graph Compact X2).

2.11. Substrate adherence

2.11.1. Morphology

Cells were seeded at 2 x 10^4 in 6 well plates. The cells were photographed on an invert microscope (Zeiss, Axiovert 135) at a magnification of x10, x20 and x32, using phase contrast to compare the morphology of the cells.

2.11.2. Rate of attachment

Cells were seeded at 4 x 10^4 cells per well in 35mm well plates in duplicate and grown under normal conditions. At specific time points the medium was removed from each well and saved separately. The cells were washed with 1ml of PBS and this was added to the saved medium. The cells which had remained attached were then detached using 1x TE and counts were obtained using a Coulter counter. The number of cells in the medium was counted in the same way and related to the volume of medium so a final cell number could be obtained.

2.12. Histochemical investigation of the cell cytoskeleton

Cells were seeded in 9 cm plates containing poly-L-lysine coated glass cover slips and grown under normal conditions for 2 days until 80% confluent. The cells were washed twice with PBS (37°C) and permeabilised by incubation with ice-cold filter sterilized MES buffer for no more than 2 minutes. The cells were washed twice with PBS (37°C) and fixed using 3.7% formaldehyde in PBS for 15 minutes at room temperature. Two more washes were carried out using PBS (37°C) and then all the liquid removed. The cells on the coverslips were incubated with 100µl of phalloidin solution (0.67ng/µl in PBS) for 20 minutes. Cells were washed twice with PBS and the coverslips mounted using Fluoromount mountant. The labelled cells were viewed using confocal microscopy (Leica TCS4D, excitation 488nm, emission 560nm ± 30nm).

2.13. Analysis of transgene protein expression

2.13.1. ELISA assay for IL-2

On day one a 75cm^2 flask was seeded at 2 x 10^5 per flask or an equivalent density in a smaller vessel so that the culture was approximately 80% confluent on day 4. Ten mls of medium was added to the flask. On day 4 a sample of the medium was removed and filtered through a 0.22µm filter and used in the ELISA assay. The total volume of medium and total cell number was also noted. Anti-IL-2 antibody was diluted to1µg/ml with coating buffer and 50µl added to each well of a maxisorp ELISA plate (Nunc). The plate was covered and incubated at 4°C overnight. The plate was washed twice with PBS/Tween 20. This was achieved by filling each well with the wash and then shaking the wash out of the wells. The first wash was for 1 minute and any further washes were shorter. After the final wash the plate was pounded on paper towels to remove all liquid and any bubbles. The plate was blocked with 200µl of PBS/10% FCS per well for 2 hours and this and all future incubations were carried out on a plate shaker at room temperature. The plate was washed as before and 100µl per well of each of the standards and samples was loaded. The IL-2 standards were prepared from a frozen stock, such that a dilution series of 200, 100, 50, 33.5, 25, 12.5 and 6.25 pg/well was achieved. Duplicates of each standard and sample were used. The plate was incubated for 3 hours before washing 3 x as before. Biotinylated anti IL-2

antibody was diluted to 2μg/ml in PBS/10% FCS and 100μl added to each well. Incubation was carried out for 45 minutes, before the plate was washed 4 times. Avidin peroxidase was diluted to 1mg/ml with PBS/10%FCS, before adding 100μl per well, and the plate was incubated for 30 minutes followed by 5 washes. An 11ml aliquot of 2,2 ABTS substrate was thawed and 11μl of hydrogen peroxide (30%) added and mixed thoroughly. Immediately, 100μl was added to each well. It is very important to be as quick and as accurate as possible, because the final reading is dependent on the volume of substrate added and the time of incubation. The colour was allowed to develop for 20-30 minutes before the plate was read at 405nm using a microtitre plate reader (Labsystems iEMS Reader MF).

2.13.2. Histochemical detection of β-galactosidase

Detection of β-galactosidase was carried out using the histochemical assay described by Macregor *et al* (1991). Cells were seeded at 2 x 10^4 and grown under normal conditions for 3 days. The medium was removed from the cells and the monolayers washed twice with PBS before being fixed at 4°C for 5 minutes with 2% paraformaldehyde/0.2% glutaraldehyde. The cells were washed twice with room temperature PBS and overlaid with the filtered substrate. The plate was placed in an incubator at 37°C overnight to develop the stain and viewed under an invert Zeiss microscope with a camera attachment.

2.13.3. Treatment of cells with ganciclovir

Cells were seeded in 35mm well plates. The following day GCV was added and this was replenished every 2 days when the medium was changed. This drug is converted into a nucleotide analogue by HSV-*tk* and is incorporated into the DNA of replicating cells. The analogue blocks replication and the cells die. The stock solution (1mg/ml) was diluted such that 1μl of GCV solution was added per ml of medium in the well. Concentrations used were in the range of 0.1 - 1.0μg/ml.

2.14. Northern blot analysis

Northern blotting involves separation of RNA molecules by electrophoresis through a formaldehyde/agarose gel and transfer of that RNA onto a membrane which is then

probed with a specific probe. The procedure enables the molecular size of a species to be determined and levels of specific RNA within different cell types to be compared.

2.14.1. Preparation of total RNA from cells

Total RNA was prepared from cells using the commercially available TRIzol™ kit. The reagent in this kit is a one phase mixture of phenol and guanidine isothiocyanate and when the cells are lysed in the buffer the RNA remains intact, while cell components are degraded. Cells were seeded in 35mm well plates and, when 80% confluent, the procedure was carried out using 1ml of reagent per well. After lysis a chloroform extraction was performed leaving the RNA in the aqueous phase, the DNA in the interphase and the protein in the organic phase. The aqueous phase was removed to a fresh tube, the RNA precipitated with isopropanol and the resulting pellet washed with 75% ethanol before being air-dried and resuspended in 50µl of RNase free water. The optical density was read at 260nm and the concentration of RNA calculated using an O.D. reading of 1 = 40mg/ml. A sample of 1µg was run on a 1% agarose gel containing 1 x TAE to check that the concentrations were correct.

2.14.2. Preparation of mRNA - method 1

Total RNA was diluted to a final volume of 500µl in RNase free water. A plastic column was loaded with 75mgs of d(T) cellulose and 5mls of 0.1M sodium hydroxide was passed through, followed by 10 mls of water and 5mls of 1 x column wash. The RNA sample was heated to 65°C and diluted with an equal volume of 2 x column wash and cooled on ice for 5 minutes before being applied to the column. The eluate was collected and heated to 65°C for 5 minutes, cooled on ice as before and re-applied to the column. The column was washed with 10mls of 2 x column wash and the RNA then eluted directly into a glass centrifuge tube using 3.5mls of elution buffer. The poly A^+ RNA was precipitated by the addition of 350µl of 3M sodium acetate (pH5.2) and 8.75mls of 100% ethanol and incubated at -20°C for 1 hour or more. The sample was spun at 15 800g at 4°C for 30 minutes. The majority of the ethanol was removed and the pellet resuspended in the remaining 1ml of ethanol, with care being taken to wash the sides of the tube. The sample was transferred to an eppendorf and spun at 15 300g

for 15 minutes at room temperature. The pellet was air dried and resuspended in 10-50µl of RNase free water.

2.14.3. Preparation of mRNA - method II

Due to difficulties in obtaining a sufficient quantity of sample with the previous method, mRNA was prepared using a maxi message maker kit (R and D Systems). The kit allows direct isolation of mRNA from cells, without initial preparation of total RNA and employs a lysis step followed by selection of the mRNA using oligo dT-latex beads. Cells (3×10^7) were used per column. Samples were sheared by passing through a 19 g needle 10 times and all spins were carried out in corex tubes. Spin times were extended by 10 minutes to aid separation of the homogenate from the supernatant. Samples were eluted from the column using 2 x 100µl of water heated to 70°C.

2.14.4. Agarose electrophoresis

All glassware and gel apparatus were treated with DEPC, to remove RNases, and rinsed with RNase free water. An 11 x 14cm formaldehyde/MOPS agarose gel was prepared and allowed to set for 2 hours. Three volumes of loading buffer II were added to each of the samples, which were heated to 65°C for 5 minutes, before transfer to ice to denature any secondary structure. The samples were separated overnight (approx. 16 hours), by electrophoresis, on the prepared gel in 1 x MOPS buffer at 21V. The buffer was recirculated to avoid depletion of the buffering capability. The gel was photographed under UV light and rinsed in 2 changes of RNase free water to remove some of the formaldehyde, which can decrease the efficiency of the probe binding.

2.14.5. Capillary blotting

The gel was capillary blotted overnight onto Hybond N+ as in section 2.3.9., (Figure 2.1.), using 20 x SSC as the transfer buffer. After marking the position of the wells, the filter was washed for 30 seconds in 5 x SSC to remove any agarose and baked at 80°C for 1.5 hours. This fixes the RNA and removes formaldehyde from the blot which could prevent the probe binding.

2.14.6. Preparation of the IL-2 probe

The probe was prepared by PCR using pBabeNeo/IL-2 as the template. This (100ng) was amplified in the presence of 10pmoles of 5′ primer (muril 4 - AGC TCG CAT CCT GTG TCA C) and 10pmoles of 3′ primer (muril5 - GCC TTA TGT GTT GTA AGC AGG or muril 6 - GCT CCT GTA GGT CCA TCA AC), 0.2mM dNTP's, 2.5 units Amplitaq DNA polymerase and 1 x buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin) by cycling at 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes with a 2 second increase in incubation time with every cycle for 30 cycles. A final extension of 72°C was carried out before the reactions were brought to 4°C. The short product was sequenced using a Sequenase® kit to confirm it was correct, while the long product was run on an agarose gel, blotted and probed with an internal oligonucleotide (muril 3 - CAC TCA AAT GTG TTG TCA GAG C) to confirm it was the IL-2 product (2.9., 2.10.). In order to ascertain that loading of the samples was even, blots were probed with a GAPDH specific probe. GAPDH is a house keeping gene which should be expressed at similar levels in all the cell types used. The probe was a 1039bp fragment (gift from Dr.T Gant), prepared by PCR using two GAPDH specific primers (forward-TCT CTG CTC CTC CCT GTT CTA, reverse-GTC CAC CAC CCT GTT CCT GTA) to amplify the region from a plasmid containing the full length cDNA (Fort et al, 1985).

2.14.7. Labelling of the Il-2 probe

The Amersham Megaprime kit was used to label long DNA probes by random priming, using ^{32}P α-dCTP. Initially, 100ng of the DNA was diluted to a final volume of 28µl with water and heated to 95°C for 2 minutes, before being placed on ice. The buffer, random hexamer primers and Klenow polymerase were added according to the protocol followed by 5µl of ^{32}P α-dCTP. The reaction was incubated at 37°C for 30 minutes. The volume was then made up to 180µl with water and the probe was heated to 95°C for 2 minutes to terminate the reaction and denature the probe and placed on ice. The probe was separated from the unincorporated nucleotide using a Nap-5 column and the hottest one or two fractions taken to be used as the probe. The probe was denatured again before use.

2.14.8. Hybridisation

The filter was prehybridised with hybridisation buffer at 45°C for 5 hours. This blocked non-specific binding of the probe to the filter. This buffer was then removed and a fresh sample of buffer containing 10% dextran sulphate added. To this the radiolabelled probe was added to a specific activity of 10^6 dpm/ml of buffer and the filter hybridised at 45°C for 20 hours. The buffer was removed and the filter put through a series of increasingly stringent washes, starting with 2 x SSC, 0.1 % SDS and followed by 1 x SSC, 0.1%SDS and ending with 0.1 x SSC, 0.1%SDS. Each wash was carried out for 20 minutes at 42°C, but these times and temperatures were increased as necessary. In general, the first two washes were carried out and then the filter wrapped in Saran wrap and set up for autoradiography using Hyperfilm™ MP (Amersham). An overnight exposure at -80°C was normal. Once the film had been developed, further washes were carried out as needed and the filter was re-exposed.

2.15. Analysis of plasmid copy number

2.15.1. Preparation of genomic DNA

Genomic DNA was prepared using a kit from Qiagen. It uses an optimised buffer system for lysis of the cells and nuclei followed by selection of the genomic DNA with a resin without the need to use phenol/chloroform. A flask ($75cm^2$) of cells was used when it had reached 80% confluency. The cells were trypsinised away from the substrate as before using 1 x TE. Fewer cells than the recommended maximum were used to prevent the column from clogging. The cells were washed according to the protocol and the resulting pellet resuspended in 2mls of PBS. The protocol was followed, with the nuclei being resuspended by vortexing for the maximum 30 seconds (otherwise the cells clump quite badly) and using an incubation time for the protease step of at least 1 hour. Once the DNA had been eluted from the column, isopropanol was added and the tube inverted ten times to precipitate the DNA. The sample was spun at 5000g at 4°C for 15 mins before the pellet was washed with 70% ethanol, a more volatile alcohol, to make it easier to dissolve in 200µl of TE pH 8.0. The sample was dissolved by shaking at room temperature overnight before being stored at 4°C. To determine the concentration of the DNA, a dilution of 1 in 100 in water was prepared

and the O.D. read at 260nm, 280nm and 320nm read using a Perkin Elmer lamda 2 spectrophotometer. To check the readings, 1μg of each sample was run on a 0.8% agarose 1 x TAE gel and stained with ethidium bromide.

2.15.2. Detection of plasmid using PCR

An aliquot of 200ng of genomic DNA was amplified in a PCR reaction containing 10pmoles of a 5′ (vec 1 - TAC GGC TAC ACT AGA AGG AC) and 3′ (vec 2 - GCT GGC TGG TTT ATT GCT GAT A) primer, 1 x buffer (20mM Tris-HCl pH 8.4, 50mM KCl), 0.2 mM dNTP, 1.5mM $MgCl_2$ and 1 unit of Taq DNA polymerase in a final volume of 100μl. The reaction was overlaid with mineral oil and cycles carried out at 94°C forl min, 55°C for 1 min and 72°C for 2 mins with an increase of 2 seconds every cycle, for 30 cycles followed by a final extension at 72°C for 7 mins. before being stored at 4°C. A 10 μl sample of each reaction was run on a 1.5% agarose gel in 1 x TAE at 50 V.

2.15.3. Restriction digest analysis of genomic DNA

A sample of 10μg of genomic DNA was placed in an eppendorf and diluted in the appropriate buffer (1 x) to a final volume of 300μl. The sample was stirred to disperse the DNA and left on ice for 3 hours. After this time 2.5 units of restriction enzyme per μg of DNA was added, the sample stirred and brought up to room temperature before transfer to a 37°C water bath. After 30 minutes a second aliquot of enzyme was added, the sample stirred and left to digest at 37°C overnight. Digests were then stored at 4°C. To concentrate the digested DNA, it was precipitated by adding 0.3 volumes of 3M sodium acetate (pH 5.2) and 2.75 volumes of 100% ethanol. The sample was mixed by inverting several times and placed on dry ice for 1 hour before being spun at 15 800g at room temperature for 15 minutes. The ethanol was removed, the pellet air dried and resuspended in 20μl TE pH8.

2.15.4. Agarose electrophoresis and Southern blotting of genomic digests

The samples were prepared for analysis by electrophoresis by the addition of 2μl of gel loading buffer I to the 20μl sample. The digests were run on a 0.8% agarose gel containing 1 x TBE gel and ethidium bromide (0.2mg/ml) and run overnight at 21 volts

with the buffer recirculating to prevent ion depletion. The gel was photographed under UV light and then prepared for blotting. The DNA was denatured by soaking the gel in 0.25M HCl for 7 minutes, followed by depurination by soaking in 0.5M NaOH, 1.5M NaCl for 30 minutes. The gel was neutralised by treatment in 0.5M Tris.HCl pH7.5, 3M NaCl for 30 minutes. A water rinse was performed between each treatment and after the final incubation. The gel was Southern blotted (Southern, 1975) onto Hybond N+ overnight using 20 x SSC as a transfer buffer (Figure 2.1., section 2.14.5.). After transfer the well positions were marked on the filter before it was rinsed for 30 seconds in 5 x SSC to remove any agarose. The DNA was fixed on the filter by treatment with 0.4M NaOH for 7 minutes before being dried. The filters were then ready for hybridisation to radiolabelled probes (section 2.14.6- 2.14.8.).

2.16. Differential display RT-PCR (DDRT-PCR)

2.16.1. Reverse transcription

A sample of 1μg of total RNA was reverse transcribed in 1 x buffer (25mM Tris-HCl pH 8.3, 37.5mM KCl, 1.5mM $MgCl_2$), dNTP, 2.5μM $dT_{11}Mg$ primer and 10mM dithiothreitol in a final volume of 20μl (Bauer et al, 1994, Liang et al, 1992). The samples were heated to 60°C for 10 minutes, followed by a 37°C incubation for 1 hour. After 10 minutes, 200 units Superscript II reverse transcriptase were added to the tube. After a further hour of incubation the samples were heated to 95°C to inactivate the enzyme and then the temperature brought to 4°C, before storing the samples at -20°C until required.

2.16.2. PCR

A 2μl sample from the reverse transcription step was used in each PCR reaction. The DNA in the sample was amplified in 1 x buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin) in the presence of 1μM $dT_{11}MG$, 0.5μM 5′ primer, 2μM dNTP, 0.037 MBq $\alpha^{33}P$ or 0.37 MBq dATP $\alpha^{35}S$ dATP, 1 unit of AmpliTaq DNA polymerase in a final volume of 20μl. The samples were cycled at 94°C for 30 seconds, 40°C for 2 minutes and 72°C for 30 seconds, followed by a final extension of 72°C for 7 minutes. The samples were brought to 4°C and stored at -20°C.

2.16.3. Polyacrylamide gel electrophoresis (PAGE)

A 6% 31cm x 38.5cm non-denaturing acrylamide (19:1 acrylamide:bis acrylamide) gel was prepared and allowed to polymerise at room temperature for at least two hours before use. A 3.5µl aliquot of each sample was mixed with 2µl of loading buffer III and 5µl of this was loaded onto the gel. The gel was run at 20W, until the bromophenol blue dye had reached the bottom of the gel. The gel was transferred to 3MM paper and dried at 80°C using a vacuum gel dryer (Hoefer DrygelSr). The dried gel was set up for autoradiography overnight using Kodak film. The gel was taped to the film and holes punched with a needle through the paper and film, so that the gel could be re-aligned with the film after it had been developed. Some of the samples were run on denaturing gels which were prepared in a similar fashion. The samples were loaded using sample buffer IV. The gels were run at 50 Watts. Contrary to usual procedure the gel was not fixed before drying, as this has been reported to inhibit reamplification of the samples.

2.16.4. Isolation of differentially expressed cDNA species from the gel

When an RNA species was differentially expressed, the autoradiograph was aligned with the dried gel using the pin holes. A new razor blade was used to cut through the film and paper to excise the differentially expressed band. The gel slice was placed in an eppendorf tube and 100µl of water added. The slice was incubated at room temperature for 10 minutes, followed by boiling for 15 minutes. The tube was spun at 15 800g for 5 minutes to pellet the debris and the supernatant transferred to a fresh tube. The sample was precipitated by the addition of 10µl of 3M sodium acetate (pH 5.2), 5µl of glycogen (5mg/ml) and 400µl of 100% ethanol at -80°C for 1 hour and spun at 15 800g for 15 minutes. The precipitated DNA was resuspended in 10µl of water.

2.16.5. Reamplification

The DNA was reamplified to prepare a sufficient amount for use in cloning. Four µl of the precipitated DNA from the previous step was placed in a tube in 1 x buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$), 2.5µM $dT_{11}MG$, 0.5µM 5′ primer (the same one as was used in the original PCR reaction), 20µM dNTP, 0.05units/µl *Taq* DNA polymerase in a final volume of 20µl. The sample was subjected to 30 cycles at

94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes with a 2 second increase in extension time with every cycle and a final extension of 72°C for 7 minutes before the reaction was brought down to 4°C. The products were viewed by electrophoresis on a 1% agarose gel containing 1 x TAE. Before ligation the insert was extracted in chloroform:isoamyl alcohol (24:1) to remove the residual polymerase activity.

2.16.6. Ligation of PCR products

The PCR products were ligated into a vector to facilitate preparation of more DNA. The pTAg vector, contained in the LigATor kit (R and D Systems), works on the principle that *Taq* DNA polymerase adds a final A base on to the end of any PCR product. The vector therefore contains a T overhang which allows easy directional cloning of the insert into the vector. The additional A base tends to be lost from the PCR product, so it is advisable to carry out the ligation within 24 hours of the PCR reaction. The insert was ligated into the vector using 2µl of the extracted PCR reaction and 50ng of vector in the presence of 1 x ligation buffer (20mM Tris-HCl pH 7.6, 5mM $MgCl_2$), 5mM dithiothreitol, 0.5mM ATP, 200 units of T4 DNA ligase at 16°C overnight.

2.16.7. Transformation

The plasmid containing the insert was transformed into the competent cells contained in the kit as described in section 2.4.1.. A 1µl sample of the ligation was mixed with 20µl of competent cells. The transformed cells were spread on L-agar plates containing ampicillin, IPTG and X-gal. The pTAg plasmid contains the lacZ gene whose transcript is able to convert X-gal into a blue product. The multiple cloning site is situated in this gene so if a piece of DNA has inserted into the plasmid the lacZ gene will be disrupted and the plaques will appear white rather than blue. The plates were incubated overnight at 37°C.

2.16.8. Plasmid preparation

White colonies were picked under sterile conditions and used to inoculate 10mls of L-broth containing ampicillin (50mg/ml). Each culture was grown overnight at 37°C in

an orbital shaker and from this plasmid was prepared and glycerol stocks made as previously described (2.4.2.).

2.16.9. Preparation of probes

The insert was amplified using M13 primers that bound in the pTAG. The plasmid DNA (100ng) was amplified in the presence of 60 pmoles of the M13 (-40) forward (GTA AAA CGA CGG CCA G) and reverse (CAG GAA ACA GCT ATG AC) primers in 1 x buffer (10mM Tris-HCl pH 8.3, 50mM KCL and 0.01% gelatin). 0.2mM dNTP and 1 unit of AmpliTaq DNA polymerase in a final volume of 100µl. The reaction was overlaid with mineral oil and cycled for 30 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes with a 2 second increase in time with every cycle. A final extension at 72°C for 7 minutes was carried out before the reaction was cooled to 4°C. The products were purified using PCR clean up columns (Qiagen) and used as probes as previously described (2.14.7.-2.14.8.).

Chapter Three

Effect of Transfection on Growth Parameters

INTRODUCTION

Studies designed to discover potential toxic effects of transfection *in vivo* have been carried out in animals (Nabel et al, 1992, Stewart et al, 1992, Zabner et al, 1994) and humans (Nabel et al, 1993). They concentrated on acute effects measured by parameters such as changes in levels of serum proteins, immune responses and physical appearance. In addition, work has been published concerning the effect of transfection, in specific cell lines, on *in vitro* and *in vivo* growth, adhesion and properties thought to be associated with metastatic potential (Melani et al, 1995, Ohira et al, 1994, Hathorn et al, 1994). Such observations have usually been made in preliminary studies where the aim of the work is to develop a gene therapy protocol (Cao et al,1995, Gunji et al, 1996, Moody et al,1994, Zhang et al, 1996).

Studying cells *in vitro* allows investigation of more subtle parameters concerned with the molecular regulation of the cell, and this is the approach taken in the work presented in this chapter. The possibility that cellular mechanisms are disrupted by the transfer of DNA is a very important issue for gene therapy which has not, so far, been adequately addressed, with implications for therapies where the protocol requires the transferred DNA to be present for the lifetime of the patient. There is some evidence that expression of a transgene can upregulate expression of other genes that would not normally be active (Han et al,1995) which implies disrupted regulation of the cell. A further point worthy of consideration is that normal tissue might take up the transgene. While protocols aim to target the transgene to defective cells, it is certainly possible that uptake and expression in normal cells could occur, so that a healthy cell might be induced to undergo apoptosis or become transformed. The aim of the work described in this chapter was to investigate possible effects of gene transfer and expression on growth parameters and to determine how generic any observed changes were likely to be. This was achieved by comparing the doubling times of a range of cell types to determine whether the transfection procedure, the transgene or the type of vector affected this parameter.

RESULTS AND DISCUSSION

The B16.F1 mouse melanoma (Fidler, 1975) and the CMT93 mouse colorectal (Franks and Hemmings, 1978) parental cell lines were compared to cells which had been transfected either with an empty vector, that did not contain a coding region (empty control vector), or one that contained a coding region and expressed the transgene (see Table 3.1.). Both a plasmid and retroviral vector system were used in these experiments (Figure 1.5.). The passage numbers quoted for each cell line are relative to the transfection event, the exceptions being marked in Table 3.1. (*). In these cases the passage number was unknown and was designated as passage 1 on receipt of the cells.

Table 3.1. Origin and vector content of each cell line.

Cell Type	Vector	origin
B16	none	single clone
B16 plus pNASSβ	empty plasmid vector	single clone
B16 plus pNASSβ/IL-2	plasmid expressing IL-2	single clone
B16 plus pNASSβ/HSV-*tk*	plasmid expressing HSV-*tk*	single clone
B16 plus pBabeNeo	empty retroviral vector	uncloned
B16 plus pBabeNeo/IL-2*	retroviral vector expressing IL-2	single clone
B16 plus pBabeNeo/IL-2 and pNASSβ/HSV-*tk* (E26)*	retroviral vector expressing IL-2 and plasmid vector expressing HSV-*tk*	single clone
CMT93	none	single clone
CMT93 plus pBabeNeo*	empty retroviral vector	uncloned
CMT93 plus pBabeNeo/IL-2LTR	retroviral vector expressing IL-2	single clone

Three types of control were employed in the growth experiments:-

1. The first consisted of a single clone that contained the empty plasmid vector in the same genomic position in every cell. This enables investigation of the effect of site

specific gene insertion on cell growth. The cells containing empty pNASSβ vector cannot be used to compare the effect of integration with the additional effect of transgene expression in the clone containing pNASSβ/IL-2, since the vector is unlikely to be inserted at the same position in both clones and, therefore, positional effects cannot be ruled out.

2. The second control was used for the retroviral vector and consisted of a pooled population of different clones, each of which had one or more copies of pBabeNeo inserted randomly into the genome. This controls for the effects of random gene insertion on cell growth.
3. The third control was used for the plasmid. It was found that B16 cells containing pNASSβ/IL-2 lost expression of the transgene with time in culture (see chapter 5). At high passage this cell line was identical to the low passage cells, except for the lack of protein expression, allowing the effect of vector insertion on growth to be studied with or without the additional consequences of transgene expression. This type of control was less useful for B16 cells containing pBabeNeo/IL-2, because the levels of expression in these cells were much lower.

3.1. Preparation of transfected cell lines

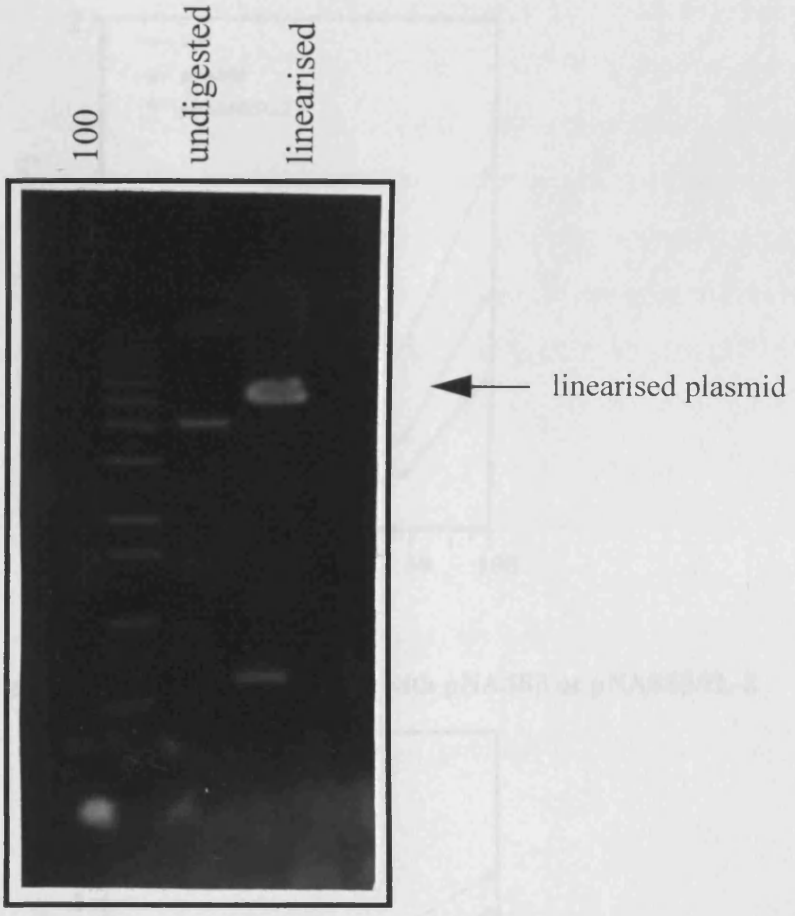
It was necessary to prepare a B16 and CMT93 cell line containing the empty retroviral vector pBabeNeo (Figure 3.1.), to use as controls for the effect of transfection (Sections 2.4., 2.5.).

3.2. Determination of doubling times

3.2.1. Effect of insertion and expression of a transgene

To assess whether transfection or transgene expression had an effect on growth parameters of the cell, doubling times (Section 2.6.) were initially determined for parental B16 cells and cells containing the pNASSβ or pNASSβ/IL-2 vector (Vile and Hart, 1994c). The number of cells was plotted against time (Figure 3.2.A.) to compare the growth of the lines, but the doubling time was calculated by plotting $\log_{10}$ of the cell number against time (Fig. 3.2.B.) The gradient of this graph in the region where

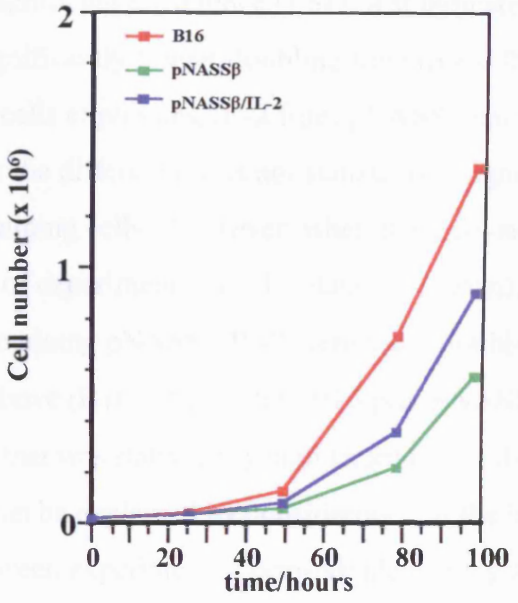
Figure 3.1. **The retroviral vector pBabeNeo/IL-2, digested with *Eco*R1**



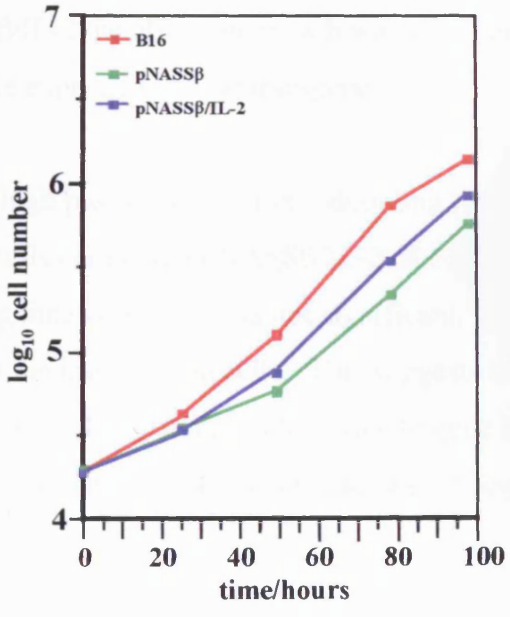
The retroviral vector, pBabeNeo/IL-2, was digested with *Eco*R1 and the fragments separated on a 1% agarose gel. The linearised plasmid was excised from the gel and purified, recircularised and used to prepare the cell lines containing the empty retroviral vector.

