SOMATIC CELL GENE THERAPY FOR DIABETES MELLITUS:

ENGINEERING A SURROGATE β-CELL

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1

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Improved methods of insulin delivery are required for the treatment of insulin-dependent diabetes mellitus (IDDM) to achieve a more physiological profile of glucose homeostasis. Somatic cell gene therapy offers the prospect that insulin could be delivered by an autologous cell implant, engineered to secrete insulin in response to glucose.

This study explores the feasibility of manipulating somatic cells to behave as a surrogate insulin-secreting β-cells.

Initial studies were conducted using mouse pituitary AtT20 cells as a model, since these cells possess an endogenous complement of enzymes capable of processing proinsulin to mature insulin.

Glucose sensitive insulin secretion was conferred to these cells by transfection with plasmids containing the human preproinsulin gene (hppI-1) and the GLUT2 gene for the glucose transporter isoform 2. Insulin secretion was responsive to changes in the glucose concentration up to about $50\mu M$. Further studies to up-rate this glucose sensitivity into the mM range will require manipulation of the hexokinase and glucokinase enzymes.

Intraperitoneal implantation of the manipulated AtT20 cells into athymic nude mice with streptozotocin-induced diabetes resulted in decreased plasma glucose concentrations. The cells formed vascularised tumours *in vivo* which were shown to contain insulin-secreting cells.

To achieve proinsulin processing in non-endocrine cells, co-transfection with a suitable enzyme, or mutagenesis of the proinsulin itself are necessary. The mutation of the human preproinsulin gene to the consensus sequence for cleavage by the subtilisin-like serine protease, furin, was carried out. Co-transfection of human fibroblasts with wild-type proinsulin and furin resulted in 58% conversion to mature insulin by these cells.

Intraperitoneal implantation of the mature-insulin secreting human fibroblasts into the diabetic nude mouse animal model gave less encouraging results than the AtT20 cells, apparently due to poor vascularisation. Cell aggregations removed from the mice at autopsy were shown to contain insulin secreting cells only at the periphery.

This thesis provides evidence that it is possible to construct, by cellular engineering, a glucose-sensitive insulin-secreting surrogate β-cell. Therefore, somatic cell gene therapy offers a feasible alternative for insulin delivery in IDDM patients.

Indexing Terms: Insulin Secretion
Proinsulin Processing
Transfection
Human Fibroblasts
AtT20 Cells.

For my mother

with love

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List of Contents

7

List of Figures

List of Tables

List of Abbreviations

ACTH	Adrenocorticotropic Hormone
ADA	Adenosine Deaminase
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	Adenosine 3',5'-Cyclic Monophsphate
cDNA	Complementary Deoxyribonucleic Acid
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Guanosine 3',5'-Cyclic Monophsphate
CSII	Continuous Subcutaneous Insulin Infusion
DMEM	Dulcecco's Modification of Eagle's medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E. Coli	*Escherichia Coli*
EDTA	Ethylene Diaminetetraacetic Acid
FCS	Fetal Calf Serum
FH	Familial Hypercholesterolaemia
GLUT2	Glucose Transporter Isoform 2
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
hppI-1	Human Preproinsulin-1
HS-tk	Herpes Simplex-thymidine kinase
IDDM	Insulin Dependent Diabetes Mellitus
IGF-1	Insulin-Like Growth Factor-1
IL-2	Interleukin-2
ILI	Insulin-Like Immunoreactivity
IRI	Immunoreactive Insulin
IRMA	Immunoradiometric Assay
LDL	Low Density Lipoprotein
MDR-1	Multidrug Resistance Gene-1
mRNA	Messenger Ribonucleic Acid
NIDDM	Non-Insulin Dependent Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test
PACE	Paired Amino Acid Cleavage Enzyme
PBS	Phosphate Buffered Saline (without Mg^{2+}, Ca^{2+})
PCR	Polymerase Chain Reaction
POMC	Proopiomelanocortin
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
SDS	Sodium Laureth Sulphate
SEM	Standard Error of the Mean
TBS	Tris Buffered Saline
TIL	Tumour Infiltrating Lymphocyte
TNF-α	Tumour Necrosis Factor-α

Chapter One

Introduction

Chapter One : Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus is a disease characterised by an absolute or relative deficiency of insulin. There are two common forms, Insulin Dependent Diabetes Mellitus (IDDM, Type I, or juvenile onset Diabetes Mellitus) and Non-Insulin Dependent Diabetes Mellitus (NIDDM, Type II, or maturity onset Diabetes Mellitus). This thesis is mainly concerned with IDDM and the approaches to its treatment.

Insulin is secreted by the β-cells found in the islets of Langerhans, in the pancreas. The β-cells of patients diagnosed with IDDM have been totally, or almost totally, destroyed. There is much speculation about the reason for β-cell dysfunction but it is generally thought that autoimmune responses (Bottazzo and Bonofacio, 1991) combined with genetic predisposition (Hitman and Marshall, 1991) and environmental influences (Szopa and Taylor, 1991) are all involved in the aetiological and pathogenic mechanisms.

Diabetes Mellitus is typically recognised as hyperglycaemia due to decreased entry of glucose into the cells, decreased utilisation of glucose by tissues which are dependent upon insulin and increased production of glucose by the liver (gluconeogenesis). As the maximum level of renal tubular reabsorption of glucose is reached, glucose is excreted in the urine (glycosuria). The volume of urine is increased owing to osmotic diuresis, and polyuria results, this in turn leads to dehydration and excessive drinking (polydipsia). Glycosuria causes substantial loss of energy, coupled with the loss of muscle and adipose tissue this causes substantial weight loss despite adequate energy intake.

12

Other metabolic effects include a decrease in protein synthesis due to diminished transport of amino acids, raised plasma fatty acids and accumulation of β-hydroxybutyric acid and acetoacetic acid (ketosis). Ketosis can lead to severe metabolic acidosis and death due to diabetic coma if insulin is not administered (Krentz and Nattrass, 1993).

1.2 Insulin Delivery

1.2.1 Conventional Therapy

For those who already have the disease insulin therapy is a life saving treatment. Before the availability of insulin, children and young adults diagnosed with *Diabetes Mellitus* would not have been expected to survive for very long. With the discovery and purification of insulin, diabetic patients could be expected to live longer and fuller lives. However even with the advancements that have been made in insulin treatment and delivery, IDDM patients still die as a result of the disease itself and especially from the complications, having a reduced life expectancy by one to two decades.

Insulin replacement is a lifetime treatment for diabetic patients and by no means a cure. There are few choices for the methods of insulin delivery. Insulin is mostly inactivated by gastric pepsin, making oral administration ineffective. Therefore, a convenient and safe method of administration for the patient providing adequate control of hyperglycaemia is required. Because of this, insulin was originally given by intermittent subcutaneous injection and this practice has continued for around seventy years.

There are at least three main areas for development of better delivery and control: manipulation of the insulin itself (Jorgensen, 1990), improved treatment regimens using conventional methods (Linde, 1991) and alternative routes of delivery.

13

Insulin can be manipulated into preparations with different absorption characteristics to give long or short acting doses. If administered judiciously these preparations can often give a peripheral plasma insulin profile which approximately mimics normal physiological insulin secretion. Protein engineering may be able to create insulins with more predictable absorption profiles, but, it remains to be seen whether they will improve glycaemic control in the long term (Jorgensen, 1990).

Subcutaneous insulin injection is the most usual route for insulin delivery. The patient injects short acting insulin before meals coupled with an evening dose of intermediate or long acting insulins to provide nocturnal cover. Injection pens and ultrafine needles have improved this delivery route although it is still inconvenient and fails to provide sustained normoglycaemia in most cases.

Other methods of exogenous insulin delivery include continuous subcutaneous infusion (CSII), intravenous insulin injection, closed loop devices (artificial pancreas), intramuscular injection, intraperitoneal injection and devices to deliver insulin via the nasal membranes and rectal mucosa. All of these methods have serious drawbacks. Continuous infusion methods require cannulation which often causes local infection and requires continuous medical attention. Absorption from nasal membranes and rectal mucosa has so far proved extremely variable and therefore unreliable (Bilous and Alberti, 1990).

The long term complications of conventional insulin therapy encountered by IDDM patients, such as retinopathy, nephropathy, neuropathy and cardiovascular disease, have caused most of the morbidity and mortality associated with the disease since the introduction of insulin therapy (Deckert *et al*, 1978). This again highlights insulin therapy as a treatment but not a cure for diabetes mellitus.

The diabetes control and complications trial (DCCT research group, 1993) showed that compared with conventional insulin therapy (one or two insulin injections, daily self monitoring of urine or blood glucose, education about diet and exercise, and three monthly clinical examinations), intensive insulin therapy (administration of insulin 3 or more times daily by injection or external pump, adjusted insulin dose according to results of self-monitoring of blood glucose performed 4 times daily, dietary intake and anticipated exercise, preprandial glucose concentrations of between 3.9 and 6.7 mmol/l, postprandial concentrations of less than 10mmol/l, monthly glycosylated haemoglobin measurements within the normal range (<6%), and monthly clinical examinations) effectively delayed the onset and slowed the progression of diabetic retinopathy, nephropathy and neuropathy in patients with IDDM. The study recommends that most patients with IDDM should be treated with closely monitored insulin regimens, with the goal of maintaining their glycaemic status as close to the normal range as safely possible.

Although these regimens offer the patient the most favourable long-term prognosis, there are considerable drawbacks to intensive insulin therapy. There is an increased risk of hypoglycaemia, the trial showed that risk increased continuously with lower monthly glycosylated haemoglobin values. Intensive therapy must therefore be used with caution in patients with repeated severe hypoglycaemic episodes or unawareness of hypoglycaemia. The other drawbacks with intensive therapy lie with patient compliance, and the cost of such healthcare. Patients are often incapable of the commitment required for intensive therapy, making compliance problematical. In the trial, intensive therapy was carried out by an expert team of diabetologists, nurses, dieticians and behavioural specialists. The time, effort and costs required were considerable. The resources needed to provide the option of

intensive therapy to every IDDM patient are not currently available, although healthcare professionals now recognise the long-term benefits of intensive therapy to the patient.

The problems encountered with conventional insulin therapy (long-term complications) and intensive therapy (compliance, costs) show us that new strategies for insulin delivery to IDDM patients must be investigated to offer them a better long-term prognosis.

1.2.2 Pancreas/Islet Transplantation

It has long been thought that transplantation of the pancreas may offer IDDM patients an alternative to insulin therapy, with freedom from injections, dietary limitations and the fear of long-term complications. However after 30 years of research and experimentation it is still not clear whether pancreas transplantation offers a feasible and safe alternative to conventional insulin therapy (Sutherland, 1994).

An important consideration for patients who have a solo pancreas transplant is that, while becoming independent of insulin, they become dependent upon continuous immunosuppressive medication that has a myriad of toxic effects. Apart from the increased risk from infections and malignant disease, side effects of the triple immunosuppression regimen, most commonly used to prevent graft rejection and recurrent type I diabetes, include azothioprine myelosuppression, cyclosporin nephrotoxicity, and steroid induced gastrointestinal bleeding and perforation. Cyclosporin and steroids also increase blood pressure and alter the lipid profile, perhaps thereby neutralising some of the cardiovascular benefits of achieving normoglycaemia (Remuzzi *et al*, 1994). Remuzzi *et al* (1994) concluded that the high failure rate and the need for a chronic immunosuppressive regimen, offsets the potential improvement in quality of life offered by solo pancreas transplantation. They conclude that there is no justification for solo pancreas transplantation, except perhaps

16

in a patient whose metabolic control is so difficult that the diabetic state itself is life threatening.

The American Diabetes Association has also suggested that pancreas transplantation is limited to individuals with end stage renal disease who have undergone or will undergo renal transplantation. The American Diabetes Association emphasised that the risk of immunosuppressive regimens may be greater than that of conventionally treated IDDM (American Diabetes Association, 1992).

For patients in end stage renal failure the issues are different. The simultaneous grafting of kidney and pancreas can be highly satisfactory for the patient, removing the dependence on both dialysis and insulin therapy. Side effects of immunosuppression are irrelevant since the patient with a renal transplant will require immunosuppressive regimen anyway. Due to a lack of a randomised comparison of pancreas transplantation and conventional insulin therapy it has been difficult to assess risk/benefit profiles for transplantation and to determine whether there is a decrease in the incidence of diabetic complications (Remuzzi *et al*, 1994). There is some evidence to suggest that the incidence of the complications of diabetes is reduced in recipients of functioning grafts. Diabetic renal disease is slowed after a successful pancreas transplant, however a significant functional advantage is not demonstrated, perhaps due to immunosuppression with cyclosporin which decreases glomerular filtration rates (Bilous *et al*, 1989).

Kennedy *et al* (1990) have reported that successful pancreas transplantation may halt the progression of motor and sensory neuropathy. However randomised control groups are needed to corroborate these data and to assess the benefits of transplantation on other complications such as retinopathy and macrovascular disease.

Pancreatic transplantation appears to have reached a point where without improved immunosuppressive regimens it is a last resort for patients already receiving immunosuppression for renal allografts. Patients receiving pancreas transplants show no proven benefits over insulin therapy with respect to the progression of diabetic complications. Whole organ pancreas transplantation is also a very expensive procedure costing around £55000, with follow up immunosuppression costing £2000 per patient per year (Kendall *et al*, 1995).

At present islet cell transplantation appears to offer better prospects than whole organ pancreatic transplantation as a future treatment for diabetic patients.

It was possible to isolate islets from animal pancreata as long ago as 1967, however, a method for isolating human islets was not available until the mid-1980's (Lacy, 1995). Lacy's group had previously shown that islets transplanted between strains of inbred rats ameliorated experimental diabetes in these animals. Further studies revealed that islet grafts could prevent or reverse early microvascular complications of the eyes and kidneys in diabetic rats.

Later Lacy's group began the first human trials with isolated islets. Isolated islets were transplanted into recipients of renal allografts already taking immunosuppressants to protect the new kidney. Some 400,000 islets were delivered to the portal vein of the patient where they lodged in smaller vessels branching from the portal vein, having direct contact with the blood. 400,000 islets proved insufficient to achieve insulin independence in the transplanted patients, though later some patients were free from insulin therapy, for limited periods, following transplantation of 800,000 islets (Lacy, 1995).

Between 1990 and 1995, approximately 145 patients world-wide received cadaveric islet transplants.

In most patients the islet grafts were unable to control plasma glucose concentrations completely or lost some or all of their activity within 3 years (Lacy, 1995). It may be that too few islets were implanted, or rejection and other immune processes may have caused the failures. In spite of the imperfect results, the routine transplantation of islets at the same time as renal allografting is being considered. This is because, although some insulin injections are required, the presence of productive islets can help keep the plasma glucose concentrations in the all-important normal range. Also the simplicity of the implantation procedure and hence the reduced expense compared to pancreas transplantation makes the reduced effectiveness of the technique more acceptable, especially considering the failure rates for pancreatic transplantation.

In contrast to the relatively poor success of islet allotransplantation, normal phasic insulin secretion and carbohydrate tolerance has been reported following islet autotransplantation. Pyzdrowski *et al* (1992) evaluated five non-diabetic patients with chronic pancreatitis who had undergone total pancreatectomy. Their own islets were harvested and autotransplanted via portal vein infusion within hours of isolation. The patients had demonstrable phasic insulin and glucagon secretion in response to intravenous injection of glucose or arginine. 4 out of the 5 patients were able to maintain normoglycaemia without exogenous insulin therapy. These data suggest that as few as 265,000 islets can provide adequate β-cell mass to provide for normal glucose tolerance.

The success of islet autografting suggests that the methods of isolation and transplantation used in the allografting of islets are effective. The autograft data also suggests that the 250,000 to 500,000 islets that can be isolated from one (donor) pancreas should be adequate to achieve normal glucose tolerance. However, 800,000+ islets per allograft (i.e. islets from at least 2 donor cadavers) appear to be necessary, indicating that the

immunosuppressive therapy may harm the transplanted islets. Indeed, cyclosporin itself has been shown to decrease insulin secretion in response to intravenous glucose (Alejandro *et al*, 1989). Also recurrence of the original autoimmune disease, destroying the new β-cells, cannot be ruled out.

If transplanted islets could be hidden from the immune system so that immunosuppression was not required, then transplants into newly diagnosed IDDM patients could be at least as successful as islet autotransplantation. These patients could essentially be cured, free from insulin therapy, immunosuppressive regimens and the fear of diabetic complications. However hiding cells from the immune system is not an easy concept to implement as they must be protected from both rejection and autoimmunity (recurrence of the original disease). Many investigators are exploring ways to encapsulate donor islets in semipermeable plastic membranes allowing the passage of glucose and insulin but not lymphocytes and antibody molecules, which are much larger than glucose and insulin.

Lacy *et al* (1991) found that encapsulating islets (suspended in alginate) in hollow, semipermeable acrylic fibres and their subsequent implantation into the abdominal cavity or under the skin of diabetic mice, resulted in normoglycaemia throughout a year of observation (half a mouse lifetime). There was good tolerance of the implant with little fibrous tissue around the surface of the fine plastic tubes. This experiment led to a safety test in humans where Scharp *et al* (1994) put fibres carrying 150-200 islets under the skin of subjects with type I and type II diabetes and control non-diabetic subjects and kept the implants in place for 2 weeks. The islets were found to be protected from both immune rejection and autoimmune destruction. Although these hollow fibre membranes hold the islets in a more compact manner than can be achieved by encapsulating each islet separately, several metres would be required to implant sufficient islets to achieve normoglycaemia.

20

Lacy *et al* (1995) reports that his group are working on a flat device, made of the same hollow fibres, approximately 12cm square which could hold up to 800,000 islets and be implanted intraperitoneally.