Figure 3.2.

A. Growth of B16 cells transfected with pNASSβ or pNASSβ/IL-2



B. Log plot of the growth of B16 cells transfected with pNASSβ or pNASSβ/IL-2



Data were plotted on an arithmetic (A) or log (B) scale. The gradient of graph B in the region where the cells were growing in log phase gave the doubling time of the cells. The doubling times in hours obtained for this set of experiments were B16 = 12.5 ± 1.0, B16 plus pNASSβ = 14.6 ± 2.1 and B16 plus pNASSβ/IL-2 = 13.8 ± 2.3, (n = 4). The cells containing pNASSβ were significantly different from the parental cells ($p < 0.05$, ANOVA and Fisher's LSD test)

the cells were growing in log phase was used to ascertain the doubling time of each cell type. Statistical analysis of these data (n = 4) using analysis of variance (ANOVA) and a Fisher's Least Significant Difference (LSD) test indicated that B16 cells containing pNASSβ had a significantly longer doubling time ($p < 0.05$) than the parental cells (Table 3.2.). The cells expressing IL-2 from pNASSβ showed a slightly slower doubling time, but the difference was not statistically significant compared to control or empty vector containing cells. However, when the analysis was repeated using an increased number of experiments (n = 11, data not shown), where only the parental cells and those containing pNASSβ/IL-2 were used, doubling times almost identical to those referred to above (B16 = 12.4 ± 0.9, B16 plus pNASSβ/IL-2 = 13.6 ± 1.5), did show a difference that was statistically significant ($p < 0.01$). The difference between the two analyses can be explained by considering that the larger set of data showed very little variation between experiments, therefore identifying a difference that was observed, but not significant using the smaller group of data. These data indicate that transfection of B16 cells with pNASSβ can slow the growth rate. In the clone containing pNASSβ/IL-2 the effect on growth was less than in the clone containing the vector alone, despite expression of the transgene.

Experiments using high passage cells gave a doubling time for B16 cells of 12.5 ± 1.7 hours and for B16 cells containing pNASSβ/IL-2 of 13.7 ± 2.7 hours (n = 6). While an increase in doubling time is seen, this is not significant. These figures are very similar to those obtained in the low passage cells. This suggests that the negative effect on doubling time seen in the low passage cells is due to gene insertion rather than IL-2 expression, because the older B16 cells containing pNASSβ/IL-2 no longer express IL-2 (see chapter 5).

3.2.2. Effect of expression of an alternative transgene on growth

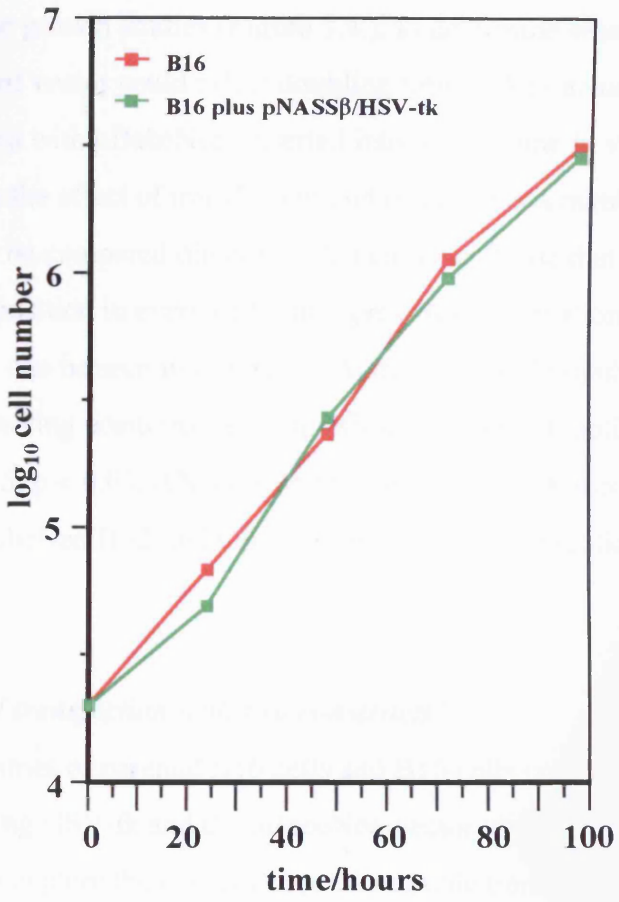
To assess whether expression of an alternative transgene had a similar effect on growth, the doubling time of a B16 cell line expressing HSV-*tk* from the pNASSβ vector (Vile and Hart, 1993b) was compared to the parental B16 cell line (Figure 3.3). The doubling time obtained for B16 cells containing pNASSβ/HSV-tk was not statistically different (ANOVA and Fisher's LSD test) to that obtained for the parental cells (n = 4). In this

Table 3.2. Doubling times of B16.F1 cell lines

	Doubling time (hours)					
Cell type	Section 3.2.1.	3.2.1.	3.2.2.	3.2.3.	3.2.3./3.2.4.	3.2.5.
B16	12.5 ± 1.0		11.5 ± 1.3	14.5 ± 1.5	13.6 ± 0.6	
B16 (high pass.)		12.5 ± 1.7				
B16 plus pNASSβ	14.6 ± 2.1*					
B16 plus pNASSβ/IL-2	13.8 ± 2.3					
B16 plus pNASSβ/IL-2 (high pass.)		13.7 ± 2.7				
B16 plus pNASSβ/HSV-*tk*			11.9 ± 2.2			
pBabeNeo					14.9* ± 1.1	
pBabeNeo/IL-2				14.9 ± 1.9		
pNASSβ/HSV-*tk*, pBabeNeo/IL-2					15.5^{+} ± 0.5	
CMT93						13.5 ± 0.5
CMT93 plus pBabeNeo						16.5 ± 0.9
CMT93 plus pBabeNeo/IL-2LTR						14.7 ± 2.9

The doubling times obtained from five sets of growth curve experiments are shown (n = at least 3). The cell lines with significantly different doubling times from parental cells are marked with * ($p < 0.05$) or + ($p < 0.01$).

Figure 3.3. Growth of B16 cells transfected with pNASSβ/HSV-tk.



Data were plotted on a log scale. The gradient of the curve in the region where the cells were growing in log phase gave the doubling time of the cells. The doubling times in hours obtained from this set of experiments were B16 = 11.5 ± 1.0, B16 plus pNASSβ/HSV-tk = 11.9 ± 2.2, (n = 4). These doubling times were not significantly different from each other (ANOVA and Fisher's LSD test)

case neither transfection, nor the expression of a transgene, caused any change in the doubling time of B16 cells.

3.2.3. Effect of a different vector system

Cells containing the retroviral vector pBabeNeo (Morgenstern and Land, 1990) were also used in the growth studies (Figure 3.4.), to determine whether transfection with a different type of vector could affect doubling times. An uncloned population of B16 cells transfected with pBabeNeo, inserted into the genome in various positions was used to look at the effect of transfection and insertion at a number of random sites. This control cannot be compared directly with a clonal cell line that has the vector inserted into the same position in every cell, but it provides information about the effect of transfection. It can be seen in Figure 3.4.A. that, a mixed population of cells containing the vector at varying positions has a significantly longer doubling time than the parental B16 cells ($n = 5$, $p < 0.05$, ANOVA and Fisher's LSD). A second clonal cell line, containing pBabeNeo/IL-2, did not show any change in doubling time (Figure 3.4.B., $n = 3$)

3.2.4. Effect of transfection with two constructs

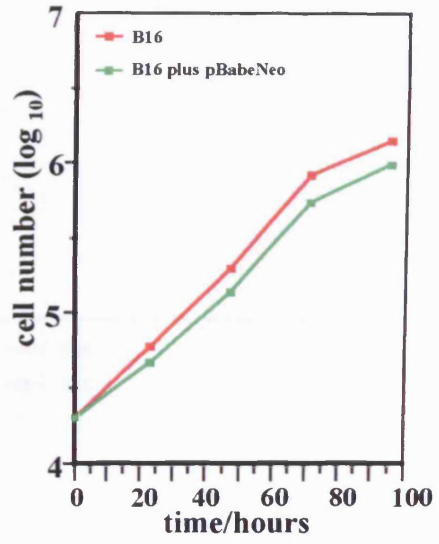
The doubling times of parental B16 cells and B16 cells containing both the pNASSβ vector expressing HSV-tk and the pBabeNeo vector expressing IL-2 were determined (Figure 3.5.) to explore the consequence of a double transfection on the cells. The growth pattern of these cells containing two vectors expressing two different transgenes was affected, with the doubling time being significantly different from that of parental cells ($p < 0.01$, $n = 5$ ANOVA and Fisher's LSD).

3.2.5. Effect of cell type

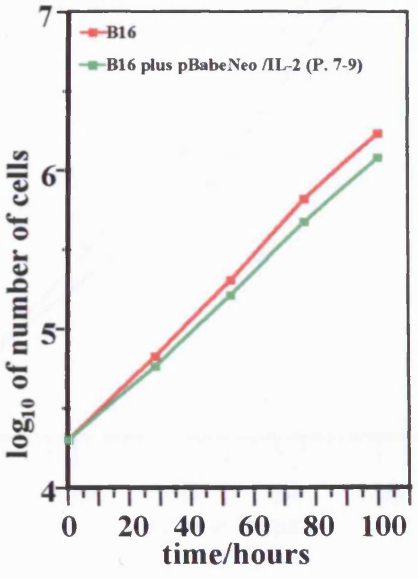
To determine if the results obtained in B16 cells represented similar trends in other cell lines, growth studies were carried out in the murine colorectal cell line CMT93 (Fig 3.6). Results show that in an uncloned cell line, transfection with pBabeNeo did not have a significant negative effect on growth of CMT93 cells. However, there did appear to be a trend towards a slower doubling time. The data from the single clone expressing IL-2 show that, in this particular case, the effect of transfection together

Figure 3.4.

A. Growth of a non clonal B16 cell population transfected with pBabeNeo/IL-2.

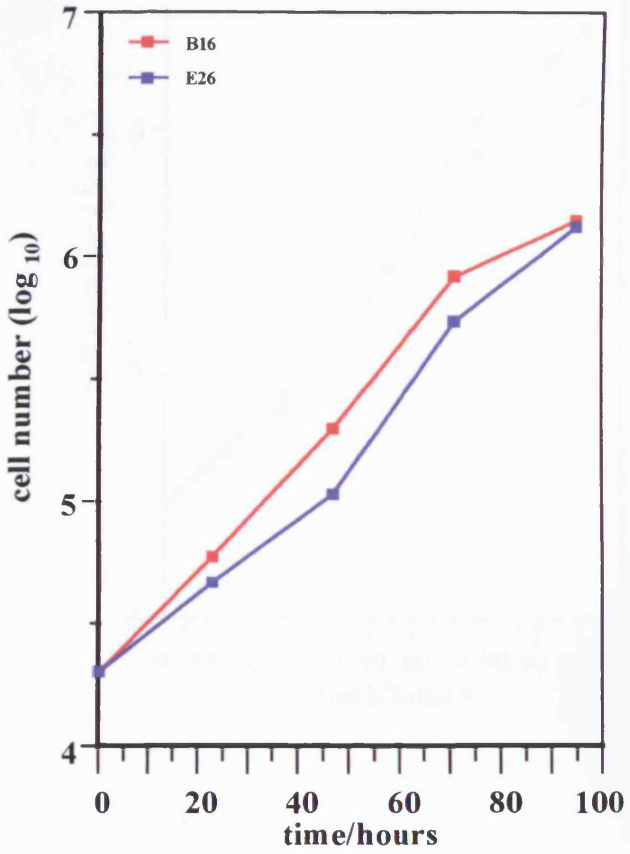


B. Growth of a B16 clonal cell line transfected with pBabeNeo/IL-2.



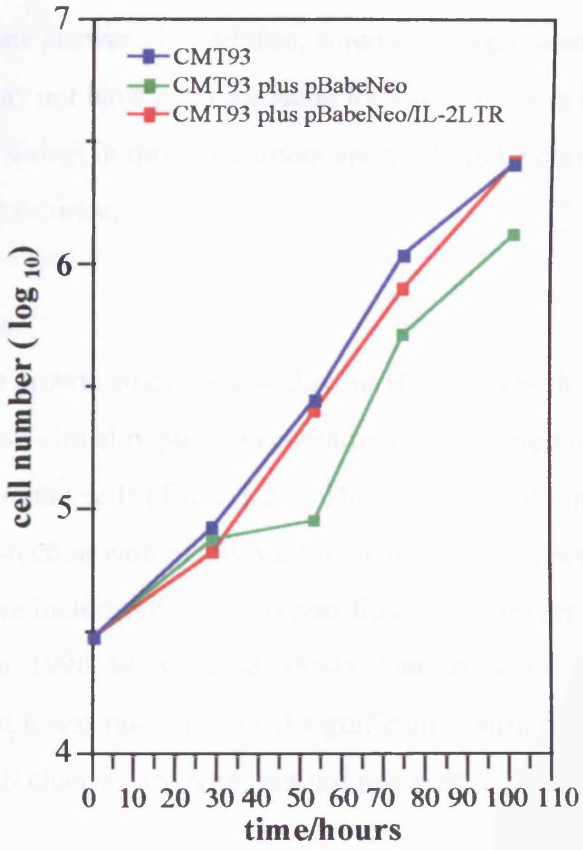
A. The doubling time of a non clonal population of B16 cells, with the vector randomly inserted into the host genome, was compared to parental cells to examine the overall effect of transfection. The doubling times were B16 = 13.6 ± 0.6 and B16 plus pBabeNeo = 14.9 ± 1.1 (n = 5). These doubling times were significantly different ($p < 0.05$, ANOVA and Fisher's LSD test).
B.The doubling time of a B16 clone containing pBabeNeo/IL-2 was compared to that of parental cells. The doubling times obtained from this experiment were B16 = 14.5 ± 1.5, low passage B16 plus pBabeNeo/IL-2 = 14.9 ± 1.9 (n = 3), indicating no significant change (ANOVA and Fisher's LSD test).

Figure 3.5. Growth of B16 cells transfected with both pBabeNeo/IL-2 and pNASSβ/IL-2 (E26).



The data were plotted on a log scale. The gradient of the curve in the region where the cells were growing in log phase gave the doubling time. These were B16 = 13.6 ± 0.6 and E26 = 15.5 ± 0.5, (n = 5). These values were significantly different from each other ($p < 0.01$. ANOVA and Fisher's LSD test).

Figure 3.6. Growth of CMT93 cells transfected with pBabeNeo or pBabeNeo/IL-2LTR.



The data were plotted on a log scale. The gradient of the curve in the region where the cells were growing in log phase gave the doubling time of the cells. These were CMT93 = 13.5 ± 0.5, CMT93 plus pBabeNeo (a non clonal population with the vector randomly inserted into the host genome) = 16.5± 0.9, CMT93 plus pBabeNeo/IL-2LTR (single clone) = 14.7 ± 2.9 (n = 3). CMT93 plus pBabeNeo exhibited a slightly longer doubling time than parental CMT93 cells, but this change was not significant.

with transgene expression is not as marked as in the cells containing the empty vector. It should be noted that the production of a single cell suspension of these cells was more difficult than for B16 cells. The CMT93 cells often remained in clumps of twos and threes, even after passage through a fine needle. All counts were carried out using a haemocytometer, so clumps of cells could be counted. However, this would not give an entirely accurate answer. In addition, when cells were seeded, the growth characteristics may not have been the same for single cells as for those in small clumps. While the trends shown in this experiment are likely to be correct, the times obtained are likely to be inaccurate.

3.2.6. Discussion

The results of the growth studies show that for B16 cells both a clone containing pNASSβ and a non-clonal population containing pBabeNeo have longer doubling times than the parental cells (Table 3.2.). There is little data in the literature concerning the effect of transfection with empty vectors that do not express a transgene, but those studies which have included this data report little, or no effect on growth (Cao et al, 1995, Gerard et al, 1996, Moody et al, 1994). One study did show a change similar to that seen here, but it was not considered significant (Ohira et al, 1994). It is therefore possible that small changes are seen, but not reported.

In the study presented here, since a B16 clone containing IL-2 in pNASSβ showed an increase in doubling time irrespective of the levels of IL-2 expression, this effect was attributed to the role of transfection, rather than to cytokine expression. Data from the experiments using B16 cells containing pNASSβ/HSV-tk showed no effect on growth (Figure 3.3.). Although the level of HSV-*tk* protein was not precisely determined, there was evidence that it was being expressed (Section 5.5). A B16 clone expressing low levels of IL-2 from pBabeNeo also showed no change in doubling time (Figure 3.4.B.). Finally, a B16 clone containing both pNASSβ/HSV-tk and pBabeNeo/IL-2 showed an increase in doubling time, which could have been due to transfection or transgene expression.

Experiments using CMT93 cells also suggested an effect of transfection. Those containing pBabeNeo or pBabeNeo/IL-2LTR showed increased doubling times, but without reaching significance.

Evidence in the literature concerning the growth effect of transgene expression *in vitro* generally concludes that there is no change in growth rate. A study in colon 26 cells showed no effect when the cells were altered to express IL-4, IL-6 or GM-CSF (Gunji et al, 1996) and another study using a number of different cell lines showed no effect of expression of a synthetic IFN (Zhang et al, 1996). Neither expression of IL-2 in a prostate adenocarcinoma cell line (Moody et al, 1994), nor IL-10 in B16.F1 cells (Gerard et al, 1996) produced an effect. A study using B16.F10 cells concluded that these cells were not affected by IL-2 expression from a plasmid vector (Ohira et al, 1994), although as mentioned above, examination of the data shows that there was approximately a 12% increase in doubling time which is similar to or greater than the significant differences presented here.

Cytokines such as IL-6 (Cao et al, 1995) in B16 cells can produce a negative growth effect, although it has been shown to have no affect in other murine melanoma cell lines (Armstrong et al, 1994). A negative effect was also observed with IL-4 in a human melanoma cell line (Melani et al, 1995), although this could have been attributable to transfection *per se*, but this was not tested. The data in the present study support this evidence that changes in doubling time can occur, but are not predictable.

The conclusion from this study is that transfection with either of the vector systems affected the doubling time of B16 and CMT93 cells to a small degree, but the extent of this effect varies for each clone and cannot be predicted. Transgene expression was not found to effect the doubling times in the cell lines tested.

It should be noted that the data obtained were treated independently in each set of experiments, resulting in some variation in the doubling time calculated for the parental B16 cell line. Since cell lines can alter their growth characteristics in response to

changes in fetal calf serum or other components of the media, or from one frozen aliquot of cells to the next, the data from the whole study period were not averaged.

3.3. Tritiated thymidine incorporation as a measure of cell number

During the growth studies, it was observed that at the end of each experiment there were always fewer B16 cells containing pNASSβ or pNASSβ/IL-2 than parental cells (Figure 3.2.A.). In the case of the pNASSβ-containing line this observation could have been due to the significantly decreased doubling time, but the difference in pNASSβ/IL-2-containing cells appeared to be greater than could be explained by any such difference. DNA synthetic activity as measured by incorporation of tritiated thymidine was used to determine cell number (Section 6.3.) to investigate this apparent difference between B16 cells and those containing pNASSβ/IL-2 (Figure 3.2.A.). This assay yielded more precise results than those obtained from the Coulter counter, particularly in the case of low cell counts. The tritium tag present in the cell lysate was proportional to the cell number, it having been established that the tritium uptake per cell was similar in all the lines used (data not shown).

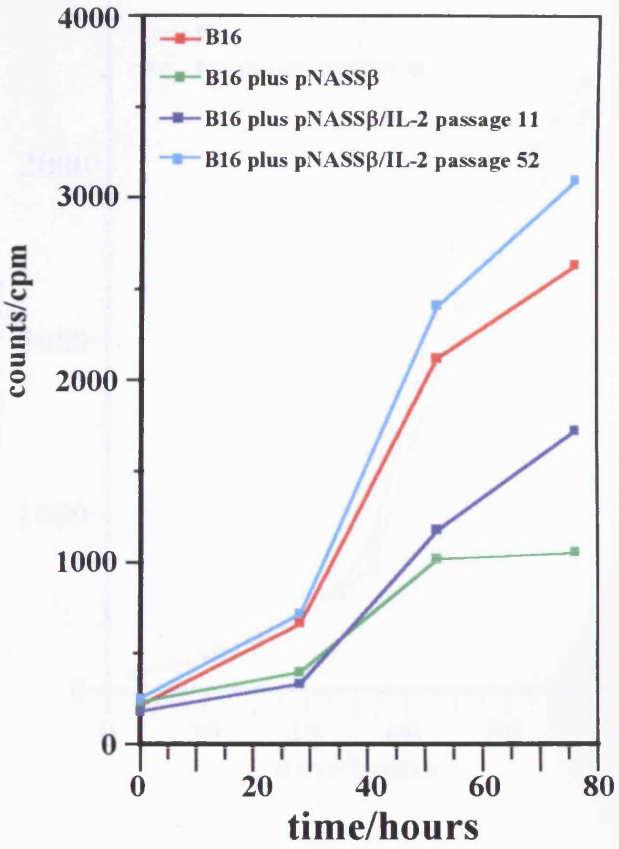
3.3.1. *Effect of insertion and expression of a transgene*

The number of parental B16 cells and low passage cells containing pNASSβ/IL-2 were compared using incorporation of tritiated thymidine. This method confirmed that, at any one time point, the number of cells expressing IL-2 was less than for the parental line (Figure 3.7.). The B16 cell line containing pNASSβ/IL-2 after at least 20 weeks in culture, and no longer expressing IL-2 (see chapter 5), incorporated tritiated thymidine in a manner similar to the parental cells. These data imply that in the low passage cells, it is not transfection with pNASSβ/IL-2 that is causing the reduced cell numbers, but overexpression of the IL-2 protein. This is in contrast to the conclusion reached in section 3.2.6. where the effect on doubling time was shown to be independent of IL-2 expression.

3.3.2. *Effect of expression of an alternative transgene*

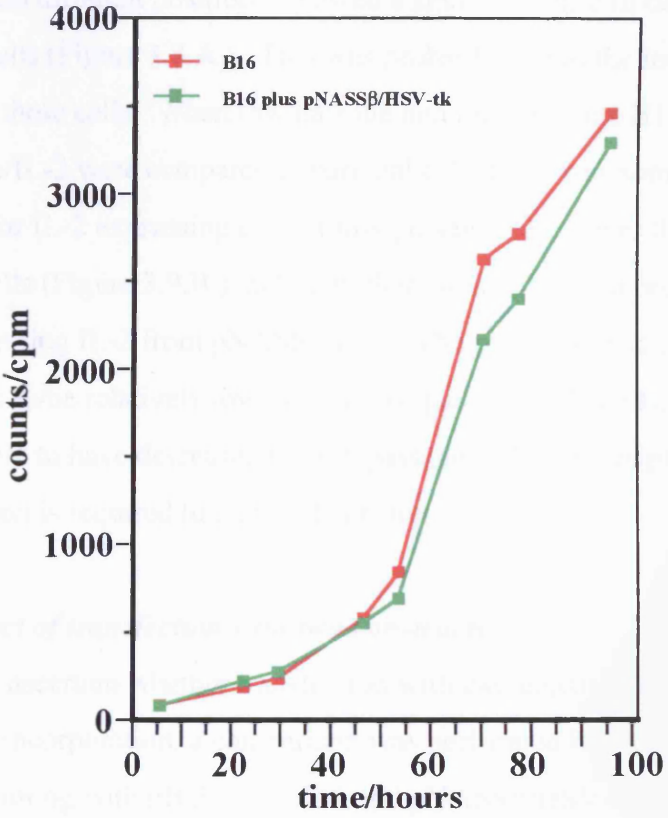
When cell numbers were compared between parental B16 cells and those expressing HSV-*tk* from pNASSβ no difference was seen (Figure 3.8). This suggests that the

Figure 3.7. Effect of transfection with pNASSβ or pNASSβ/IL-2 on B16 cell numbers.



Cells containing pNASSβ/IL-2 were used at a low and high passage to compare the effect of transfection and IL-2 expression in low passage cells, to that of transfection alone in the high passage cells, where expression of IL-2 is lost. IL-2 expressing cells had significantly lower cell numbers ($p<0.05$) than high passage cells of the same lineage, but any difference between them and parental cells did not reach significance. The control cells containing empty vector were significantly different ($p<0.05$) from both parental and high passage pNASSβ/IL-2 containing cells.

Figure 3.8. Effect on B16 cell numbers of transfection with pNASSβ/HSV-tk.



Tritiated thymidine incorporation was used to determine relative cell numbers. These cell types were not significantly different (ANOVA).

lower cell numbers seen with the low passage IL-2 expressing B16 cells is a consequence of overproduction of IL-2, not over production of any protein, although absolute levels of HSV-*tk* production were not determined (Section 5.5.).

3.3.3. *Effect of a different vector system*

The B16 cell line consisting of a mixed population of cells containing pBabeNeo inserted into different positions, showed a small decrease in cell number compared to parental cells (Figure 3.9.A.). This was probably due to the increase in doubling time shown by these cells. When low passage and high passage B16 cells containing pBabeNeo/IL-2 were compared to parental cells there was some indication that numbers for IL-2 expressing cells at low passage were lower than those for the high passage cells (Figure 3.9.B.), although there was not such a pronounced effect as in B16 cells expressing IL-2 from pNASSβ/IL-2. The production of IL-2 from pBabeNeo was later found to be relatively low even in low passage cells and could not be shown conclusively to have decreased in high passage cells (see chapter 5). Therefore, further investigation is required to clarify this result.

3.3.4. *Effect of transfection with two constructs*

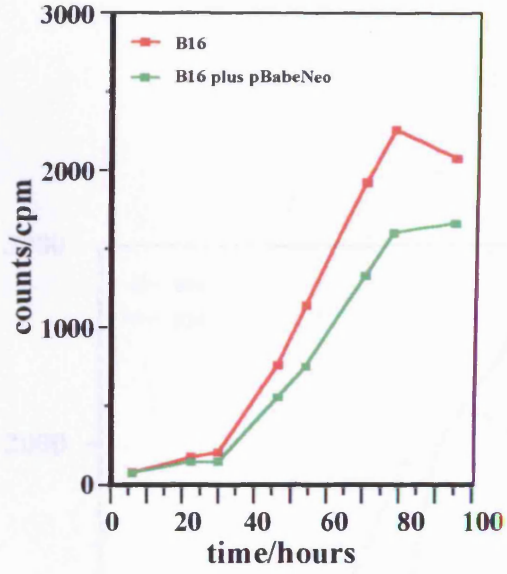
In order to ascertain whether transfection with two constructs affected tritiated thymidine incorporation, a comparison was performed between parental B16 cells and those containing both pBabeNeo/IL-2 and pNASSβ/HSV-*tk*. The increased incorporation in the parental line (Figure 3.10.) could be related to doubling times which were significantly longer for these cells.

3.3.5. *Effect of supplementing the medium with IL-2*

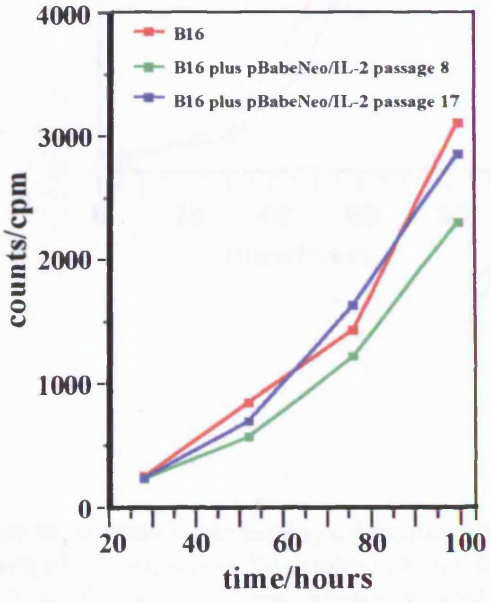
The evidence from the tritiated thymidine study (3.3.1.) suggests IL-2 is having an effect on cell number when expressed from pNASSβ. An experiment was carried out whereby recombinant IL-2 was placed in the medium of parental B16 cells at a similar concentration (150pg/ml/10^6cells) to that found in the medium of cells containing pNASSβ/IL-2 (passage 6), to determine whether this would affect cell numbers in the parental cell line. Figure 3.11. shows no such effect occurred. This result contrasts with published data where addition of IL-2 to the medium of B16.F10 cells caused

Figure 3.9.