Once all of the problems with the encapsulation devices and their implantation are overcome there is still a major limitation to islet transplantation: the lack of human donor pancreases. In America alone there are more than 700,000 type I diabetic patients and more than 2 million type II diabetics treated with insulin therapy. However, fewer than 5000 donor cadavers are available each year and only approximately 1000 pancreases are ever recovered. Thus there will never be enough donor islets for more than a tiny percentage of diabetic patients to benefit.

There are several of areas of research investigating ways of making up the shortfall in donor islets. Some investigators are exploring implantation of islets from aborted foetuses. It is possible that precursor cells able to give rise to islets could be isolated and induced to produce islets in quantity (Lacy, 1995).

Xenotransplantation is another consideration. Pig islets are a particularly attractive option as they are readily available and the insulin they produce is similar to that of human insulin. The possibility of islets from genetically modified transgenic pigs which have reduced immunogenicity in man is awaited with interest.

Techniques for pig islet isolation are already in place and the procedure awaits the perfection of the method of transplantation where islets can be hidden from the immune system by encapsulation. The encapsulation procedure should also reduce the possibility of the transmission of diseases between species. This is currently one of the major barriers to the use of transgenic pig organs, such as hearts, in xenotransplantation (Lacy, 1995).

Another way to overcome the shortfall in donor islets for transplantation, would be to use insulin secreting cells engineered from animal insulinoma tumour cell lines. There are several immortal rat and mouse β-cell tumour lines which, given an appropriate encapsulation device, could be engineered to deliver insulin in the same way as healthy β-cells. These tumour cell lines have been found to lose glucose-stimulated insulin secretion after a period of time in culture, however they can be engineered by transfection with a number of genes to resume glucose-stimulated insulin secretion and further engineering to replace the rodent insulin gene with the human insulin gene could make these cells an attractive alternative to pig islets in encapsulated xenografts (Ferber *et al*, 1994; Normington *et al*, 1996; Efrat, 1996). A further option for the physiologic replacement of insulin could be the development of a fully artificial pancreas. However devising a glucose sensor that is small, durable and accurate is a major problem and further problems with biocompatability of implanted plastic devices, especially in contact with the blood flow, are likely to arise.

In summary, islet transplantation in biocompatible encapsulation devices looks to be a promising solution to providing a more physiologic replacement of insulin in IDDM than conventional insulin therapy and thus helping to prevent the long-term complications of such treatment. However, there is a severe shortage of donor islets and there will never be enough for such a treatment to become available to all diabetic patients. The main alternative to the use of donated human islets is xenotransplantation of pig islets. Xenotransplantation is fraught with technical, safety and ethical considerations and is some way from clinical use. Therefore, other methods of delivering insulin to IDDM patients in a physiologically relevant manner must be investigated. One such method could be somatic cell gene therapy.

1.3 Gene Therapy for Insulin Delivery

1.3.1 Gene Therapy

Gene therapy is a novel form of drug delivery that uses the patients own cells to produce a therapeutic agent. There are two main approaches to gene therapy. First, genes may be inserted into somatic cells, that is any body cell other than a germ cell. This method raises no major ethical issues because it is essentially the same as organ transplantation; any changes in the genotype of a cell population will be confined to the recipient. The second, germ-line therapy, involves the introduction of genes into fertilised eggs. In this case the genes will be distributed in both somatic and germ cells and hence will be passed on to future generations. This is a much more difficult ethical area because the technique alters the genetic makeup of future populations (Weatherall, 1991).

Somatic cell gene therapy is a medical intervention based on the modification of genetic material of living cells. Cells may be modified *ex-vivo* for subsequent administration or may be altered directly *in vivo* (Centre for Biologics Evaluation and Research, 1991).

The applications of gene therapy are not limited to rare inherited diseases but extend potentially to common acquired disorders, including cancer, heart disease, and the acquired immunodeficiency syndrome.

Gene Delivery

The method of delivering genes to the target cells, *in vivo* or *ex vivo* is of major importance in gene therapy and is a problematical area. Most gene therapy to date has involved the use of viruses as carriers of the genes (Blau and Springer, 1995). Retroviruses can be manipulated to lack virtually all viral genes, except those required for infecting mammalian cells. The viruses must be prepared so that they are so defective that after they infect the

23

appropriate target cell they cannot replicate or infect other cells. Retroviruses are easily generated, the infected viruses can be extensively characterised in tissue culture before being injected into a patient, and the stable integration of the virus into the chromosome ensures its retention by the cell (Miller,1989). The risk that retroviral integration into the cell genome could inactivate host tumour-suppressor genes or activate proto-oncogenes appears to be minimised by reducing the efficiency of infection so that at most one virus infects a cell. However, the risk of insertional mutagenesis has been a major safety concern when approving clinical trials. As vectors, retroviruses have two major limitations. First, they can only harbour genes up to approximately 7 kilobases. Secondly, retroviruses infect only dividing cells (Miller *et al*, 1990). For this reason, retroviruses are more useful in *ex vivo* gene therapy.

Adenoviruses are the second most commonly used viral vector for gene delivery (Blau and Springer, 1995). Adenoviruses readily infect non-dividing cells, making infection of tissues more efficient, although if delivered *in vivo* they may infect all tissues, including germ line cells, affecting subsequent generations (Berkner, 1988). Adenoviruses have been found to remain extrachromosomal, thereby reducing the chance of disrupting the cell genome and activating oncogenes (Emery and Malcom, 1995). By not integrating into the genome, regular reapplication may be required for continuous therapeutic advantage, however this is in some respects useful as treatment could be withdrawn should any adverse reaction occur. Adeno-associated virus (AAV) is a naturally defective parvovirus that is non-pathogenic. When AAV infects a cell in the absence of a helper virus it integrates into the host genome and becomes latent. Upon subsequent wild type adenovirus or wild type herpes virus infection, the AAV genome is rescued and is excised from the chromosome to yield a lytic infection (Carter, 1990). The AAV genome is small (4.7kb) and vector construction

24

removes all but the 0.2kb of the genome required for integration into the host genome. The 4.5kb available for a cDNA insert is too small for many therapeutic genes, although the AAV will package larger inserts albeit inefficiently (Flotte *et al*, 1993). Insertional mutagenesis is a risk with vectors that integrate into the host cell genome. However, the scale of this risk is yet to be determined.

There are non-viral methods of gene delivery. Liposomes, or lipid vesicles, which combine readily with cell membranes, are being used to deliver genes, sometimes as aerosols (Felgner *et al*, 1987).

Other methods of non-viral gene delivery include direct injection of DNA into the target cells, which is mostly useful for *in vivo* delivery to heart and muscle. The disadvantages of this method is that relatively few cells take up the DNA and thus only small quantities of protein are produced.

Physical transfection methods, such as electroporation, and chemical methods such as calcium phosphate co-precipitation, may be useful in *ex vivo* gene therapy.

Clinical Trials

More than 100 studies of human gene therapy are currently approved by the appropriate regulatory agencies in the United States and a number of other countries (Friedmann, 1996). The diseases treated, genes used, target tissues and types of vector used are shown in table 1.1 (Blau and Springer, 1995).

The first transfer of genetically manipulated cells, in 1989, involved gene marking of tumour infiltrating lymphocytes (TIL) rather than gene therapy. Tumour infiltrating lymphocytes isolated from malignant melanoma tumours were grown up in large numbers *in vitro* and manipulated to constitutively secrete cytokines such as interleukin-2 (IL-2). The cytokine

Table 1.1 Gene therapy protocols that have been approved for clinical trials.

Disease	Gene or Product Delivered	Target	Vector
Inherited Diseases			
Alpha$_1$-antitrypsin deficiency	Alpha$_1$-antitrypsin	Respiratory tract	Liposomes
Chronic granulomatous disease	p47^{PHOX} (oxidase)	Myeloid cells	Retrovirus
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator	Respiratory tract	Adenovirus, adenovirus associated virus, liposomes
Familial hypercholesterolaemia	Low-density-lipoprotein receptor	Hepatocytes	Retrovirus
Fanconi's anaemia	Complementation group C gene	Haemopoetic progenitors	Retrovirus
Gauchers disease	Glucocerebrosidase	Peripheral-blood cells or haemopoetic stem cells	Retrovirus
Hunter's syndrome	Iduronate-2-sulphatase	Lymphocytes	Retrovirus
SCID (ADA deficiency)	Adenosine deaminase	Lymphocytes	Retrovirus
Acquired Diseases			
Human immunodeficiency virus	HIV-cleaving ribozyme; antisense to HIV TAR (transactivation response element) along with transdominant negative Rev (regulator of HIV gene expression)	Lymphocytes	Retrovirus
Peripheral-artery disease	Tumour angiogenesis factor	Endothelial cells	Plasmid (plasmid coated balloon catheter)
Rheumatoid arthritis	Interleukin-1-receptor antagonist	Proliferating synovial cells	Retrovirus
Cancer	Tumour repressor genes (eg p53)	Lung, liver, squamous cell carcinoma	Retrovirus, Adenovirus
	Herpes virus thymidine kinase followed by glanciclovir treatment (toxic only to cells expressing herpesvirus thymidine kinase)	Brain, ovarian tumours	Retrovirus, Adenovirus
	Antisense RNA to c-fos and c-myc RNA	Breast tumour	Retrovirus
	Antisense RNA to insulin-like growth factor-1 RNA	Brain tumour	Retrovirus
	Multidrug resistance (MDR-1) for chemiprotection	Haemopoetic cells	Retrovirus
	Tumour necrosis factor	Tumour infiltrating lymphocytes	Retrovirus
	B7 cofactor to induce T-cell co-stimulation	Melanoma	Retrovirus
	HLA-B7 to induce immune response	Melanoma (HLA-B7 negative)	Retrovirus
	Cytokines Interleukin-2,4 and 7; granulocyte-macrophage colony-stimulating factor	Tumours of brain, prostate, lung, colon, skin, kidney	Retrovirus, liposomes
	Interferon-γ to induce immune response to tumour	Malignant melanoma	Retrovirus

secreting TIL's were then given back to the patient resulting in an improved prognosis (Rosenberg *et al*, 1988).

The first true gene therapy was given to a 4 year old girl with severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency (Blaese *et al*, 1990). ADA deficiency causes an increase in 2-deoxyadenosine which is toxic to T and B cells causing SCID. Conventional treatment involves weekly injections of bovine ADA or bone marrow transplantation. The gene therapy protocol utilised the patients own lymphocytes infected with a retrovirus containing the human ADA and neomycin genes (neomycin was used for selection). Infusions of the transduced cells were administered monthly as well as the weekly bovine ADA injections. The two children treated in this study showed significant improvements in immune function, with increased cell circulating ADA levels and increased lymphocyte counts (Parker and Gelfand, 1991).

Other early gene therapy trials began with diseases such as familial hypercholesterolaemia, Duchenne muscular dystrophy and cystic fibrosis. The initial clinical trial results of these diseases have recently been published.

Severe Combined Immunodeficiency

Blaese *et al* (1995) reported the follow up of the 1990 trial described above, where the ADA gene was transferred using a retroviral vector into the T cells of 2 children with SCID using *ex vivo* gene therapy. Gene treatment was stopped after 2 years, however, integrated vector and ADA gene expression in T cells persisted and after 4 years the dose of bovine ADA remained at half the pre-trial dose for each patient. The trial has shown no evidence of malignancy or other problems caused by the retroviral-mediated gene transfer, which remains a central concern in the use of these vectors.

In a different trial two patients with SCID received both peripheral blood lymphocytes and bone marrow cells transduced with the human ADA gene (Bordignon *et al*, 1995). This group showed that after 2 years of treatment there was long term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene. This resulted in normalisation of the immune repertoire and restoration of cellular and humoral immunity in the two patients. The successful transfer to the bone marrow progenitor cells appeared to produce a functional multilineage progeny, which may have many other applications in the treatment of other genetic and acquired diseases.

Familial Hypercholesterolaemia

Familial hypercholesterolaemia (FH) is an inherited disease caused by defects in the gene encoding the low density lipoprotein (LDL) receptor. Patients homozygous for this disease develop severe hypercholesterolaemia and early onset coronary artery disease (CAD). Treatment and therapy for the disease are inadequate with orthotropic liver transplant offering the best success. The success of liver transplantation led to the suggestion of a strategy for gene therapy directed at the liver (Grossman *et al*, 1995). An *ex vivo* approach was adopted in which autologous hepatocytes were genetically corrected with recombinant retroviruses while in culture and subsequently transplanted back into the liver via the portal circulation. Preclinical experiments in a rabbit model of FH were very successful with stable engraftment of hepatocytes expressing the transgene leading to a 20-40% decrease of serum cholesterol (Chowdhury *et al*, 1991) and studies in dogs and non-primates confirmed the safety and feasibility of ex vivo gene therapy directed to liver (Grossman *et al*, 1992; 1993). The pilot study of this technique in humans was performed in 5 patients (Grossman *et al*, 1995). Significant and prolonged reductions in LDL cholesterol were demonstrated in 3 out of the 5 patients, and persistent gene expression was observed lasting at least 4 months after

gene therapy. There were variable metabolic responses to the stable engraftments of the genetically modified hepatocytes in the 5 patients compared to the results obtained with the animal models. This must be expected as there is substantial environmental and genetic heterogeneity in the human population compared with an inbred colony of rabbits. Further gene therapy trials should expect variability of results in humans compared to the animal models used.

The ability of the genetically corrected hepatocytes to repopulate the liver was deemed a limiting factor in this *ex vivo* gene therapy trial. Grossman *et al* (1995) suggested substantial modifications to the procedure, with perhaps the use of in vivo approaches to liver-directed gene therapy to increase the efficacy of gene delivery, before expansion of this trial.

Duchenne's Muscular Dystrophy

A trial involving myoblast transfer in the treatment of Duchenne's muscular dystrophy has recently been reported (Mendell *et al*, 1995). Duchenne's muscular dystrophy is an X-linked disorder caused by deficiency of the protein dystrophin (Hoffman *et al*, 1987). Animal studies have shown that myoblast transfer, where skeletal muscles were injected with donor cells capable of fusing with host muscle fibres and providing dystrophin, could be successful in replacing the missing gene (Partridge *et al*, 1989). This led to human trials where donor-derived dystrophin transcripts were demonstrated one month after myoblast injection (Gussoni *et al*, 1992). Other trials failed to identify donor-derived dystrophin or messenger RNA (Law *et al*, 1992; Kaparti *et al*, 1993; Tremblay *et al*, 1993) and no improvement in strength was observed in any controlled study.

The most recent trial has also failed to demonstrate improved strength following myoblast transfer monthly for 6 months (Mendell *et al*, 1995).

The efficiency of the delivery of the dystrophin gene by donor myoblast transfer and fusion is in doubt. Results so far indicate that other delivery methods may be required to increase the efficacy of the technique.

Cystic Fibrosis

One of the diseases that has caused most of the media attention to gene therapy is cystic fibrosis. Cystic fibrosis is an autosomal recessive disorder, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, affecting 1:2500 live white births (Boat *et al,* 1989). The disease is characterised by abnormal salt and water transport. A variety of epithelial tissues are affected by the disease, including airway, pancreatic, sweat ductal, and gastrointestinal epithelia, leading to abnormal airway secretions, impaired mucociliary clearance, chronic bacterial infections, bronchiectasis, and premature death. Since lung disease is the major cause of morbidity and mortality (Quinton, 1990), the initial gene therapy efforts have been directed toward the lung disease. The transfer of a single copy of the normal CFTR gene into all epithelial cells affected by cystic fibrosis might be expected to correct airway function. However, gene therapy for this disease will require direct *in vivo* delivery of the gene to the airways of patients with cystic fibrosis as the *ex vivo* manipulation and reimplantation of airway epithelium could not incorporate all areas of the lungs. Liposome-mediated gene transfer has been the vector system selected for initial clinical studies in the United Kingdom, while clinical safety and efficacy trials in the United States will utilise adenovirus-mediated gene transfer (Johnson, 1995).

It has previously been suggested in an *in vitro* study that as few as 6-10% of cystic fibrosis airway epithelial cells must be corrected in order to restore normal Cl⁻ transport function to an entire epithelial sheet (Johnson *et al,* 1992). Efficient gene transfer using adenovirus-mediated gene transfer has been reported *in vivo* in cotton rats (Rosenfeld *et al,* 1992).

30

However *in vivo* gene transfer to the airway epithelia of non-human primates tends to be of low efficiency and patchy (Engelhardt et al, 1993).

In vitro studies on human cystic fibrosis airway epithelia have shown correction of the Cl⁻ permeability defect using adenovirus-mediated transduction of CFTR at a low multiplicity of infection (Rich *et al*, 1993). Three patients treated by Zabner and colleagues (1993) were reported to have corrected Cl⁻ transport function in the nasal epithelium following adenovirus-mediated gene transfer at a multiplicity of infection as low as 1.

The most recent human trial has reported inefficient CFTR gene transfer by adenoviral-vector-mediated transfer. Inability of the procedure to correct functional defects in the nasal epithelium, and local inflammatory responses which limited the dose of adenovirus that could be administered to overcome the inefficiency of gene transfer, were reported (Knowles *et al*, 1995). This study contrasts with the results obtained by Zabner et al (1993) and the discrepancies highlight the need for standardised methods for measuring the efficiency of gene transfer. Knowles *et al* (1995) concluded that adenoviral-vector-mediated gene transfer to nasal epithelium affected by cystic fibrosis is inefficient. They suggested targeting of adenoviral vectors to basal cells, or differentiation of basal cells to columnar. Another option is modification of the vector so that it becomes tropic for columnar cells. If nasal epithelium is typical of all human airway regions with respect to inefficiency of adenovirus-mediated gene transfer, the development of other vectors will be necessary.