A. Effect on cell numbers of a non clonal B16 cell line following transfection with pBabeNeo.

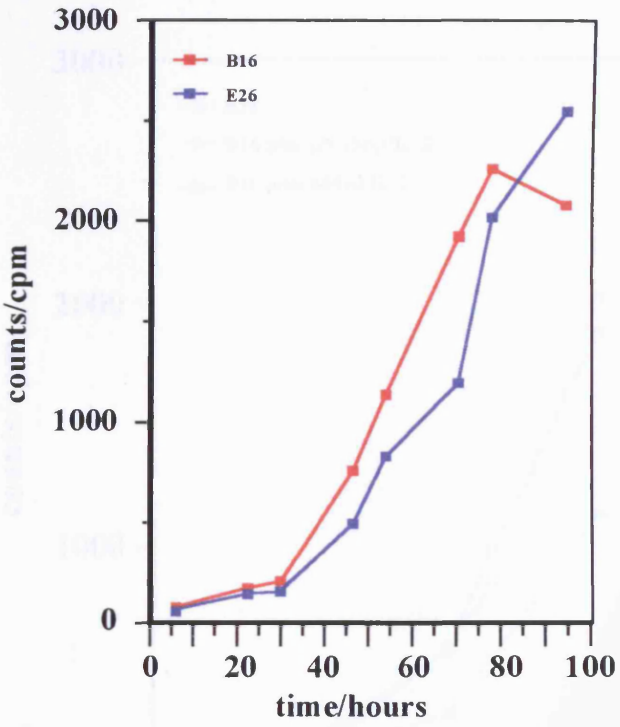


B. Effect on B16 cell numbers following transfection with pBabeNeo/IL-2.



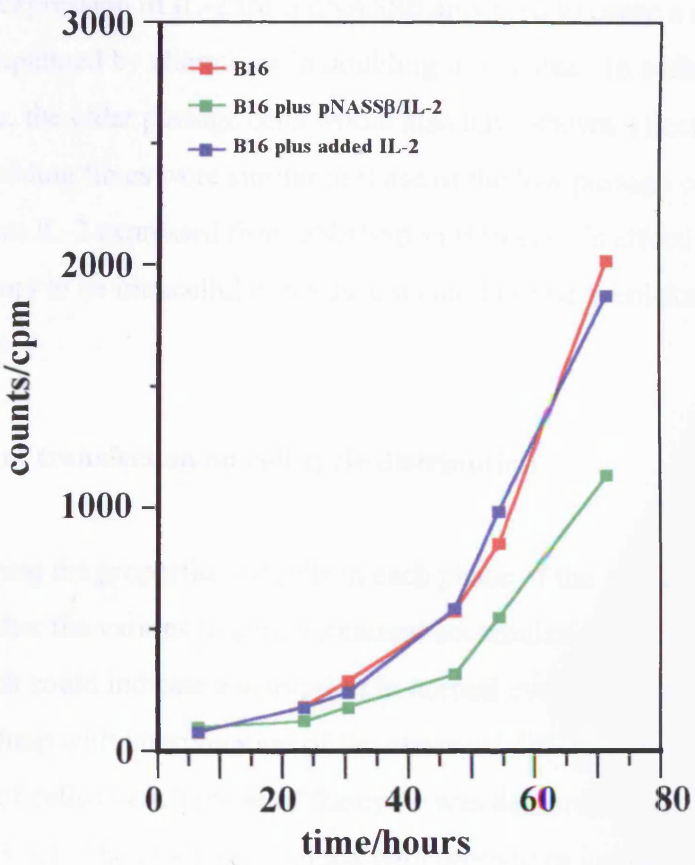
Tritiated thymidine incorporation was used to determine relative cell number.
A. A cell line consisting of a mixed population of cells, each cell of which had the vector, pBabeNeo, inserted into a random position of the host genome, was used to monitor the effect of transfection on B16 cells. The transfected cells showed significant changes in cell number ($p<0.001$).
B. Cells containing pBabeNeo/IL-2 were used at a low (passage 7-9) and high (16-18) passage to investigate the effect of IL-2 expression in addition to transfection, in a single clone. The low passage cells containing pBabeNeo/IL-2 exhibited decreased cell numbers compared to the parental cell line and this difference was significantly different from the high passage cells of the same lineage ($p<0.05$).

Figure 3.10. Effect on B16 cell numbers of transfection with pNASSβ/HSV-tk in combination with pBabeNeo/IL-2.



Tritiated thymidine incorporation was used to determine relative cell numbers. The cell line transfected with two constructs (E26) exhibited a significant difference in cell numbers (ANOVA and Fischer's LSD test) when compared to the parental line ($p<0.005$).

Figure 3.11. Effect on B16 cell numbers of supplementing the medium with IL-2.



Tritiated thymidine incorporation was used to determine relative cell numbers. Parental B16 cells were compared to B16 cells grown in medium supplemented with IL-2 and also to B16 cells containing pNASSβ/IL-2 (n = 2). The amount of IL-2 added to the medium was similar to that secreted by B16 cells expressing IL-2 from pNASSβ/IL-2. No difference was observed between parental cells with or without the IL-2 supplement.

them to grow faster (de Galdeano et al, 1996, Palomares et al,1997). The data presented here suggest that the effect of IL-2 expressed from the transgene on B16.F1 cells is mediated by intracellular mechanisms (see section 3.5.).

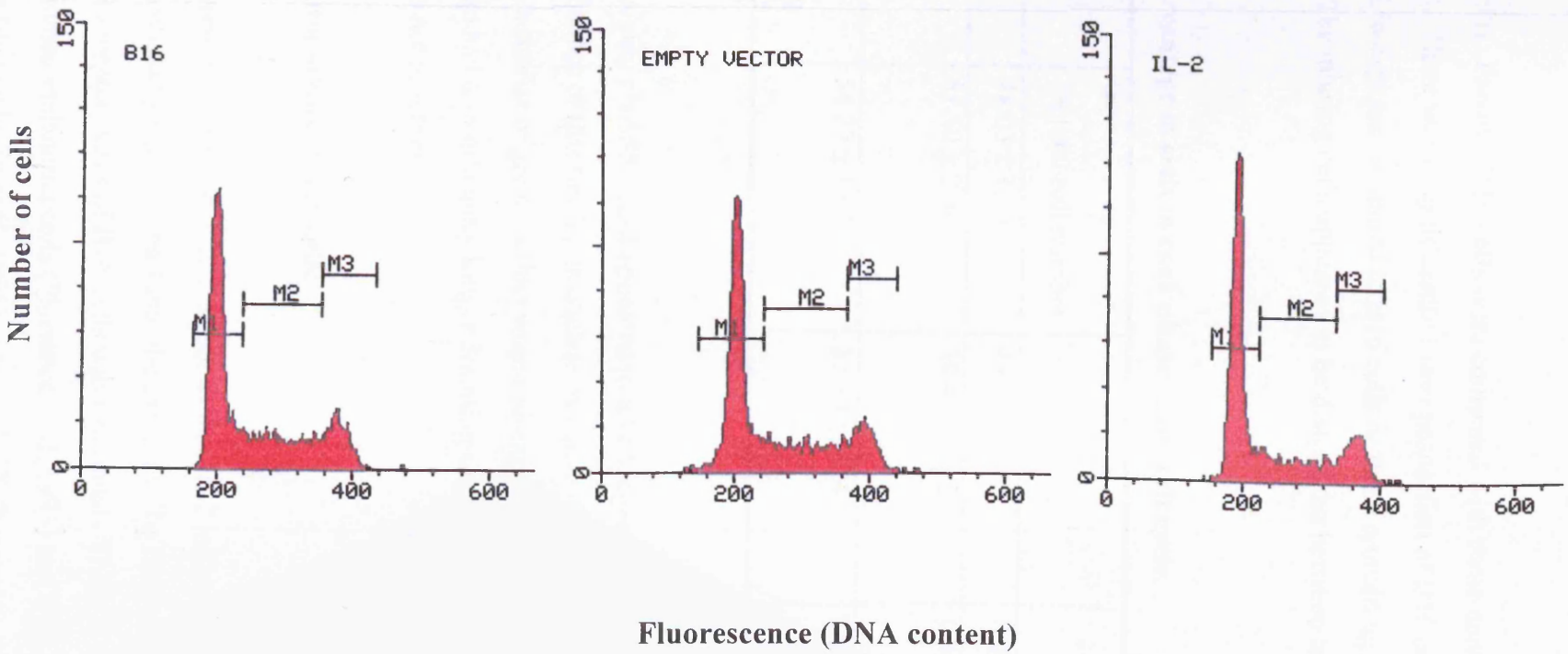
3.3.6. Discussion

Transfection caused a small decrease in growth rate as determined by tritiated thymidine incorporation, which supported the observed alterations in doubling times. However, expression of IL-2 from pNASSβ appeared to cause a change greater than could be explained by alterations in doubling time alone. In addition, if this was responsible, the older passage cells would also have shown a decrease in cell numbers, as their doubling times were similar to those of the low passage cells. This finding suggests that IL-2 expressed from pNASSβ in B16 cells is affecting cell numbers. The effect appears to be intracellular, because it could not be mimicked by adding IL-2 to the medium.

3.4. Effect of transfection on cell cycle distribution

By monitoring the proportion of cells in each phase of the cell cycle, it was possible to assess whether the various treatments caused accumulation of cells in any particular phase, which could indicate a disruption in normal cycling. It was anticipated that such data might help with interpretation of the observed differences in growth. The proportion of cells in each phase of the cycle was determined using flow cytometric analysis (2.3.5.). The DNA was stained with propidium iodide which fluoresces under laser light (488 nm). The level of fluorescence emitted from the nucleus provides an estimate of DNA content. When a cell is triggered into cycling it moves out of G_0 to G_1. During this phase RNA levels increase and essential replication proteins are made. As the cell starts to replicate its DNA it enters S-phase, and when the DNA content has doubled at G_2 the cell divides through M phase returning to the start of G_1. If DNA content is plotted against number of cells the percentage in each phase of the cell cycle can be calculated (Figure 3.12). Those in the G_2/M region on the right of the histogram will have double the content of those in G_1, while those in S-phase will be in-between

Figure 3.12. Distribution of cells in each phase of the cycle.



Propidium iodide was used to label the DNA. The proportion of cells in each phase of the cycle was calculated using the Cell Fit computer package. A significantly lower proportion of cells containing pNASSβ/IL-2 were present in S-phase compared to parental cells ($p < 0.01$, ANOVA and Fisher's LSD test) M1 = G_1, M2 = S and M3 = G_2/M.

(Ormerod, 1990). Parental B16 cells were compared with those containing pNASSβ or pNASSβ/IL-2. There was a significantly lower proportion of B16 cells containing pNASSβ/IL-2 in S-phase compared to B16 cells or those containing the empty vector (Table 3.2.). The missing cells appeared to be distributed between both the other phases.

Table 3.2. Percentage of cells in each phase of the cell cycle.

Cell Type	G_1 % total cell number	S	G_2/M
B16	48.03 ± 1.35	43.03 ± 0.55	8.97 ± 0.81
B16 plus pNASSβ	51.30 ± 2.46	39.40 ± 3.12	9.33 ± 2.30
B16 plus pNASSβ/IL-2	54.27 ± 1.95	31.40 ± 3.24	14.37 ± 3.16

n = 3

B16 cells containing pNASSβ/IL-2 appeared to take longer to pass through G_2/M and G_1. The significance of this finding is unclear, because if it is indicative of changes in doubling time, a similar or greater effect would be expected in the empty vector control cells, which also had a significantly longer doubling time compared to the parental cells. This was not observed.

3.5. IL-2 receptor status of B16 cells

The evidence presented (Section 3.3.5.) suggests that IL-2 influences the cell when generated intracellularly before export into the medium. To investigate this matter further the IL-2 receptor status of B16 cells was examined. There is evidence in the literature that human melanoma cells (Plaisance et al, 1993) and B16.F10 mouse melanoma cells (de Galdeano et al, 1996) express the IL-2 receptor, which consists of three subunits p55, p75 and p64. The α–subunit (p55) binds IL-2 with low affinity, and the complex of β-subunit (p75) and γ-subunit (p64) bind IL-2 with intermediate affinity. The low and intermediate affinity receptors combine to produce a receptor

with high affinity. Interleukin-2 is able to bind to 2 sites, one on the p55 chain and the other on the p75 chain. Two separate methods were used to look at the status of the α and β subunits in B16.F1 cells.

3.5.1. Examination of IL-2 Receptor-α status by flow cytometric analysis

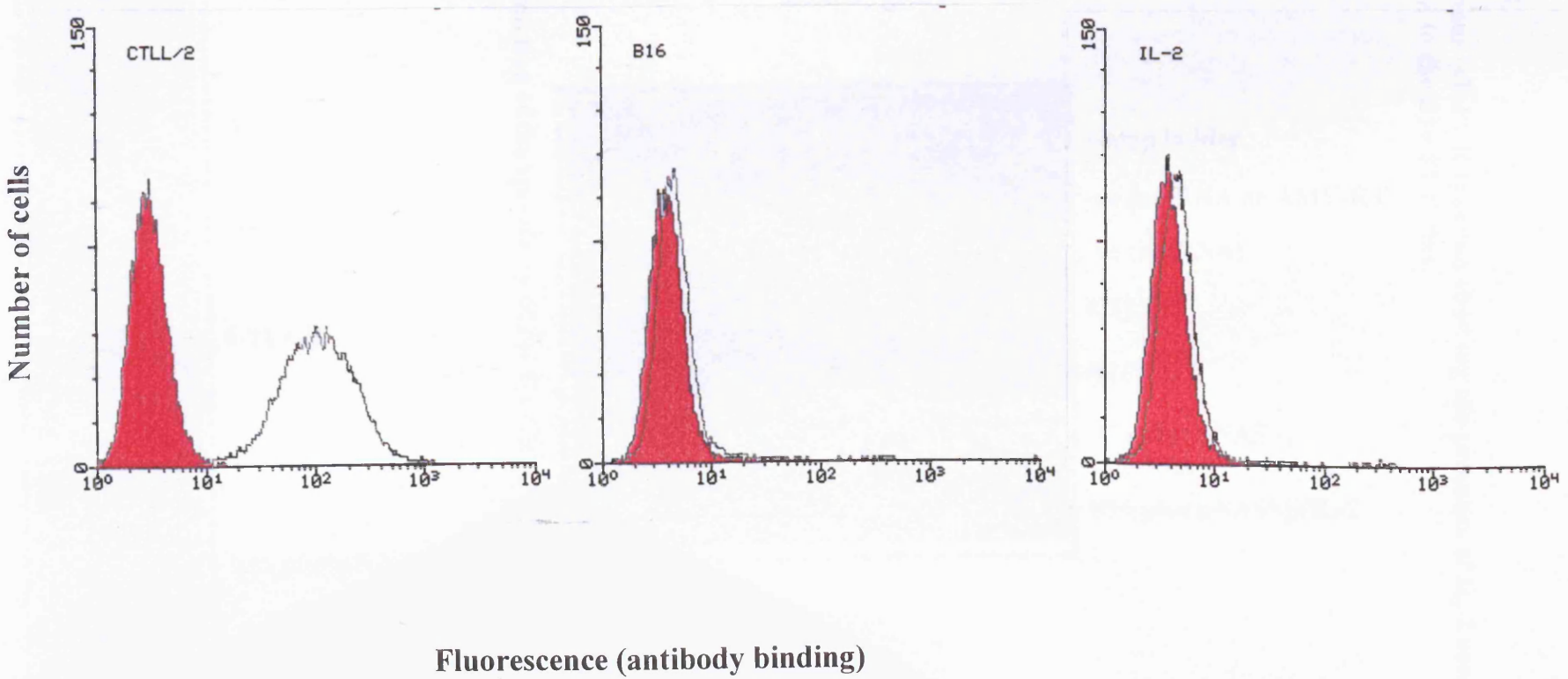
Parental B16 cells and those containing pNASSβ/IL-2 were incubated with a FITC conjugated antibody to murine IL-2 receptor-α subunit (2.3.6.). The fluorescence of labelled and unlabelled cells was compared using flow cytometric analysis. Antibody binding is indicated by a shift in the histogram along the y-axis, caused by increased levels of fluorescence. A mouse CTLL/2 cytotoxic T-lymphocyte cell line, which is IL-2 dependent and therefore expresses IL-2 receptor, was used as a control. The flow cytometric analysis showed that CTLL/2 cells expressed IL-2 receptor-α, but that parental B16 or B16 pNASSβ/IL-2 cells did not (Figure 3.13).

3.5.2. Examination of IL-2 Receptor -β status by RT-PCR

Total RNA was prepared from the cell lines CTLL/2, B16 and B16 containing pNASSβ/IL-2 (2.3.6.), for use in a continuous RT-PCR reaction in conjunction with two primers (murilRc 1 and 2) specific for the murine IL-2 receptor-β (de Galdeano et al,1996). The RT-PCR products were analysed by agarose gel electrophoresis and a product of the expected size of 350bp was observed in the CTLL/2 sample lane. Products were not observed in any other lanes (3.14.A.). The gel was blotted onto Hybond N+ and hybridised with a third oligonucleotide (murilRc 3) specific for a sequence within the region of the expected product, to confirm that the observed band was specific for the IL-2 receptor β subunit. A single band in the CTLL/2 track was observed (Figure 3.14.B.) confirming the product was specific for IL-2 Rc-β (2.3.7., 2.3.8.).

The results from these two experiments confirm that the B16.F1 cells used here did not express IL-2 receptor on the cell surface and that expression of IL-2 did not upregulate expression of the receptor. It also confirms that IL-2 expressed from the plasmid vector works intracellularly, rather than via an extracellular autocrine route, because without

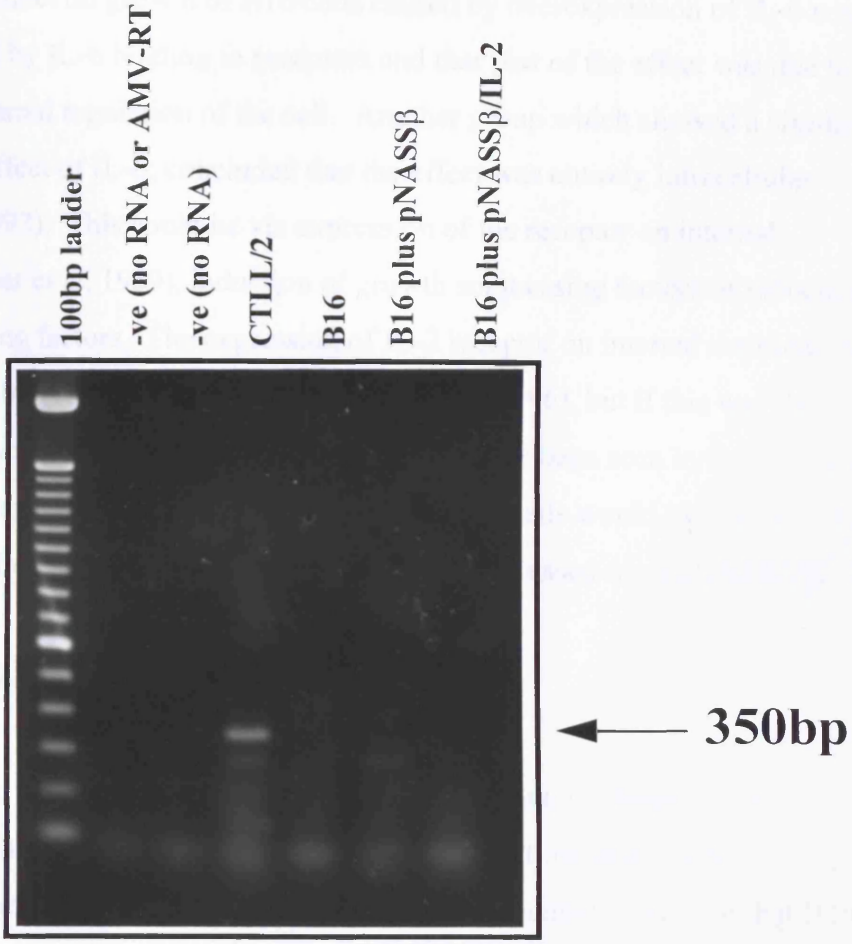
Figure 3.13. FACScan analysis of IL-2 receptor α levels in B16 cells.



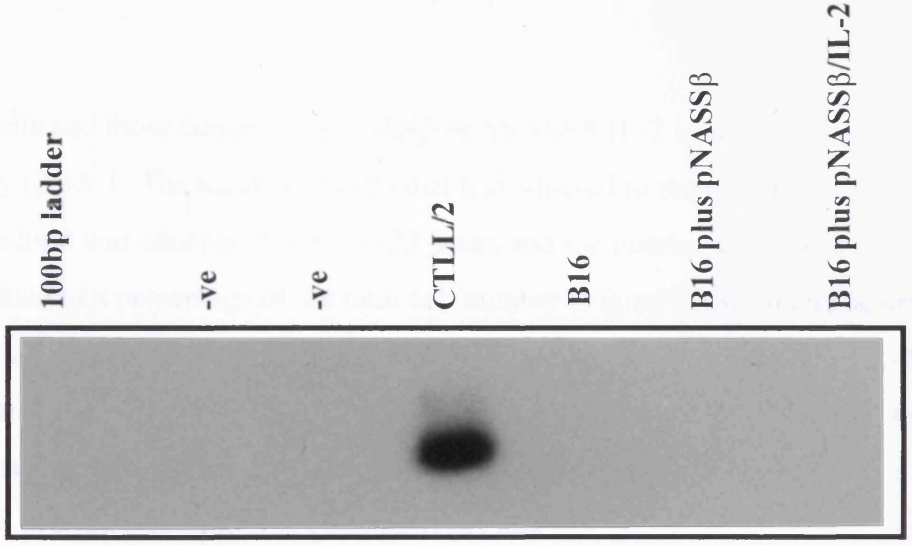
Parental B16 cells and those containing pNASSβ/IL-2 were incubated with a FITC labelled anti- IL-2 receptor α antibody. The level of fluorescence (black outline) was compared to that obtained with a control mouse IgG antibody (red). An increase in fluorescence indicated the receptor was present. A CTLL/2 mouse cell line was used as a positive control.

Figure 3.14.

A. Continuous RT-PCR reaction showing the presence of IL-2 receptor β in CTLL/2 cells but not in the B16.F1 clones.



B. Confirmation of the specificity of the IL-2 Rc β PCR product.



The gel shown in A. was blotted onto Hybond N+ and probed with an internal oligonucleotide specific to IL-2 Rc-β to confirm the identity of the PCR product.

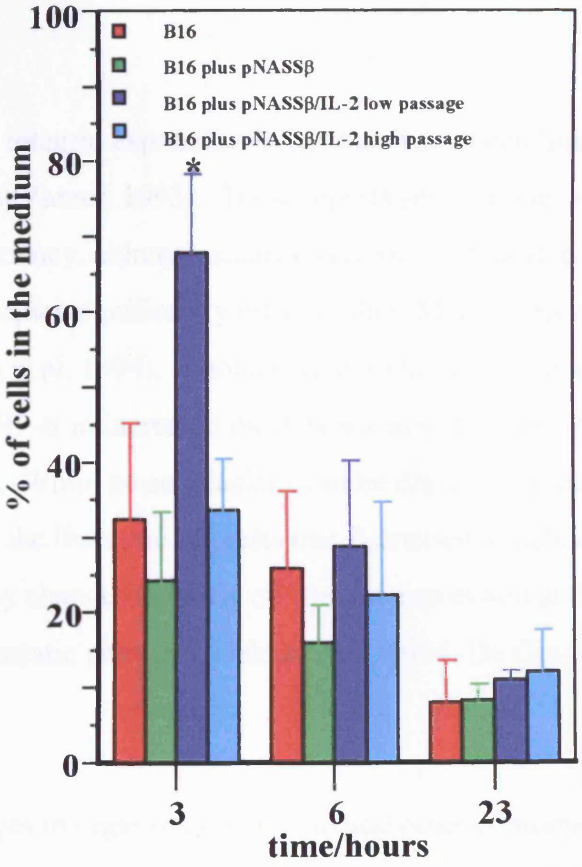
the receptor, the protein in the medium could not influence the cells. This interpretation is partially supported by data from Cao et al (1995), who showed that the negative effect on growth of B16 cells caused by overexpression of IL-6 was only partly caused by IL-6 binding to receptors and that part of the effect was due to IL-6 affecting the internal regulation of the cell. Another group which showed a similar negative growth effect of IL-6, concluded that the effect was entirely intracellular (Porgador et al, 1992). This could be via expression of the receptor on internal membranes (Dunbar et al, 1989), induction of growth suppressing factors or reduction of growth promoting factors. The expression of IL-2 receptor on internal membranes of B16.F10 cells has been demonstrated (de Galdeano et al, 1996), but if this was the case in the B16.F1 cells used in this study, a product should have been seen in the RT-PCR assay for the β-receptor. The lack of IL-2 receptor in B16 cells would explain why IL-2 supplemented medium did not mimic the effect of IL-2 expressed from pNASSβ/IL-2.

3.6. Substrate Adherence

It was hypothesised that a possible cause of the lower cell counts observed for B16 containing pNASSβ/IL-2 might be an increased attachment time or an increased lag time or a combination of the two. Indeed visual evidence seemed to suggest that B16 pNASSβ/IL-2 cells took longer to adhere to the substrate as compared to parental B16 cells.

B16 cells and those containing pNASSβ or pNASSβ/IL-2 were seeded at the same density (2.3.9.). The number of cells that had adhered to the substrate, or remained in the medium was counted at 3, 6 and 23 hours and the number of cells in suspension was calculated as a percentage of the total cell number (Figure 3.15). It can be seen that at 3 hours there was a much higher proportion of unattached B16 pNASSβ/IL-2 cells compared to parental cells, but that the older cells no longer expressing the transgene attached at a similar rate to parental cells. Cells containing the empty vector also behave in a manner similar to parental B16 cells.

Figure 3.15. Rate of adherence of B16 cells and those containing pNASSβ or pNASSβ/IL-2.



Cells were plated at a density of 3 x 10^4 cells in 35mm wells and counts performed at 3, 6 and 23 hours (n = 3). The number of cells in the medium is shown as a % of the total cell number (cells in suspension plus adherent cells). Low passage B16 cells containing pNASSβ/IL-2 had a significantly higher proportion of cells in the medium at 3 hours (*) compared to the other cell types ($p < 0.001$).

These data suggest that at least part of the explanation for the difference in growth of the IL-2 expressing cells is an increase in the time the cells take to adhere to the substrate, despite the fact that once the cells begin to divide they do so at a similar rate to the parental cells. Changes in adhesive interactions such as decreased homotypic adhesion and adhesion to ECM components in a B16 tumour derived cell line were seen after modification of MHC expression via transfection, and this was related to possible alteration of expression of molecules of the β_1 integrin family (De Giovanni et al, 1994).

Changes in integrin expression in tumours have been linked to the malignant phenotype (Juliano and Varner, 1993). These reports suggest one possibility for the change in plating efficiency, although studies have shown that B16.F10 cells expressing IL-2 as a transgene do not significantly differ in their MHC expression from wild type B16.F10 cells (Ohira et al, 1994). Another study in human melanoma cell lines has implicated IL-1 and TNF-α in increased motility via modification of integrin expression (Dekker et al, 1994). While no conclusions can be drawn from the *in vitro* data shown here, evidence in the literature suggests that decreased attachment to ECM components can be caused by changes in levels of integrin expression and that this can be related to altered metastatic potential (Dekker et al, 1994, De Giovanni et al, 1994, Smith et al, 1996).

Since changes in organisation of actin and other components of the cell cytoskeleton may relate to changes in adhesion (De Giovanni et al, 1994) and metastatic ability (Ze-ev et al, 1985), it seemed appropriate to investigate actin organisation of B16 cells expressing IL-2 (2.3.10.).

3.7. Changes in the actin cytoskeleton

It has been shown that B16.F1 cells have a higher level of organised actin stress fibres than the more metastatic B16.F10 cells (Sadano et al, 1994) and that deformability of B16.F1 cells, which is a function of the cytoskeleton, is related to metastatic potential (Ochalek et al, 1988). The cytoskeleton was visualised by staining with phalloidin,

which binds specifically to actin. By using FITC conjugated phalloidin, the cells could be viewed using confocal microscopy. The resulting patterns of fluorescence showed that there did appear to be a change in the structure of the cytoskeleton as the staining in the B16 cells containing pNASSβ/IL-2 was less organised in its localisation in the cell (Figure 3.16).

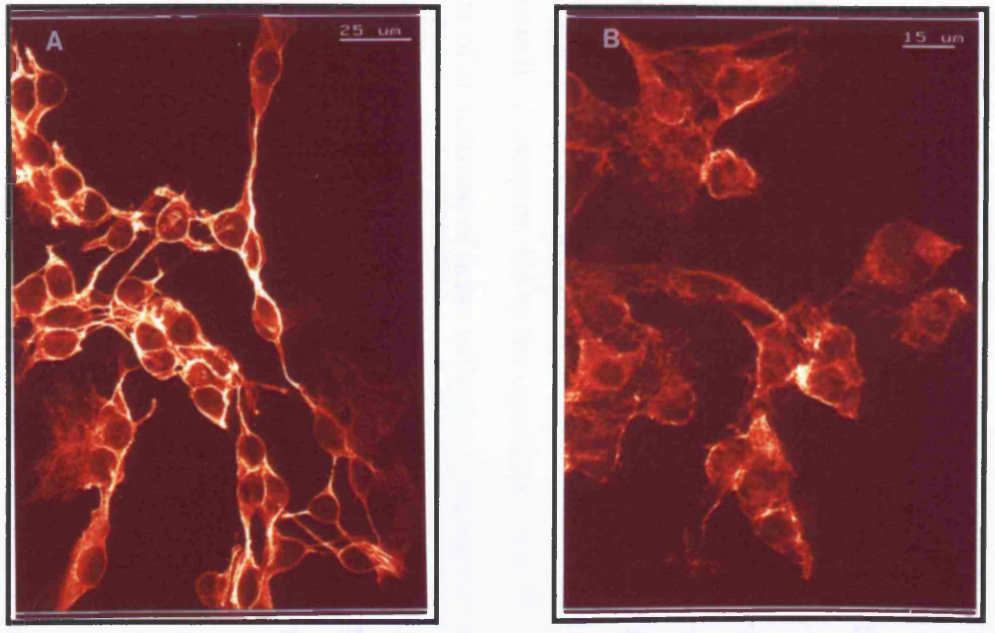
Inhibition of adhesion stabilisation can be affected by calcium concentration, calmodulin concentration, and inhibitors of protein kinase C and tyrosine kinases. Protein kinase C is involved in regulation of cell adhesion to the ECM and focal contact formation, while tyrosine kinases regulate focal adhesion and stress fibre assembly. An intact cytoskeleton is required for adhesive stabilisation, and phosphorylation is important in cytoskeletal organisation and rearrangement. For example integrin regulation is tyrosine kinase dependent. If IL-2 affects phosphorylation via tyrosine kinases or PKC, it could affect the cytoskeleton (Smith et al, 1996). B16 cells modified to express IL-6 have been shown to have increased adhesion to fibronectin via the integrin receptor (Sun et al, 1992). Additionally, IL-1 has been shown to affect the phosphorylation and redistribution of talin in fibroblasts, possibly via a protein serine/ threonine kinase. However, this did not effect the plating efficiency of the cells (Qwarnstrom et al, 1991). Given these observations it seems possible that IL-2 could modify components of the cytoskeleton.

CONCLUSION

The work described in this chapter investigated whether transfection or transgene expression had an effect on cell growth. It was determined that transfection with an empty plasmid or retroviral vector could effect the doubling times of B16.F1 cells and the retroviral vector also had a negative effect on the growth of CMT93 cells.

Expression of the HSV-*tk* transgene from the plasmid vector had no effect on the growth of B16.F1 cells. The effect of IL-2 transgene expression on doubling time was more difficult to interpret. The B16.F1 cells expressing this protein from pBabeNeo did not show any change in doubling time, while both CMT93 cells expressing IL-2

Figure 3.16. Actin cytoskeleton in B16 cells



Actin cytoskeleton was stained using FITC-conjugated phalloidin. A.parental B16 cells. B. pNASSβ/IL-2 transfected cells. The fluorescence in B appears to show less organisation than in A.

from pBabeNeo/IL-2LTR and B16.F1 cells expressing the gene from pNASSβ were affected. However, in CMT93 cells, the effect on growth was greater in cells containing the empty vector. In the B16.F1 cell line, older cells no longer expressing IL-2 showed the same doubling time as low passage cells, implying that the difference in doubling time was due to the transfection, rather than the IL-2 expression. Overall, it appears that insertion of a vector into the genome of a cell may have the potential to slow the growth rate of that cell. The effects seen here were relatively small and any effect of expression of a transgene on the doubling time of the cell was less than that of transfection *per se*.

Although only a small negative effect on doubling time was seen in B16.F1 cells expressing IL-2 from pNASSβ, cell numbers of this particular clone were always low when compared to other clones. Tritiated thymidine incorporation confirmed that a difference existed and comparison with the same cells no longer expressing IL-2 implicated this protein as the cause of the change. Placing IL-2 in the medium of B16.F1 parental cells did not affect cell growth and this fact and the observation that these cells did not express IL-2 receptor, led to the conclusion that IL-2 affects the cell intracellularly. The rate of attachment of these cells was investigated and it was found that B16.F1 cells expressing IL-2 from pNASSβ took longer to adhere to the flask surface, providing at least part of the explanation for the decreased cell numbers. Preliminary results suggested that the actin cytoskeleton in these cells may have been altered, contributing to the altered attachment rate.