Liposome mediated CFTR gene transfer is an alternative to adenovirus-mediated transfer. A recent trial where patients received liposome complexed with complementary DNA encoding CFTR, found that delivery to the nasal epithelium was more successful than that of Knowles *et al* (1995) using adenoviral gene transfer. Caplen *et al* (1995) reported the detection of plasmid derived mRNA in the majority of nasal biopsy samples taken from the

treated subjects four days after treatment. There was also a modest restoration (20%) of the response to low Cl⁻ perifusion following CFTR cDNA administration. The absence of any adverse clinical or histological changes that correlate with the treatments and the lack of a systemic immunological response is encouraging with respect to the safety of the procedure. Caplen *et al* (1995) concluded that although improvement to the efficiency of gene delivery and optimisation of gene expression are required, studies in the lower airway are now of importance. The nasal epithelium is a useful initial tissue for monitoring the safety and efficacy of CFTR gene transfer, but there are anatomical and physiological differences between the upper and lower airways which make studies in the lungs necessary.

Cancer

Table 1.1 shows that many of the protocols approved for gene therapy trials are directed at the treatment of cancer. The potential applications for the treatment of cancer by gene therapy include:

1. Enhancement of the immunogenicity of the tumour by inserting genes for cytokines, allogeneic surface antigens, co-stimulatory molecules, and inhibition of tumour growth factor with insulin like growth factor 1 (IGF-1).

2. Production of genetically altered immune cells to increase antitumour efficacy by the insertion of immune stimulatory factors (e.g. IL-2) or genes that encode antibody to tumour specific receptors to enhance tumour-immune cell interactions.

3. Insertion of a "sensitivity" or "suicide" gene into the tumour; the herpes simplex thymidine kinase (HS-tk)/glanciclovir treatment, cytosine deaminase/5-fluorocytidine or genes that disrupt DNA repair (e.g. antisense DNA polymerase).

4. Blocking of oncogene expression; antisense oligonucleotides or ribozymes.

5. Insertion of tumour suppression genes.

6. Genetic protection of tissues from systemic toxicities of chemotherapy; e.g. by insertion of an MDR-1 gene (Culver, 1994).

One of the first gene therapy protocols for cancer was directed at brain tumours. Murine fibroblast cells producing retroviral vectors were directly implanted into growing tumours. The gene that was transferred to the tumour cells was the HS-tk gene, which confers sensitivity to the antiherpes drug glanciclovir, resulting in cell death (Oldfield *et al*, 1993). Animal experiments have shown that 60% of tumour cells were transduced and complete tumour destruction resulted. Tumour cells negative for HS-tk were probably destroyed by the little understood "bystander effect" (Ram *et al*, 1993). Five of the eight human patients receiving this treatment showed evidence of antitumour efficacy with a decrease in size and cystic changes in the tumour.

Another approach to the treatment of brain tumours targets the methods that tumours use to hide from the immune system. This method uses an antisense copy of insulin-like growth factor-1 (IGF-1) to block tumour cell production of IGF-1. The injection of these genetically altered cells into animals results in immunologic rejection of the cells as well as non-genetically altered tumour cells at other sites in the body (Trojan *et al*, 1993).

Genetic manipulation of haemopoeitic stem cells to protect these cells from the toxic effects of chemotherapy may be possible. The insertion of the multidrug resistance type-1 (MDR-1) gene into haemopoeitic stem cells would be performed before high-dose, myelosuppressive chemotherapy. The use of *ex vivo* retroviral vector-mediated insertion of the MDR-1 gene into murine marrow cells has demonstrated significant protective effects *in vivo* in animals treated with chemotherapy drugs (Sorrentino *et al*, 1992). This treatment may be particularly useful with the high dose chemotherapy used to treat brain tumours and disseminated breast and ovarian cancer.

Deletion of tumour suppressor genes can, theoretically, be corrected at a genetic level by insertion of a normal copy of the gene. Likewise, the overexpression of an oncogene may be blocked by insertion of an antisense gene that blocks oncogene expression.

A study to investigate these techniques in endobronchial lung cancers has been approved. Retroviral vectors will be used to transfer a normal copy of the p53 gene to lung cancers that are deficient in the p53 tumour suppressor gene (Fujiwara *et al*, 1993).

Similarly, in lung cancers that overexpress the K-ras oncogene, a vector containing an antisense K-ras vector will produce mirror image RNA molecules that will bind the molecules being produced by the oncogene. These RNA:RNA hybrids will then be degraded by the cell. Experiments in animals have shown that both of these approaches can result in destruction of the injected tumour *in vivo*.

The most common clinical trials of human gene therapy for cancer involve the injection of gene-modified human autologous or allogeneic tumour cells (Culver, 1994). The initial experiments are an attempt to immunise tumour bearing patients against their own tumour by injecting genetically altered cells that stimulate host antitumour immunity. *Invitro* insertion of genes for IL-2, tumour necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), or B7 costimulatory molecules by means of retroviral vectors into melanoma, neuroblastoma, colorectal, and renal carcinoma cells have been approved (Gansbacher *et al*, 1992a; Gansbacher *et al*, 1992b; Rosenberg *et al*, 1992a; Rosenberg *et al*, 1992b). These "tumour vaccines" are produced after surgical resection of the tumour, the cells are modified and once they have been shown to produce the inserted gene product, they are reinjected subcutaneously into the patient.

Non-viral mediated gene transfer to tumours *in vivo* has been of some success. Liposomes, injected directly into the tumour, delivered a foreign HLA-B7 gene in an attempt to increase

immunogenicity of malignant melanoma. Of three patients treated, one has responded well, with regression of the injected lesion and other non-treated lesions. Also there was no evidence of toxicity in any of the patients (Nabel *et al*, 1993).

Another approach to the genetic therapy of cancer is gene transfer into T-lymphocytes. T-lymphocytes are critical for the prevention and elimination of tumours and have been grown from tumour biopsies. The gene for tumour necrosis factor-α has been inserted into these tumour infiltrating lymphocytes (TIL) in an effort to increase their antitumour efficiency (Rosenberg *et al*, 1990). Trials have been blighted by poor efficiency of gene transfer into human TIL, and down-regulation of cytokine expression by the TIL (Hwu *et al*, 1993).

Other Diseases

There are many other diseases in which gene therapy may be of clinical relevance in the long-term. In many diseases research into the feasibility of gene therapy begins with animal models of the disease.

In the rat Parkinson's disease model, *ex vivo* therapy, with autologous myoblasts manipulated to express tyrosine hydroxylase, caused a significant chronic reduction of the asymmetric rotational behaviour in the rats (Jiao *et al*, 1993).

Haemophilia is another disease which could benefit from the development of effective gene therapy. Both skin fibroblasts (Palmer *et al*, 1989) and primary keratinocytes (Gerrard *et al*, 1993) have been manipulated to produce factor IX. Factor IX is used to treat the clotting deficiency found in patients with haemophilia B. Both studies used retroviral-mediated gene transfer and showed the presence of biologically active factor IX in the culture medium of the manipulated cells. Transplantation of these cells into nude mice, or encapsulated cells into normal mice and rats resulted in detectable levels of factor IX in the circulation of these animals.

A similar experiment has used retroviral-mediated gene delivery of human factor VIII to transfect primary fibroblasts in culture. Factor VIII is the clotting factor that is deficient in patients with haemophilia A. Again implanted fibroblasts delivered factor VIII to the circulation in mice, although the efficiency of the delivery to the circulation was strongly dependent on the site of implantation (Dwarki *et al*, 1995).

Other diseases where gene therapy may have an application are being identified. For example, it may be possible to use genetic approaches to regulate gene expression so that aqueous humor production may be specifically targeted in glaucoma patients (Wax & Patil, 1994).

The gene therapy literature is growing explosively, with many basic and clinical investigators devoting their efforts to the study of gene therapy for their particular field of interest. In contrast to many previous medical revolutions such as anaesthesia, antibiotic therapy and organ transplantation, gene therapy has an upside-down history. The conceptual advance has become widely accepted and firmly established as a medical principle before even a single instance of clinical efficacy has been demonstrated (Friedmann, 1996).

Although the publications reviewed herein involve some degree of successful gene transfer and even prolonged expression of the potentially therapeutic gene, true therapeutic efficiency has not been convincingly demonstrated. The optimism and expectations for speedy clinical success were unrealistic and a number of recent reports have recognised the need for realism in the reporting of the modest successes achieved (Friedmann, 1994; Verma, 1994; Rigby, 1995; Brenner, 1995; Leiden, 1995; Dorin, 1996; Touchette, 1996). These reports use such headings as "gene therapy: not ready for prime time" and "optimism tempered with reality". The reports outline the problems encountered with human trials

which were not anticipated and the pitfalls of current treatments. However, these reports all agree that the initial studies present encouraging "proofs of principle" that the available techniques do permit some gene transfer into human tissue, albeit inefficiently. They report that gene therapy is not developed enough as a field to deliver on its promises. Gene therapy requires development to create more efficient gene delivery systems and better expression systems. Also clinical trials must be carried out with the same criteria expected of other clinical studies to ensure that the results cannot be misinterpreted.

1.3.2 Insulin Delivery by Gene Therapy

Somatic cell gene therapy is a potential method for insulin gene therapy for insulin dependent diabetes mellitus. An *ex vivo* approach is the most likely method to be of use, however, *in vivo* techniques may become feasible. Many of the *ex vivo* techniques discussed above have involved the delivery of a therapeutic protein, absent from the host due to a disease state. However these therapeutic proteins (factor VIII in haemophilia, adenosine deaminase in SCID) are not tightly regulated in the body and are not deleterious in excess. Insulin secretion is one of the most tightly regulated events in the body. Both hyperglycaemia and hypoglycaemia can be fatal and hence *ex vivo* gene therapy is complicated by the requirement of tight control and secretion of insulin in a physiologically relevant manner. For this reason an *ex vivo* approach is recommended so that the manipulated cells can be extensively characterised *in vitro* before reimplantation to the host. Another problem with insulin is that it is produced as a prohormone which is cleaved by specific endopeptidases in the β-cell before secretion. The machinery required for a cell to process prohormones is only found in neuroendocrine cells, presenting problems in obtaining biopsies of primary cells for manipulation.

37

For somatic cell gene therapy for insulin delivery to become a viable alternative to conventional treatments, a non-endocrine cell must be genetically manipulated to behave like a β-cell, i.e. a surrogate β-cell must be produced.

Surrogate β-cells must meet a number of criteria to be useful for insulin delivery by somatic cell gene therapy: the cells must secrete insulin in amounts similar to those of normal β-cells; undertake regulated synthesis and secretion of insulin in response to physiological secretagogues; grow efficiently in culture to allow propagation of large numbers; operate a regulatory mechanism to control cell proliferation when transplanted *in vivo* (over proliferation could lead to hypoglycaemia); and the phenotype of the cells must remain stable in culture and *in vivo*.

From these criteria we can see that the pancreatic islet β-cell is a highly specialised secretory cell, and manipulating an easily accessible cell type to behave as a β-cell is not going to be an easy task.

The first steps in engineering a surrogate β-cell are making the chosen cells secrete insulin, getting the cells to process the proinsulin to fully mature insulin and then conferring stimulus -secretion coupling of glucose stimulated insulin secretion.

Neuroendocrine cell lines such as murine AtT20 pituitary cells contain the necessary machinery for processing of proinsulin and regulated secretion (Moore *et al*, 1983). However in non-endocrine cell lines stably transfected with the preproinsulin gene, insulin-like material is secreted constitutively as proinsulin (Moore *et al*, 1983).

There are two methods for achieving processing in non-endocrine cells. The use of mutagenesis to alter the preproinsulin dibasic cleavage sites to the consensus sequence for a single enzyme such as furin. Furin is a subtilisin-like serine protease expressed

38

endogenously in almost all cells. Furin will cleave dibasic sites of the consensus sequence Arg^{-4}, Xaa^{-3}, Lys/Arg^{-2}, Arg^{-1}, $\downarrow$, Xaa^{+1}. The alternative to mutagenesis is overexpression of furin in the cells to improve the mostly inefficient processing of the non-consensus insulin cleavage sites. There has been some success with the mutagenesis of proinsulin and subsequent processing in non-endocrine cells. Since the present study was started Yanigita *et al* (1993) have found that mutated rat proinsulin I was processed to mature insulin by a variety of non-endocrine cell lines such as Chinese hamster ovary (CHO), liver cells (Hep G2) and fibroblasts (NIH 3T3). Human proinsulin cDNA mutated for consensus cleavage by furin was found to be processed by human kidney cells (Groskreutz *et al*, 1994). Also Vollenweider *et al* (1995) have shown that co-transfection with cDNAs for wild-type human proinsulin with furin in monkey kidney (COS7) cells resulted in 60% of the insulin-like material being secreted as mature processed insulin.

Engineering of glucose stimulated insulin secretion in non-endocrine cell lines is not as advanced as the engineering of processing ability. The mechanism of glucose stimulated insulin secretion is still being studied in β-cells and is not yet fully understood.

At present, efforts to confer glucose stimulated insulin secretion have been limited to the use of neuroendocrine cells such as the murine AtT20 pituitary cell line and rodent insulinoma/β-cell tumour cell lines.

Insulin secreting AtT20 cells do not exhibit glucose stimulated insulin secretion despite low levels of glucokinase activity (Hughes *et al*, 1991). The AtT20 cells were later found to be completely lacking in the expression of the high affinity glucose transporter GLUT2 (Hughes *et al*, 1992). Co-expression of insulin and GLUT2 in AtT20 cells has more recently been shown to confer some glucose stimulated insulin secretion (Hughes *et al*, 1993).

Insulinoma/β-cell tumour cell lines have been shown to lose glucose stimulated insulin secretion when cultured *in vitro* (Ferber *et al*, 1994). These cells appear to down-regulate the expression of GLUT2 and increase the ratio of the glucose phosphorylating enzyme hexokinase relative to glucokinase. Stable transfection with the GLUT2 gene has been shown to restore glucose stimulated insulin secretion in insulinoma cells in culture (Tiedge *et al*, 1993). Since generation of the main signals (as yet unknown) to promote insulin gene transcription, translation and secretion appear to require glucose metabolism, it follows that GLUT2 and glucokinase would be required in non-endocrine cells to confer glucose stimulated insulin secretion. However, it is likely that further manipulations, in some non-endocrine cell types, would be necessary to confer glucose stimulated insulin secretion and glucose regulation of insulin biosynthesis. Fibroblasts and keratinocytes and myoblasts would be the most convenient cell types for somatic cell gene therapy. These cells are easy to biopsy, robust and grow well in culture. However these cell types are so unlike β-cells that they present a particularly formidable challenge when trying to engineer a surrogate β-cell. Hepatocytes are closer to β-cells than some other non-endocrine cell types and share a developmental origin with islet cells. The liver can be biopsied, although the technique is more invasive than obtaining, for instance, fibroblasts.

In principle the liver is a good choice for replacement of insulin as it is the main target organ for insulin action and the principal effector organ in maintaining glucose homeostasis and ketogenesis (Kolodka *et al*, 1995). As discussed earlier islet allografting has also shown some success in the portal circulation and the liver.

The liver has been the target of research into *in vivo* gene therapy for insulin delivery. Hepatic expression of insulin has been shown to alleviate the effects of severe diabetes in transgenic mice (Valera *et al*, 1994) and normoglycaemia has been achieved in rats after

40

recombinant retroviral-transduction of the liver *in vivo* with the insulin gene (Kolodka *et al*, 1995). Hepatoma cell lines *in vitro* have also been shown to secrete insulin following transfection, some of this insulin is secreted as mature processed insulin (Vollenweider *et al*, 1992; Simpson *et al*, 1995). However insulin production and secretion was found not to be regulated. Indeed, the control of glucokinase in the liver is regulated at least partly by insulin, which contrasts with the islet β-cell where glucokinase is mainly dependent on glucose influx into the cell (Tiedge & Lenzen, 1995).

It may be possible to use other cell types to deliver insulin, one such suggestion is the follicular cells of the thyroid, which have a large protein-synthetic capacity, sensitivity to hormone regulation and good blood flow contact (O'Malley & Ledley, 1993). However such cells again require a fairly invasive biopsy procedure for harvesting, and present different challenges in that they already secrete hormones.

When a suitable surrogate β-cell has been created a method of containing the cells on implantation must be found. The cells must either be engineered in such a way that they cannot proliferate, or be contained in a physical way.

Kawakami *et al* (1992) engineered insulin secreting fibroblasts co-transfected with a BALB/c mouse T-cell differentiation antigen CD8.2. The administration of an antibody to CD8.2 to mice implanted with the co-transfected cells resulted in a recurrence of hyperglycaemia and apparently complete immunological removal of the transplanted cells.

Methods for physically containing the implanted cells follow on from attempts to encapsulate allogeneic islets for transplantation. Although with somatic cell gene therapy the cells do not need to be hidden from the immune system, the methods and materials developed for encapsulating islets, such as alginate beads, could be useful for containing the autologous insulin-secreting cells. Another method which may be useful is the use of

collagen to form a tissue-like fabric around fibroblasts which have been engineered to secrete insulin. These tissue-like lattices have been found to be useful in skin grafting (Bell *et al*, 1979).

1.4 Aims and Objectives

As we have discussed, the first steps towards *ex vivo* gene therapy for insulin delivery involve the ability to make cells which will secrete mature insulin.

The aim of this work is to explore the feasibility of insulin delivery by genetic engineering of an endocrine cell type (murine pituitary AtT20) and a non-endocrine cell type (1BR.3.G human fibroblasts) to produce surrogate insulin-secreting β-cells. The specific objectives set are defined in each of the chapters and are summarised below.

Chapter three describes the mutagenesis of the dibasic cleavage sites of the human preproinsulin gene to the consensus cleavage sequence for furin. Thus the mutant proinsulin should be processed by endogenous furin when transfected into non-endocrine cells.

Chapter four addresses the problem of conferring glucose stimulated insulin secretion to AtT20 pituitary cells by expression of GLUT2. The chapter also examines the effects of implanted insulin-secreting AtT20 cells into streptozotocin diabetic nude mice.

Chapter five is involved with the creation of a human fibroblast which will secrete mature insulin. In this chapter the wild type human preproinsulin gene is co-transfected with furin to facilitate processing in the constitutive pathway of secretion. The effect of implanting the resulting cell lines into streptozotocin diabetic nude mice is examined.

Overall this thesis aims to make steps towards the creation of a functional surrogate β-cell, in a cell line that is easily accessible by biopsy and can be reimplanted into a variety of sites.

Chapter Two

Materials and Methods

Chapter Two : Materials and Methods

2.1 Suppliers of materials

Molecular biology reagents were supplied by Gibco BRL, Paisley, Scotland. Bacterial culture medium was from Difco, East Molesley, Surrey, U.K. Radiochemicals were obtained from Amersham International, Amersham, U.K. Other chemicals were of analar grade and supplied by BDH Ltd, U.K., Fisons, U.K., and Sigma, U.K., unless otherwise stated.

The rat anterior pituitary (corticotrophic) cell line AtT20d16v was a gift from Dr. R.E. Mains, John Hopkins University, Baltimore, USA. The human skin fibroblast cell line 1BR.3.G was obtained from the European Collection of Animal Cell Cultures, U.K. Recipes for solutions used in this chapter are given in appendix 1.

2.2 Cell Culture

2.2.1 Cell Culture Media and Supplements

DMEM (Dulbecco's modification of Eagles medium) with Glutamax 1, RPMI 1640 media, L-Glutamine and G418 sulphate were purchased from Gibco BRL, Paisley, Scotland. Fetal calf serum was from PAA Laboratories, Austria. PBS (phosphate buffered saline) tablets were from Flow Laboratories, Scotland. Hygromycin B was from Novobiochem-Calbiochem Ltd, Beeston, Nottingham, U.K. Plasticware was Nunclon from Nunc Inter-med, Denmark.

DMEM and RPMI 1640 were obtained as 500ml sterile 1x solutions containing 2.0g/l or 3.7g/l bicarbonate respectively, the DMEM contained Glutamax 1 which is L-Alanyl-L-

Glutamine. Media were supplemented by the addition of fetal calf serum to 10% (v/v) and L-Glutamine (RPMl 1640 only) to 2mM. For selection the appropriate amount of G418 sulphate or Hygromycin B was also added as described in section 2.5.4. The supplements were sterilised by filtration and added to 500ml of basic medium. Supplemented Media were stored at 5°C for a maximum of four weeks.

Fetal calf serum was stored in 50ml aliquots at -20°C. L-Glutamine was made up as a 200mM stock and stored in 5ml aliquots at -20°C. G418 sulphate was made up to 50mg/ml and Hygromycin B to 25mg/ml, both were stored as 5ml aliquots at -20°C.

2.2.2 Maintenance and Propagation of Cell Lines

The cell lines were routinely cultured in 80cm^3 flasks (Nunc) with 14mls of Dulbecco's modified eagles medium (DMEM) with Glutamax supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% carbon dioxide, 95% air. Cells were passaged when roughly 90% confluent.

Passaging was performed using a 2.5% trypsin solution (Sigma, U.K.) diluted in PBS, 1mM EDTA . Medium was removed and the cells were then rinsed with 10ml of sterile PBS. 1.5ml of diluted sterile trypsin in PBS (final concentration 0.25%) was then added. After 2-3 minutes at room temperature the trypsinisation reaction was stopped by adding 10mls of complete medium. The suspension of cells was then disaggregated by pipetting and approximately 0.5ml was added to a fresh flask with 13.5ml of warmed complete medium. The flask was then swirled gently to disperse the cells evenly. All cells were split 1 to 20 and passaged weekly during routine culture.

For long term storage the cells were frozen and stored in liquid nitrogen. The cells were trypsinised as described above and the cells recovered by centrifuging the suspension at

1000rpm in a bench top centrifuge for 5 minutes. The pellet of cells was then resuspended at a concentration of 10^7 cells/ ml of 90% fetal calf serum, 10% DMSO. The resulting suspension was aliquoted into a well labelled cryotube, which was placed in an insulating container and stored at -70°C overnight. The cryotubes were then transferred to tanks of liquid nitrogen for long term storage. When required the cryotubes of cells were rapidly thawed at 37°C, the cells were then transferred to a sterile 15ml centrifuge tube and 10ml of prewarmed medium was added dropwise. The cells were then pelleted by centrifugation at 1000rpm for 5 minutes, the supernatant was discarded. The pellet of cells was then resuspended in prewarmed complete medium and placed in an 80cm^3 flask for routine culture.

2.3 Plasmid Construction

2.3.1 Cloning Strategy

Two mammalian expression vectors were obtained for the sub-cloning work. pCB7 conferring resistance to hygromycin B and pCMV conferring resistance to neomycin (see appendix 2). The two vectors are suitable for co-transfection and double selection. The furin cDNA (2.4kb) was cut out of pBS-PACE by restriction enzyme digest using Sal I and Sst I. pCB7 was cut with the same restriction enzymes to linearise, and a simple sticky ended forced orientation ligation was carried out. The human preproinsulin cDNA (500bp) was cut out of pMtNeoIhppI-1 with Hind III and Xba I. pCMV was linearised with the same restriction enzymes and a sticky ended forced orientation ligation was performed to generate pCMV-hppI-1. pCB7 was also linearised with Hind III and Xba I and the same preproinsulin fragment ligated in to generate pCB7-hppI-1. These constructs allow

46

neomycin resistant pLK444/GLUT2 to be co-transfected and double selected with pCB7-HppI-1 (hygromycin resistance) into rat pituitary AtT20 cells. pCB7-PACE (hygromycin resistance) can be co-transfected and double selected with neomycin resistant pCMV-hppI-1 into human 1BR.3.G fibroblasts.

2.3.2 Large Scale Preparation of Plasmid DNA

A single colony of the bacteria containing the plasmid of interest was picked from a fresh plate and used to innoculate 5ml of LB broth, supplemented with ampicillin at 50µg/ml, in a 15ml centrifuge tube. This was incubated overnight at 37°C with shaking. This culture was used to innoculate 400ml of LB broth with antibiotic in a 2L flask which was incubated at 37°C with vigorous shaking. The cells were harvested by centrifugation at 5000rpm for 10 minutes at 4°C in a Sorvall RC5B centrifuge. The supernatant was removed and the pellet resuspended in 10ml of a solution containing 50mM Tris.HCl pH 7.5, 10mM EDTA in a centrifuge tube. 10ml of 0.2M sodium hydroxide, 1% SDS was added and the tube was mixed gently and incubated on ice for 10 minutes. 10ml of 2.55M potassium acetate pH 4.8 was added, the tube was mixed well, and centrifuged at 20,000rpm for 20 minutes at 4°C. The supernatant was removed to a fresh tube and allowed to reach room temperature before 0.6 volumes of isopropanol was added. The solution was mixed, left for 15 minutes, then centrifuged at 3000rpm for 15 minutes in a bench top centrifuge. The supernatant was then discarded and the pellet resuspended in 30ml TE pH 8.0. 30g of caesium chloride was added with 3ml of ethidium bromide (10mg/ml) and the solution was loaded into a Beckman Quickseal Ti70 tube. The caesium chloride gradient was formed by centrifugation at 55000rpm for 17 hours followed by 40000rpm for one hour in a Beckman L8M ultracentrifuge. The lower supercoiled plasmid-containing band in the gradient was located

47

under UV light and removed with a 19 gauge needle fitted to a 20ml syringe. The plasmid solution was diluted with 2 volumes of water in a centrifuge tube and 1 volume of isopropanol added. Following careful mixing the tube was centrifuged at 3000rpm for 15 minutes to precipitate the DNA. The pellet was resuspended in 400μl TE pH 8.0 and extracted with equal volumes of phenol twice before being extracted with equal volumes of phenol:chloroform, chloroform and water-saturated ether. Finally the DNA was reprecipitated with 2.5 volumes of absolute ethanol for 10 minutes and collected by centrifugation. The DNA was then resuspended in TE pH 8.0 in a volume suitable to the pellet size and the A_{260} was recorded to calculate its concentration (an A_{260} of 1 is equivalent to 50μg/ml DNA).

2.3.3 Restriction Enzyme Digests and Agarose Gel Electrophoresis

Restriction enzyme digests were carried out according to the manufacturers instructions in the buffers supplied (Gibco, Paisley, Scotland; Promega,U.K.; New England Biolabs, U.K.). These digests were then loaded onto agarose gels. Agarose gels (0.7%-1.2%) were prepared by dissolving agarose in TBE buffer (45mM Tris.HCl, 45mM boric acid, 1mM EDTA pH 8.0) by boiling and then adding ethidium bromide to a final concentration of 0.5μg/ml. Gels were then poured into Hybaid horizontal electrophoresis tanks and run in TBE buffer at approximately 5V/cm. Samples were mixed with 0.1 volume of loading buffer (25% (w/v) ficoll 400, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol) before loading onto the gel.

2.3.4 DNA Modification

Recessed 3' ends of DNA were made blunt using Klenow fragment of DNA Polymerase I. 2 units of Klenow fragment was added to 1µg of DNA in 25µl of nick translation buffer containing 80µM dNTPs and incubated at room temperature for 30 minutes. The enzyme was inactivated by heating to 70°C for 5 minutes. To prevent recircularisation of plasmids in ligation reactions, phosphatase treatment is required. Calf intestinal phosphatase was usually added to the restriction digest at 1:20 dilution (0.25U/µl). The reaction was inactivated by gel purification of the plasmid and subsequent extraction with phenol. Where this was not possible shrimp alkaline phosphatase was used. This can be inactivated by heating to 65°C for 10 minutes.

2.3.5 Gel Purification of DNA Fragments

To separate fragments of plasmid and purify DNA, slices of agarose gel containing the relevant DNA were removed from the gel and finely chopped. These were then mixed with an equal volume of phenol in a microcentrifuge tube and quick frozen in liquid nitrogen for 10 minutes. The tube was then centrifuged in a microcentrifuge for 15 minutes. The aqueous layer was removed to a fresh tube and the organic layer back extracted with an equal volume of TE pH 8.0. The aqueous layers were combined, extracted with an equal volume of phenol:chloroform and subsequently chloroform and then precipitated by the addition of 0.1 volume of 3M sodium acetate pH 7.0 and 2.5 volumes of absolute ethanol. Precipitated DNA was recovered by centrifugation in a microcentrifuge for 15 minutes. The pellet was resuspended in about 10µl of TE pH 8.0.

2.3.6 Ligation

Ligations were carried out using T4 DNA ligase (Gibco BRL, Paisley, Scotland) and the ligation buffer supplied according to the manufacturers instructions. The resulting mixture was then used to transform competent cells with selection by an appropriate antibiotic.

2.3.7 Preparation of Competent Bacteria and Transformation

Competent *E. coli* cells were prepared by Hanahan's method (Hanahan, 1983). A glycerol stock of the relevant strain of *E. coli* cells (eg XL1 Blue, DH5α) was plated on to a SOB agar plate. After incubation overnight at 37°C a colony of cells was resuspended in 1ml of SOB medium and used to innoculate 100ml of SOB medium in a 500ml flask. The flask was incubated with shaking at 37°C until the A_{550} was between 0.4 and 0.5. The culture was then chilled in ice water for 20 minutes before centrifugation at 2400rpm in 2x50ml tubes for ten minutes in a pre-cooled MSE coolspin centrifuge. The cell pellets were resuspended in 24ml of ice-cold FSB and incubated on ice for fifteen minutes. The cell suspension was centrifuged as before and the pellet resuspended in 8ml of ice-cold FSB. 280μl of dimethyl sulphoxide was added and the tubes left on ice for five minutes. To store these competent cells they were then immediately snap frozen in liquid nitrogen and stored at -70°C. When required for transformation the cells were thawed on ice and 200μl of cells was added to the relevant DNA in a 15ml tube on ice and incubated for 40 minutes. The mixture was then heated to 42°C for 90 seconds in a waterbath and then returned to ice for 2 minutes. The mixture was then spread onto an LB agar plate containing the relevant antibiotic. The plates were then incubated overnight at 37°C.

2.3.8 Small Scale Preparation of Plasmid DNA

Single colonies of bacteria from a fresh transformation plate were used to innoculate 5ml of LB broth supplemented with the appropriate antibiotic in a 15ml tube. The tubes were incubated overnight at 37°C with shaking. 1.5ml of the resulting culture was poured into a microcentrifuge tube and the cells pelleted by centrifugation for 2 minutes. The cell pellets were resuspended in 100µl of a solution containing 50mM glucose, 25mM Tris.HCL, 10mM EDTA pH 8.0 and mixed thoroughly. 200µl of 0.2M sodium hydroxide, 1% (w/v) SDS was added, the tubes were mixed by inversion and left to stand for 5 minutes. 150µl of 2.55M potassium acetate pH 4.8 was added, the solution was mixed vigorously and the cell debris were pelleted by centrifugation for 3 minutes. The cleared supernatants were removed and subjected to 3 sequential extractions with equal volumes of phenol, phenol:chloroform, and water saturated ether, before precipitation with 2.5 volumes of absolute ethanol and 0.1 volumes of 3M sodium acetate. The pellets were washed with 70% ethanol and resuspended in 10-50µl of TE pH 8.0. The DNA was then analysed by restriction enzyme digestion with the appropriate enzymes followed by agarose gel electrophoresis and ethidium bromide staining.

2.4 Mutagenesis

2.4.1 Transformation of E.Coli with M13K19HppI-1 DNA

E. coli cells (XL1 Blue or HB2154 as required) were plated out on to GMM agar from glycerol stock and incubated at 37°C overnight. A colony was dispersed in 5ml of 2xTY medium and incubated overnight at 37°C with shaking. 200µl of this overnight culture was then used to innoculate 15ml of 2xTY medium. The culture was then incubated at 37°C

with shaking until early log phase (A_{550}=0.4). The culture was then chilled in ice water for twenty minutes, before centrifugation at 2400rpm for 10 minutes in a pre-cooled MSE coolspin centrifuge. The supernatant was poured off and the cells were resuspended in 0.5 volumes of ice-cold 100mM calcium chloride and left in ice water for 30 minutes. The suspension was then centrifuged as before and the cell pellet resuspended in 0.1 volumes of the ice-cold 100mM calcium chloride. 200μl of these competent cells was added to the DNA in a 15ml Falcon tube on ice. The cells and DNA were incubated on ice for 40 minutes before heating to 42°C in a water bath for ninety seconds and cooling on ice for two minutes. 3ml of molten H-top agar at 45°C and 200μl of a saturated culture of HB2151 cells were added and this mixture poured onto an H-agar plate. The agar was allowed to set and the plate was incubated overnight at 37°C.

2.4.2 Single Stranded Template Preparation

Phage plaques obtained from transformation were used to infect 1.5ml cultures of *E.coli* diluted from and overnight culture of (XL1-Blue) cells in 2xTY medium. These cultures were then incubated at 37°C with shaking for 5 hours. The infected cultures were then transferred to microcentrifuge tubes and centrifuged for one minute to pellet the cells. 1ml of the supernatant was added to 200μl of freshly prepared 2.5M sodium chloride, 20% polyethylene glycol 6000, and the solutions were mixed by vortexing and left at room temperature for 15 minutes. Precipitated phage particles were pelleted by centrifuging in a microcentrifuge for 5 minutes, the supernatant was aspirated and the pellet was resuspended in 100μl of TE pH 8.0 (10mM Tris.HCl, 1mM EDTA). This resuspension was then subjected to 3 sequential extractions with phenol, phenol:chloroform and water saturated ether. Phage DNA was then precipitated by the addition of 0.1 volumes of 3M sodium

acetate pH 7.0 and 2.5 volumes of absolute ethanol. The precipitate was recovered by centrifugation for 5 minutes in a microcentrifuge, air dried and resuspended in 10μl of TE pH 8.0.

2.4.3 DNA Sequencing

Single stranded DNA was sequenced using a Sequenase kit (USB). All solutions required were provided except the radiolabelled α^{35}S-dATP which was purchased from Amersham International, Amersham, U.K. An oligonucleotide which is complementary to a sequence at the 3' end of the coding strand of the hppI cDNA was used as a sequencing primer. Single stranded DNA (0.5μg) was mixed with 0.5pmol of primer in 40mM Tris.HCl pH 7.5, 20mM magnesium chloride, 50mM sodium chloride in a total volume of 10μl. Primer and DNA were then annealed by heating to 70°C for 5 minutes and cooled to room temperature over at least thirty minutes. After cooling, 2μl of dilute labelling mix (as supplied), 1μl of 100mM DTT, 0.5μl (5μCi) of α^{35}S-dATP (1000 Ci/mmol) and 2 units of Sequenase^{TM} polymerase diluted 1:8 in ice-cold TE pH 8.0 were added and the reaction incubated at room temperature for 5 minutes. 4 fresh tubes were then prepared each containing 2.5μl of one of the termination mixes ddA, ddC, ddG or ddT (as supplied). 3.5μl of the labelling reaction mixture was added to each termination tube and the tubes were incubated at 37°C for 5 minutes. The reaction was then stopped by adding 4μl of stop solution (as supplied) and the samples were then heated to 90°C for 2 minutes and immediately loaded on to a 5% polyacrylamide gel containing 7M urea and electrophoresed in TBE at 2500V in a Hybaid tank. When the bromophenol blue dye had reached the bottom of the gel, the gel was removed and fixed for 20 minutes in 10% methanol, 10% acetic acid. The gel was

transferred to a sheet of Whatman 3MM paper and dried before autoradiography overnight at -70°C.