Chapter Four

Investigation of the effect of transfection on mRNA expression, using differential analysis

INTRODUCTION

One of the aims of this project was to determine whether transfection or transgene expression causes any changes in the regulation of the cell's genes. The approach described in Chapter 3 involved using well informed "guesses" as to the parameters most likely to be affected. This approach does not allow conclusions to be drawn as to the underlying change in regulation. An alternative is to use a technique which indicates the extent to which gene regulation is altered and allows identification of particular alterations.

Fundamental cellular processes, including cell cycle control, differentiation and cell transformation are affected by alterations in gene expression. A technique which allows comparison of gene expression between cell types is differential display reverse transcription-PCR (DDRT-PCR). This technique was first described by Liang and Pardee (1992a) and it permits mRNA expression in two or more tissues or cell lines to be compared, so enabling the identification of mRNA species which have been up or down regulated in one cell compared to another.

Total RNA is prepared from the cells of interest and reverse transcribed, using a primer that binds to the poly A^+ tail of the RNA species. Twelve 3′ primers, which anneal to the poly A^+ tail are used to reverse transcribe the mRNA . The oligomers have the sequence $dT_{11}VN$, where position N can be any nucleotide and V can be A, C or G (Table 4.1.).

Table 4.1. Primers used in the reverse transcription step of a DDRT-PCR reaction

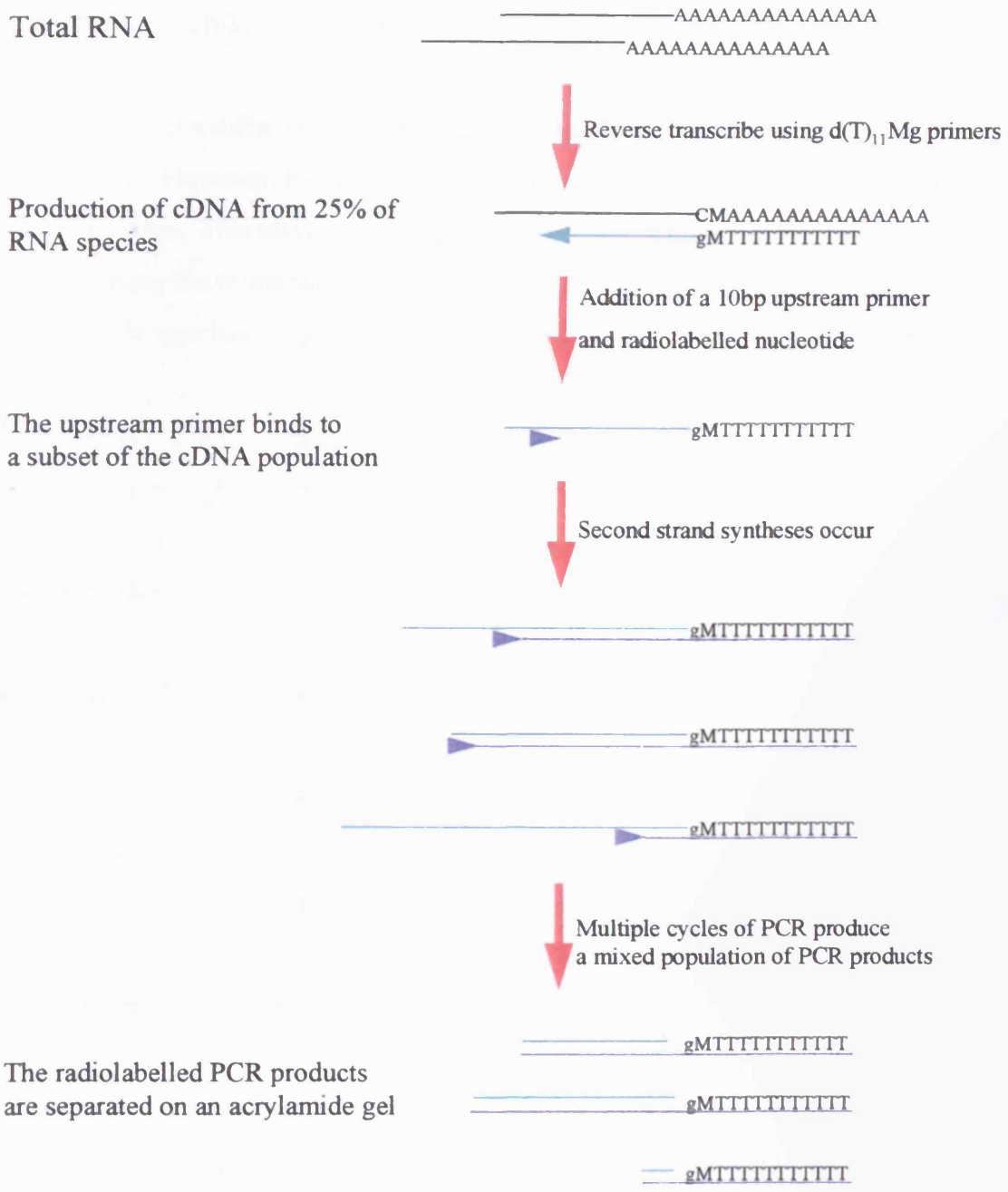
The twelve 3′ primers used to reverse transcribe mRNA			
$dT_{11}GG$	$dT_{11}GA$	$dT_{11}GT$	$dT_{11}GC$
$dT_{11}AG$	$dT_{11}AA$	$dT_{11}AT$	$dT_{11}AC$
$dT_{11}CG$	$dT_{11}CA$	$dT_{11}CT$	$dT_{11}CC$

It is these two positions which determine the mRNAs to which the primer anneals, e.g. $dT_{11}AC$ will reverse transcribe all mRNA species ending in $GTdA_n$.

The cDNA prepared in the first step, which theoretically should represent one twelfth of the cell's mRNA population, is amplified in a PCR reaction (Figure 4.1.). The same 3′ 13mer that was used in the reverse transcription is used in the PCR reaction. It has been estimated that approximately 15 000 mRNA species are expressed in a cell. To have a good probability of amplifying each one at least once, a minimum of 25 different 5 ′ primers are required, each of which has to be used in combination with each of the reverse transcription primers. These PCR primers are 10bp in length. The 10mer primers are able to prime a sequence containing up to 4 mismatches, providing those mismatches are at the 5′ end of the primer. This can effectively cut down the sequence length to 6bp and thus increases the number of possible places it can bind. This ensures that as many mRNA species as possible are amplified. Twenty six possible 5′ primers have been described (Bauer et al, 1993), each of which has a 50% GC content and low self complementarity. Each one of the primers will bind to a fraction of the cDNA molecules produced in one reverse transcription reaction i.e. any cDNA that contains the complement of this 10 bp sequence will be amplified.

The PCR reaction is carried out in the presence of radiolabelled dATP and the products separated on an acrylamide gel. The PCR products will vary in size and products up to 500bp can be resolved on these gels. It has been estimated that 120-150 bands per sample can be analysed on a gel and so the conditions used in the DDRT-PCR reaction have been tailored to achieve this. If fewer bands are seen on each gel, the number of different primer combinations has to be increased to compensate. If too many bands occur, they begin to merge into one another and it becomes very difficult to pick individual bands. A pattern of bands is seen in each sample lane, representing the RNA population in each sample, and each band corresponds to one PCR product that has been produced in the reaction. To compare two or more samples, the DDRT-PCR reaction is carried out on both samples and the corresponding reactions run in parallel on the gel. For example, if population A expresses the poly A^+ RNA encoding tyrosinase, but population B does not, then the band (or bands) which correspond to this RNA species in population A will be absent in population B. Only bands which are absent in one sample compared to the second sample should be considered as true

Figure 4.1. Differential display RT-PCR reaction



The diagram shows the principle of DD-RTPCR. If the reaction is carried out using two separate RNA populations, the final products can be compared on the gel and any differences noted.

positives. Bands that change in intensity can be investigated, but the change is more likely to be an artifact created by the PCR reaction.

At this stage, if a difference is observed in the banding pattern, the identity of the band is unknown. However, it can be excised from the gel, reamplified and cloned into a plasmid vector. This allows the band to be verified as a differentially expressed mRNA, using the technique of northern blotting. The sequence can be determined, and any possible matches identified using data bases such as EMBL and GENBANK.

It can be seen that this technique not only has the potential to pick out any differences between samples, but allows the identification of these differences. It, therefore, appeared to be a technique suitable to use in the search for any effects transfection and transgene expression might have on cells.

4.1. Method development

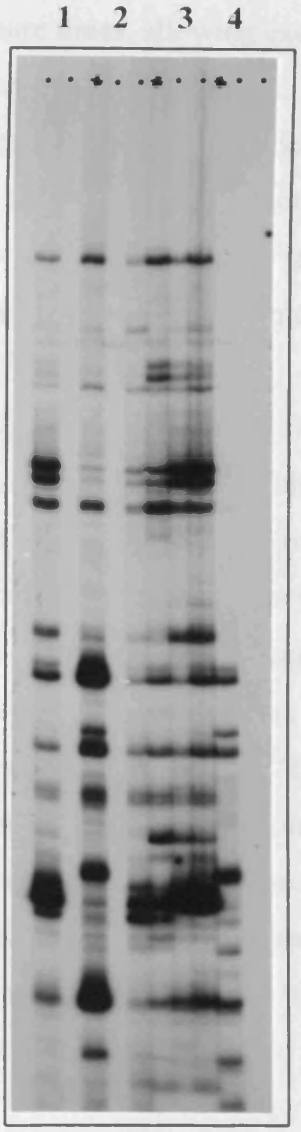
The technique of DDRT-PCR is one which requires a certain amount of optimisation. Parameters such as quality of RNA, type of radioisotope and temperatures used in the PCR protocol can all contribute to the success or failure of the technique.

4.1.1. Method of RNA preparation

Total RNA has been shown to give similar banding patterns to those achieved with mRNA. This implies that the $dT_{11}VN$ primers are preferentially annealing to the mRNA in the total RNA sample, rather than binding to ribosomal DNA (Liang, 1993). Thus, total RNA was used in these experiments.

The quality of the RNA was found to be important. Initially, RNA was prepared using TRIzol™, but duplicate sample reproducibility was low (Figure 4.2.). The fact that most samples worked well implies that the RNA was intact. However, when a modified version of a protocol described by Chomczynski and Sacchi (1987) was used, the reproducibility improved, suggesting that there may have been an inhibitor present in the TRIzol™ preparation.

Figure 4.2. Loss of reproducibility



1 - Parental B16 (3 lanes)
2 - B16 clone (3 lanes)
3 - B16 plus PNASSβ/IL-2 (3 lanes)
4 - negative (2 lanes)

A DDRT-PCR reaction using total RNA prepared using TRIzol™. Three reactions per cell type and two negative lanes, labelled with P^{32} are shown. In each third lane (*) the sample was prepared using a different RNA preparation from the first two lanes. This was done in order to minimise false positives. It can be seen that in two cases (1b and 2a) one of the duplicates has been lost.

4.1.2. Choice of isotope

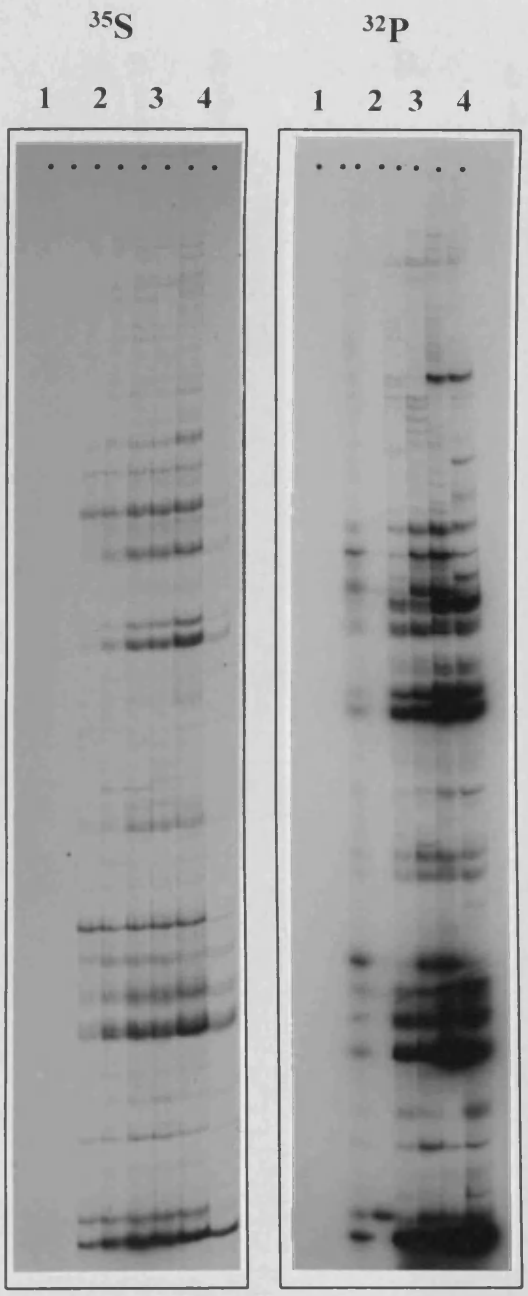
Three different isotopes can be used in DDRT-PCR reactions (Callard et al, 1994, Utans et al, 1994). Some work has been carried out using ^{32}P. This isotope has the advantage of short exposure times, allowing excision of bands from wet gels (Hadman et al, 1995) and gives intense banding patterns. In addition, it is a relatively inexpensive isotope, since its high energy reduces the amount needed per reaction. This isotope was tried in some of the early reactions, but while the banding patterns obtained were darker when compared to ^{35}S (Figure 4.3.), the increased precautions needed to handle this isotope made the experiment laborious.

Probably the most commonly used isotope for DDRT-PCR has been ^{35}S, which has the advantage of being safe to use on the bench with minimal screening. However, this isotope has been reported to contaminate PCR machine blocks (Trentmann et al, 1995), a finding supported by observations during this work, despite the reactions being carried out under mineral oil. This contamination was very difficult to remove and therefore, use of this isotope was less than ideal. A good compromise is ^{33}P, which has an intermediate energy level between ^{35}S and ^{32}P . This isotope gave clearly defined bands, there was no problem with contamination of the PCR machine and ^{33}P could be used on the bench with the same precautions used for ^{35}S. The only disadvantage with the use of this isotope is that it is relatively expensive. However, the small amounts needed per reaction, compared to ^{35}S, not only bring the cost into line with this isotope, but reduces the level of isotope required and so improves safety.

4.1.3. Reverse transcription primers

It has been reported that the penultimate base of the reverse transcription primer is degenerate and it is the final base which confers specificity. Similar banding patterns have been obtained using a single primer, $T_{12}CA$, or a mix of primers, $T_{12}MA$ (Liang et al, 1993). This was observed in this study (Figure 4.4.) where a similar pattern was obtained using $dT_{11}GG$ and a mixture of the three primers $dT_{11}GG$ (AP1), $dT_{11}AG$ (AP2) and $dT_{11}CG$ (AP3). Thus, it would appear that nearly as many mRNA species are detected with the degenerate primer combination and the number of RT reactions is reduced substantially. For this reason the reactions in this work were performed with a

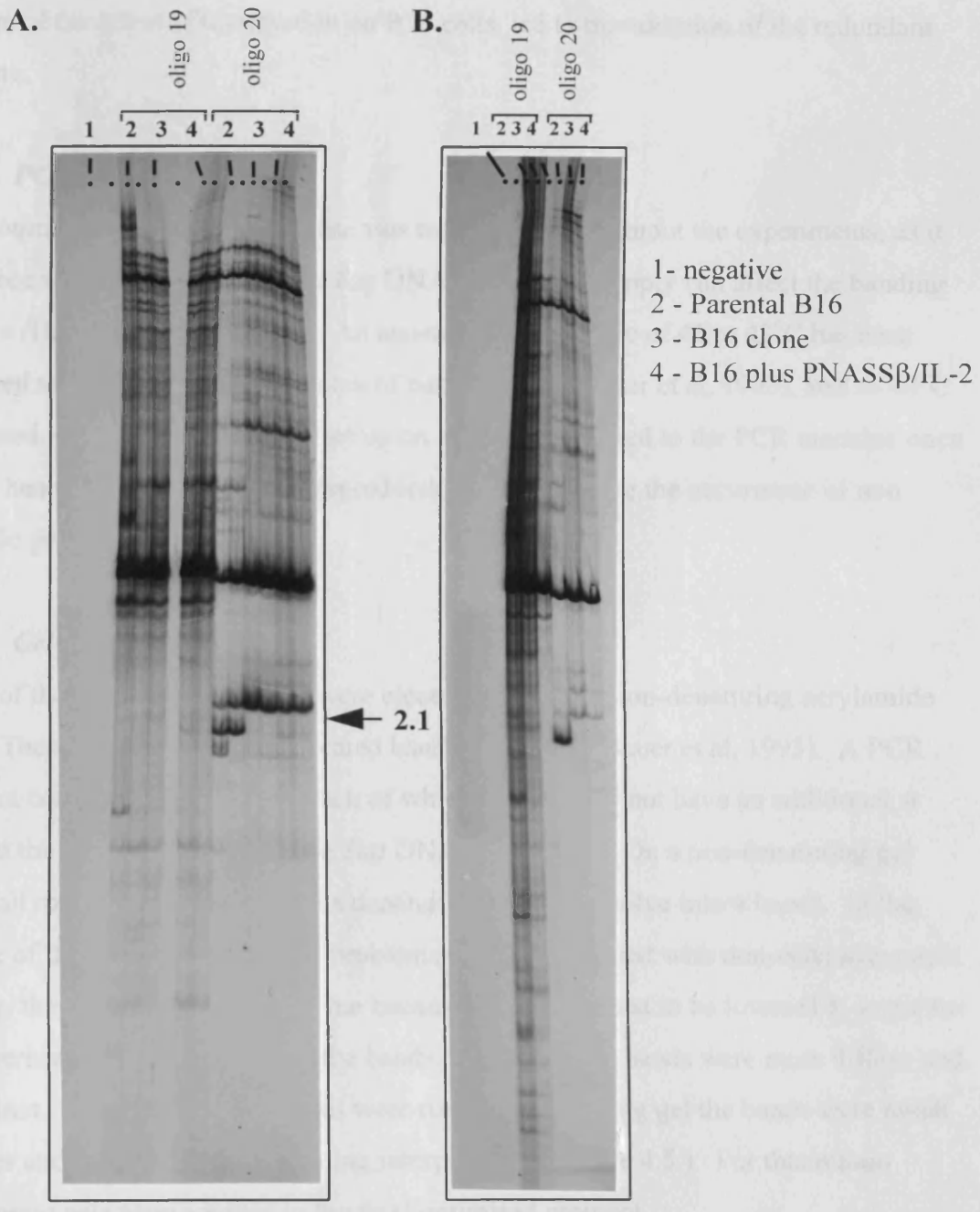
Figure 4.3. Comparison of labelling with ^{35}S and ^{32}P



1 - negative (2 lanes)
2 - Parental B16 (2 lanes)
3 - B16 clone (2 lanes)
4 - B16 plus PNASSβ/IL-2 (2 lanes)

A comparison of the two panels, prepared using primer $dT_{11}GG$ and oligo 7, shows that the basic pattern is similar, but the PCR products labelled with ^{32}P are more prominent against the background and the fainter bands are easier to identify. However, the signal from this isoptope is fuzzy, making band excision imprecise. Samples were run in duplicate.

Figure 4.4. Banding pattern obtained from a single or combination of reverse transcription primers



The banding pattern obtained using oligo 19 or 20 in conjunction with $dT_{11}MG$ (A.) was compared with that obtained using $dT_{11}GG$ alone (B.). It can be seen that the patterns are similar suggesting there is little benefit in using all twelve RT primers individually. The differentially displayed band 2.1. was excised from the gel and reamplified. Samples 2, 3 and 4 were run in duplicate in A.

combination of the primers $dT_{11}MG$. Although others have suggested that all twelve primers should be used (Bauer et al, 1994) to obtain a complete spectrum of mRNA expression, time constraints and the need to obtain an indication, rather than a complete picture of the effect of transfection on B16 cells, led to the adoption of the redundant primers.

4.1.4. PCR parameters

The source of *Taq* DNA polymerase was maintained throughout the experiments, as it has been shown that variability in *Taq* DNA polymerase supply can affect the banding pattern (Haag and Raman, 1994). An annealing temperature of 40 to 42°C has been reported to give the optimum number of bands per gel (Bauer et al, 1993), and so 40°C was used. All the reactions were set up on ice and transferred to the PCR machine once it had heated to 85°C to increase reproducibility by avoiding the occurrence of non specific priming.

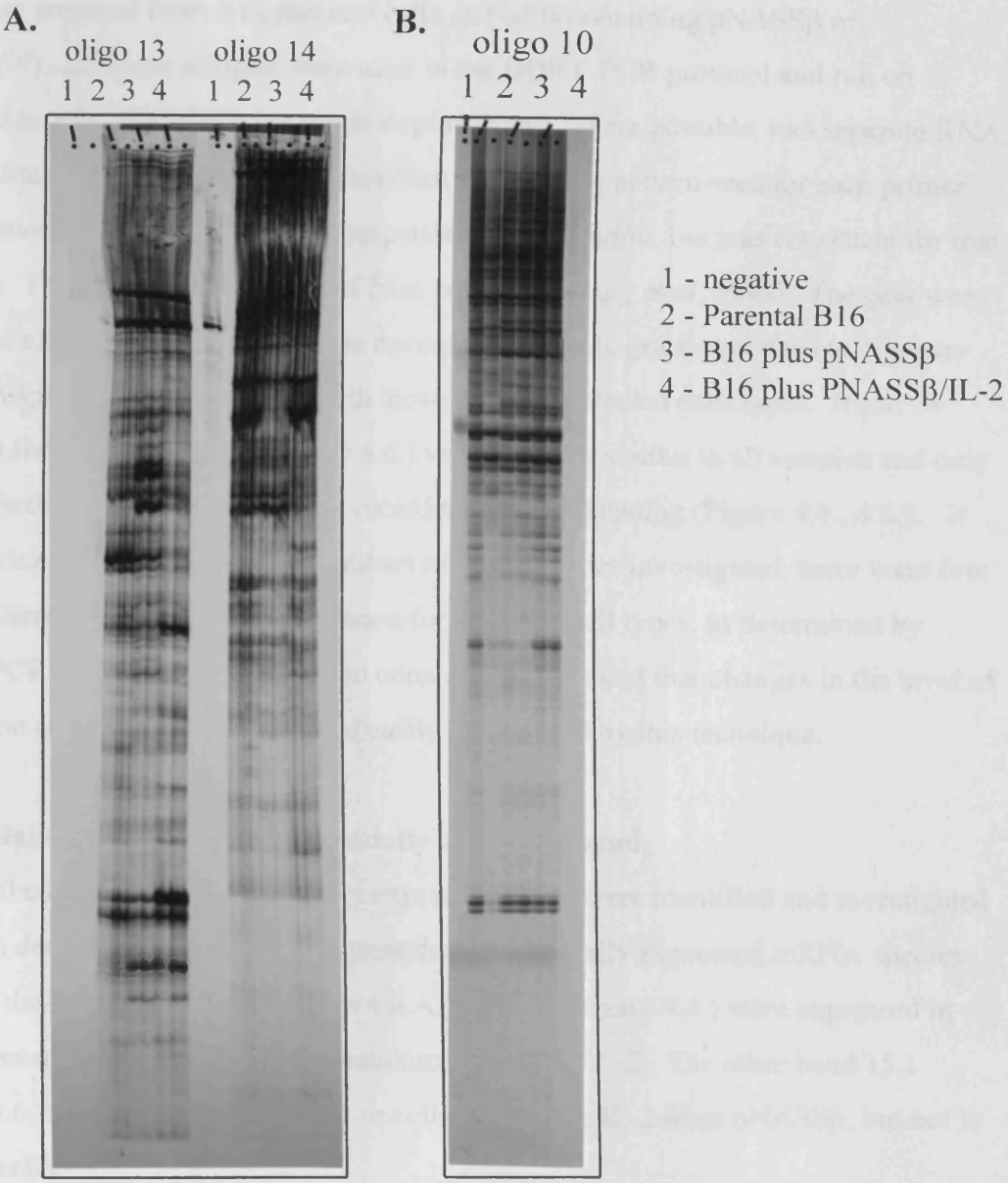
4.1.5. Gel type

Most of the reactions carried out were electrophoresed on non-denaturing acrylamide gels. These gels give less complicated banding patterns (Bauer et al, 1993). A PCR product consists of two strands, each of which may or may not have an additional A base at the 3' end, added on by the *Taq* DNA polymerase. On a non-denaturing gel these all run together, whereas on a denaturing gel they resolve into 4 bands. In the course of the experiments several problems were encountered with non-denaturing gels. Firstly, they took much longer to run because the voltage had to be lowered to avoid the gel overheating and distorting of the bands. Secondly, the bands were more diffuse and indistinct. When the same samples were run on a denaturing gel the bands were much sharper and more distinct, facilitating interpretation (Figure 4.5.). For this reason denaturing gels were adopted in the final optimised protocol.

4.2. Differences highlighted by DDRT-PCR

The DDRT-PCR reactions were carried out (Section 2.16.) with the modifications described. The reverse transcription primer set $dT_{11}MG$ was used in all of these

Figure 4.5. DDRT-PCR samples run on a non-denaturing or denaturing gel.



A. shows duplicate DDRT-PCR reactions labelled with ^{33}P and separated on a non-denaturing gel. Reactions were carried out using dT11MG and oligo 13 or oligo 14 and samples run in duplicate. The resolution is poor with smearing occurring in the lanes. B. shows duplicate DDRT-PCR reactions labelled with ^{33}P and separated on a denaturing gel. Reactions were carried out using $dT_{11}MG$ and oligo 10.

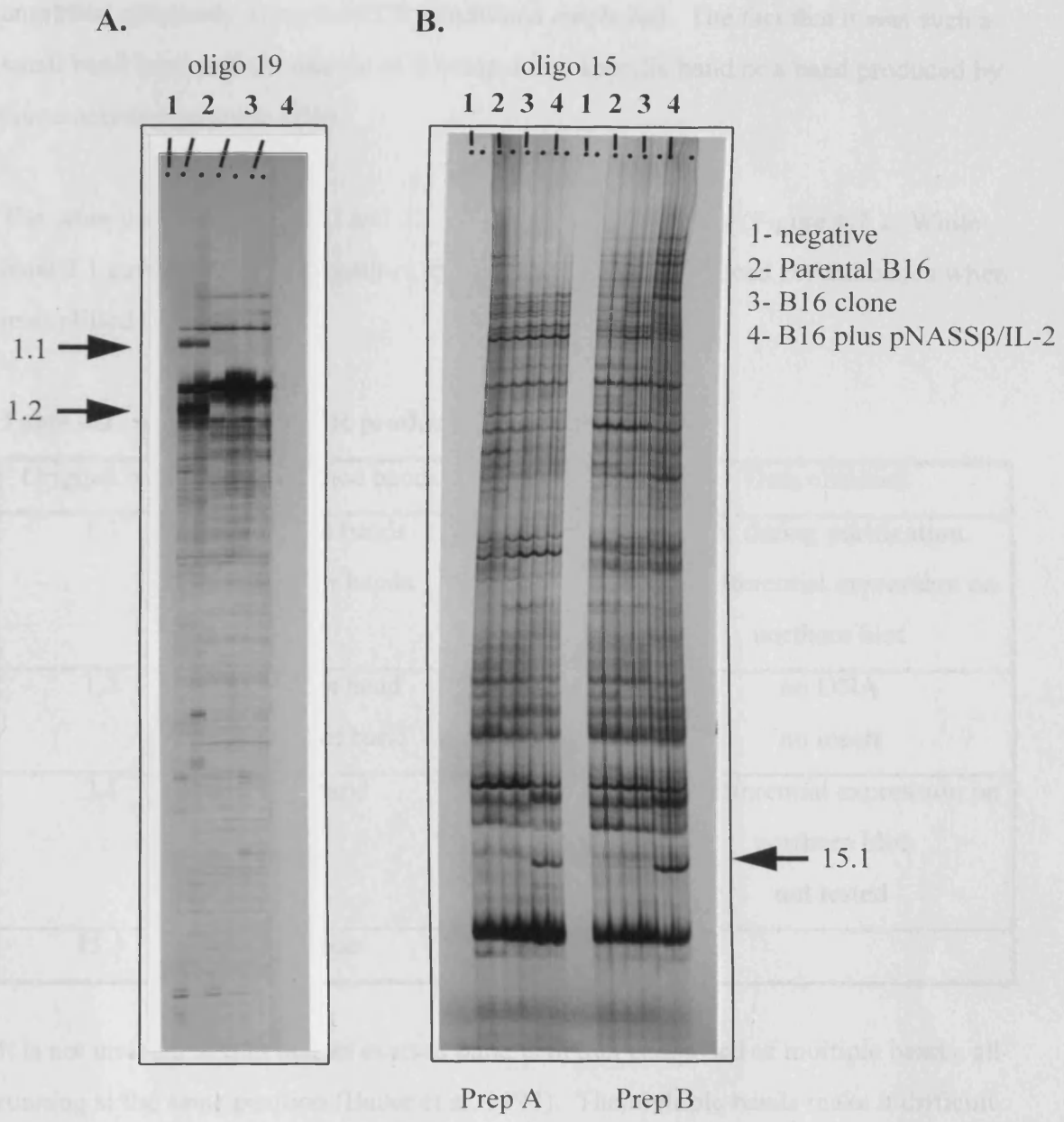
reactions and the cDNA was amplified using 20 different 5′ primers (Appendix). This represents just under a quarter of the possible combinations of primers and therefore, suggests that nearly a quarter of the possible RNA species has been investigated. Total RNA was prepared from B16 parental cells and cells containing pNASSβ or pNASSβ/IL-2. These samples were used in the DDRT-PCR protocol and run on acrylamide gels. Samples were run in duplicate and, where possible, two separate RNA preparations were used in order to show that the banding pattern seen for each primer combination was not an artifact of one particular preparation, but was consistent for that cell line. This reduces the number of false positives (Liang et al, 1993). The gels were dried and exposed to X-ray film. The developed autoradiograph was used to compare the RNA species in parental cells with those of the transfected cells types. It can be seen that the banding pattern (Figure 4.6.) was generally similar in all samples and only four differences were seen that were considered worth pursuing (Figure 4.4., 4.6.). It was concluded from this that in the subset of RNA species investigated, there were few major differences in the RNA expression for the three cell types, as determined by DDRT-PCR. This does not take into consideration the fact that changes in the level of expression could occur, which cannot easily be detected by this technique.

4.3. Isolating DNA from a differentially displayed band.

As described above four differentially expressed bands were identified and investigated further to determine whether they represented differentially expressed mRNA species. Three of these bands. 1.1, 1.2 (Figure 4.6.A.) and 2.1 (Figure 4.4.) were expressed in B16 parental cells, but not in cells containing pNASSβ/IL-2. The other band 15.1 (Figure 4.6.B.) was seen consistently in cells expressing IL-2 from pNASSβ, but not in parental cells.