2.4.4 Oligonucleotide Site Directed Mutagenesis

The double primer technique with Eco K selection was used to introduce the desired mutations into the processing sites of hppI-1 (see introduction to chapter 3). M13K19hppI-1 single stranded template was prepared in XL1blue $E.coli$ cells and sequenced as described above. Mutagenic oligonucleotides complementary to the single strand obtained in the template preparation (as determined by sequencing) were synthesised by Alta Bioscience, University of Birmingham. Mutant oligonucleotides were Moli 1 (D3540) and Moli 2 (D3541) and the selection oligonucleotide was Sel 2 (D144) (sequences of the oligonucleotides can be found in appendix 3). Crude oligonucleotides were resuspended in 200µl of water and extracted with water saturated butanol 5 times. The A_{260} was then measured and the concentration adjusted to 10pmol/µl. In the mutagenesis reaction (figure 2.1) the template DNA (M13K19hppI-1) was annealed with the 3 primers, second strand synthesis then produces the mutant strand. This strand has the selection mutation where the Eco K site in the mp19 polylinker is mutated to an Eco B site by the Sel 2 oligonucleotide and should contain the two desired mutations in the hppI-1 cDNA. The reaction mixture also contains wild type strands still harbouring the Eco K site. When the mixture is transformed into the HB2154 F', repair⁻, Eco K⁺ strain of $E.coli$ the wild type strand is restricted by the Eco K enzyme, while the mutated strand with the new Eco B site is not. The HB2154 strain is also repair⁻, thus the mismatched bases will not be repaired. The HB2151 $E.coli$ lawn is repair⁺ to minimise any unwanted mutations. The resulting plaques will all carry the selection mutation, however, they may not all contain both of the other

Figure 2.1 The oligonucleotide site-directed mutagenesis reaction. The primers are annealed to M13K19hppI-1 and second strand synthesis extends from the selection oligonucleotide (Sel2) to the mutant oligonucleotides complementary to the hppI-1 gene (Moli1 & Moli2) to produce the mutant strand. The mutant strand will have the selection mutation (Eco K mutated to Eco B) and may have either of the hppI-1 mutations, both of them or neither.
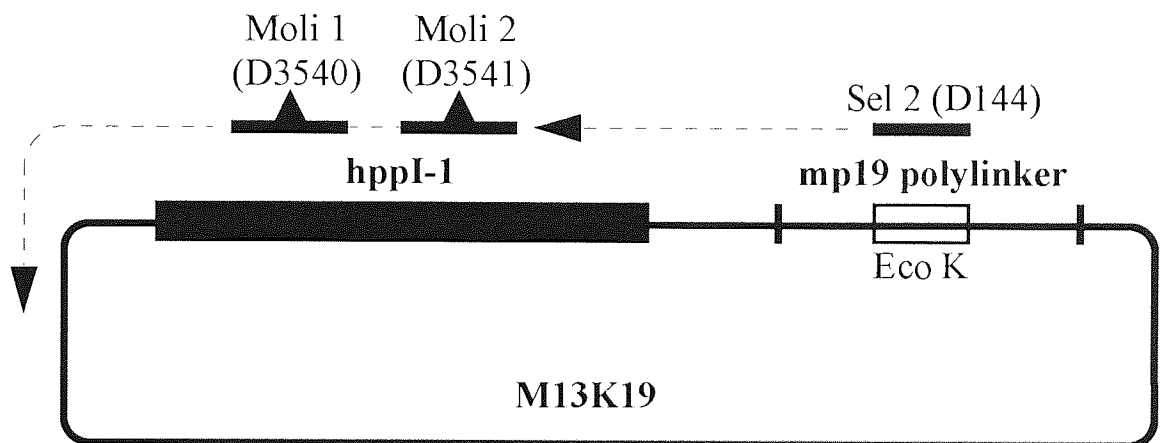
mutations, but will be a mixture of either the Moli 1 mutation or the Moli 2 mutation, both mutations or neither. The desired double mutant must be identified by screening the plaques using DNA sequencing of template preparations from each plaque. In the mutagenesis reaction 1μg of M13K19hppI-1 template DNA was mixed with 10pmol of each of Moli 1, Moli 2 and Sel 2 oligonucleotides in 10μl of 10mM Tris.HCl pH 8.0, 10mM magnesium chloride. Template and primers were annealed by heating to 80°C and allowing to cool to room temperature for over 30 minutes. Double stranded DNA was then synthesised by adding all 4 deoxyribonucleotides and ATP to 250μM, 1 unit of klenow fragment of DNA polymerase 1 and 10 units of T4 DNA ligase. The reaction was incubated at 14°C for 4 hours. The mutagenesis mixture was then used to transform competent HB2154 *E.coli* cells with a lawn of HB2151 *E.coli*. Template preparations from plaques obtained from this transformation were sequenced to screen for the required double mutant.

2.4.5 PCR mutagenesis

PCR mutagenesis was also used in the mutagenesis of the processing sites of hppI-1. In this technique the hppI-1 cDNA was sub-cloned into the EcoR1 restriction site of the Sp64T-R vector (Kreig & Melton, 1984). Orientation analysis was required to identify positive clones with the insert in the correct orientation. A positive clone was grown up and DNA prepared on a large scale for use as the template DNA in the PCR mutagenesis. The PCR was performed as 3 separate reactions involving 6 oligonucleotide primers (the sequences of the oligonucleotide primers can be found in appendix 3). The diagram in figure 2.2 shows the 3 fragments generated by the PCR reactions with overlapping complementary sequences. These fragments were then annealed together to give the mutated gene intact. Mutagenic oligonucleotides were synthesised by Alta Bioscience, University of Birmingham.

56

Figure 2.2 Schematic of the PCR mutagenesis and the fragments created by each reaction. The 3 reactions were carried out by priming the Sp64T-RhppI-1 with the appropriate primers and amplifying the fragment according to the profiles given in section 2.4.5.

Sp6 primer   CMoli 3   CMoli 4
D5770   D4794   D4795

→   →   →   EcoR1

| Sp64T-R | | |
| polylinker | **hppI-1** | |

        EcoR1   ←   ←   ←

                    Moli 3   Moli 4   hppI-1 end
                    D5936   D5937   D6019

D5770   D4794   D5937   D6019
       Reaction 1   Reaction 2   Reaction 3

                    D5936   D4795
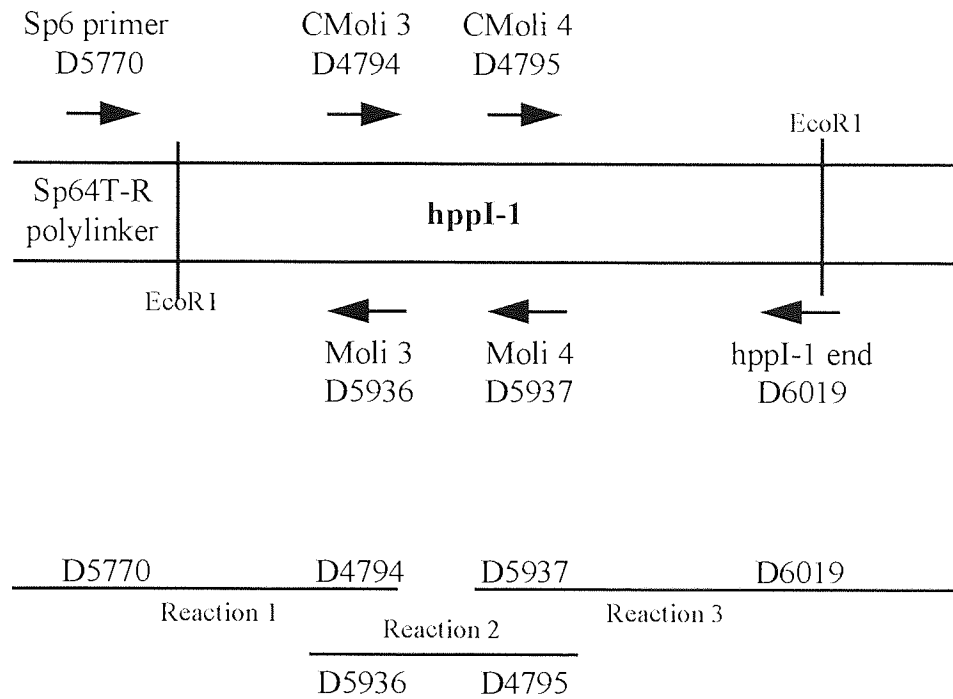
Oligonucleotides were resuspended in sterile water to a concentration of 10pmol/μl. To titrate for the optimum Mg^{2+} concentration for the mutagenesis reaction 1μl of 1μg/μl template DNA was mixed with 20μl of each of the two primers required for each reaction, 4μl dNTP (20mM), 20μl 20x vent polymerase buffer, 2μl vent DNA polymerase (5U/μl) and 115μl of sterile water. 45μl of this mixture was then added to each of four tubes containing 1μl, 2μl, 3μl, and 5μl of 100mM MgCl$_2$ and 4μl, 3μl, 2μl, and 0μl of sterile water respectively, giving tubes with final Mg^{2+} concentrations of 2mM, 4mM, 6mM, and 10mM. The mixture was covered with 2 drops of mineral oil and centrifuged briefly to separate the layers. Amplification was then carried out using the following profile:

	95°C	5 minutes	
	95°C	15 seconds	
Annealing temperature	°C	25 seconds	} 4 cycles
	72°C	1 minute	
	95°C	15 seconds	
Annealing temperature	°C	25 seconds	} 20 cycles
	72°C	1 minute	

The annealing temperatures were obtained by calculating the Td of the primers using the Wallace rule and using a temperature 2°C below the Td for annealing (The Wallace rule gives the mid point temperature on the temperature versus dissociation curve (Td) for short oligonucleotides. Td(°C) = 4x(number of G or C bases) + 2x(number of A or T bases)). The amplifications were separated on an agarose gel to check for the required band. The band was then gel purified by cutting out of the gel and soaking in 1ml of distilled water to remove the TBE running buffer. The gel was then placed in a 0.6ml tube on a plug of siliconised glass wool and frozen for 10 minutes at -70 °C. The bottom of the tube was then pierced and it was placed inside a 1.5ml tube and centrifuged for 10 minutes in a microfuge. The inner tube was then discarded and the eluate was extracted once with

phenol:chloroform and precipitated with 0.1 volumes of 3M NaAc and 2.5 volumes of absolute ethanol. The precipitated DNA was resuspended in 30μl of TE and run out on an agarose gel which was stained with ethidium bromide to check the quality and quantity of the purified DNA. Once each reaction of the PCR mutagenesis was complete the fragments were annealed by mixing equal quantities of each fragment with 15μl 2.5mM MgCl$_2$, 20μl 10x Taq buffer and sterile water to 157μl. This mixture was overlaid with mineral oil and heated to 80°C for 5 minutes then cooled slowly to 30°C. 2μl 2mM dNTPs and 1μl Taq DNA polymerase were then added and the mixture incubated at 37°C for 30 minutes. 20μl of each of primer required to amplify the annealed product were then added and the following profile was used for amplification:

	72°C	2 minutes
	95°C	5 minutes
	95°C	15 seconds
Annealing temperature	°C	25 seconds
	72°C	2 minutes

} 25 cycles

The annealing temperature was again calculated as 2°C below the Td for the primers used in the amplification. The amplification was then run out on an agarose gel to check for the required sized product. The annealed product was then gel purified as described above for the mutagenic fragments. The purified product was then digested with the EcoR1 restriction enzyme and repurified. The cut and purifed product was then ligated into the Sp64T-R vector for sequencing. The final stages of the PCR mutagenesis were achieved with the help of Dr. H. Cragg, A. Hart and Dr. N.A. Taylor at the University of Aberdeen.

2.5 Generation of Stably Transfected Cell Lines

2.5.1 Cell Lines Used

AtT20d16v - Mouse anterior pituitary cell line .

1BR.3.G - Human fibroblast cell line.

2.5.2 Calcium Phosphate Co-precipitation and Transfection of Cells

24 hours prior to transfection, cells (AtT20d16v) were replated at approximately 8×10^5 cells per 10cm dish. 30μg of the required DNA in 0.9ml of 1mM Tris.HCL pH 8.0, 0.1mM EDTA was placed in a 5ml universal and 0.1ml of 2.5M calcium chloride was added. This solution was added dropwise to 1ml of 2xHBS in a second 5ml universal with continual agitation. The mixture was left at room temperature for 30 minutes to allow a precipitate to form. 1ml of the resulting suspension was added to each plate of cells to be treated. The cells were then incubated for 6 hours in standard culture conditions. The medium was then removed and 2mls of 10% (v/v) glycerol in serum free medium was added for one minute. This was then removed and the cells were then washed twice with serum free medium. Complete medium was then added and the cells incubated in standard conditions for 48 hours. The cells were then split into 4-5 10cm dishes and after 12-16 hours recovery were transferred into selection media.

2.5.3 Polybrene/DMSO Mediated Transfection

24 hours prior to transfection cells (1BR.3.G) were replated at approximately 5×10^5 cells per 10cm dish. 20μg of the required DNA (10μg of each of the required DNAs for double transfection) was added to 3mls of complete medium in a 5ml sterile universal. 3μl of a

60

10mg/ml sterile solution of polybrene (hexadimethrine bromide or 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) was added and the solution was mixed. The medium was then removed from the plates of cells and replaced with the DNA/polybrene mixture. The plates of cells were then incubated for 6 hours in standard culture conditions. Every hour the plates were rocked to ensure the DNA/polybrene mixture remained evenly distributed. After 6 hours the DNA/polybrene medium mixture was aspirated and replaced with 5mls of 27% (v/v) DMSO in complete medium. The plates were then incubated for 4 minutes. The cells were washed three times with serum free medium to remove the DMSO and DNA/polybrene mixture. The cells were then fed with complete medium and incubated for 48 hours in standard conditions. Each plate of cells was then split into 4-5 10cm dishes using routine subculture techniques and left to recover for 12-16 hours before being transferred into selection medium.

2.5.4 Selection of Transfectants

Cells transfected with a plasmid conferring neomycin resistance were incubated in medium containing G418 at 0.5mg/ml. Cells transfected with a plasmid conferring hygromycin resistance were incubated in medium containing hygromycin B at 125μg/ml. After 3-4 weeks, resistant colonies were transferred individually into 6 well plates and allowed to proliferate. These colonies were then analysed for the expression of the required genes.

2.6 Analysis of mRNA

2.6.1 Single Step RNA Isolation

mRNA was prepared from semi-confluent plates of cells by the method described by Chomczynski and Sacchi, 1987. Cell monolayers were washed with PBS and 1ml of solution D added. The resulting lysate was transferred to eppendorf tubes and 100μl of 2M sodium acetate pH4.0 added and the tubes vortexed. 1ml of water saturated phenol and 200μl of 49:1 chloroform:isoamyl alcohol was then added and the tubes vortexed thouroughly. The tubes were cooled on ice for 20 minutes prior to centrifugation for 20 minutes at 4°C in a microfuge. The aqueous top layer was then transferred to a fresh tube and precipitated for at least an hour in 1 volume of isopropanol at -20 °C. The precipitate was collected by centrifugation at 4°C for 20 minutes in a microfuge and dissolved in 300μl of solution D. A further precipitation in 1 volume of isopropanol was performed overnight at -20 °C. The precipitate was collected as before and washed with 75% (v/v) ethanol in DEPC treated water. The pellet was then dried and resuspended in 50μl of 0.5% (w/v) SDS in DEPC treated water. A 5μl sample was electrophoresed on a 1% agarose gel to check the quantity and quality of the preparation. RNA was then stored at -70°C until required.

2.6.2 Northern Blotting

The gel was prepared by boiling 5.25g of agarose in 262.5ml of water, then when cooled to 60°C adding 35ml of 10xMOPS buffer (1M MOPS, 0.5M sodium acetate, 0.1M EDTA) and 52.5ml of 37% (v/v) formaldehyde solution. The RNA samples were prepared by

adding 10µl of RNA preparation at the desired concentration to 30µl of denaturing solution (1100µl formamide, 220µl 10xMOPS, 330µl formaldehyde) and heating to 60°C for 5 minutes. The gel was poured in a Biorad Horizon 20.25 gel set and the wells were rinsed before loading. 2µl of loading buffer (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, 1mM EDTA) was added to each sample and these samples were loaded. The gel was electrophoresed in 1xMOPS buffer at approximately 100V for 3-4 hours. The gel was then placed on a wick soaked in 20xSSC over a reservoir, a piece of Hybond-N filter cut to size was placed over the gel avoiding bubbles. Filters were then covered with layers of filter paper and dry paper towels cut to size. Finally, a weight was placed at the top of the pile and the RNA was left to transfer onto the filters overnight. Filters were then fixed by exposure to 1200J of U.V. light. Filters were then prehybridised in JAP buffer for at least three hours. (JAP buffer was prepared by adding 300mls of a solution of 27g/l NaH_2PO_4, 83g/l Na_2HPO_4, 1.86g/l EDTA, to 150mls of 20% SDS and 4.5 ml of 10mg/ml stock of denatured, sonicated herring sperm DNA). Radioactively labelled cDNA probes were prepared using the Pharmacia Oligolabelling Kit and α^{32}-P dCTP. Probes were denatured at 95°C for 5 minutes before adding to the prehybridising filters. Hybridisation was carried out at 65°C for about 16 hours. Filters were washed twice at room temperature in 2xSSC, 0.1% SDS for 15 minutes, followed by one wash at 50°C in 0.1xSSC, 0.1% SDS for 30-60 minutes. Filters were then sealed in Saran Wrap and exposed to X-ray film (Fuji) at -70°C overnight or for longer if the signal was weak.

2.7 *In Vivo* Studies

2.7.1 Animal Care

The animals were maintained in a sterilised air conditioned room at $22 \pm 2^{\circ}C$, with a light cycle of 12 hours light (0800-2000) and 12 hours dark. Animal handlers wore overalls, gloves and footwear at all times. A standard rodent pellet diet (SDS Economy Rodent Breeder, Special Diet Services (SDS), Witham, Essex, U.K.) and tap water were provided *ad libitum* throughout the study unless decreed otherwise by experimental procedure.

2.7.2 Blood Sampling

Blood samples of approximately $60\mu l$ for determination of plasma glucose and insulin were obtained from the cut tail tip of conscious mice into microfuge tubes pretreated with 500U/ml heparin. Blood samples were stored on ice throughout the procedure. The plasma was separated by centrifugation at 15000g for 30 seconds. $20\ \mu l$ was stored at $-20^{\circ}C$ for insulin assay, and 5 to $10\mu l$ used immediately for glucose analysis.

2.7.3 Induction of Diabetes

Diabetes was induced in overnight fasted mice by intraperitoneal injection of 200mg/kg streptozotocin dissolved in citrate buffer as a 30 mg/ml solution. The streptozotocin solution was kept on ice between solubization and injection to minimize the deterioration of anomers. Induction of diabetes was confirmed by the development of non-fasting plasma glucose concentrations of approximately 12mmol/l and above after 4 days.

64

2.7.4 Implantation of Cells

Trypsinised cells were counted using a haemocytometer, pelleted by centrifugation and resuspended in minimal volumes of complete medium. The cell suspensions were then administered to concious mice by intraperitoneal injection via a 25 gauge needle. The mice received a maximum volume of 300µl of cell suspension per mouse. The total number of cells delivered varied for each experiment as described in subsequent chapters. Following implantation the weight, food intake and plasma glucose concentrations of the animals were monitored once or twice per week.

2.7.5 Glucose Tolerance Tests

Oral glucose tolerance tests (OGTT) were performed on fasted animals. The glucose dose used was 2g/kg body weight, administered as a 40% w/v solution (i.e 5ml/kg) by oral gavage. Blood samples were usually obtained immediately before (i.e time 0) and 30 and 60 minutes after administration of the glucose load. The plasma was separated for glucose assay.

2.7.6 Removal of Implants and Tumours

At the end of the implant experiments the mice were killed by cervical dislocation and autopsies were carried out. Any visible tumours or cell aggregations were dissected out and preserved in 10% formaldehyde in PBS for histological examination.

2.7.7 Histological Processing

Tumour sections were processed and stained by Nicola Carless (Research Technician), Islet Research Laboratory, Worcester Royal Infirmary, Worcester.

Tissue pieces were fixed in 10% formal saline for a minimum of 24 hours, placed in processing cassettes and processed on the Shandon 2 litre processor (Shandon Scientific Instruments, Runcorn, Cheshire, U.K.) on a 22 hour cycle. This automated process dehydrates the tissue by passing them through a series of graded alcohols up to 100%, and clearing with chloroform. The dehydrating process removes all the water from the tissue and permits it to be placed in a non-aqueous clearing agent (e.g. chloroform, xylene, toluene) which is miscible with melted paraffin. Following embedding in paraffin wax, the tissue was cut on a sledge microtome to 3-5μm sections.

2.7.8 Haematoxylin and Eosin Staining

Haematoxylin and eosin staining was performed to demonstrate general cell morphology. The sections were hydrated through xylene to distilled water, by a series of graded alcohols decreasing in strength. They were then placed in haematoxylin (Mayer's Haematoxylin) for 6 minutes. Differentiation was carried out by acid alcohol for 30 seconds (1% hydrochloric acid in 70% alcohol), followed by blueing up (changing the pH of the stain from acid to alkali causing a red to blue colour change) of sections in running tap water. The sections were then immersed in eosin for 3 minutes, washed in tap water and then dehydrated through a series of alcohols back to xylene. Finally the sections were mounted in Pertex mounting medium (Cellpath plc, Hemel Hempstead, Herts, U.K.). This process gives blue nuclei, pink cytoplasm and orange red blood cells.

2.7.9 Immunohistochemical Staining

Immunohistochemical staining was carried out to demonstrate the presence of insulin. A DAKO prediluted guinea pig anti-insulin antibody (DAKO Ltd, High Wycombe, U.K.) was used in conjunction with a DAKO Strept ABComplex/HRP Duet Kit (mouse/rabbit) to stain the tumour sections for insulin secreting cells. Paraffin sections were prepared as described above, cut to 3µm, floated onto distilled water and picked up onto alcohol clean slides. Sections were dried on to the slides and placed at 55°C overnight. The sections were then carefully dewaxed through graded alcohols. Endogenous peroxidase was blocked by incubating for 30 minutes in peroxidase blocking solution (see section 3.5). Sections were then washed well in running tap water and rinsed in tris-buffered saline (TBS) pH 7.6. Excess buffer was removed and a ring drawn around the section with a DAKO pen (supplied with the kit) in order to create a moat to hold the solutions on the tissue section. Serum blocking solution (10% non-immune serum (goat) (Zymed Laboratories Inc, California, U.K.) was then applied to the section for 10 minutes in a moist chamber (surgipath slide rack with damp blotting paper), excess was tipped off and the surrounding area gently wiped. Insulin antibody was applied for 60 minutes in the moist chamber. The sections were then washed well in stirring TBS for 5-10 minutes. After removing excess buffer from around the section, biotinylated goat antibody working solution (bottle one, duet kit) was applied to the section and left for at least 30 minutes in the moist chamber. Washing in TBS was then repeated and Strept ABComplex/HRP working solution (bottle two, duet kit) was added to the section for 30 minutes in the moist chamber. After a further washing step in TBS, sections were visualised with DAB solution (Sigma, U.K.) for 15 minutes at room temperature monitoring the colour intensity

67

throughout (if necessary DAB intensity can be highlighted using a copper sulphate solution). After washing well with tap water, sections were counterstained in Mayer's Haemalum for 60 seconds, then washed and blued in running tap water. Sections were then dehydrated and cleared through graded alcohols and finally mounted in Pertex mounting medium. A crisp, insoluble, stable dark brown reaction indicates the presence of insulin in the cell cytoplasm and the nuclei are stained blue. Microwave antigen retrieval was performed with some of the sections to enhance insulin demonstration. Following blocking of the endogenous peroxidase, sections were placed in citrate buffer and microwaved at full power until the solution boiled, after cooling to room temperature the protocol was continued as described.

2.8 Analyses

2.8.1 Insulin Radioimmunoassay

The general principle of radioimmunoassay (RIA) is based on the immunogenic properties of polypeptide hormones such as insulin (Ag); hence a specific antibody (Ab) can be raised when the hormone is administered to a species in which the hormone bears sufficient structural difference from the endogenous hormone to induce an immune response. A standard preparation of purified hormone can be labelled with a radioisotope (Ag* - in this case ^{125}I-Insulin), and a known amount added to a limiting quantity of antibody. Unlabelled test hormone is then added; the antibody shows no preference in binding to Ag or Ag*, and since Ab and Ag* are both known constant values, the amount of Ag*-Ab complex formed is inversely proportional to the concentration of Ag (unknown).

Two different insulin antibodies were used throughout this project. A specific human insulin antibody (Linco Research Inc, St Louis, USA) and a non-specific insulin antibody (Sigma,U.K.). The specific human antibody had the following crossreactivity:

Human Insulin	100%
Human Proinsulin	<0.2%
Des 31,32 Proinsulin	<0.2%
Des 64,65 Proinsulin	76%
Rat Insulin	<1.0%

The Sigma insulin antibody crossreacts extensively with the insulins and proinsulins of a number of species including rat/mouse I and II and human.

Insulin RIA was used to measure insulin released from transfected cells *in vitro*, therefore, all samples were collected in serum free cell culture medium.

Human insulin standards were prepared by serial dilution of human actrapid insulin (NovoNordisk, U.K.) in serum free cell culture medium to give final concentrations of 10ng/ml to 0.16ng/ml. Insulin antibody was diluted in assay buffer (5g/L BSA in PBS) to the manufacturers recommended dilution (Linco antibody) or 1:45000 (Sigma antibody). ^{125}I-Insulin was diluted in assay buffer to give approximately 12000 counts per minute in 100μl. The following tubes were set up in triplicate:

Total Counts	-empty
Blank	-300μl buffer
Zero	-200μl buffer, 100μl antibody
Standards	-100μl buffer, 100μl standard, 100μl antibody
Samples	- 100μl buffer, 100μl sample, 100μl antibody

After mixing, 100μl of labelled insulin was added to all the tubes which were mixed and covered before incubation overnight at 4°C. 100μl of 1% γ-globulin in PBS was then added to all the tubes except the total counts. After a short incubation, 1ml of 21% PEG6000 in PBS was added to the same tubes and they were mixed thoroughly. After a 20 minute

incubation at 4°C the tubes were centrifuged at 3000rpm in a bench top centrifuge for 30 minutes. The supernatant was discarded and the tubes were drained dry for 1-2 hours. The precipitates were then counted on an automatic gamma counter (LKB-Wallac 1282 CompuGamma, London) for one minute per tube.

2.8.2 Insulin Immunoradiometric Assay (IRMA)

Insulin IRMA was used to determine plasma insulin concentration of diabetic nude (nu/nu) mice implanted with insulin secreting cells. Blood samples were collected from the mice and separated plasma stored in aliquots of 25μl or 50μl at -20 °C until assayed.

A Medgenix IRMA kit for human insulin was purchased from Lifescreen, Watford, U.K. The principle of the assay kit was based on coated tube separation and the oligoclonal system, in which several monoclonal antibodies directed against distinct epitopes of insulin are used. The capture antibodies are attached to the inner surface of the plastic tube. Standards and samples added to the tubes at first show low affinity for the capture antibody and the addition of the second monoclonal antibody, the tracer antibody, radiolabelled with ^{125}I completes the oligoclonal system and triggers the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration.

Coated tubes were labelled in duplicate and 50μl of standard or sample was pipetted to the bottom of each tube. Samples were not available in duplicate and only single tubes of the samples were assayed. 50μl of the tracer was then added to each tube and two empty tubes (not coated) for total counts. The racks of tubes were agitated by hand to mix the contents. After 2 hours incubation at room temperature the tubes were aspirated and washed out with 2ml of washing solution (supplied with the kit) except the total counts tubes. The wash step was repeated and the tubes thoroughly aspirated before counting on an automatic gamma

counter (LKB-Wallac 1282 CompuGamma, London, U.K.) for 1 minute per tube. Determination of insulin concentration of samples was determined by interpolation of counts divided by total counts (%) values. The assay specifically detected human insulin with no cross reactivity with human proinsulin or rat/mouse insulin or proinsulin. The intra-assay variation was calculated at around 4% and the inter-assay variation around 10%.

2.8.3 ACTH Immunoradiometric Assay (IRMA)

ACTH IRMA was used to determine plasma ACTH concentrations of diabetic nude (nu/nu) mice implanted with ACTH secreting AtT20 pituitary cells. Blood samples were collected from the mice and separated plasma stored in aliquots of 25µl or 50µl at -20 °C until assayed. A Euro-Diagnostica ACTH IRMA kit was purchased from Europath Ltd, Cornwall, U.K. The assay used site-specific polyclonal antibodies in which the peptide hormone is quickly sequestered by binding of highly avide non-competing antibodies: one from sheep and the other from rabbit. They protect ACTH from degradation by proteases allowing the assay to be performed in plasma. One of the antibodies is a highly purified mono-specific radioiodinated sheep IgG with specificity to the amino terminal region of ACTH. The rabbit antibody reacts non-competitively with the C-terminal region. Separation of bound and free labelled sheep anti-ACTH antibody is achieved by the addition of a second reagent consisting of a sheep anti-rabbit IgG antiserum. It only precipitates radiolabelled sheep antibody bound to the ACTH-rabbit complex. Radioactivity present in the resulting precipitate collected by centrifugation is directly proportional to the concentration of ACTH in the sample. Standards were set up in triplicate and samples as single tubes. 100µl of standard (as supplied) or sample was added to each tube, samples were made up to 100µl with distilled water. 100µl of the ACTH (^{125}I) complexing reagent

71

was added to each tube and to three empty tubes for total counts. The tubes were vortexed gently and incubated for three hours at room temperature. 100μl of precipitating reagent (as supplied) was then added to each tube, the tubes were vortexed and a further 30 minutes incubation carried out. Following addition of 1ml of wash solution (as supplied) tubes were centrifuged at 3000rpm for 20 minutes at 4°C in a bench top centrifuge. The supernatant was aspirated and a further 1ml of wash solution added. The centrifugation step was repeated and the supernatant well aspirated from the pellet. Tubes were then counted on an automatic gamma counter (LKB-Wallac 1282 CompuGamma, London, U.K.) for 1 minute per tube. ACTH concentration of the samples was determined by interpolation of the binding/maximum binding (%) values. The assay had an intra-assay variation of around 3% and an inter-assay variation of around 5%. The sensitivity of the assay was calculated at 0.8pg/ml.

2.8.4 Plasma Glucose Assay

Glucose was assayed by an automated glucose oxidase procedure (Stevens, 1971) using a Beckman Glucose Analyser (Beckman Instruments, High Wycombe, Bucks, U.K.). The analyser uses the oxygen rate method to assay glucose; an oxygen electrode within the reaction well compares oxygen utilisation by the sample with oxygen utilisation of a standard glucose solution (a standard of 8.3mmol/l glucose was used). The rate of oxygen utilisation is directly proportional to the concentration of glucose in the solution:-

$$\beta\text{-D-glucose} + O_2 \xrightarrow[\quad H_2O \quad]{\text{glucose oxidase}} \text{Glucuronic acid} + H_2O_2$$
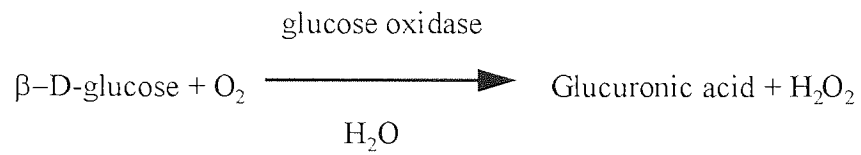
The H_2O_2 is removed by two further reactions to ensure it cannot yield back any oxygen to the solution:-

$$H_2O_2 + C_2H_5OH \xrightarrow{\text{catalase}} CH_3CHO + 2H_2O$$

$$H_2O_2 + 2H^+ + 2I \xrightarrow{\text{molybdate}} I_2 + 2H_2O$$
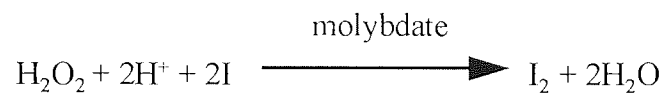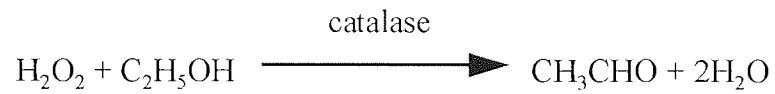
Chapter Three

Plasmid Construction and Mutagenesis

Chapter Three : Plasmid Construction and Mutagenesis

3.1 Introduction

Plasmid Construction and Cloning

Without the advances made in the field of molecular biology in the last 25 years, gene therapy could not even be contemplated. In this project the construction of plasmids containing the genes of interest provides the basis for the creation of novel cell lines expressing insulin and furin or GLUT2.

Both molecular biology and biomedical research have experienced revolutionary change with the development of gene manipulation. Gene manipulation refers to a variety of complicated *in vivo* genetics as well as to *in vitro* techniques. In most western countries there is a precise legal definition of gene manipulation as a result of government regulation to control it. In the UK gene manipulation is defined as 'the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid or any other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation' (Old & Primrose, 1989). *In vitro* gene manipulation was first made possible in the early 1970's with the simultaneous development of techniques for the transformation of *E.coli*, cutting and joining of DNA molecules and the monitoring of such reactions. The first cloning experiments were reported in 1972-3 (Jackson *et al*, 1972: Lobban & Kaiser, 1973) and were quickly followed by many similar experiments. Monitoring the progress of cloning experiments was originally performed by velocity sedimentation in sucrose gradients. Gel electrophoresis has now entirely superseded this method. As well as its analytical applications gel electrophoresis is

75

extensively used preparatively for purification of specific DNA fragments. It was reported in 1972 that the migration rates of the DNA molecules in an agarose gel were inversely proportional to the logarithms of the molecular weights (Aaij & Borst, 1972) and in 1979 that plotting fragment length against the reciprocal of mobility gives a straight line over a wider range than the semi-logarithmic plot (Southern, 1979a,b). These methods allow accurate size determination of DNA molecules which is fundamental in cloning experiments. Prior to 1970 there was no method available for cutting a DNA molecule into discrete fragments. However, a restriction endonuclease from *H. influenzae* Rd was then discovered that was to become the prototype for a large number of restriction endonucleases which are fundamentally important in the manipulation of DNA. These enzymes recognise a particular target sequence in a duplex DNA molecule and break the polynucleotide chains within, or near to, that sequence to give rise to discrete DNA fragments of defined length and sequence (Kelly & Smith, 1970; Smith & Wilcox, 1970). By 1985 several hundred such restriction endonucleases had been identified and at least partially characterised (Kessler *et al*, 1985), and present day DNA technology is almost totally dependent on the ability to cut DNA molecules at specific sites using restriction endonucleases.