DNA was isolated from these bands as described in the methods. A sample of the precipitated DNA was used in a PCR reaction to amplify the band of interest. The same combination of primers was used as had been added in the original DDRT-PCR reaction. A sample of the PCR reaction was run on an agarose gel to determine whether the reaction had worked and obtain an estimate for the product size. Despite several attempts, band 15.1 was not successfully reamplified. This could have been

Figure 4.6. Differentially expressed bands



The differentially expressed bands (arrows) were excised from the gel and reamplified. Samples were analysed in duplicate. Gel A shows two bands, 1.1 and 1.2, which are expressed in the parental cell line only, while gel B shows a band only expressed in B16 cells containing pNASSβ/IL-2. Two different RNA preparations were used to confirm this difference. Samples were run in duplicate.

because it was a non-specific band or because contaminants in the gel inhibited the PCR reaction. It is possible that band 15.1 was less than 100bp, too small to be amplified efficiently using the PCR conditions employed. The fact that it was such a small band increased the chance of it being a non specific band or a band produced by contaminating genomic DNA.

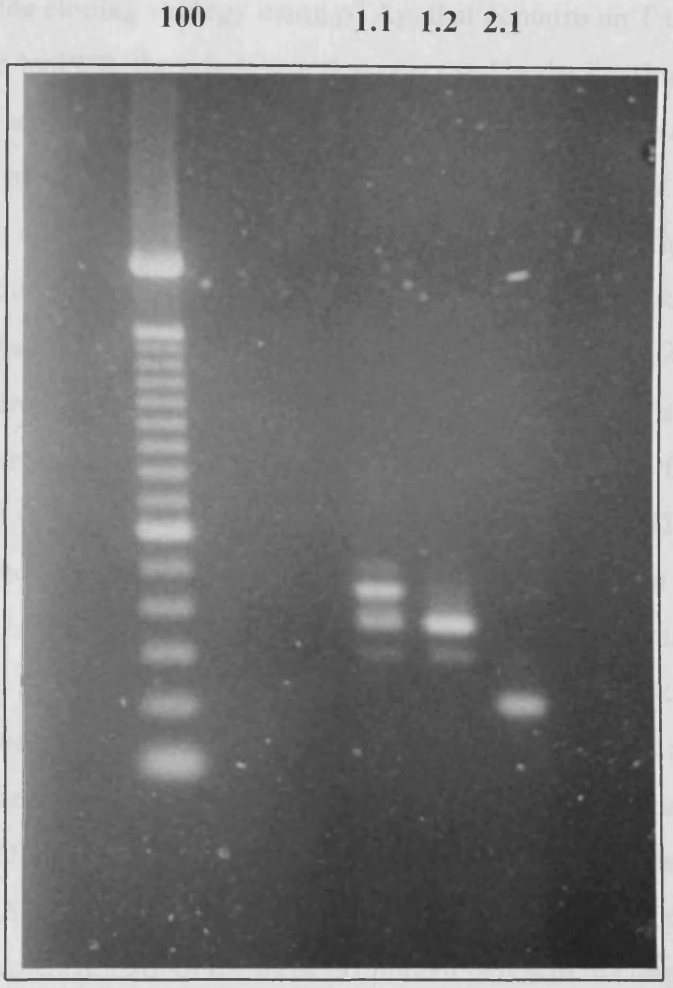
The other three bands 1.1, 1.2 and 2.1 were cloned successfully (Figure 4.7.). While band 2.1 gave an individual product, the other two bands produced several bands when reamplified (Table 4.2.).

Table 4.2. Reamplified PCR products

Original band	Reamplified bands	Cloned bands	Data obtained
1.1	2 main bands 2 minor bands	1.1.1 1.1.2	lost during purification no differential expression on northern blot
1.2	1 main band 1 minor band	1.2.1 1.2.2	no DNA no insert
2.1	1 band	2.1.1 2.1.2	no differential expression on northern blot not tested
15.1	none	none	

It is not unusual to find that an excised band is in fact composed of multiple bands, all running at the same position (Bauer et al, 1993). The multiple bands make it difficult to determine which band is the original differentially displayed band. The difference in the size (approx. 50bp between each) of the multiple bands seen here is quite large. However , they were excised from the top part of the gel, where resolution is poorer. It is also possible that some of the bands were produced by inappropriate binding of the primers in the reamplification reaction.

Figure 4.7. Reamplified bands excised from DDRT-PCR gel



DNA eluted from three differentially displayed bands, 1.1, 1.2 and 2.1 was reamplified using a PCR reaction and 10µl of the product electrophoresed on a 1% agarose gel in 1 x TAE. Both 1.1 and 1.2 appeared to be a composite of several products. These products were cloned into pTAg.

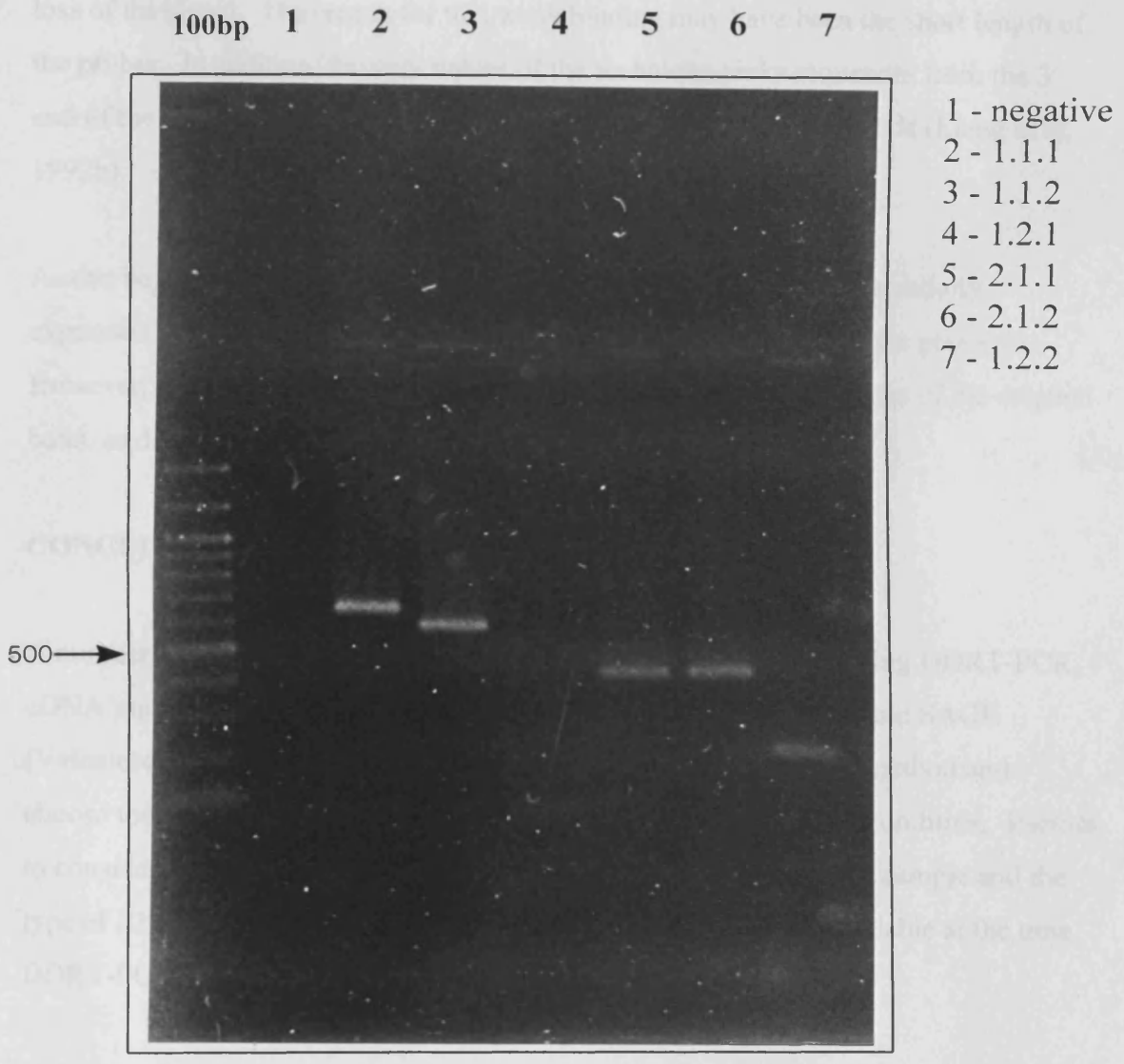
4.4. Cloning the differentially displayed bands

The fact that *Taq* DNA polymerase adds an extra A onto the 3′ end of the PCR product was used in the cloning strategy using pTAg, that contains an T overhang. After amplification by PCR, the whole reaction was used in the ligation, rather than cloning a single band isolated from an agarose gel, to increase the chances of cloning the correct DNA fragment. A blue/white selection strategy was used to pick clones which contained the vector with the insert. Since the ligation was inefficient, all the white colonies produced were picked and used to prepare glycerol stocks. This gave six colonies for band 1.1, three for band 1.2 and 2 colonies for band 2.1. Two clones from each band were chosen and used to prepare plasmid. If the chosen clones did not represent all the original bands, plasmid DNA could be prepared from further clones. Each plasmid was used in a PCR reaction using M13 primers (M13(-40) forward, M13 reverse) that bound within the plasmid. If no insert was present a band of 209bp was produced. A larger band implied that the vector contained the reamplified band. This band could be compared to that obtained in the original reamplification reaction. If the second reaction produced a band 209bp larger than the original it suggested that the vector contained the reamplified band. One of the six plasmid preparations failed to produce any DNA (1.2.1). Of the other five, four contained inserts, but clone 1.2.2 did not (Figure 4.8.). The two clones picked from the band 1.1 reaction were different and appeared to represent both of the most prominent bands in the original reamplification (Figure 4.7.). The two clones picked from the band 2.1 reaction were the same size and appeared to represent the single band seen in the original reamplification.

4.5. Confirmation of a differentially displayed RNA.

The PCR reactions, performed to identify the insert length, were purified and used as probes in a northern blot experiment. Both 1.1.2 and 2.1.1 were isolated in this way, but 1.1.1 was lost during the purification. The experiment was carried out with the two remaining probes, representing the smaller band reamplified from band 1.1 and the single product reamplified from band 2.1. Total RNA prepared from parental B16 cells and those containing pNASSβ or pNASSβ/IL-2 was prepared and electrophoresed on a

Figure 4.8. Amplification of the cloned DDRT-PCR bands for use as probes



The differentially displayed bands were cloned into pTAg. Multiple clones were picked and grown up. For each excised band plasmid was prepared from two individual clones. The insert in these plasmids was amplified using PCR in conjunction with the M13 forward and reverse primers. If the plasmid contains no insert a band of 209bp is produced. It thus follows that the reamplified band should be 209 bp longer than that seen when it was originally reamplified.

formamide gel. The gel was blotted onto Hybond N^+. The blots were hybridised with one of the probes 1.1.2 or 2.1.1. The blots were also probed with GAPDH in order to check that the loading of the RNA samples was even. The DDRT-PCR probes bound weakly to the blots and only one low stringency wash was carried out to avoid complete loss of the signal. The reason for this weak binding may have been the short length of the probes. In addition, the very nature of the technique picks sequences from the 3′ end of the mRNA, which is AT rich and will thus form less strong bonds (Liang et al, 1992b).

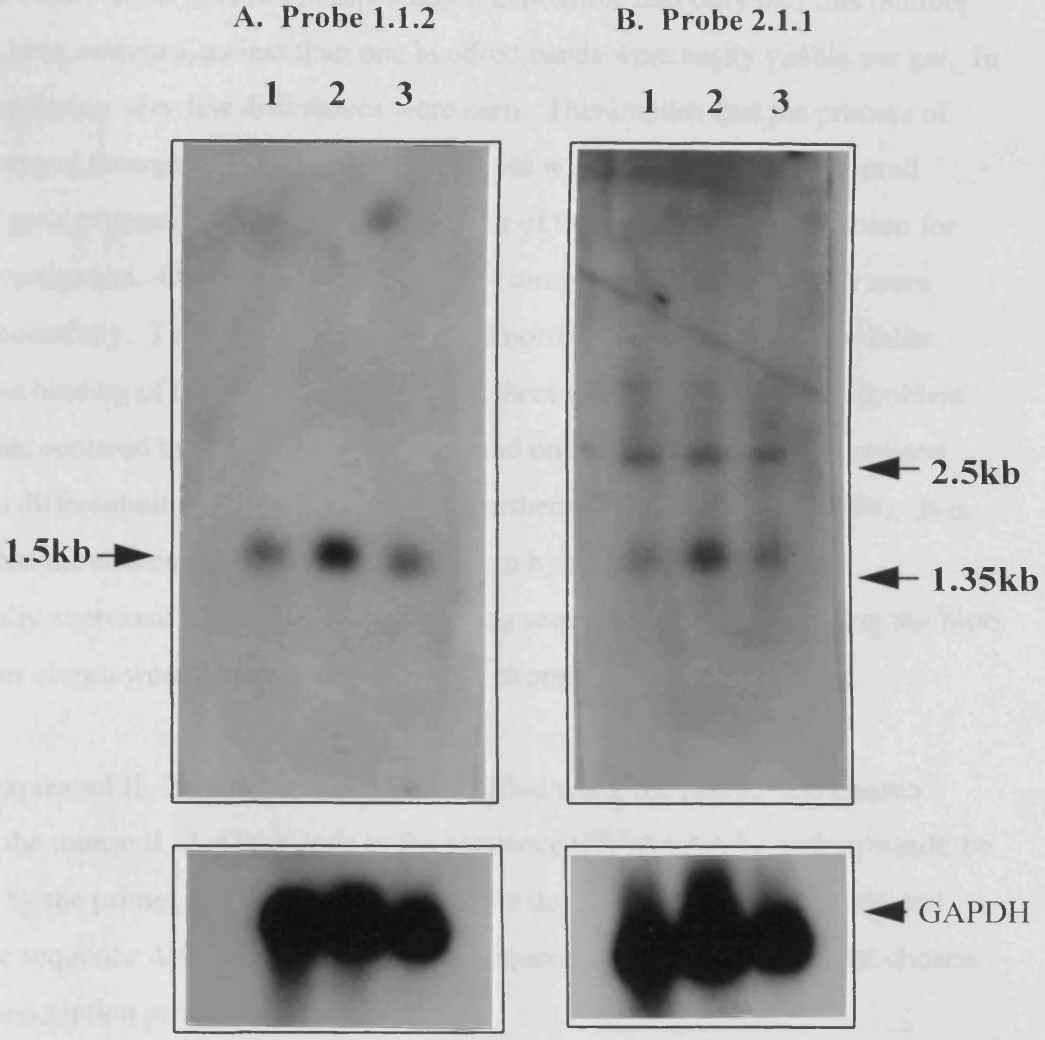
As can be seen from Figure 4.9., neither of the probes bound to a differentially expressed mRNA on the northern blots, implying that they are both false positives. However, it is also possible that the probe did not represent the sequence of the original band, as discussed below.

CONCLUSION

A number of RNA fingerprinting techniques are now available including DDRT-PCR, cDNA microarray (DeRisi et al, 1996), RAP-PCR (Vogt et al, 1997) and SAGE (Velculescu, 1995). It is important to look at the advantages of each method and choose the one which is most applicable to the specific experimental condition. Factors to consider are availability of specialised equipment, amount of tissue sample and the type of RNA to be investigated. For this study, of the techniques available at the time, DDRT-PCR seemed the most appropriate method.

There are several drawbacks to the method. For example, it produces a high number of false positive results, and underrepresents some mRNA species (Liang et al, 1994). However, it was used in preference to other protocols, because it allows the comparison of more than two samples and requires smaller amounts of RNA. It gave an overall impression of the extent to which the expression of genes had been altered by transfection, allowing any genes altered in such a way to be sequenced and identified. This has the advantage of allowing changes in currently unknown genes to be detected.

Figure 4.9. Northern blot to confirm differential mRNA expression



1 - B16
2 - B16 plus pNASSβ
3 - B16 plus pNASSβ/IL-2

Total RNA prepared from parental B16 cells and those containing pNASSβ or pNASSβ/IL-2 was separated by gel electophoresis and the RNA blotted onto Hybond N^+. The blot was probed with one of the differentially displayed bands previously isolated and cloned into pTAg. Gel A. was probed with band 1.1.2 and produced a band of approximately 1.5kb in length. Gel B. was probed with band 2.1.1 and produced two bands of approximately 1.35 and 2.5kb in length. None of the bands was differentially expressed. Both gels were probed with GAPDH to confirm that the loading was equal.

The theoretical percentage of the total number of mRNA species investigated was just under 25%, which translates to approximately 3000 mRNA species. By estimating the number of bands on the gels run in this study it is possible that only half this number may have been screened, as less than one hundred bands were easily visible per gel. In this subpopulation very few differences were seen. This implies that the process of transfection and transgene expression in a cell does not appear to alter the overall pattern of gene expression to a large extent. Four of the differences were chosen for further investigation. One of these could not be reamplified, but three others were cloned successfully. Two of these used to probe northern blots, proved to be false positives as binding of the probe was seen in all three samples. This type of problem has been encountered by another group who found only 10 % of the probes isolated bound to a differentially expressed mRNA on northern blots (Bauer et al, 1994). It is possible that the chosen clone used in the northern hybridisation was not the differentially expressed band, but a contaminating sequence and that screening the blots with further clones would produce a differential expression pattern.

The overexpressed IL-2 transgene was not identified using the primer sets chosen. Although the mouse IL-2 cDNA ends in the sequence $CTAAAAAA_n$, and so would be amplified by the primer combination $dT_{11}MG$, the transgene insert is truncated and ends in the sequence $AAATAAAAAA_n$. This sequence would not bind to the chosen reverse transcription primer set.

The high number of false positives seen with this technique could be due to contaminating cDNAs, which co-purify with the band of interest. During cloning, any of these products may be inserted into the vector such that the clone picked may not be the major band seen on the gel. Thus multiple bands must be cloned and sequenced to find the most abundant cDNA, that corresponds to the original band. Various methods have been suggested for reducing the number of false positives, including modifying the RT primers (Liang et al, 1994) and producing fluorescently labelled restriction enzyme fragments from the excised bands, which can be analysed to detect the most frequent pattern (Smith et al, 1997). A dot blot screening procedure has also been proposed to overcome this problem using radiolabelled cDNA isolated from the

original band or the DDRT-PCR reaction (Callard et al, 1994). Recently, a modified protocol was devised which amplifies the differential band directly from the gel and ligates the product into a plasmid, which is used to transform bacteria. Colony PCR is used on multiple clones and those containing the correct size product are dotted onto filters and probed with cDNA from the tissues of interest. This allows a high throughput of samples for less work and eliminates false positives during the procedure (Corton and Gustafsson, 1997).

A problem with the protocol described here is that there is always the possibility that the reverse transcription step will be primed by a 5′ primer rather than the $dT_{11}MN$ primer. This increases the possibility of amplifying an RNA species such as ribosomal RNA or nascent RNA, that does not contain a poly A^+ tail, and therefore does not give a true picture of the genes that are being expressed. The $dT_{11}MN$ primer is used to bias binding to the 3′ end of the molecule. This reduces the potential number of amplified bands and minimises artefacts caused by genomic contamination or cDNA quality. Several possibilities have been described for overcoming the problem of 5′ : 5′ products, including labelling the 3′ primer instead of the PCR product, so any 5′ : 5′ PCR products will not be labelled (Hadman et al, 1995), or designing the primers with a high AT content to bias binding towards the 3′ UTS (Graf et al, 1997).

The limited results obtained suggest that transfection and transgene expression do not alter the mRNA fingerprint dramatically in the B16.F1 sub-population of cells screened. However, the tendency of DDRT-PCR to produce a high number of false positives has hampered efforts to identify any of the small number of changes seen.

Chapter Five

Loss of expression of a transgene

INTRODUCTION

In order to ascertain whether transgene expression was a factor in the changes in growth parameters described in chapter three, it was necessary to establish whether the cells used were expressing the inserted transgene. It became apparent that the initial level of IL-2 expression from pNASSβ/IL-2 in B16 cells was high, but that the level decreased with increasing time in culture. Other B16 clones and a second cell line, were investigated to discover whether the expression loss was a characteristic of pNASSβ in this particular clone, or if the reduction in expression was seen in other transfected B16 cell lines or other model systems.

RESULTS AND DISCUSSION

5.1. Determination of initial interleukin-2 protein levels using an ELISA assay

The level of IL-2 expression from pNASSβ/IL-2, pBabeNeo/IL-2 or pBabeNeo/IL-2LTR, inserted into the B16 or CMT93 cells, was determined by performing an ELISA assay on the culture medium. Cells were seeded such that at the time of sampling the number was equivalent for all cell lines. The total amount of IL-2 produced is given to account for differing volumes of medium used, since it was necessary, in some cases, to decrease the volume of culture medium to give an IL-2 concentration within the limits of the sensitivity of the ELISA assay. Levels of IL-2 produced by the different cell lines at the lowest possible passage number are shown in Table 5.1. The initial levels of IL-2 were highest in B16 cells containing pNASSβ/IL-2. The levels expressed by both B16 and CMT93 cells containing their respective retroviral vectors were less than 20% of these levels. This could be due to the influence of enhancer elements at the site of insertion, which would vary between sites or be a feature of the vector. However, it was unknown how many passages these cells had passed through since transfection and so it is also possible that initial levels of expression were higher but had decreased in the cells used in these experiments. The B16 clone containing the plasmid vector was specifically chosen from several similar clones because it produced the highest levels of IL-2 (Vile and Hart, 1994c), also indicating that initial level of expression depends

Table 5.1. IL-2 levels produced by cell lines containing the IL-2 transgene

Cell Type	Initial level of IL-2 pg/10^6/72 hrs
B16 containing pNASSβ/IL-2 (passage 6)	3000-4000
B16 containing pBabeNeo/IL-2 (passage 4)	350-450
B16 containing pBabeNeo/IL-2 plus pNASS/HSV-tk (E26) (passage 6)	200-250
CMT93 containing pBabeNeo/IL-2LTR (passage 4)	650-850

The data on this graph are estimated from Figures 5.1, 5.2, 5.3 and further data not presented.

upon position of insertion. Expression from pBabeNeo/IL-2 in the cells transfected with this construct in addition to pNASSβ/HSV-*tk* (E26) was very low, barely reaching the lower threshold of detection of the assay (data not shown).

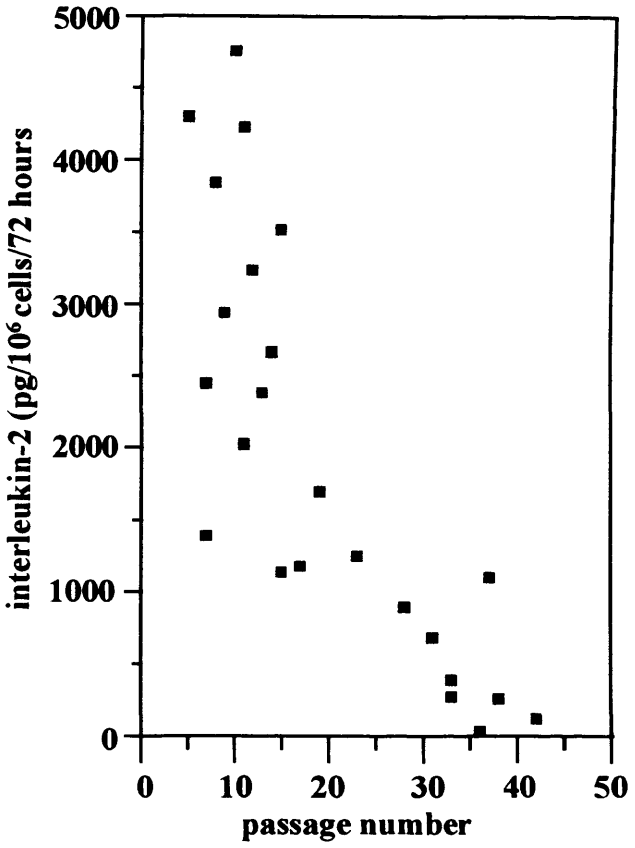
5.2. Production of IL-2 from pNASSβ/IL-2 with increasing passage number

The amount of IL-2 produced by low passage (passage 6-10) B16 cells containing pNASSβ was greater than 4000pg/10^6 cells/72 hours (Figure 5.1.). However, when higher passage cells were used the levels of IL-2 were significantly reduced. By passage 15 the amount of IL-2 produced had dropped by half and by passage 40, the equivalent of 20 weeks in culture, the levels had dropped to less than 10% of the original amount. For this reason, it was decided that, where possible, no growth studies would be carried out using cells beyond passage 15.

5.3. Production of IL-2 from pBabeNeo/IL-2 in B16 cells with increasing passage number

To investigate whether IL-2 transgene expression was lost when expressed from a retroviral construct, culture medium from B16 cells of increasing passage number containing pBabeNeo/IL-2 was also assayed. Unfortunately, the initial level

Figure 5.1. Interleukin-2 protein levels in B16 cells containing pNASSβ/IL-2.



Cells were seeded in a 75cm^2 flask and on day 3 the medium was sampled and used in an ELISA assay. Individual data points are shown.

of IL-2 protein production was near the lower threshold of sensitivity of the ELISA assay. As can be seen in Figure 5.2. the figures obtained for each passage number were very variable and this made it difficult to draw any firm conclusion as to whether transgene expression was lost in these cells. Levels of expression were too low in E26 cells to look for any changes in expression.

5.4. Production of IL-2 from pBabeNeo/IL-2LTR in CMT93 cells

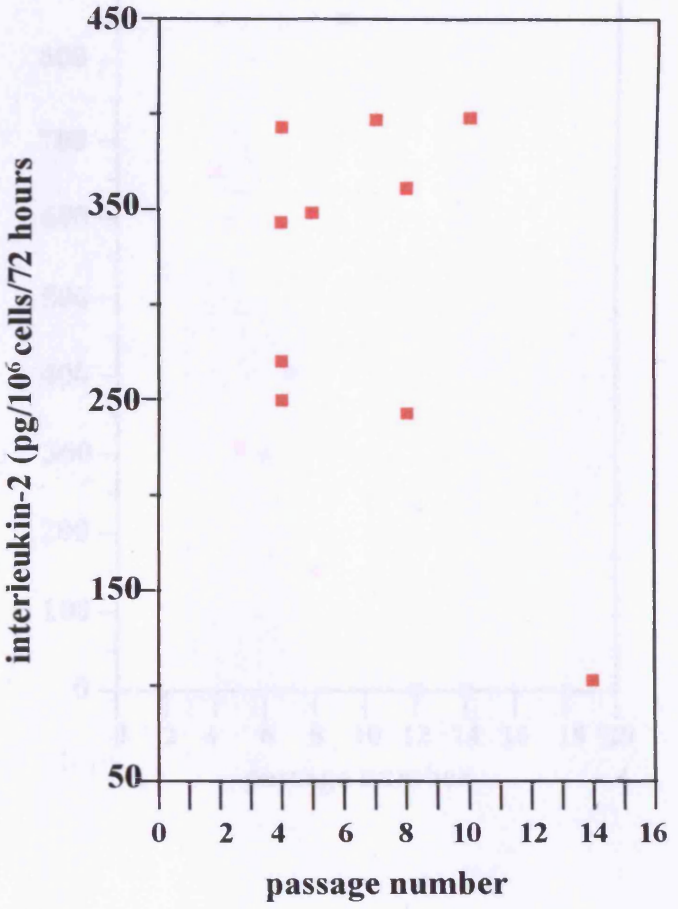
In order to investigate whether loss of expression of the IL-2 transgene was occurring in a different cell line, IL-2 protein production was monitored in the CMT93 cell line containing pBabeNeo/IL-2LTR. It was found that expression of the cytokine from the retroviral vector was lost with time such that by passage 14, the level of IL-2 was too low to be detected by ELISA assay (Figure 5.3.).

5.5. Production of HSV-*tk* from pNASSβ/HSV-*tk* in B16 cells

A cell line expressing HSV-*tk* from pNASSβ was investigated to determine whether expression of a different transgene was lost from this vector. Cells were treated with ganciclovir (GCV). Parental B16 cells are not affected by the drug, but cells that contain pNASSβ/HSV-*tk* should metabolise the prodrug to its toxic form, provided the transgene is being expressed. Low passage cells were treated with GCV and this drug completely inhibited cell growth, showing that the transgene was being expressed. The experiment was repeated using both low and high passage cells in order to assess whether transgene expression was lost. It was observed that both sets of cells were killed by GCV (Figure 5.4.). This shows that high passage cells continued to express HSV-tk.

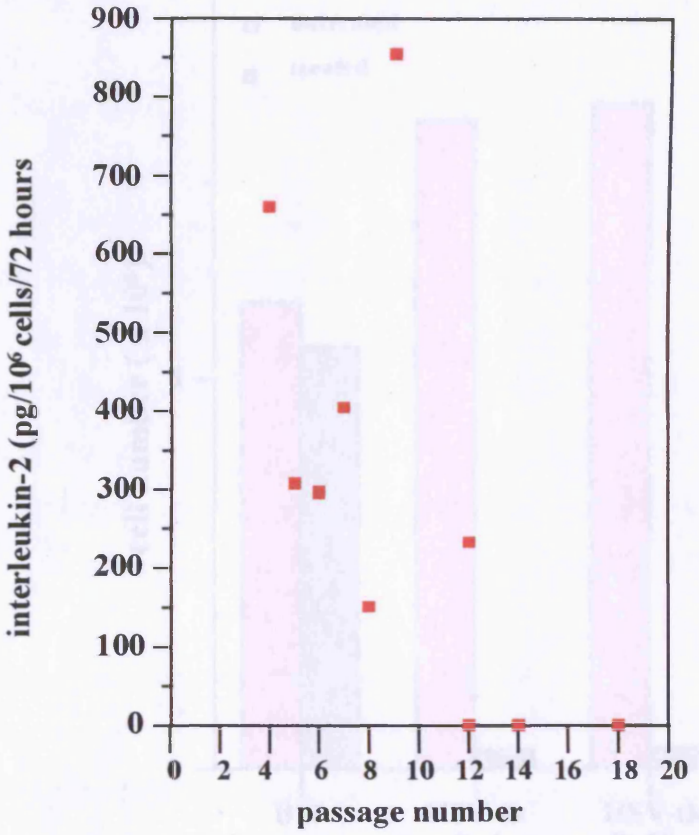
A complicating factor in this HSV-*tk*/GCV model is the presence of the bystander effect. It is possible that in the high passage cells fewer cells are expressing HSV-tk,

Figure 5.2. Interleukin-2 protein levels in B16 cells containing pBabeNeo/IL-2.



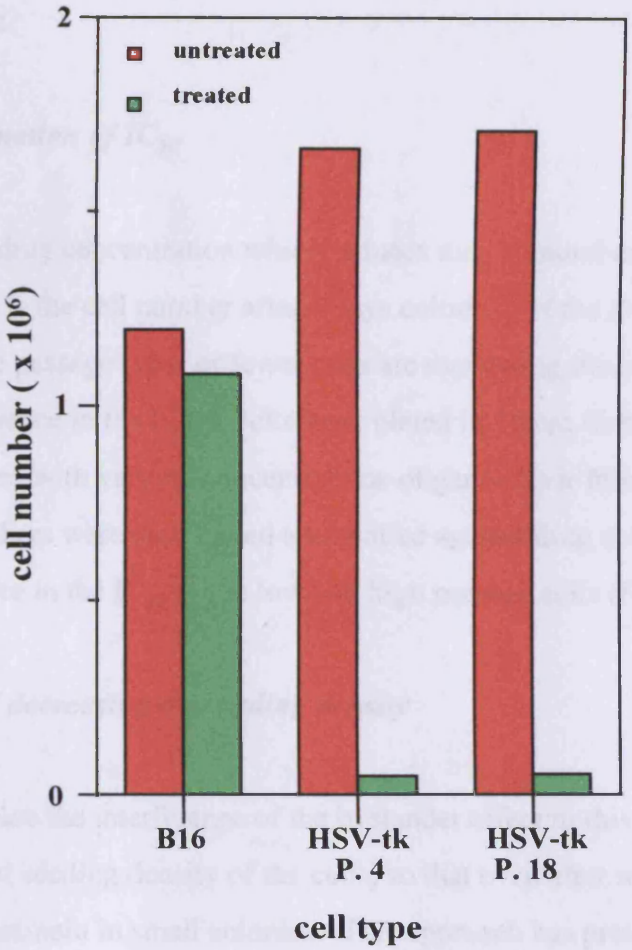
Cells were seeded in a 75cm^2 flask and on day 3 the medium was sampled and used in an ELISA assay. Individual data points are shown.

Figure 5.3. Interleukin-2 protein levels in CMT93 cells containing pBabeNeo/IL-2LTR.



Cells were seeded in a 75cm^2 flask and on day 3 the medium was sampled and used in an ELISA assay. Individual data points are shown.

Figure 5.4. Effect of treatment with ganciclovir on B16 cells expressing HSV-tk.



Cells were treated with ganciclovir (1μg/ml) to determine the effect on growth in low (passage 4) and high (passage 18) passage B16 cells containing pNASSβ/HSV-*tk*. This is a preliminary result from a single experiment.

but those that are, metabolise ganciclovir and pass the metabolite to adjacent cells, where it terminates DNA synthesis. This would mask the decrease in expression of HSV-tk. To attempt to determine whether this was the case two further experiments were carried out.

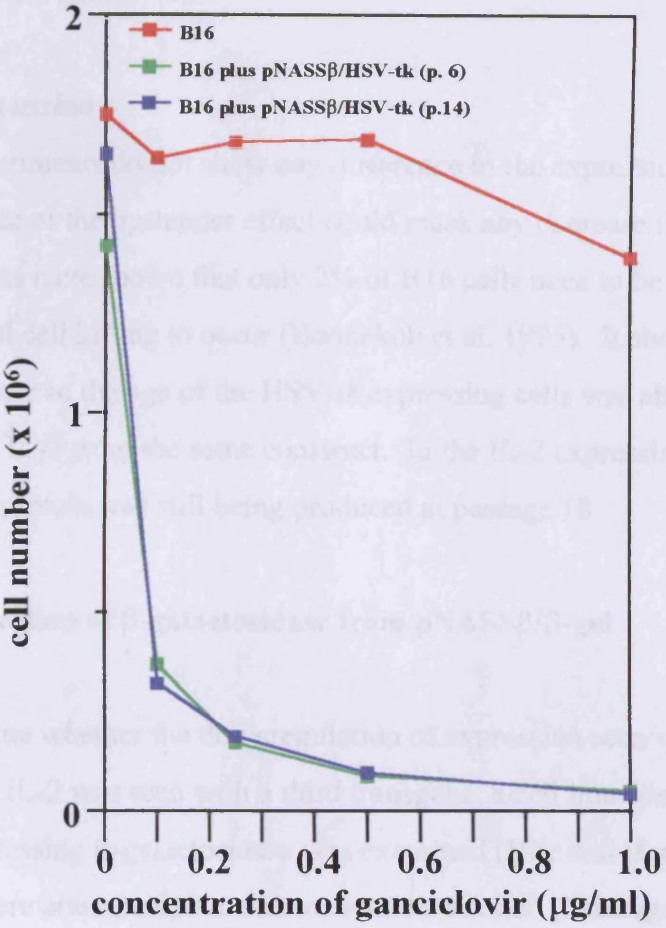
5.5.1. Determination of IC_{50}

The IC_{50} is the drug concentration which reduces the cell number by 50% (in this experiment this is the cell number after 4 days culture) . If the levels of expression are lower in the late passage cells, or fewer cells are expressing the transgene, this might result in a difference in the IC_{50}. Cells were plated in 35mm dishes and after 24 hours in culture, treated with varying concentrations of ganciclovir from 0 - 1.0μg/ml for 3 days. Cell numbers were determined and plotted against drug concentration. There was no difference in the IC_{50} of the low and high passage cells (Figure 5.5.).

5.5.2. Effect of decreasing the seeding density

One way to reduce the interference of the bystander effect in this experiment was to reduce the initial seeding density of the cells, so that even after several days in culture the cells would remain in small colonies. This approach has previously been shown to reveal differences in the degree of cell death in a culture when different percentages of HSV-tk expressing cells were added to a culture of parental cells (Elshami et al, 1997). The approach relies on the fact that the toxic metabolite is transferred between cells rather than being excreted into the medium, as outlined in the introduction (Section 1.6.2.). The ganciclovir metabolite can only be passed between cells in a colony. If some of these colonies have formed from a cell that had lost expression, none of the cells in this colony would be affected, because they would not be in close enough proximity to any cell that could pass on the toxic metabolite. A second possibility is that all the cells have a lower level of expression, which would be less likely to produce a difference in this experiment, as long as the level of expression is above the threshold required to produce an effect. A cell producing a lower level of HSV-tk would still

Figure 5.5. IC_{50} for ganciclovir in B16 cells expressing HSV-*tk*



B16 cells containing pNASSβ/HSV-*tk* were treated with ganciclovir at concentrations of 0, 0.1, 0.25, 0.5 and 1.0μg/ml of medium. A comparison of low and high passage cells was made to determine whether there was any difference in the susceptibility of the cells to ganciclovir that could be attributed to a loss of HSV-*tk* expression in the high passage cells. The diagram represents data from one experiment.

metabolise the GCV, and affect adjacent cells in the small colony. A general decrease in transgene expression in all cells would be more likely to produce a change in the IC_{50} and this was not seen. Cells were treated as in the previous section. As can be seen in Figure 5.6., both the high and low passage cells were killed to an equal extent by treatment with ganciclovir.

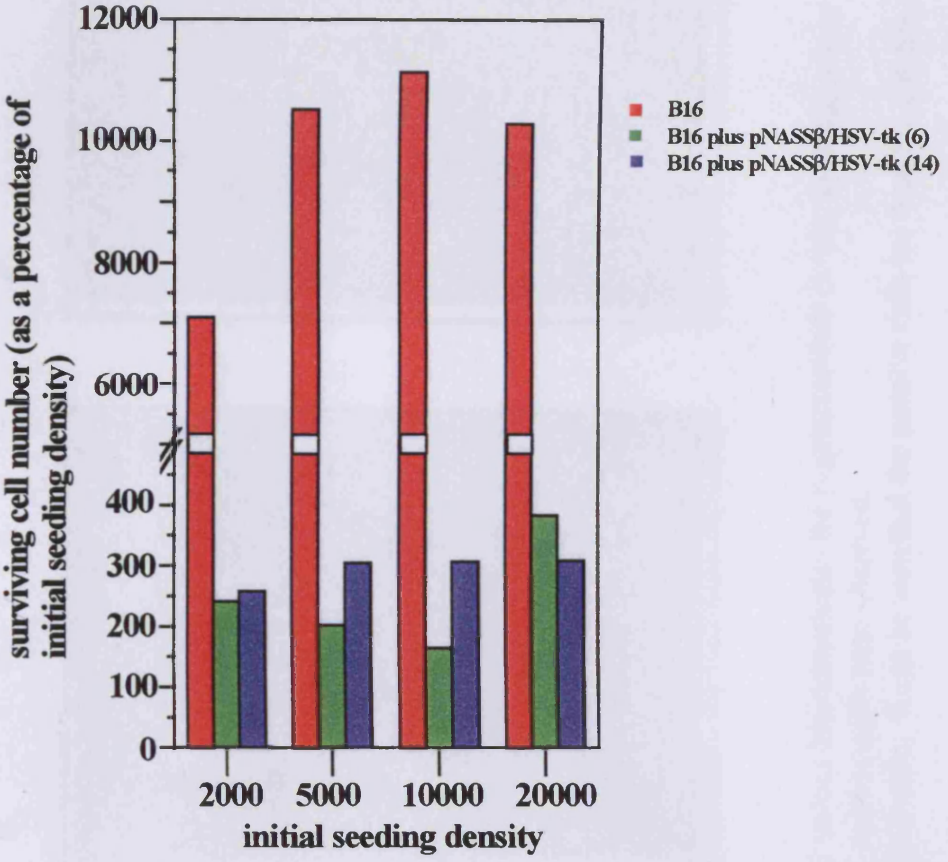
5.5.3. Discussion

These experiments do not show any difference in the expression level of HSV-*tk*, but the presence of the bystander effect could mask any decrease in expression. Experiments have shown that only 2% of B16 cells need to be expressing HSV-tk for almost total cell killing to occur (Bonnekoh et al, 1995). It should also be noted that the difference in the age of the HSV-*tk* expressing cells was about half that of the cells expressing IL-2 from the same construct. In the IL-2 expressing cells a substantial amount of protein was still being produced at passage 18.

5.6. Production of β-galactosidase from pNASSβ/β-gal

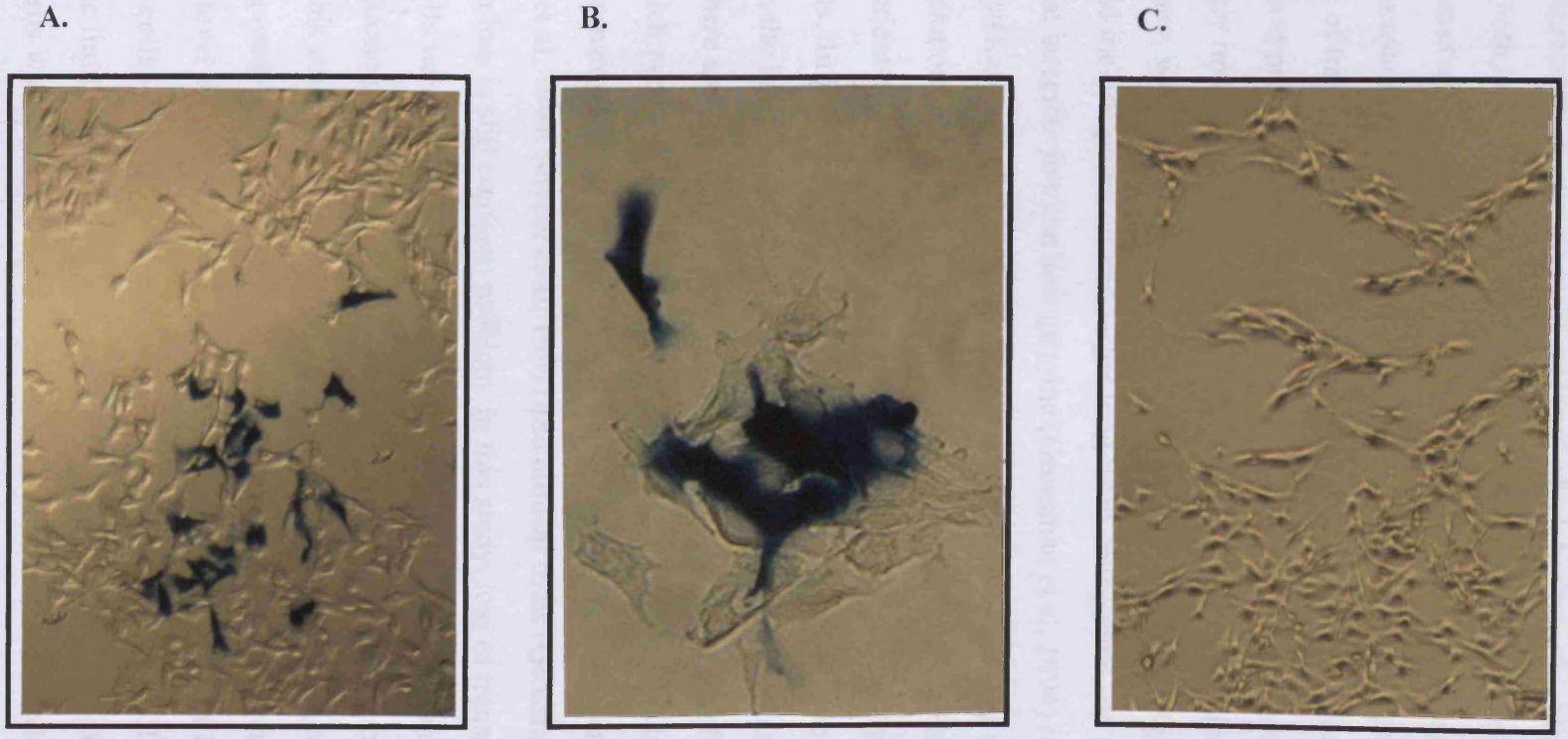
To determine whether the downregulation of expression seen with the constructs expressing IL-2 was seen with a third transgene, a cell line containing the pNASSβ vector expressing β-galactosidase was examined (Vile and Hart, 1993a). Several types of assay were attempted, but a histochemical method (Macregor et al, 1991) was the only one which was able to detect expression of this protein in the low passage cells (passage 6), where small colonies of cells expressed β-galactosidase (Figure 5.7.). The level of expression varied slightly between these colonies, although all cells in a colony had a similar level of expression. Expression was not apparent in high passage cells (passage 23). This suggests that other transgenes expressed from pNASSβ also exhibit a reduction in expression with increasing time in culture. However, the small number of positive cells in the lowest passage culture make it difficult to directly compare the rates of expression loss with pNASSβ/IL-2.

Figure 5.6. Effect of seeding density on susceptibility of B16 cells to ganciclovir



B16 cells containing pNASSβ/HSV-*tk* were seeded at varying densities, to investigate the effect this had on the susceptibility of low (passage 6) and high (passage 14) passage cells to ganciclovir (0.5μg/ml).

Figure 5.7. Expression of β-galactosidase in low and high passage cells



A. B16 cells (passage 6) containing pNASSβ/β-gal stained histochemically for β-galactosidase (x 10), showing colony of positive cells. Two 35mm wells were scanned and 18 colonies of positive cells were observed.
B.Another smaller colony (x 32) in the same passage 6 cells. It can be seen that the positive cells are found in patches, but not all the cells in the vicinity are positive.
C. shows B16 cells containing pNASSβ/β-gal at passage 23. None of these cells stained positively for β-galactosidase.

CONCLUSION

Loss of expression of a transgene is an issue which has been highlighted in the literature both *in vivo* (Dong et al, 1996, Kay et al, 1994, Palmer et al, 1991) and *in vitro* (Chen et al, 1997, Doll et al, 1996, Duch et al, 1994, Hoeben et al, 1991, Lange and Blankenstein, 1997, Melani et al, 1995). As protocols are improved with respect to efficiency of transduction and specificity of targeting, and initial levels of protein expression approach desired levels, maintaining expression levels will become increasingly important. Loss of transgene expression is incompatible with long term gene therapy, which requires the vector to remain active for the life time of the patient. This would include protocols that target long lived cells (Neve and Geller, 1996), use vectors that integrate into the host genome (Grossman et al, 1994) or are maintained episomally (Kaplitt et al, 1994). If long term expression cannot be achieved readministration of the vector will be necessary. This is undesirable from the point of view of patient comfort and safety, with each new course of therapy potentially exerting side effects. In some instances, repeated administration may not be possible. As detailed in the introduction, this is the case with adenovirus administration (Section 1.8.2.), where an immune reaction is initiated in the patient by the first dose of the vector which prevents further administration. While some groups have seen transgene expression maintained for 6 months or more in animal studies (Kiem et al, 1995, Rettinger et al, 1994, Snyder et al, 1997) and clinical trials (Grossman et al, 1994), expression loss is still common problem. In this study, loss of transgene expression in B16.F1 cells was demonstrated from the vector pNASSβ, containing either IL-2 or β-gal. A reduction of HSV-tk expression from this vector could not be demonstrated, although this could have been due to the bystander effect masking a reduction in the cell killing assay. A western blot method could clarify the question of whether there is a reduced level of protein production. A decrease in expression of IL-2 from pBabeNeo in B16.F1 cells was not observed, but even at the lowest passage, IL-2 production was close to the limits of detection for this assay. A similar construct expressing IL-2 in CMT93 cells was observed to lose expression. As mentioned previously (section 5.1.), the passage number of the B16.F1 cells containing pBabeNeo/IL-2 relative to transfection was unknown. It is possible these cells started with a greater level of

expression, but when the ELISA was performed in this study the cells had a greatly reduced expression level, because they had previously been cultured for some weeks. The fact that cells expressing β-gal appear to either express the protein strongly or not at all, suggests an on-off mechanism of downregulation. However, there is no experimental evidence for this hypothesis.

There are several explanations for why transgene expression is lost with time. An immune response could account for the initial loss of expression, with viral proteins (McDonald et al, 1997) or the transgene product (Wells et al, 1997) being recognised as foreign antigens, leading to destruction of the cell by immune effector cells. In the case of AAV vectors, initial expression levels result from large numbers of unintegrated vector, which are rapidly lost, leaving a small number of integrated vectors expressing the transgene (Doll et al, 1996). Similarly, other episomal vectors will be lost during replication, unless they are modified to replicate episomally, or are used to modify cells which are not dividing, such as airway epithelium (Dong et al, 1996) or skeletal muscle (Naffakh et al, 1996).

While some cases of loss of expression can be easily explained, many are thought to be due to mechanisms occurring at the DNA level. The loss of transgene expression from two constructs used in this study is consistent with the idea that loss of expression is a common occurrence. The decrease cannot be attributed to an immune response, or loss of an episomal construct. In the following chapter, the mechanism of transgene downregulation in pNASSβ/IL-2 is explored further. Defining the mechanism could improve the design of vectors to allow maintenance of transgene expression, a major benefit in the process of developing gene therapeutic protocols.

Chapter Six

Investigation of loss of transgene expression

INTRODUCTION

The experimental work described in this chapter was designed to determine the mechanism of IL-2 transgene expression loss from pNASSβ/IL-2. Such information could be useful in understanding why some constructs lose expression, while others do not. This is an important step in the goal of designing effective vectors for use in gene therapy.

Levels of protein expression in a cell can be regulated at the level of transcription or translation, whether from an endogenous or introduced gene. One additional explanation which could apply to the latter, is that the vector carrying the transgene is recognised as foreign DNA and is ejected from the host genome (Stone et al, 1986). If this occurs randomly within a population of cells, it would result in a gradual decrease in transgene protein levels as seen in this study. Experiments were carried out to determine whether such a mechanism was operative in the B16.F1 model.

RESULTS AND DISCUSSION

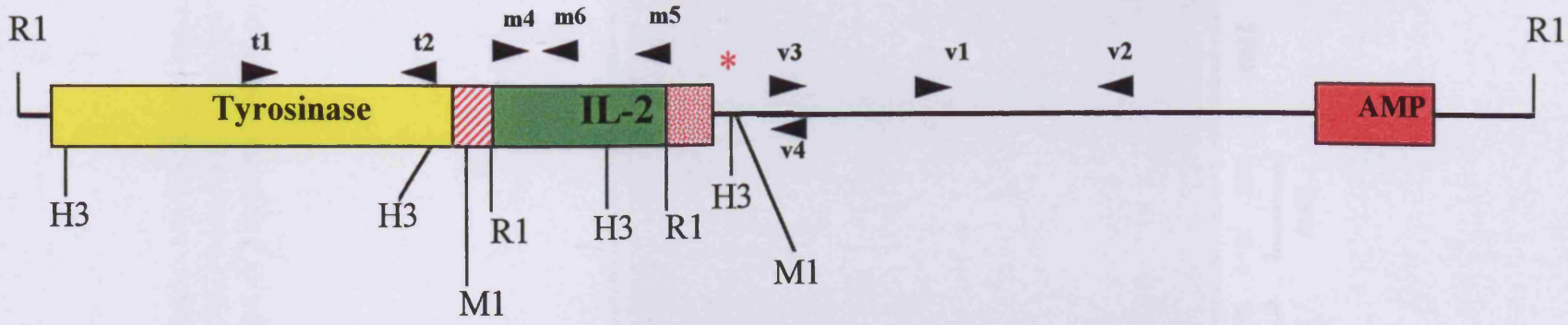
6.1. Presence of pNASSβ/IL-2 in the host genome

Two methods were used to investigate whether loss of the vector or transgene from the genome of B16.F1 cells containing pNASSβ/IL-2 was the explanation for the decreased protein levels.

6.1.1. Genomic PCR to detect integrated vector

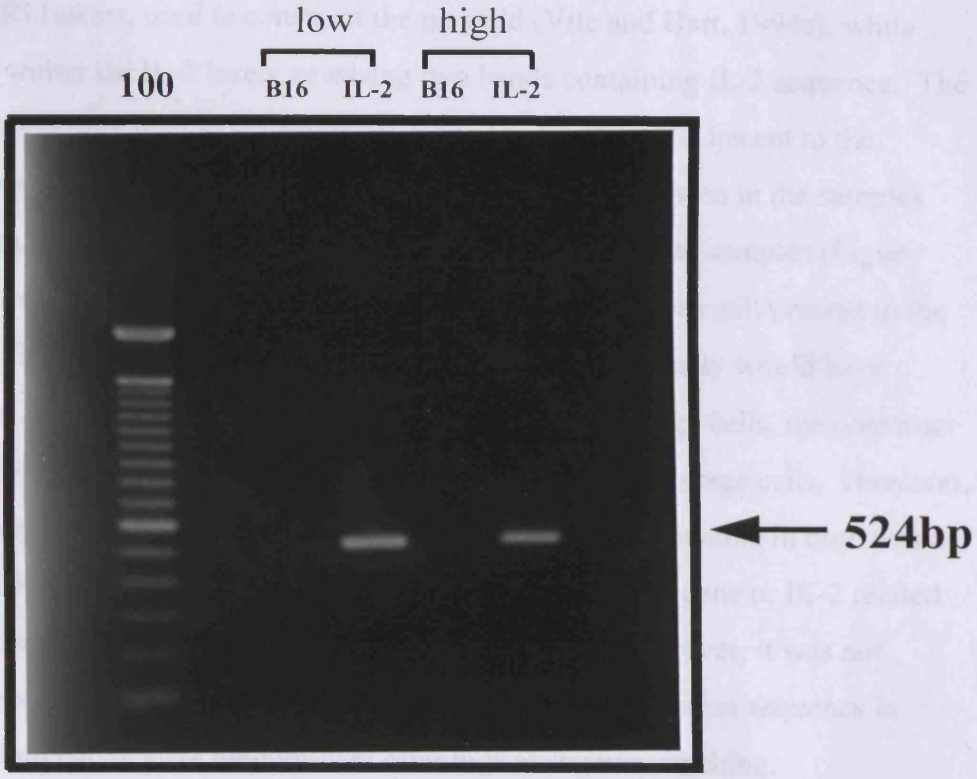
Genomic DNA, prepared from the B16 clone containing pNASSβ/IL-2, was used in a PCR reaction containing primers specific for the vector sequence (vec1 and 2, Figure 6.1.). The expected product of 524bp was seen in both low and high passage cells (Figure 6.2.), confirming that the vector pNASSβ was still present in the high passage cells.

Figure 6.1. Position of the oligomer primers used to analyse pNASSβ/IL-2



A scheme showing the positions of relevent restriction sites (*Eco*R1 (R1), *Hind*III (H3) and *Msp*I (M1)) and primer binding sites relative to the first base of pNASSβ. The tyrosinase promoter (-2540 to -46) drives expression of the IL-2 transgene. The lined box ▨ represents the splice donor sequence, while the dotted box ▩ represents the poly A signal. Four primers which bind in pNASSβ (vec (v)1-4), three primers specific for IL-2 (muril (m) 4-6) and two primers specific for the tyrosinase promoter (t 1 and 2) are shown. A possible point of recombination is shown (*).

Figure 6.2. Confirmation of the presence of pNASSβ in the genome of B16 cells.



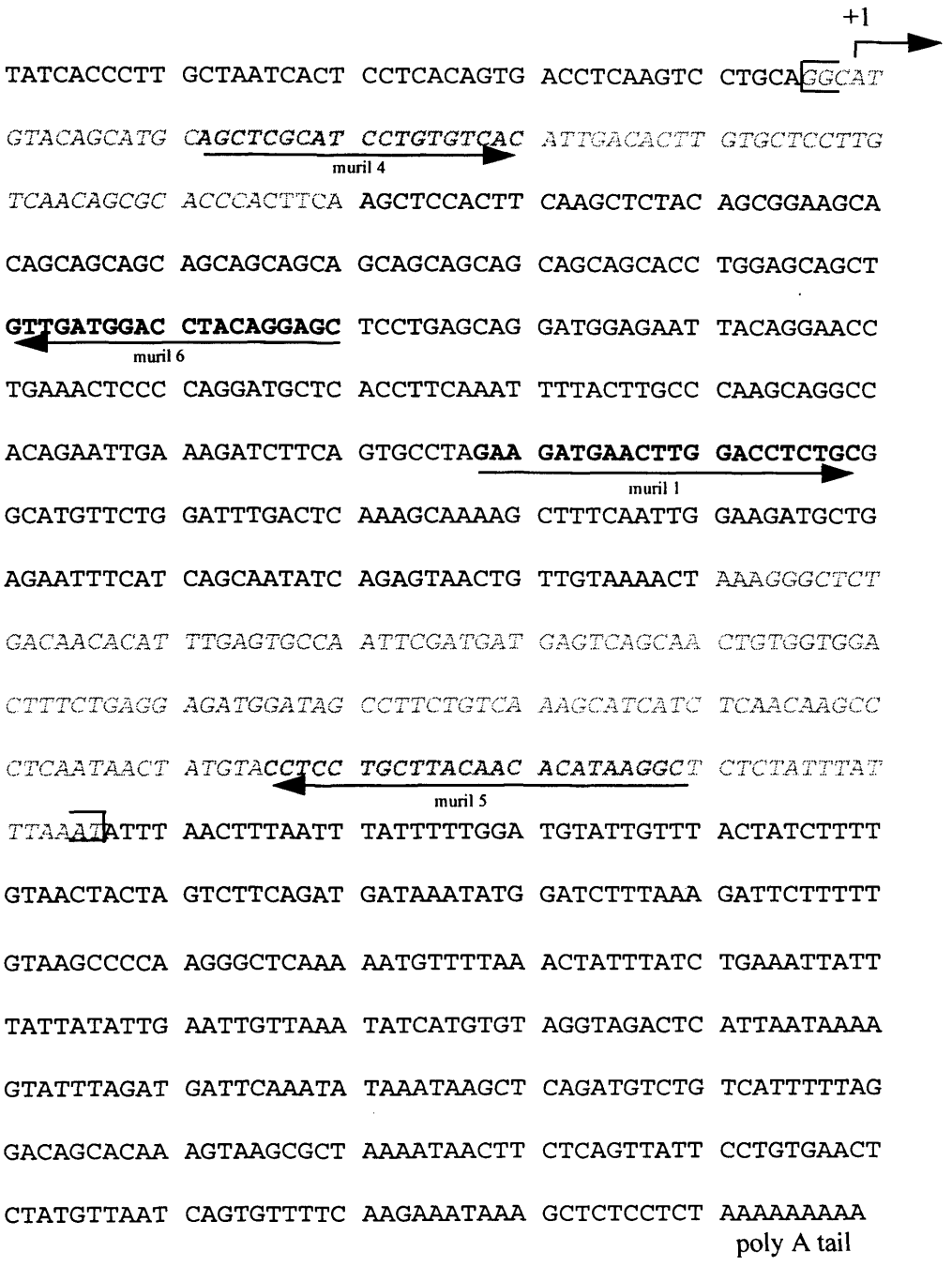
Genomic DNA (100ng) was amplified in a PCR reaction using primers (vec 1 and vec 2) specific for pNASSβ. The expected product of 524bp was seen in low and high passage B16 cells containing the vector, but not in parental B16 cells.

6.1.2. Restriction digest to release IL-2 transgene

Genomic DNA, isolated from B16 clones, was digested with *Eco*RI or *Hind*III. These enzymes should digest the mouse IL-2 construct into predictable fragment sizes. When the digested DNA was probed with a 159bp IL-2 specific probe (muril 4 to 6, Figure 6.1. and 6.3.), the probe bound to these bands, which were then visualised by autoradiography. As can be seen from Figure 6.1., *Eco*RI is predicted to excise the insert in the RI linkers, used to construct the plasmid (Vile and Hart, 1994c), while *Hind*III cuts within the IL-2 insert, releasing two bands containing IL-2 sequence. The 159bp probe will only bind to the fragment of the IL-2 transgene adjacent to the tyrosinase promoter. In digests with either enzyme, bands were seen in the samples prepared from cells containing pNASSβ/IL-2, and not in any other samples (Figure 6.4.). This confirmed that the transgene as well as the plasmid was still present in the host genome, in high and low passage cells. A loss in band intensity would have suggested that, despite being present in some of the higher passage cells, the construct had been lost from the high passage cells compared to the low passage cells. However, this did not appear to be the case, with band intensity being comparable in both sets of cells. It should have been possible to detect the endogenous IL-2 gene or IL-2 related sequences existing in the mouse genome (Fuse et al, 1984). However, it was not possible to predict the size of the endogenous band because the intron sequence is unknown and no bands were predominant after high stringency washing.