DNA molecules can be joined by a number of methods to create artificially recombinant molecules. *E. coli* and phage T4 encode an enzyme, DNA ligase, which seals nicks between adjacent nucleotides in a duplex DNA chain (Olivera *et al*, 1968; Gumport & Lehman, 1971). When terminals created by a restriction endonuclease that creates cohesive ends associate, the joint has nicks (nicking is the process of creating single-stranded breaks in the DNA molecule) a few base pairs apart in opposite strands. Purified DNA ligase can then repair these nicks to form an intact duplex. Ligation is therefore another procedure fundamental to gene manipulation and cloning.

Many early attempts to transform *E. coli* were unsuccessful, however, it was eventually discovered that treatment of *E. coli* cells with $CaCl_2$ allowed uptake of DNA from bacteriophage λ (Mandel & Higa, 1970). Later Cohen *et al* (1972) showed that $CaCl_2$ treated *E.coli* cells can also receive plasmid DNA. Many workers have made attempts to improve the efficiency of the transformation procedure, examining the factors and conditions governing the uptake of the DNA into the cells. More recently such an examination of these factors has produced a set of conditions for optimal efficiency that is applicable to most *E.coli* strains with efficiencies of 10^7 to 10^8 transformants per 10μg of DNA (Hanahan, 1983).

Having created a recombinant plasmid with the gene of interest sub-cloned into a suitable restriction site, this plasmid must be prepared in a larger quantity and purified for use in further experiments. Methods for large scale preparation involve growing the selected *E. coli* cells in a large volume of medium and then lysing them to release the plasmid DNA without too much contaminating chromosomal DNA and without shearing or nicking the plasmid DNA. Isopycnic centrifugation of cleared lysates in a solution of caesium chloride containing ethidium bromide (EtBr) (Radloff *et al*, 1967) can be performed to separate covalently closed circular plasmid DNA from chromosomal DNA and open plasmid circles and purify them. There are several alternative procedures for achieving the purification of plasmid DNA from the transformed host cells. Most of these protocols have evolved in an attempt to overcome the difficulties encountered and improve the yields obtained with other protocols particularly when using higher molecular weight plasmids.

Mutagenesis

Mutants are an essential prerequisite for many genetic studies. There is now a multitude of methods available for mutagenesis of specific target sequences or individual bases in cloned DNA sequences.

In vitro site directed mutagenesis has become a fundamental tool of gene manipulation. Mutagenesis is often performed in a single stranded system such as the vectors derived from the filamentous phage M13. DNA fragments are readily cloned into M13 for mutagenesis using M13mp vectors (Messing *et al*, 1977). The underlying strategy behind many methods of mutagenesis is to anneal a mutagenic primer to a single stranded template such as M13, the primer is then extended with klenow fragment of *E.coli* DNA polymerase I using deoxynucleotide triphosphates in the presence of T4 DNA ligase to ligate the 3' end of the new strand to the 5' end of the oligonucleotide primer. After transfecting competent *E. coli*, the heteroduplex DNA gives rise to the mutant and wild type progenies. Theoretically this process should give rise to a frequency of mutants of around 50% (Carter, 1986). The double priming method for oligonucleotide site directed mutagenesis involves partial extension from the mutagenic oligonucleotide and a second oligonucleotide 5' to the mutagenic oligonucleotide to protect the mismatch after extension and ligation from removal of the mismatch by 5'>3' exonucleases *in vivo* (Norris *et al*, 1983; Zoller & Smith, 1984). The desired mutant progeny is identifiable by the analysis of the DNA sequence of the clones obtained from transformation. Several more elaborate procedures for increasing the frequency of mutants obtained using M13 have been described (Carter, 1986). One such procedure involves elimination of progeny phage derived from the template strand of M13 by preparation of the template in a *dut ung* host strain of *E.coli*. This results in a few deoxyuridine residues being incorporated into the template in place of thymidine, the plus

78

strand can hence be destroyed *in vitro* by treatment with uracil glycosylase and alkali giving mutant frequencies in the region of 70-90% (Kunkel *et al*, 1985). The use of repair deficient host strains and amber or Eco K selection gives similar mutant frequencies to the Kunkel procedure. In amber selection, one primer is used to generate the mutation of interest and a second primer is used to remove an amber mutation previously introduced into gene IV of M13. After extension and ligation from these two primers, the heteroduplex DNA is used to transfect a repair deficient, non-supressor strain of *E.coli*. The disadvantage of amber selection is that once the selectable marker is removed, further mutations cannot be constructed using selection (Kramer *et al*, 1984). Eco K selection overcomes this disadvantage, allowing a series of mutations to be constructed by cycling between two markers. The markers used are restriction modification systems found in K strains (Eco K) and B strains (Eco B) of *E.coli*. In the reaction one oligonucleotide creates the desired mutation and the other is used to remove one selectable marker (Eco K or Eco B site) and at the same time generate a second selectable marker (Eco B or Eco K site) adjacent to the first. When transfected into the relevant Eco K$^+$ or Eco B$^+$ host strain of *E.coli* the non-mutated wild type strand will be restricted and degraded resulting in phage being produced from the mutant strand only (Carter *et al*, 1985). A proportion of the phage produced by this method will be derived from DNA strands only mutated at the Eco K site, thus not carrying the desired mutations. A further advantage of the Eco K selection method is that it decreases the probability of displacement of the other mutagenic oligonucleotide by the klenow enzyme by protecting its 5' end. The use of a repair deficient strain ensures the host will not repair the mismatch in the DNA from the mutagenesis reaction. Using a repair$^+$ strain minimises random mutations which may occur in lawn bacteria. In this chapter

M13K19hppI-1 was used as a template for a double primer approach with Eco K selection for oligonucleotide site-directed mutagenesis.

The polymerase chain reaction (PCR) has revolutionised many of the techniques used in molecular biology and expanded the possibilities of research in the biological sciences and medicine.

PCR amplification involves two oligonucleotide primers that flank the DNA segment to be amplified. These primers hybridise to the opposite strands of the target sequence and are orientated so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products are also complementary to and capable of binding primers, the cycle can be repeated after a denaturation step. Repeated cycles of denaturation, priming and extension cause rapid exponential accumulation of the specific target fragment i.e. 10 cycles amplifies the target sequence 1000 fold (Old & Primrose, 1989).

The very first PCR experiment was performed by Dr. Kjell Kleppe, a postdoctoral fellow in Dr. Har Gobind Khorana's research group, around 1969. However despite further successes by another group member, Dr. Ian J. Molineux, between 1970 and 1972 the group did not pursue research into large scale automation of DNA amplification. This decision was taken for several reasons including the lack of a thermostable DNA polymerase and the difficulty in synthesising large amounts of primers. These problems made the possibility of specific amplification of genomic DNA seem unfeasible. In addition, DNA cloning, which had just begun became the preferred method for producing large quantities of DNA (Templeton, N. S., 1992). Modern PCR technology was therefore initially developed by Dr. K. B. Mullis at the Perkin-Elmer Cetus Corporation in April 1983 (Mullis, 1990). The first publications utilising PCR appeared in 1985 and in 1989 DNA polymerase was

named molecule of the year and PCR, major scientific development of the year (Guyer, 1989). The step that revolutionised the automation of PCR was the discovery of a thermostable DNA polymerase from the thermophile *Thermus aquaticus* (Taq). Taq is not inactivated by the heat denaturation step meaning that fresh klenow enzyme need not be replenished at each cycle enabling amplification to be performed at higher temperatures increasing the specificity, sensitivity and yield of the product significantly (Sakai *et al*, 1988). PCR has provided an alternative to many conventional gene cloning techniques and mutagenesis is not an exception. The concept of overlap extension allows PCR to be used for introducing site directed mutations into the centre of a DNA fragment and was first reported in 1988-89 (Higuchi *et al*, 1988; Ho *et al*, 1989). This concept also allows joining or 'splicing' of different genes by PCR known as gene SOEing or 'splicing' by overlap extension (Higuchi *et al*, 1988; Ho *et al*, 1989; Sarkar and Sommer, 1990; Perrin, 1990). In overlap extension (figure 3.1) for mutagenesis a point mutation is introduced into the 3' primer for the 5' upstream DNA fragment. The same point mutation is placed in the 5' primer for the downstream DNA fragment. The first PCR amplification is performed on the overlapping DNAs separately. The amplification products are then combined, primers removed, and the DNA strands denatured and renatured. A population of DNAs will anneal so that they will join only at the short region of the overlap. Primer extension can then be performed in the 5'>3' direction on the DNAs that overlap in a manner producing exposed 3' ends. The extended DNA is then denatured and renatured in the presence of primers from the outermost ends of the original overlapping DNA fragments amplifying the fully extended overlapping DNA with the internal site directed mutation (Templeton, 1992). The main advantage of PCR mutagenesis by overlap extension above other mutagenesis techniques is the efficiency in terms of the yield of mutants which should be 100%,

81

Figure 3.1 Internal site specific mutagenesis by the PCR overlap extension technique.
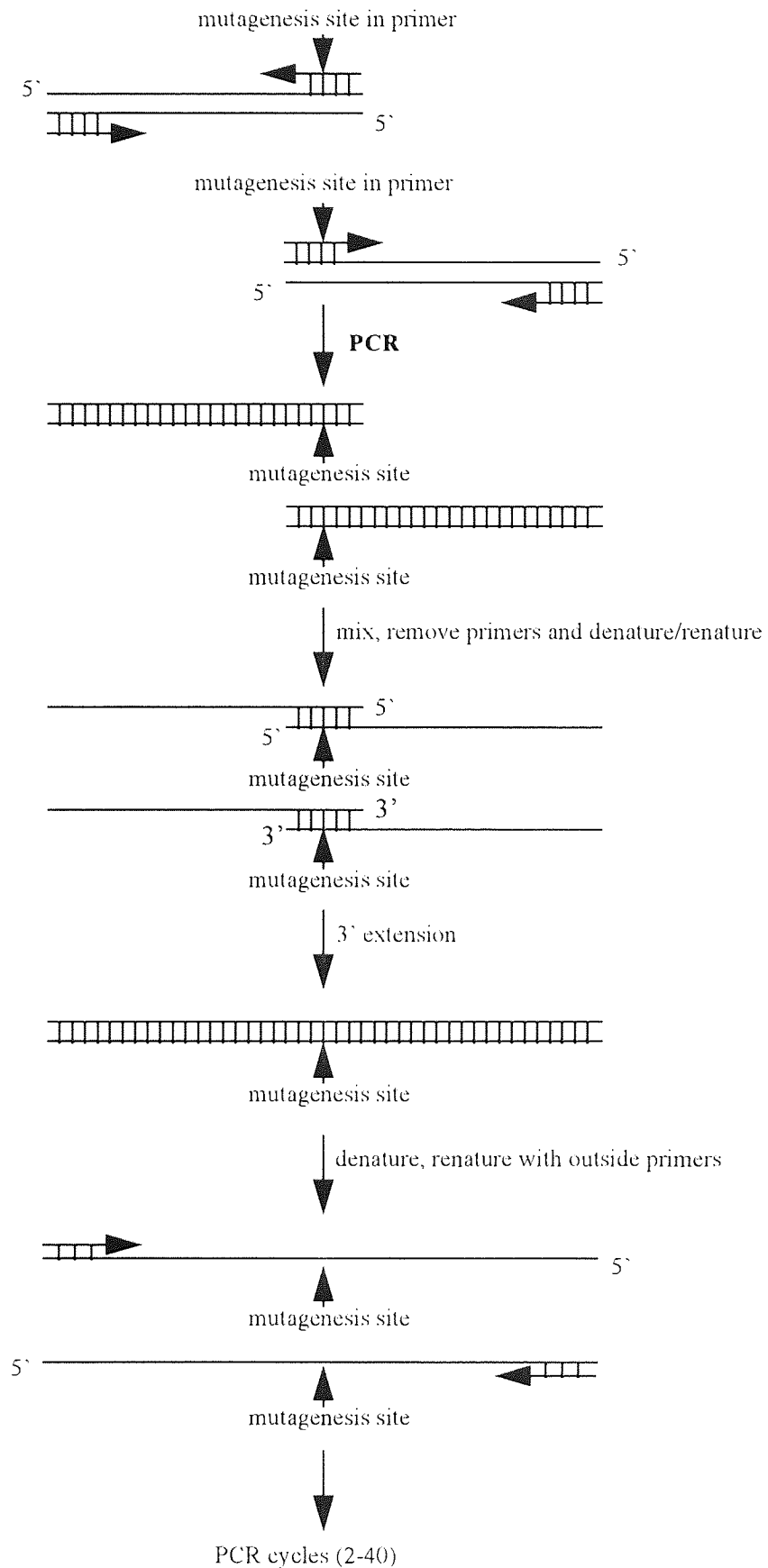
compared to 50-80% using the conventional techniques (Ho *et al*, 1989; Wu *et al*, 1987). This increase in efficiency will obviously lead to a saving in both time and materials making PCR the method of choice for site-directed mutagenesis wherever possible.

The array of techniques available for the manipulation of DNA is constantly growing, enabling the consideration of entirely new applications in preventative and therapeutic medicine. Gene therapy is such an application, using molecular biology techniques as a tool to manipulate somatic cells to express gene products which will benefit a patient lacking such a product *in vivo*.

Aims

This chapter describes the construction of plasmids and the mutagenesis of the human preproinsulin gene. The objectives of the plasmid construction section are straightforward, namely to prepare plasmids suitable for use in the transfection of mammalian cell cultures with the purpose of creating insulin-secreting cell lines. The vectors chosen to sub-clone the human preproinsulin gene must contain strong promoters to ensure that high expression of the hormone is achieved. High expression of the other genes of interest is also desirable. The vectors must also have genes for ampicillin resistance and neomycin or hygromycin resistance for selection in bacteria and mammalian cells respectively. The constructed plasmids will then be ready to use in mammalian expression systems, in this case, when transfected into mammalian cell lines cultured *in vitro*.

The aims of the mutagenesis section of this chapter were to mutate the cleavage sites in the proinsulin prohormone molecule to a consensus sequence that is cleaved by an endoprotease that is found in virtually all cell types. Proinsulin is normally processed to mature insulin in the β cells by two specific endoproteases PC2 and PC3. However to achieve processing of the proinsulin in non-endocrine cell lines it would be advantageous to use an endoprotease

83

already present in that cell type. Furin is an endoprotease found in virtually all cell types tested for its presence and has the consensus cleavage site Arg^{-4}, Xaa^{-3}, Lys/Arg^{-2}, Arg^{-1}, $\updownarrow$, Xaa^{+1}. Proinsulin with cleavage sites adjusted to this sequence by site-directed mutagenesis would be cleaved by endogenous furin in non-endocrine cell lines (Yanigita et al, 1992; Yanagita et al, 1993; Groskreutz et al, 1994; Vollenweider et al, 1995). This step would increase the number of cell types feasible for use in gene therapy for insulin delivery and possibly reduce the number of genes needed to manipulate a non-endocrine cell to process proinsulin to mature insulin. Insulin biosynthesis is discussed further in chapter 5.

3.2 Methods

For details of the techniques mentioned in this section see chapter 2. Maps of all the plasmids, both commercially obtained vectors and constructed plasmids, can be found in appendix 2. Sequences of the primers used in the oligonucleotide site directed mutagenesis and the PCR mutagenesis can be found in appendix 3.

3.2.1 Plasmid Construction

Four plasmids were required for the transfection studies. pLK444/GLUT2 was the kind gift of Dr. G. Gould of the Department of Biochemistry at the University of Glasgow. This plasmid was already in the form of a mammalian expression system and contained the human GLUT2 gene under the control of the β-actin promoter and genes for neomycin resistance and ampicillin resistance (Brant *et al*, 1994). pLK444/GLUT2 was grown up in a large scale plasmid preparation and purified by caesium chloride gradient ready to use in transfection studies.

Three plasmids were actually constructed for use in the transfection studies, pCB7-hppI-1 and pCMV-hppI-1 containing the human preproinsulin gene and pCB7-PACE containing the furin gene.

To construct pCB7-hppI-1 and pCMV-hppI-1, the human preproinsulin gene was cut out of pMtNeoIns (Stewart et al, 1993) using unique Hind III and Xba I restriction enzyme sites. The resultant fragment was gel purified by the phenol squeeze method and subcloned into the commercial mammalian expression vector pCMV (Invitrogen, Abingdon, U.K.) and pCB7 (Dr. M. Roth, University of Texas, USA). These vectors were linearised, using unique Hind III and Xba I restriction sites in the polylinker region, and also gel purified by the phenol squeeze method. The vector and insert were then ligated overnight at 16°C using

DNA ligase (Promega, U.K.). Ligation mixes were used to transform competent bacteria and plated onto ampicillin L-agar plates for incubation overnight at 37°C. Resulting colonies were grown up in ampicillin L-broth and minipreparations of DNA from them were analysed by restriction enzyme digestion to detect positive (hppI-1 insert containing) clones. This was a forced orientation sticky ended ligation thus all positive clones would have incorporated the insert in the correct orientation.

pCB7-PACE was constructed similarly by obtaining the furin cDNA from pBS-PACE (pBS-PACE was the kind gift of Dr. J. W. M. Creemers, Centre for Human Genetics, University of Leuven, Belgium) by an Sst I and Sal I restriction enzyme digestion followed by gel purification by the phenol squeeze method. The pCB7 vector was linearised using Sst I and Sal I unique restriction enzyme sites in the polylinker region and gel purified by the phenol squeeze method. The vector and insert were ligated using a simple sticky ended forced orientation ligation with DNA ligase overnight at 16°C. The ligation mix was used to transform competent bacteria. Resulting colonies were grown up and minipreparations of DNA were analysed by restriction enzyme digests to detect insert-containing clones.