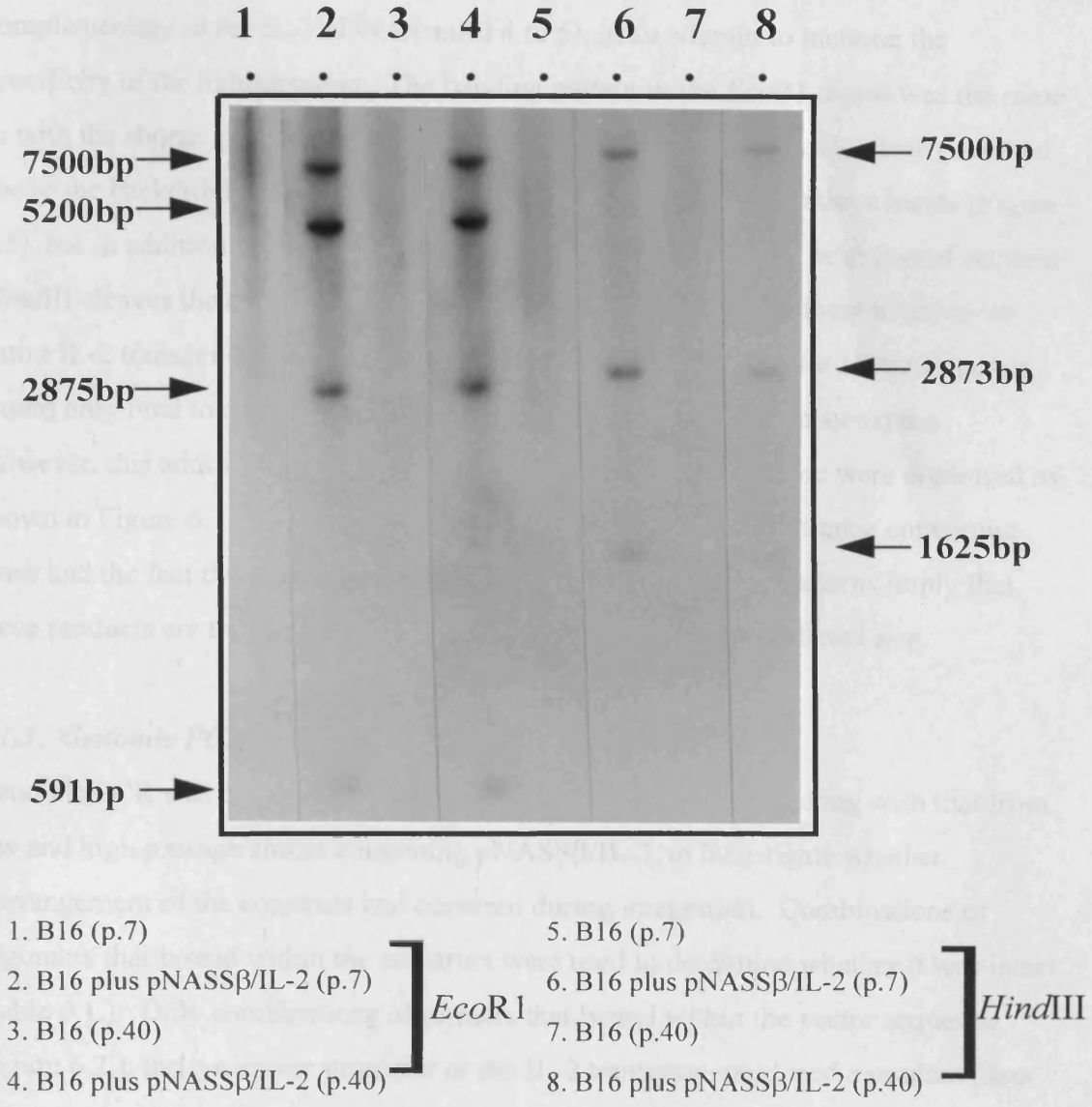
Although the bands obtained from the digests were specific for the IL-2 insert when probed with the 159bp fragment, the predicted size of the fragments did not correspond with the sizes of these bands. In the *Eco*R1 digest, the expected band of 591bp was observed, but three additional bands were also seen. The *Hind*III digest released three bands instead of the predicted single band. Partial digestion was a possibility, but seemed unlikely due to the long digestion time and the excess of enzyme used. In addition the theoretical band sizes that would have been released by partial digestion did not correspond to the actual fragments either. One difficulty with predicting the restriction fragment sizes was that it was not clear at which site the plasmid recombined when it inserted into the host genome. The fact that IL-2 was expressed, implies that the transgene and at least part of the tyrosinase promoter region were intact, but it is

Figure 6.3. Mouse IL-2 cDNA sequence



The sequence of mouse IL-2 cDNA. The sequence of the IL-2 transgene (561bp) is enclosed by brackets [ ]. The muril primers used in RT-PCR and PCR reactions are marked in bold and the sequence in italics was confirmed by manual sequencing.
Yokota et al, 1985, EMBL/GENBANK accession no. MMIL2T

Figure 6.4 Confirmation of the presence of the pNASSβ/IL-2 in high passage cells.



Genomic DNA (10μg) was digested with EcoR1 or HindIII and the samples separated on a 0.8% agarose gel. The resulting blot was hybridised with a 159bp probe specific for mouse IL-2. The bands sizes are as follows: EcoR1, 7500bp, 5200bp, 2875bp, 600bp (which corresponds to the predicted 591bp fragment), HindIII, 7500bp, 2875bp, 1625bp.

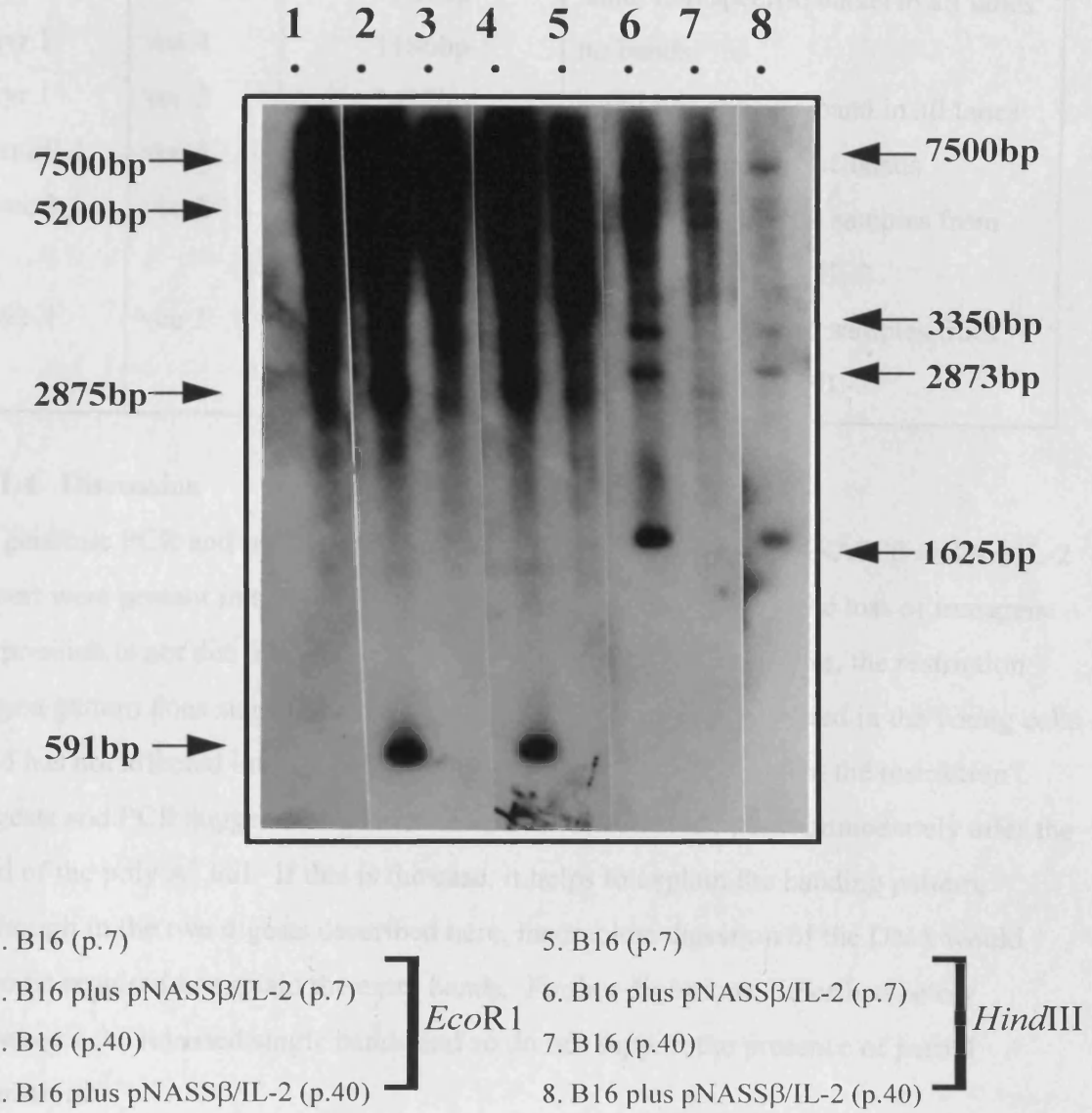
possible that the plasmid could have recombined at a point that left the restriction sites in a different position to that shown in Figure 6.1. in relation to each other.

The blot used in Figure 6.4 was reprobed with a longer 528bp fragment, complementary to the IL-2 cDNA (muril 4 to 5), in an attempt to increase the specificity of the hybridisation. The banding pattern in the *Eco*R1 digest was the same as with the shorter probe, although the larger *Eco*RI bands were not as clearly defined above the background. The *Hind*III digest also produced the same three bands (Figure 6.5), but in addition a fourth band was also observed. This would be expected because *Hind*III cleaves the transgene into two fragments. The 528bp fragment binds to the entire IL-2 transgene, and so would detect both fragments, while the 159bp fragment would only bind to one half of the transgene when digested with this enzyme. However, this additional band is not of the predicted size if the gene were organised as shown in Figure 6.1.. Both the specificity of the bands for the transgene containing lanes and the fact that two different probes give similar banding patterns imply that these products are transgene-specific, despite not being of the predicted size.

6.1.3. Genomic PCR to determine arrangement of the construct

Genomic PCR was carried out on DNA prepared from B16 cells, along with that from low and high passage clones containing pNASSβ/IL-2, to investigate whether rearrangement of the construct had occurred during integration. Combinations of oligomers that bound within the construct were used to determine whether it was intact (Table 6.1.). Only combinations of primers that bound within the vector sequence (Figure 6.2.), the tyrosinase promoter or the IL-2 transgene, produced a product (data not shown). The products resulting from use of the primer complementary to the tyrosinase promoter were not specific for the transgene because they were seen in all lanes, indicating that the endogenous copy of this tyrosinase gene had been amplified. Any primer combination that involved two different segments of the construct (see coloured fragments in Figure 6.1.) did not produce a product. It would appear that all the components are present and intact, although not necessarily in the original order, but the tyrosinase promoter should still be upstream of the IL-2 transgene to allow expression to occur.

Figure 6.5. Confirmation of the presence of the pNASSβ/IL-2 in high passage cells.



Genomic DNA (10μg) was digested with EcoR1 or HindIII and the samples separated on a 0.8% agarose gel. The gel was Southern blotted and the blot hybridised with a 159bp probe specific for mouse IL-2. The bands sizes are as follows: EcoR1, 7500bp, 5200bp, 2875bp, 600bp (which corresponds to the predicted 591bp fragment), HindIII, 7500bp, 3350bp, 2875bp, 1625bp.

Table 6.1. Genomic PCR to investigate the pNASSβ/IL-2 construct

5′ primer	3′ primer	expected product	product
tyr 1	tyr 2	1024bp	expected product seen in all lanes
tyr 1	muril 5	1806bp	small non specific bands in all lanes
tyr 1	vec 4	1186bp	no bands
tyr 1	vec 2	2427bp	small non specific band in all lanes
muril 4	vec 4	911bp	multiple non specific bands
vec 3	vec 2	1263bp	expected product in samples from B16 plus pNASSβ/IL-2
vec 1	vec 2	524bp	expected product in samples from B16 plus pNASSβ/IL-2

6.1.4. Discussion

A genomic PCR and restriction digest study confirmed that both pNASSβ and the IL-2 insert were present in the low and high passage cells. Therefore, the loss of transgene expression is not due to loss of part or all of the construct. However, the restriction digest pattern does suggest rearrangement. This has already occurred in the young cells and has not affected initial levels of expression. The evidence from the restriction digests and PCR suggest that recombination occurred at the point immediately after the end of the poly A^+ tail. If this is the case, it helps to explain the banding pattern, although in the two digests described here, incomplete digestion of the DNA would also be required to explain the extra bands. Further digestions, described below (section 6.3.) released single bands and so do not support the presence of partial digestion.

Restriction digests of genomic DNA should also give an idea of copy number. If the chosen restriction site cleaves only once in the vector, then the next site will be at a random point in the genomic DNA. The distance to the next site will vary, depending on the integration site. This means that for each integration, a different band size should be seen. Unfortunately, in these experiments all the restriction sites used occurred twice within the vector, and so the same size bands would be released, regardless of the integration site. Multiple copies of the fragment would give a more

intense signal than a single copy, but a dilution series of plasmid DNA was not run with these samples. In this case, the additional possibility of rearrangement would have confounded attempts to investigate copy number further.

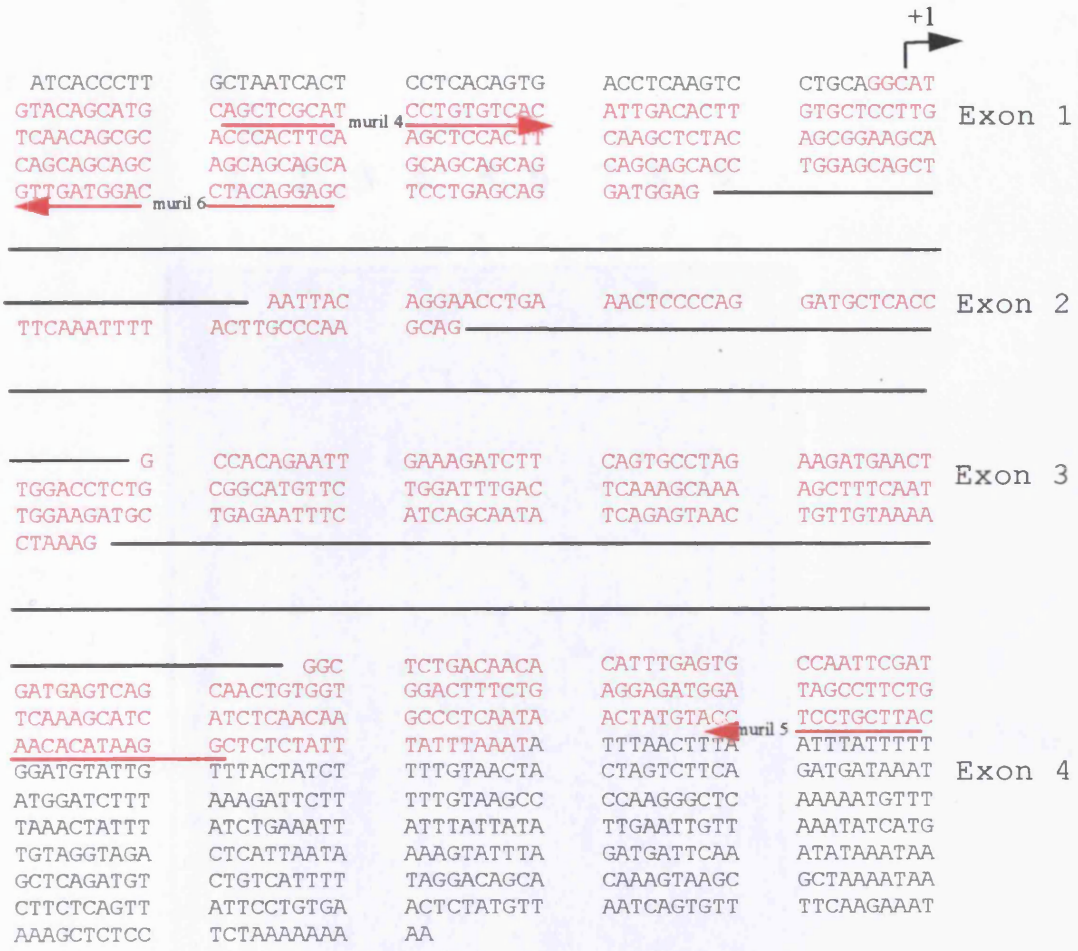
6.2. Investigation of mRNA levels in B16 cells containing pNASSβ/IL-2

Another possible explanation for reduced protein levels is a decrease in the level of translation of the transcript (reviewed by Gray and Hentze, 1994), or alternatively, a decrease in the availability of the transcript. This could be caused by a change in the stability of the mRNA or in a decrease in transcription. The latter option has been seen to occur in several transgene systems (Hoeben et al, 1991, Rettinger et al, 1994). Having established that the construct was intact, the next step was to investigate whether the decrease in IL-2 transgene protein was due to a translational effect, or reflected a difference in the level of mRNA.

6.2.1. Continuous RT-PCR

The level of mRNA in low and high passage cells was investigated, using a semi-quantitative RT-PCR reaction. Total RNA from parental cells and those containing pNASSβ/IL-2 was used with two oligomers specific for murine IL-2 and the products analysed by Southern blotting (muril 4 and 5, Figure 6.3). The blot was hybridised with an internal oligomer specific for murine IL-2 (muril 6, Figure 6.3.). The final autoradiograph was analysed using densitometry. The relative intensity of each IL-2 band was normalised using GAPDH, which compensated for any variation in the amount of template that was added to the RT-PCR reaction. The possibility of genomic contamination was eliminated by the use of a reverse transcription primer (muril 5) that bound in exon 4 of the genomic DNA. This meant that if any genomic DNA was present, a much larger product would be formed, due to the presence of 3 introns found in the genomic sequence, but not present in the cDNA insert (Figure 6.6.). It can be seen (Figure 6.7., lanes 5 and 6) that there was a marked decrease in the level of IL-2 mRNA in the high passage cells compared to the low passage cells. This shows that the level of protein is decreasing in the high passage cells because there is a decrease in the

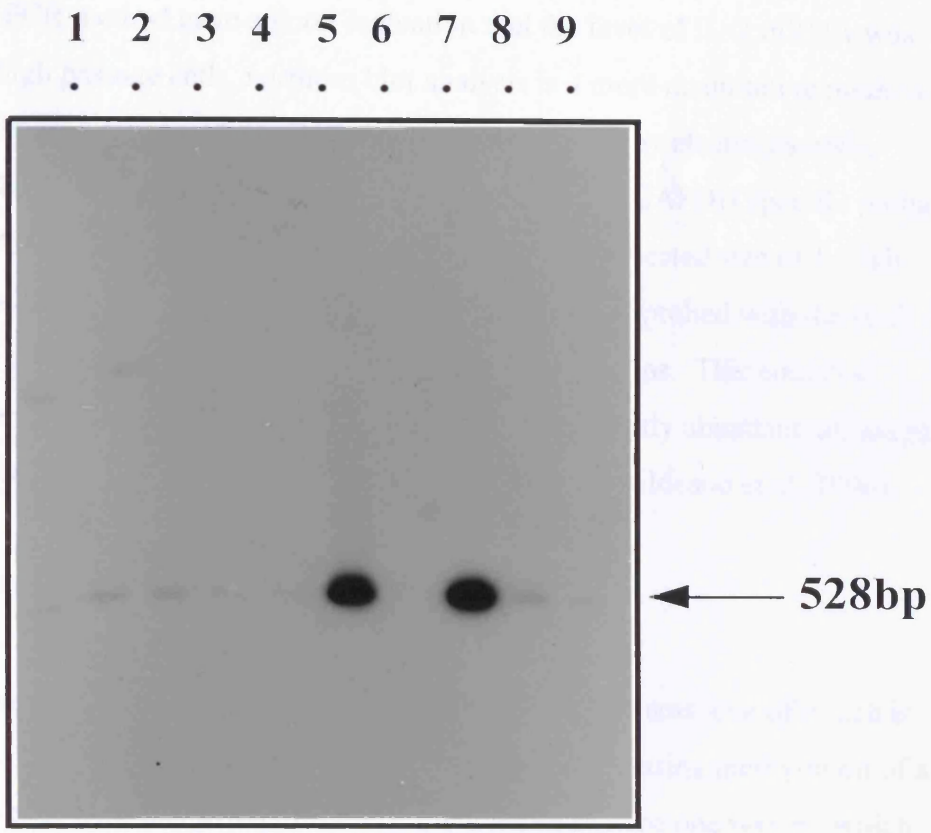
Figure 6.6. Gene structure of mouse IL-2



The mouse IL-2 gene consists of 4 exons. The IL-2 sequence contained in pNASSβ and pBabeNeo is highlighted in red. The start of translation is shown (+1). The oligomers used to make the 159bp (muril 4 and 6) and 528bp (muril 4 and 5) IL-2 specific probes are also indicated.

Fuse et al, 1984

Figure 6.7. MSH upregulated IL-2 mRNA production in B16 cells that have lost transgene expression



1. negative
2. B16
3. B16
4. B16, 10^{-6} M MSH
5. B16 plus pNASSβ/IL-2 (p.11)
6. B16 plus pNASSβ/IL-2 (p.42)
7. B16 plus pNASSβ/IL-2 (p.42), 10^{-6}M MSH
8. B16 plus pNASSβ/IL-2 (p.42), 10^{-9}M MSH
9. B16 plus pNASSβ/IL-2 (p.42), 10^{-11}M MSH

A continuous RT-PCR reaction was carried out using a two primers specific for murine IL-2. The products were separated by electrophoresis and blotted onto Hybond N^+. The blot was hybridised with a third primer, specific for mouse IL-2, that bound within the sequence of the PCR product. This confirmed that the PCR product was specific for IL-2. The band intensity was normalised using a parallel GAPDH reaction (data not shown). Quantitation of data showed that MSH induced levels of IL-2 mRNA five times greater than the levels found in passage 11 cells.

level of mRNA. This experiment does not distinguish between reduced production or a decrease in stability of the mRNA.

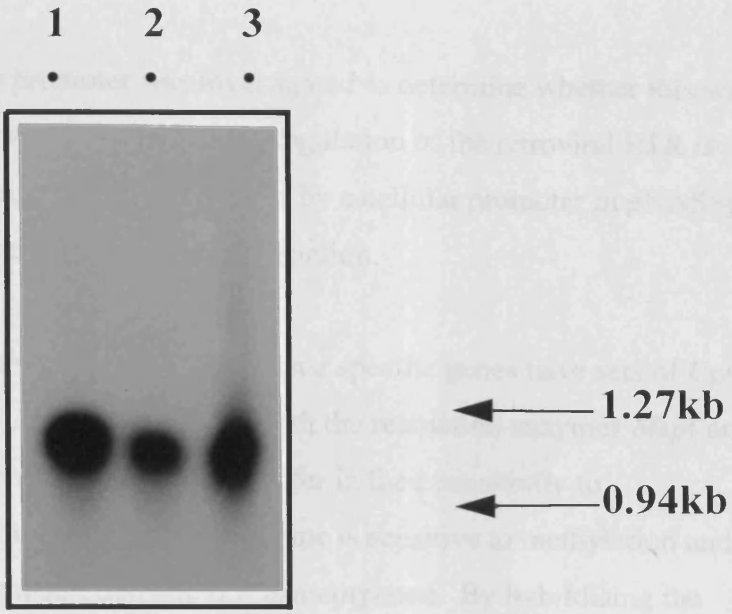
6.2.2. Northern blot

While the RT-PCR method gave a good indication that the level of IL-2 mRNA was decreasing in high passage cells, northern blot analysis is a more quantitative method. Messenger RNA was prepared from B16 clones and separated by electrophoresis, blotted and hybridised with a 528bp IL-2 specific or a 1039bp GAPDH specific probe. Despite the GAPDH probe producing a specific band of the expected size of 1.27kb (Figure 6.8.), a band was not produced when the same blot was probed with the IL-2 specific probe, despite several changes in experimental conditions. This could be because, even using mRNA, the IL-2 message was not sufficiently abundant, although other workers have obtained a signal using poly A^+ RNA (de Galdeano et al, 1996).

6.3. Methylation study

A cell can regulate transcription of its genes via several mechanisms, one of which is methylation of CpG dinucleotides in the promoter region. Increasing methylation of a transgene promoter, with successive rounds of replication, may be one way by which expression of foreign DNA is lost. Methylation of DNA can affect the binding capacity of certain transcription factors (Tate and Bird, 1993), although the decrease in activity may not be sufficient to explain the inactivity of the promoter *in vivo*. Alternatively, methylated DNA may bind transcriptional repressors, which modify the chromatin structure and prevent transcription occurring (reviewed by Kass et al, 1997). This is an attractive model because it would be possible for large stretches of DNA to be coordinately downregulated. If a transgene was inserted into an already inactive region, it would be susceptible to downregulation. The retroviral LTR has been found to be inactive or downregulated by methylation in some cell types (Challita and Kohn, 1994, reviewed by Szyf, 1996). In some cases this is at least partly due to methylated regulatory elements in the LTR enhancer, binding repressor proteins (reviewed by Lund et al, 1996). A study in fibroblasts has shown that the position of insertion is important in determining whether an inserted gene is shutdown by methylation, and that once

Figure 6.8. Confirmation of intact mRNA on northern blot



1. B16
2. B16 plus pNASSβ/IL-2 (passage 8)
3. B16 plus pNASSβ/IL-2 (passage 41)

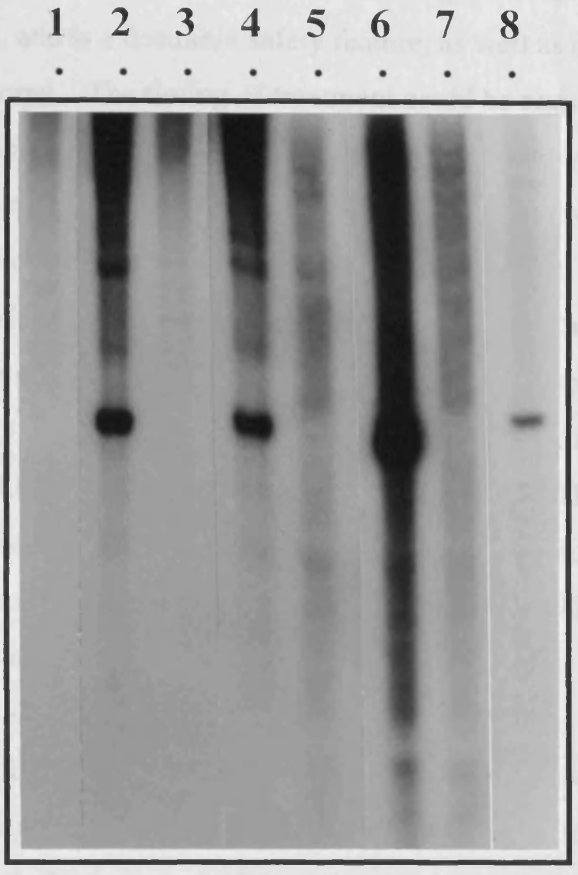
A northern blot carrying mRNA from B16 parental cells (9.1μg) and cells containing pNASSβ (4μg) and pNASSβ/IL-2 (4.1μg) was hybridised with GAPDH to confirm that the mRNA was intact (1.27kb). A signal was not obtained when a probe specific for IL-2 was used. The expected product size of 0.94kb is indicated.

expression is lost, it can be reinstated by treatment with 5-azacytidine, a DNA methylase inhibitor (Hoeben et al, 1991). Downregulation by methylation cannot be reversed by this treatment in all cell types, which may reflect whether methylation is the cause of the shutdown, or is merely a secondary event (reviewed by Lund et al, 1996).

Methylation of the tyrosinase promoter was investigated to determine whether this was the cause of the transgene downregulation. Downregulation of the retroviral LTR is ruled out because transgene expression is promoted by a cellular promoter in pNASSβ, but this promoter could still be susceptible to methylation.

DNA is methylated at CpG base pairs and many tissue specific genes have sets of CpG in their promoters. Genomic DNA was digested with the restriction enzymes *Msp*I and *Hpa*II, which cleave at the same CCGG site, but differ in their sensitivity to methylation of the internal C base. The *HpaII* enzyme is sensitive to methylation and will not cleave the DNA at its restriction site if it is methylated. By hybridising the DNA with an IL-2 specific probe, the methylation status of the construct was determined. If the DNA was unmethylated both enzymes should release a fragment of 990bp. If the DNA had become methylated in high passage cells, the *HpaII* would not recognise the site and would, therefore, produce a larger band. The same band of 3100bp was obtained from DNA of both high and low passage cells, which was specific for the samples prepared from B16 cells containing pNASSβ/IL-2. As with other earlier digests, using different enzymes, this band was not of the predicted size. Any other bands occurred in all lanes and were considered to be non-specific (Figure 6.9.). Larger bands were not seen in the high passage sample digested with *Hpa*II. Although the released band is not of the predicted size, the fact that both enzymes give the same banding pattern suggests that a change in methylation status is not the explanation for decreased expression.

Figure 6.9. Methylation study by restriction digest analysis



1. B16 (p.7)
2. B16 plus pNASSβ/IL-2 (p.7)
3. B16 (p.40)
4. B16 plus pNASSβ/IL-2 (p.40)

*Msp*I

5. B16 (p.7)
6. B16 plus pNASSβ/IL-2 (p.7)
7. B16 (p.40)
8. B16 plus pNASSβ/IL-2 (p.40)

*Hpa*II

Genomic DNA (10μg) was digested with the restriction enzymes *Msp*I and *Hpa*II. Both recognise the sequence CCGG, but *Msp*I is methylation insensitive while *Hpa*II is methylation dependent. If DNA is unmethylated both enzymes will produce the same sized band, but if the DNA has become methylated HpaII will fail to digest the DNA at these sites and larger bands will be produced. By hybridising the blot with an IL-2 specific probe, methylation status in the region of the IL-2 gene can be studied. High passage (40) cells were compared with low passage (7) cells.

6.4. Reactivation of transgene expression by MSH

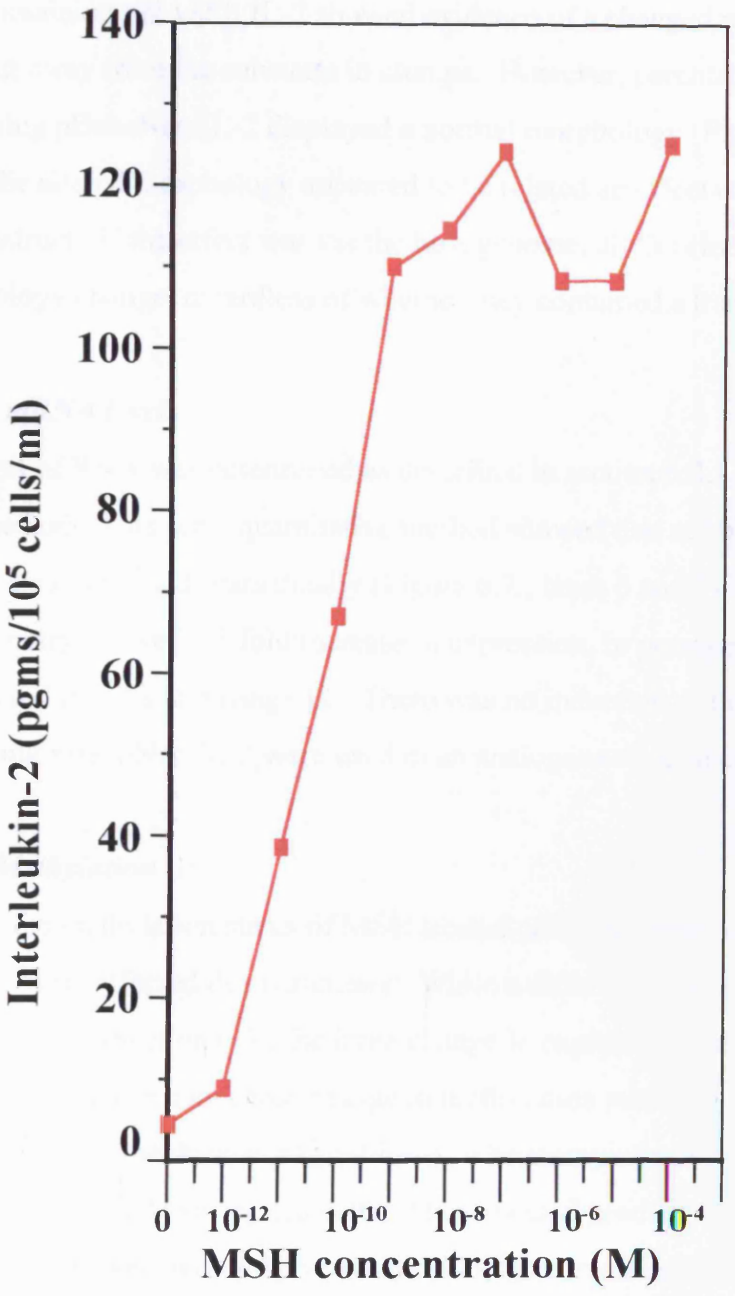
One of the areas under investigation in gene therapy is that of inducible promoters (Suzuki et al, 1996). The ability to up and downregulate expression of a transgene increases control, and is a desirable safety feature, as well as increasing the possibility of successful therapy . The timing of treatment could be regulated, and if expression of a transgene was lost the possibility exists for re-expression by treatment with an inducer. Melanocytes or melanoma cells can be influenced by several extracellular factors, which determine whether melanin production occurs. Melanocyte stimulating hormone is one of the best characterised of these. It is a product of the pro-opiomelanocortin gene and is produced in the posterior pituitary (Hearing and Tsukamoto, 1991). When the peptide binds to its receptor, it increases melanocyte proliferation (de Luca et al, 1993) and melanin production. The latter is caused by an increase in the levels of tyrosinase protein, which is thought to be due, at least partially, to an increase in transcription of the gene (Haganson et al, 1989), but also to post translational regulation.

Preliminary work by another member of the group, in which MSH was added at varying concentrations to cell culture, showed that this peptide could upregulate expression of the IL-2 transgene in B16 cells containing pNASSβ/IL-2, after expression had been lost. This piece of information was used in this study to investigate further the loss of expression, by examining the mechanism by which MSH caused the re-expression of IL-2.

6.4.1. Protein levels

Cells were treated with varying concentrations of MSH for 2 days, and an ELISA assay was performed on the medium to ascertain the level of IL-2 protein being expressed by the cells. It was found that B16 cells of high passage, containing pNASSβ/IL-2, that had lost transgene expression, were induced to re-express IL-2 (Figure 6.10.). The maximum induction, of approximately 100 fold in passage 45 cells, was obtained with treatment of 10^{-7}M MSH. This level was 10 times that of cells at passage 11. When the experiment was repeated using B16 cells containing pBabeNeo, there was some

Figure 6.10. Effect on IL-2 protein levels of treatment with MSH



B16 cells containing pNASSβ/IL-2 (passage 45) were treated with MSH (10^{-12} to 10^{-4} M) for two days. The level of the IL-2 transgene protein in the medium was measured using an ELISA assay

evidence of induction, but this was only in the region of a doubling in expression levels. Since these levels were close to the limit of sensitivity of the assay, this probably did not represent a signal induction. Interestingly, when treated with MSH, the B16.F1 cells containing pNASSβ/IL-2 showed evidence of a changed morphology, with cells growing away from the substrate in clumps. However, parental cells, and those containing pBabeNeo/IL-2 displayed a normal morphology (Figure 6.11. a and e). Thus, the altered morphology appeared to be related an effect of MSH on the cells via the construct. If the effect was via the host genome, all the clones would show this morphology change, regardless of whether they contained a transgene construct.

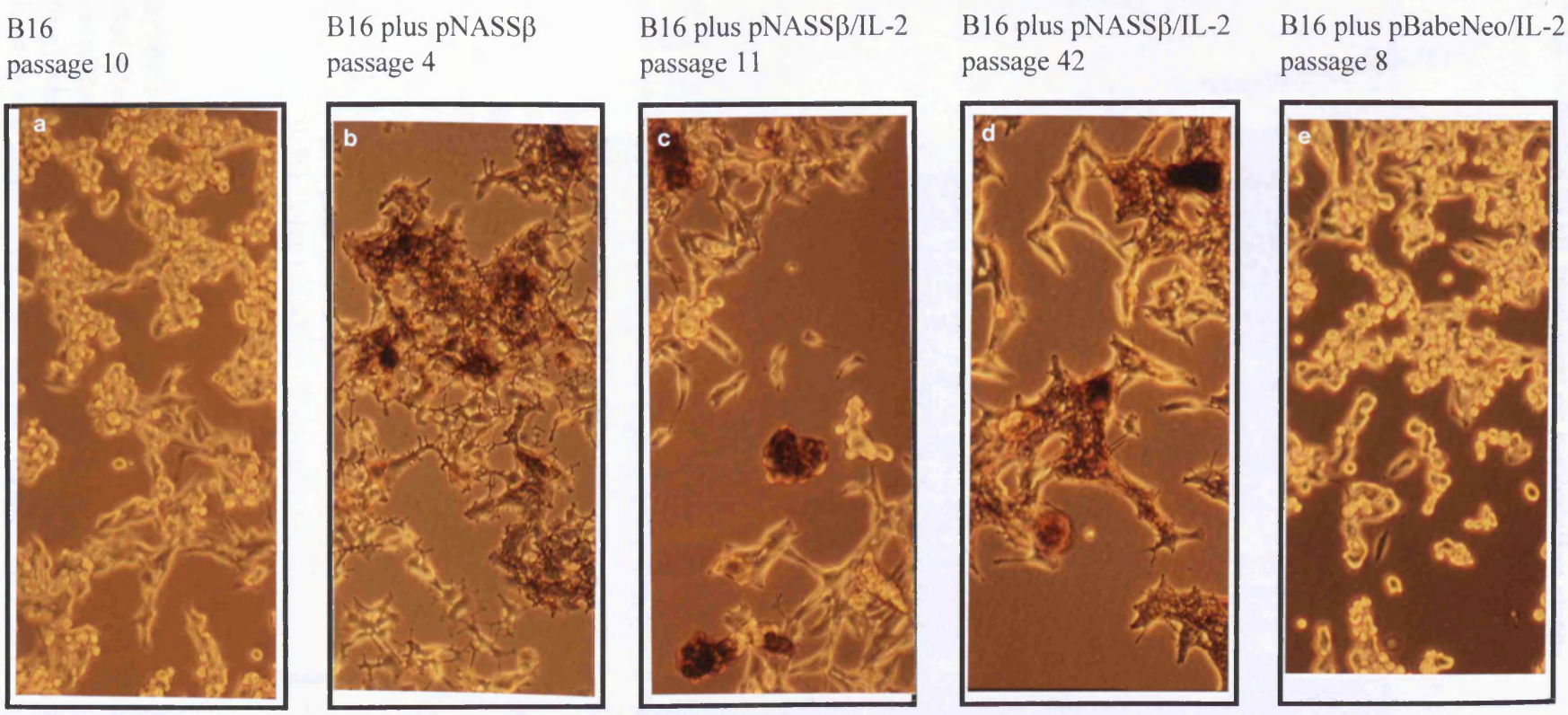
6.4.2. mRNA levels

The level of RNA was determined as described in section 6.2.1. using a continuous RT-PCR method. This semi-quantitative method showed that at 10^{-6}M MSH, the level of mRNA also increased dramatically (Figure 6.7., lanes 6 and 7). Analysis by densitometry showed a 5 fold increase in expression, in passage 42 cells, above the level found in cells at passage 11. There was no induction of mRNA when B16 cells containing pBabeNeo/IL-2 were used in an analogous experiment (data not shown).

6.4.3. Methylation

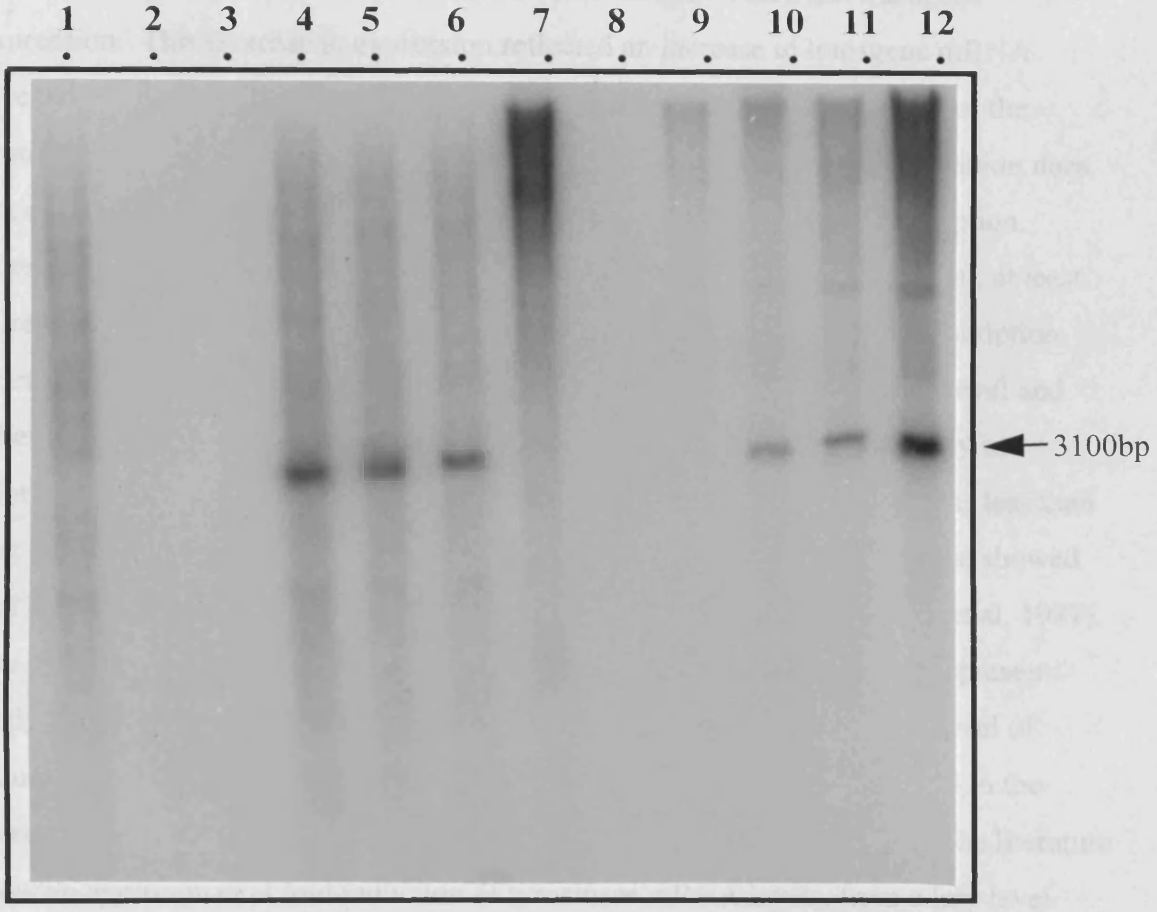
Finally, the methylation status of MSH treated cells was investigated to determine whether MSH affected this parameter. While a difference had not been observed in the previous study (Section 6.3), the large change in expression induced by MSH could have caused a more noticeable change in methylation status, that would indicate that this could be the mechanism of shutdown. The methylation study was carried out as in section 6.3., using MSH treated cells. The expected product of 990bp was not seen, but a product of 3100bp was observed in all samples containing the IL-2 transgene. Although larger bands were observed, they were seen in all lanes, regardless of passage number and MSH treatment (Figure 6.12). If MSH had changed the methylation status of the promoter, high molecular weight bands would be expected to disappear, which was not the case. Overall, MSH did not appear to affect the methylation status of the construct.

Figure 6.11. Effect on morphology of B16 cells after treatment with MSH



Cells were treated with 10^{-6}M MSH for 48 hours before being photographed using a Zeiss invert microscope. a and e display normal morphology, while b, c and d all display dense patches of dark cells induced by MSH treatment.

Figure 6.12. Effect of MSH treatment on methylation status



1. B16 p.7
2. B16 p.7 10^{-6} MSH
3. B16 plus pNASSβ
4. B16 plus pNASSβ /IL-2 (p.7)
5. B16 plus pNASSβ/IL-2 (p.42)
6. B16 plus pNASSβ IL-2 (p.42), 10^{-6} MSH

*Msp*I

7. B16 p.7
8. B16 p.7 10^{-6} MSH
9. B16 plus pNASSβ
10. B16 plus pNASSβ /IL-2 (p.7)
11. B16 plus pNASSβ/IL-2 (p.42)
12. B16 plus pNASSβ IL-2 (p.42), 10^{-6} MSH

*Hpa*II

The methylation status of high passage B16 cells containing pNASSβ/IL-2 was investigated after treatment with MSH, to determine whether MSH induced expression of the transgene by altering methylation of the promoter. Genomic DNA (10μg) was digested with *Msp*I or *Hpa*II and the products separated by gel electrophoresis and blotted. The blot was hybridised with a 528bp region specific for mouse IL-2. A specific band of 3100bp was seen, irrespective of therestriction enzyme used..

6.4.4. Discussion

The work described in this section has shown that MSH was able to induce high levels of IL-2 transgene protein in a B16.F1 clone that had previously lost transgene expression. This increase in expression reflected an increase in transgene mRNA levels. The size of the induction was 5 fold at the mRNA level and 10 fold at the protein level as compared to the expression seen at passage 11. This observation does not exclude the possibility of further levels of control in addition to transcription. These results are in accordance with studies in the literature, which show that, at least in part, MSH induction of endogenous tyrosinase occurs at the level of transcription. There is conflicting data as to the percentage increase in tyrosinase protein level and whether this is directly influenced by the increased mRNA levels. One study in Cloudman S-91 melanoma cells showed that the increase in protein level was less than that of mRNA (Hoganson et al, 1989), while another study, in the same cells, showed that activation of pre-existing protein molecules was also occurring (Fuller et al, 1987). The increase in catalytic activity of existing tyrosinase is not relevant to the present study. However, it seems clear that, MSH does affect mRNA levels. The level of induction seen in the model used here is difficult to compare to that reported in the literature, because the initial levels of IL-2 expression are high. Reports in the literature show an approximate 4 fold induction of tyrosinase mRNA levels, from a low level base line, on treatment with equivalent amounts of MSH, which suggests the levels of induction seen in our model are well above that seen in Cloudman S-91 cells (Hoganson et al, 1989) or B16 cells (Ganss et al, 1994, Englaro et al, 1995).

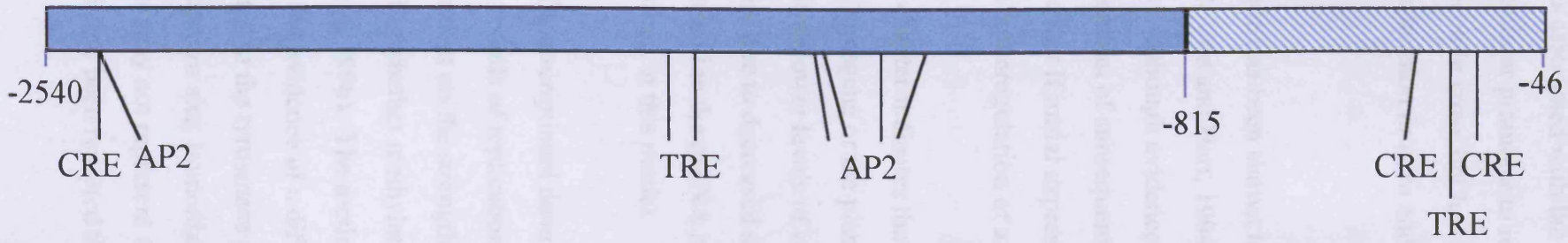
The mechanism by which MSH upregulates the IL-2 transgene is unclear. Melanocytes can be regulated through activation of adenylate cyclase, the diacylglycerol/protein kinase C pathway or tyrosine kinase activity of cell surface receptors. MSH causes increased cAMP levels in a cell via adenylate cyclase, although there is evidence that protein kinase C may influence MSH-induced signalling pathways in human (de Luca et al, 1993) and mouse cells (Siegrist et al, 1995). It has been shown that upregulators of cAMP, such as forskolin, cause increases in the levels of tyrosinase mRNA (Ganss et al, 1994) and tyrosinase is upregulated in response to PKC (Mahalingham et al, 1997). Additionally, the cAMP inducer, theophylline, increased IL-2 production in a tissue

specific manner in both pNASSβ/IL-2 and pBabeNeo/IL-2 (Miller et al, 1995), implying that the tyrosinase promoter is induced by cAMP. However, in the present study MSH did not affect expression from the 769bp tyrosinase promoter fragment found in pBabeNeo/IL-2. Therefore, it would appear that upregulation by MSH is not occurring via cAMP. A comparison of the promoter fragments indicates that the longer promoter fragment in pNASSβ contains an AP2 binding site, that is not present in the shorter promoter present in the retroviral construct (Figure 6.13.). This element potentially responds to cAMP and phorbol esters. One possibility is that the cAMP is the normal inducer of the tyrosinase promoter, via an element at -126 to -80 (Bertolotto et al, 1996). However, in the downregulated promoter a repressor protein is bound and blocks the normal site of induction. MSH may be able to work via a second pathway, causing binding of transcription factors at a second site, either via cAMP or another pathway, thus allowing induction. It has been reported in the literature that in a B16 model, cAMP (via an MSH analogue) causes AP1 activation via a MAP kinase pathway, but that, additionally, cAMP induces AP2 (Englaro et al,1995). Miller et al (1995) have reported that the AP2 element in pNASSβ may well be inactive as it fails to respond to TPA, but Englaro et al (1995) showed that AP2 induction occurred in response to cAMP, but did not respond to TPA. This indicates that there is a functional AP2 site in the tyrosinase promoter. It is unclear whether the two papers are studying the same AP2 site. Another alternative is that MSH works via a different signalling pathway, which can only take effect through the longer promoter fragment. Electophoretic mobility shift assays or footprinting would be a logical step in the search to find which element is active in MSH-induced cells containing the longer 2494bp tyrosinase promoter fragment.

CONCLUSION

The need to produce vectors which function effectively *in vivo* has been highlighted in the literature (Bonifer et al, 1996, Lund et al, 1996). Vector design can affect the likelihood of downregulation, with retroviral LTRs apparently more prone to inactivation than cellular promoters (Rettinger et al, 1994). Interaction between

Figure 6.13. Putative transcription factor binding sites in a fragment of the mouse tyrosinase promoter



The diagram depicts a 2496bp fragment of the mouse tyrosinase promoter. The whole fragment is found in pNASSβ, while the area indicated by ▨ is the part of the promoter contained in pBabeNeo. Putative transcription factor binding sites are shown. The longer fragment contains several possible AP2 binding sites that are not found in the shorter 769bp fragment. The consensus sequences used were as follows: TRE - TGAGTCA, CRE - TGACGTCA, AP2 - CCCCAGGC. All the putative sites shown have 1 or 2 mismatches.

promoter elements within the construct can affect expression levels and tissue specificity (Vile et al, 1994a). The pNASSβ vector was designed with a cellular promoter to avoid problems associated with the LTR, while pBabeNeo was designed with a reverse orientation cellular promoter to reduce regulatory element interaction. While expression is often seen *in vitro*, but lost *in vivo* (reviewed by Lund et al, 1996), this model shows loss of expression *in vitro* and has provided an opportunity to investigate the mechanism

The initial level of expression has been shown to be dependent on the point of insertion (Naviaux and Verma, 1992, Vile and Hart, 1994c), possibly due to the location of nearby enhancer sequences. Although evidence in the literature suggests that initial expression levels are independent of subsequent loss of expression (Duch et al, 1994), it seems reasonable to assume that if initial expression can be affected by the position of insertion, it is possible that downregulation of a transgene may be affected too.

The work presented in this chapter indicates that the loss of IL-2 transgene expression is not caused by loss of the transgene or the plasmid construct. The drop in protein levels is, at least in part, due to lower levels of mRNA. It was not determined whether these low mRNA levels were due to decreased stability of the molecule or decreased transcription. Studies to determine the mRNA half life could determine whether message stability was important in this model.

One possible mechanism of transcriptional downregulation was that the promoter was methylated with successive rounds of replication. The degree to which methylation affects transcription is dependent on the strength of the promoter (reviewed by Tate and Bird, 1993). The evidence for whether methylation is a causal or secondary event is conflicting (reviewed by Szyf, 1996). The methylation status of the tyrosinase promoter was investigated, but evidence of a difference was not observed. An *Msp*I restriction site was not located in the tyrosinase promoter, but a site was found in the SV40 splice donor/splice acceptor site, immediately upstream of the IL-2 transgene sequence. Events at this site may not represent the methylation status of the promoter further upstream, although it has been reported that a relevant change in methylation

status has been detected this close to the start site of a gene (Challita and Kohn, 1994). The possible rearrangement that has occurred means the location of the site is unknown, although it is still likely to be upstream of the IL-2 transgene. Rearrangement would also explain why the band size in the Southern blots differed from the predicted size. Use of a second enzyme, SmaI, might confirm that methylation had not taken place. Alternatively, treatment of the cells with the demethylating agent 5-azacytidine would indicate whether methylation was playing a role in downregulation.

In addition to those investigated in this study, there are other mechanisms by which the transgene could be downregulated. An analogous mechanism to methylation is acetylation, which has been shown to down regulate the LTR and ITR (Chen et al, 1997). It was found that when histones became hypoacetylated, the viral genes were silenced. This could be reversed by butyrate treatment, which inhibits histone deacetylase. It is possible that downregulation relies on a combination of interacting factors. The fact that tissue specific elements often perform differently after stable integration compared to these in transient constructs, points to chromatin structure and distal regulatory elements playing an important role (reviewed by Bonifer et al, 1996). Binding of proteins to methylated DNA can affect the structure of chromatin, but there is no reason why other co-repressors, such as histone acetylases, could not work in conjunction with methylation. Changes in acetylation status of DNA is associated with movement from an unorganised to a nucleosomal structure, and could bring repressor proteins nearer to the transgene, thus causing downregulation. This model has multiple components each of which contribute to the probability of transgene shutdown. It assumes that gene regulation is a fluid process, which may explain why transgene shutdown is relatively unpredictable.

It was not clear whether the IL-2 gene was gradually, coordinately switched off in all B16.F1 cells, or whether a sudden loss of expression randomly took affect (Duch et al, 1994). Both would have had the result of gradual decrease in the expression of the transgene from a culture. If the on-off mechanism occurred it implies that, within a single model, the timing of shutdown in each cell is an unpredictable event. Whatever the final answer, it is clear that the elements contained in the tyrosinase promoter were

not sufficient to provide tissue specific, position independent long term expression. This is the goal of gene therapists designing vectors and it is likely to require increased understanding of the intricate regulation of chromatin structure, transcriptional promotion and post transcriptional regulation. This work has provided evidence that subtle changes in these regulatory mechanisms, rather than large scale changes, can cause loss of transgene expression.

It has also been shown that MSH was able to reverse the mechanism of downregulation. It may have reversed the shutdown mechanism, or overridden this mechanism and induced expression while the original repression was still in place. Induction required the presence of the longer section of tyrosinase promoter and did not occur in the construct containing the 769bp promoter fragment. While the finding has shed some light on the mechanism of downregulation, the exact details remain elusive. However, the finding that downregulation can be reversed is important as it reinforces evidence in the literature that vectors can be designed which are regulated more tightly than many currently used in gene therapy (Miller and Whelan, 1997). Such control could involve incorporation of an optimal combination of promoter and repressor elements so that expression could be initiated when required and re-induced if necessary after down-regulation.

Chapter Seven

General Discussion

As described in the introduction, current safety requirements for gene therapy are rigorous. Data has to be presented on the potential toxicity of the components of a protocol, and on the effect of transferring the transgene vector into the body. However, the parameters used to define toxicity are currently designed to highlight whole organ or body toxicity. After initial safety concerns, the procedures and components used in gene therapy have been deemed to be relatively safe in this respect, although recent articles reviewing the possible transfer of vectors to the germ line (Boyce, 1998) and concerns over new vector design (Putman, 1998), suggest that safety concerns are clearly still high on the agenda.

Direct *in vivo* transfer of a transgene to its target cell is an approach, which, at least conceptually, is appealing. It removes the need for manipulation of the patient's cells and, in many cases, requires minor surgical intervention. It has the potential to deliver genes to the necessary location so they can be expressed by cells in their normal context, thus increasing the likelihood of achieving a beneficial result.

There is little data concerning the effect of transfection or transgene expression on cell regulation, an important issue in both target and non-target cells. While, in some cases, the aim is to destroy the target tissue, other protocols aim to maintain normal function of the target tissue for the patient's life time. Additionally, *in vivo* protocols currently use vectors which have the potential to transfect normal cells. Safety concerns will become more prominent as treated patients progress to live a normal life-span and the possibility of treating non-fatal conditions is considered. Both these situations might uncover detrimental long term effects, caused by the treatment.

The first aim of this project was to investigate the effect of transfection and transgene expression on cell regulation with a view to gaining information on the long term safety of gene therapy procedures. The study carried out in Chapter three showed that transfection is able to alter one parameter i.e. the doubling time of cells. The observed alterations occurred with two vector systems and in two different cell lines. The fact that different systems showed similar trends, suggested that the potential for change was not restricted to one model. The random nature of the effect of transfection is

likely to be due to the position of insertion of each individual vector into the host genome. Disruption of the particular gene or alterations caused by changing the spatial arrangement of *cis* acting regulatory elements could cause changes in gene regulation. These changes could lead to disruption of cell regulation and at worst, a move towards transformation. One way to prevent this is to develop site specific insertion vectors, so the foreign DNA always inserts into a site that had previously been shown to cause no cellular disruption.

In addition to this general change caused by transfection, expression of the IL-2 transgene was observed to cause changes in cell adhesion. The effect was only seen in the cell line expressing high levels of IL-2, and was specific to IL-2. This model highlights the potential for unexpected interactions to occur when a gene is overexpressed in a cell. It would seem appropriate for gene therapists to bear in mind the potential for this to occur, and to realise that *in vitro* testing of cell growth parameters may be a suitable safety study that could add useful toxicity information. However, it may be the case that models may not always be good predictors of problems in patients. Care should also be taken to reproduce conditions faithfully. For example, if B16 cells had merely been grown in the presence of IL-2, the adhesion change would not have been observed. It was pertinent that the data obtained from the DDRT-PCR implied that, in the sub-population studied, few changes had occurred in gene expression and yet the effect on cell growth in the pNASSβ/IL-2 containing cells was marked.

The second area studied in this thesis was that of loss of transgene expression. The results presented in chapter five show that this occurs in several systems, which concurs with the view in the literature that expression loss is widespread.

By investigating the mechanism of expression loss in one system, it was hoped to gain an insight into how to improve vector design. It became clear that loss of expression was due to changes in the level of mRNA. There are many possible mechanisms by which this may have occurred, such as the interaction of the vector with the host genome regulatory elements and chromatin. Nucleosomal organisation and correct

spacing of regulatory elements has been shown to be required for prolonged transgene expression (Bonifer 1996). It appears that, while short promoters can confer tissue specificity, much longer promoter fragments are required for position-dependent expression. This is the case with the tyrosinase promoter, where 15kb of promoter sequence is needed for position independence (Porter and Meyer, 1994), requiring much larger fragments of promoters to be included in a vector than those described in the present study. At the same time it is important to avoid an influence of the vector on the status of the host genes as, for instance, altered methylation may trigger changes in host gene expression which could disrupt cell regulation (Syzf, 1996).

The fact that this work shows that expression could be induced after loss opens up another avenue of vector development. The potential to include inducible promoters in a vector would allow induction of the transgene at controlled time points and thus overcome expression loss. Some steps have been made towards developing inducible promoters. One study reports on the inclusion of multiple cAMP elements (Suzuki et al, 1996). Another possibility is the use of non mammalian systems which would use inducers that would not disrupt the cell regulation of the patient (Miller and Whelan, 1997). Any inducible system would have to use an inducer that could be administered repetitively without causing toxicity.

The conclusion reached from the work presented here is that transfection and transgene expression can affect the host cell, but equally, the host cell can cause changes in the regulation of the vector. A consequent recommendation might be to design site directed insertion vectors. Alternatively, the vector could be designed to replicate episomally or be contained in a human artificial chromosome. By using a strategy that does not disrupt important genes, interruption of cell regulation by the vector and downregulation of transgene expression by host cell mechanisms could be avoided. In addition, either larger promoter fragments or defined elements should be included in the vectors to provide a greater chance of maintaining position and tissue dependent transcription. Perhaps the ideal vector would contain one or more transgenes regulated by inducible promoters, allowing for specific timing of expression and providing the potential for upregulation in response to expression loss. In the event of toxicity

occurring, the transfected cells could be eliminated by use of another inducible promoter which would control a transgene encoding a suicide gene. More research needs to be carried out into the effect of transfection and transgene expression in both target and non-target cells and patients will have to be monitored until it is clear whether gene therapy has detrimental long term side effects.

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Appendix

Oligonucleotide primers

DDRT-PCR primers

	5' *3'*
AP1	TTTTTTTTTTTGG
AP2	TTTTTTTTTTTAG
AP3	TTTTTTTTTTTCG
oligo 1	TACAACGAGG
oligo 2	TGGATTGGTC
oligo 3	CTTTCTACCC
oligo 4	TTTTGGCTCC
oligo 5	GGAACCAATC
oligo 6	AAACTCCGTC
oligo 7	TCGATACAGG
oligo 8	TGGTAAAGGG
oligo 9	TCGGTCATAG
oligo 10	GGTACTAAGG
oligo 11	TACCTAAGCG
oligo 12	CTGCTTGATG
oligo 13	GTTTTCGCAG
oligo 14	GATCAAGTCC
oligo 15	GATCCAGTAC
oligo 16	GATCACGTAC
oligo 17	GATCTGACAC
oligo 18	GATCTCAGAC
oligo 19	GATCATAGCC
oligo 20	GATCAATCGC

M13 primers

M13 (-40) forward	GTAAAACGACGGCCAG
M13 reverse	CAGGAAACAGCTATGAC

Mouse IL-2 primers

muril 1	GAAGATGAACTTGGACCTCTGC
muril 4	AGCTCGCATCCTGTGTCAC
muril 5	GCCTTATGTGTTGTAAGCAGG
muril 6	GCTCCTGTAGGTCCATCAAC

Mouse tyrosinase primers

tyr 1	GAGAGCTTCCTTATTCCAGC
tyr 2	CTTGGAGTTTGTACATAGCC

pNASSβ primers

vec 1	TACGGCTACACTAGAAGGAC
vec 2	GCTGGCTGGTTTATTGCTGATA
vec 3	TGCCTAATGAGTGAGCTAACTC
vec 4	GAGTTAGCTCACTCATTAGGCA

Mouse IL-2 Receptor β primers

	5'	*3'*
murilRc 1	CTCCGTGGACCTCCTTGACATAAATGTGG	
murilRc 2	TGTTTCGTTGAGCTTTGACCCTCACCTGG	
murilRc 3	TGACAACCTTCGCCTGGTGGC	

GAPDH primers

RT-PCR

forward	CCACCCATGGCAAATTCCATGGCA
reverse	TCTAGACGGCAGGTCAGGTCCACC

GAPDH probe preparation

forward	TCT CTG CTC CTC CCT GTT CTA
reverse	GTCCACCACCCTGTTCCTGTA