Positively identified clones for each of the plasmids constructed were grown up on a large scale and the prepared DNA purified by caesium chloride gradient ready for use in the transfection of mammalian cell cultures.

3.2.2 Oligonucleotide Site-Directed Mutagenesis

A double primer technique with Eco K selection was chosen to introduce the desired mutations to the processing sites of hppI-1. The hppI-1 cDNA in the M13K19 vector was used as the template for the mutagenesis reaction. M13K19 is a modified M13 phage and contains the mp19 polylinker with a 30 base pair insert coding for an Eco K restriction site.

The single stranded template was prepared in a F'. repair$^+$ strain of *E.coli*, XL1 blues. F' cells carry the genes necessary for the formation of an F pilus, necessary for DNA transfer between conjugating bacteria. As M13 phage infect through the F pilus, host bacteria must be of the F' strain. The single stranded preparation of M13K19hppI-1 was sequenced to determine which coding strand of the DNA had been obtained. Mutagenic oligonucleotides were then synthesised by Alta Bioscience, University of Birmingham. Two mutant oligonucleotides and the selection oligonucleotide, Moli 1 (D3540), Moli 2 (D3541) and Sel 2 (D144) were used in the annealing reaction with the M13K19hppI-1 template (see figure 2.1). Second strand synthesis, extension and ligation were then performed and the resulting double stranded DNA was used to transform the HB2154 strain of *E.coli* (F', repair$^-$, Eco K$^+$)with a lawn of HB2151 *E.coli* (F', repair$^+$, Eco K$^+$). Plaques obtained from this transformation will all carry the Eco K selection, however, there will be a mixture of the hppI-1 mutations. Some will have no hppI-1 mutations, some the Moli 1 mutation, some the Moli 2 mutation and some will be carrying both of the desired mutations. Single stranded template preparations from the plaques were screened by sequencing for the desired double mutant.

3.2.3 PCR Mutagenesis

Owing to the failure of the oligonucleotide site directed mutagenesis, a further attempt to obtain the required mutant was made using the relatively new technique of PCR mutagenesis.

The PCR mutagenesis was performed in three reactions using six separate primers (see appendix 3 for sequences of the oligonucleotide primers) with Sp64T-RhppI-1 as the template DNA (figure 3.2).

Figure 3.2 Schematic of the PCR mutagenesis and the fragments created by each reaction. The 3 reactions were carried out by priming the Sp64T-Rhppl-1 template with the appropriate primers and amplifying the fragment according to the profiles given in below. The 3 overlapping fragments generated were then annealed and amplified with the Sp6 primer and the hppl-1 end primer.
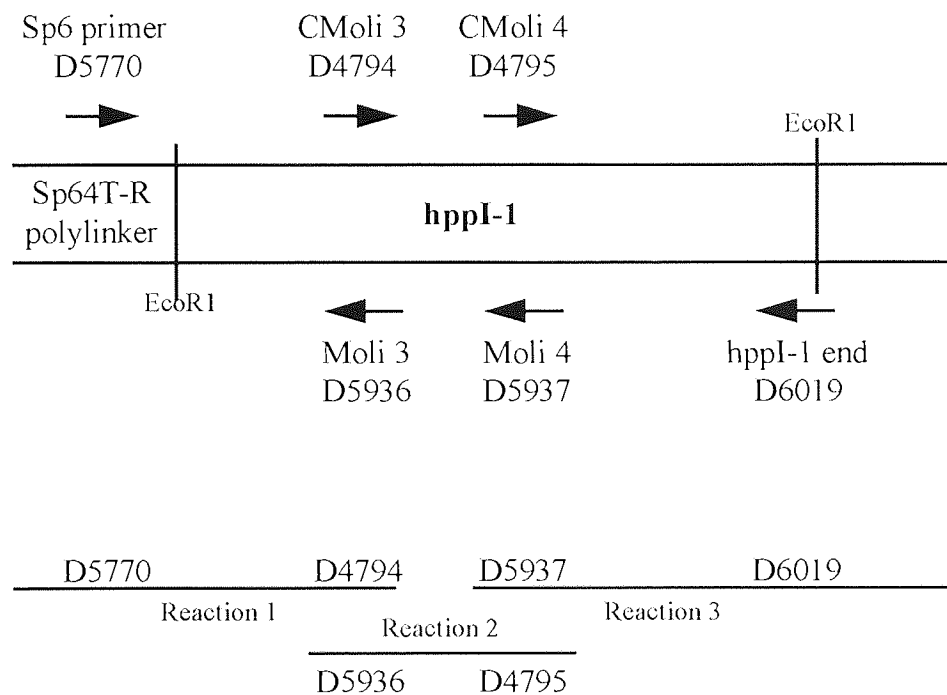
Reaction one was from the 5770 Sp64T up primer to the 4794 (moli3) primer (mutagenic).

This reaction gave a 250 base pair fragment when amplified using the following conditions:

96°C	10 minutes
96°C	15 seconds
52°C	20 seconds } 25 cycles
72°C	45 seconds
72°C	2 minutes

Reaction two was from D5936 (cmoli 3) to D4795 (moli 4). Both primers are mutagenic

for regions in the cleavage sites of hppI-1. This reaction gave a fragment approximately 100

base pairs in length when amplified using the following conditions:

96°C	10 minutes
96°C	15 seconds
52°C	20 seconds } 4 cycles
72°C	45 seconds
96°C	15 seconds
62°C	20 seconds } 20 cycles
72°C	45 seconds

Reaction three was from D5937 (cmoli 4) to D6019 (hppI-1 end). The complementary

mutagenic primer cmoli 4 and a primer to the end of the hppI-1 gene ending in an EcoRI

restriction site. This reaction gave a fragment approximately 100 base pairs in length when

amplified using the following conditions:

96°C	10 minutes
96°C	15 seconds
39°C	20 seconds } 4 cycles
72°C	45 seconds
96°C	10 seconds
56°C	25 seconds } 20 cycles
72°C	20 seconds
72°C	10 minutes

The three fragments were then annealed using the conditions and steps described in chapter two to give a 450 base pair product. This product was then digested with EcoRI and gel purified. A ligation was then set up between EcoRI digested linearised Sp64T-R vector and the EcoRI mutated product fragment. The resulting plasmid was sequenced to ensure the correct mutations were incorporated and that no unwanted mutations had occurred.

3.3 Results

3.3.1 Ligation

Ligations were set up as described in the methods to create the plasmids pCB7-PACE, pCB7-hppI-1 and pCMV-hppI-1. Following overnight incubation at 14°C, an aliquot of the ligation mixture was removed and electrophoresed on an agarose gel along with equal quantities of vector without ligase, vector with ligase and vector and insert without ligase (figure 3.3a-c). In figure 3.3a the pCB7 vector in lane 1 corresponds to 6.0kb on the DNA marker ladder. In lane 2 the pCB7 vector is incubated with ligase as in the ligation reaction, however, the vector remains linear and there is no evidence of supercoiling indicating that the phosphatase treatment was successful in preventing recircularisation of the vector. Lane 3 shows the vector and insert together without ligase, the furin insert corresponds to approximately 2.6kb compared to the marker ladder. There is no evidence of supercoiling or ligation. Lane 4 shows the ligation reaction mixture, the DNA is supercoiled and the insert band is depleted in comparison to lane 3 indicating successful ligation has occurred. Transformation with this reaction mixture should give positive colonies, lane 2 confirms that the colonies will not contain recircularised vector. Figure 3.3b similarly shows the pCB7 vector in lane 1 at approximately 6.0kb. Lane 2 confirms that the vector has not religated to itself indicating successful treatment with phosphatase. Lane 3 shows the 0.5kb hppI-1 insert and the pCB7 vector without ligase. There is no evidence of ligation. Lane 4 shows the ligation reaction mixture. There is supercoiled DNA, insert depletion and the faint band at 1kb shows insert dimerisation. Again there has been successful ligation with no evidence of vector recircularisation. Figure 3.3c shows the same as figure 3.3b but with the 5.5kb

Figure 3.3 Agarose gel electrophoresis for analysis of ligation reactions. Lane 1 shows vector without DNA ligase, lane 2 shows vector incubated with DNA ligase, lane 3 shows vector and insert without DNA ligase and lane 4 shows vector and insert incubated with DNA ligase. The lane marked M shows the gel marker DNA ladder. a) shows the pCB7 vector with the furin insert, b) shows the pCB7 vector with the hppI-1 insert and c) shows the pCMV vector with the hppI-1 insert.
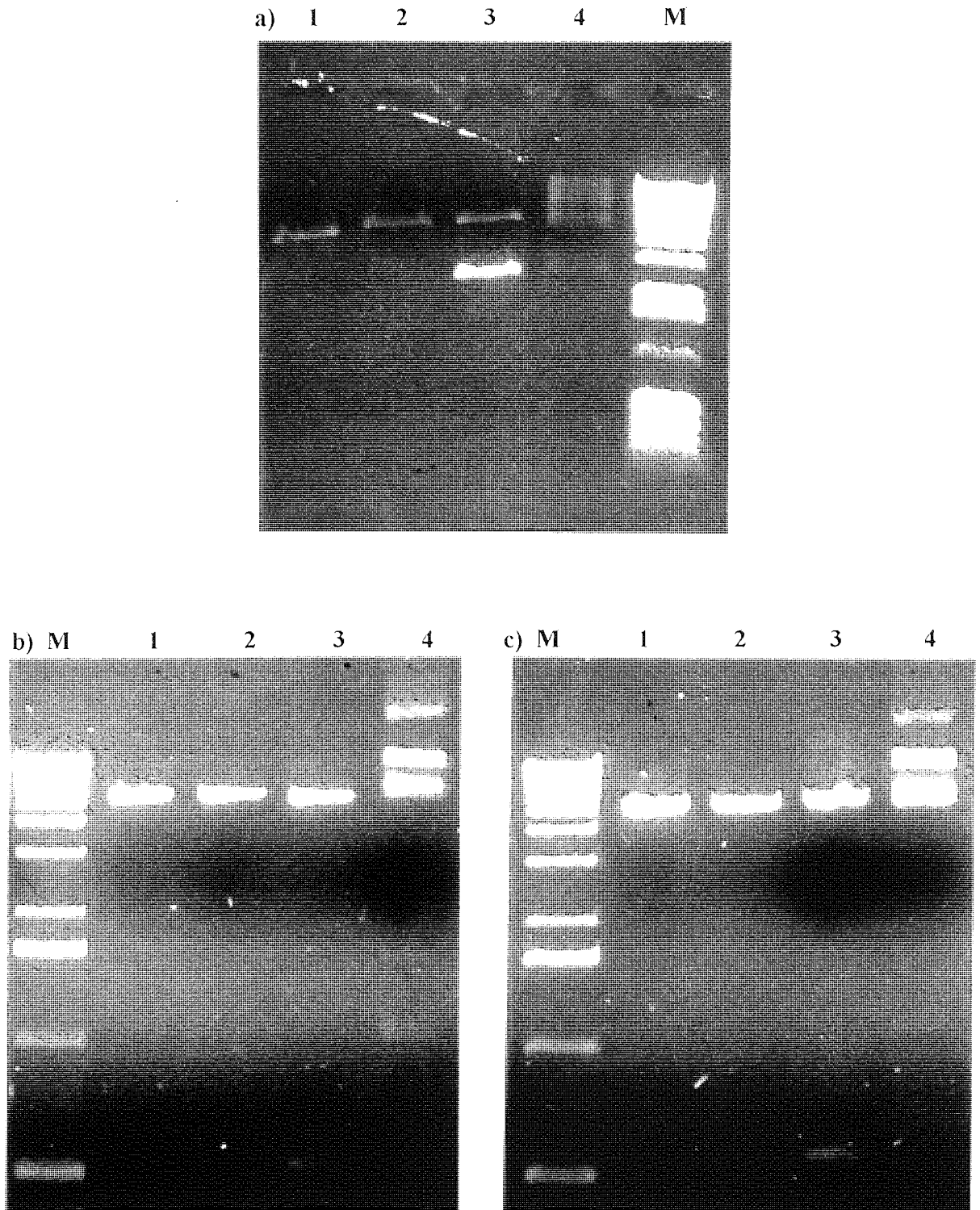
pCMV vector and the hppI-1 insert. The ligation mixture shows supercoiling and insert depletion indicating successful ligation and lane two confirms that there is no vector recircularisation. All three ligations have been successful and transformation into suitably competent *E.coli* should yield many positive colonies.

3.3.2 Transformation

On transformation of the ligation mixtures into competent *E.coli* cells with ampicillin selection the following colonies were obtained. The pCB7-PACE ligation yielded 13 colonies, the pCB7-hppI-1 ligation yielded 26 colonies and the pCMV-hppI-1 ligation 42 colonies. Colonies from these transformations were picked and grown up in 5ml cultures for small scale preparation of the plasmid DNA to screen for the desired plasmid.

3.3.3 Minipreparations of DNA

Preparations of DNA from the colonies obtained at transformation were digested with restriction enzymes and separated by agarose gel electrophoresis to detect positive clones. Figure 3.4a shows 4 minipreps digested with 4 different restriction enzymes from the pCB7-PACE ligation. Lanes 1-4 show digestion with the Bam H1 restriction enzyme which gives 3 bands of 6051bp, 1651bp and 780bp in clones (lanes 1-3). Lanes 5-8 show digestion with the Kpn 1 restriction enzyme giving a single band in positive clones of 8458bp (lanes 5-7). Lanes 9-12 show digestion with the Eco R1 restriction enzyme giving 2 bands of 4836bp and 3822bp in positive clones (lanes 9-11). The final digestion (lanes 13-16) was with the Xba 1 restriction enzyme and gave 2 bands of 6024bp and 2434bp in the 3 positive clones (lanes 13-15). The pCB7-PACE plasmid is present in the first 3 lanes of each digestion, the

Figure 3.4 Agarose gel electrophoresis of restriction enzyme digests of miniprep DNA to screen for the desired plasmids. 3.4a shows 4 minipreps from the pCB7-PACE ligation digested with Bam H1 (lanes 1-4), Kpn 1 (lanes 5-8), EcoR1 (lanes 9-12) and Xba 1 (lanes 13-16). The first 3 minipreps contain the desired pCB7-PACE plasmid and the fourth is a contaminant. 3.4b shows the minipreps from the pCB7-hppI-1 ligation digested with Hind III and Xba 1 to cut the hppI-1 insert from positive clones. All the minipreps screened contain the hppI-1 insert at 0.5kb. 3.4c shows minipreps from the pCMV-hppI-1 ligation digested with Hind III and Xba 1 to remove the hppI-1 insert from positive clones. 11 of the 12 clones screened contain the 0.5kb insert.

a)

b)

c)
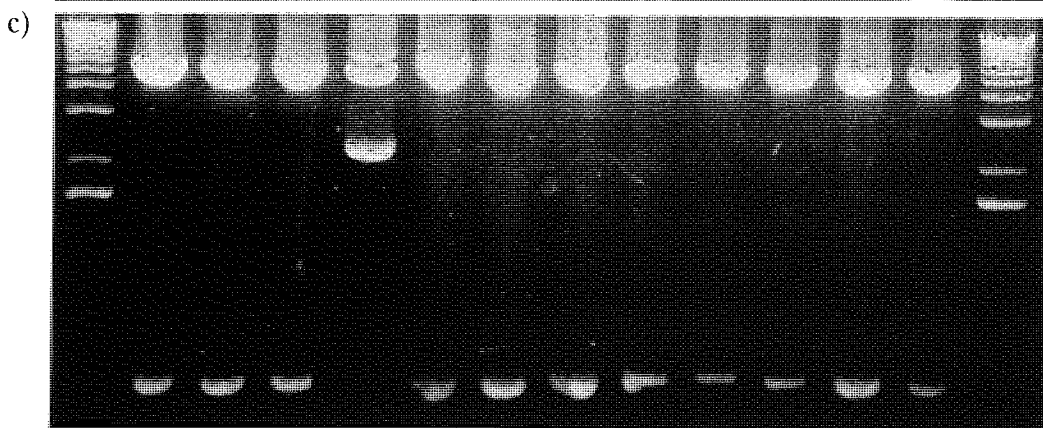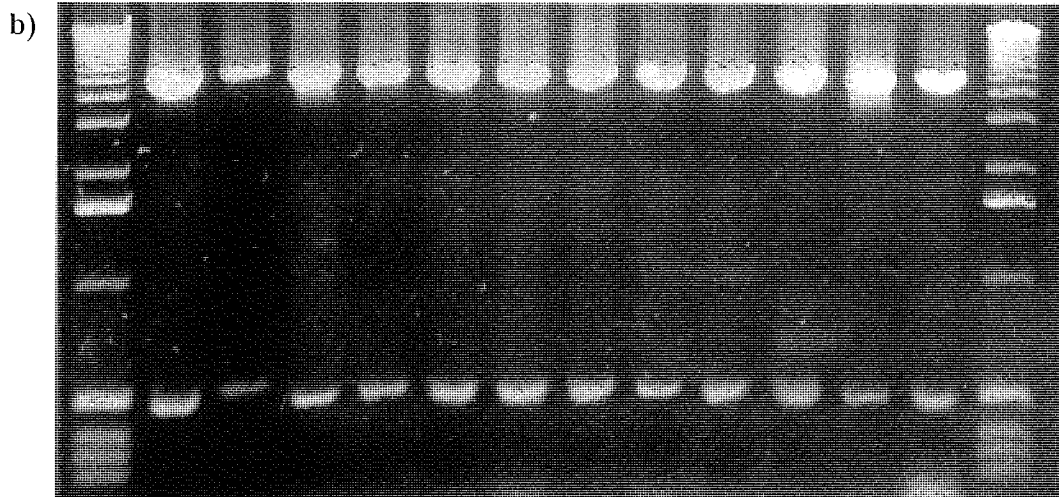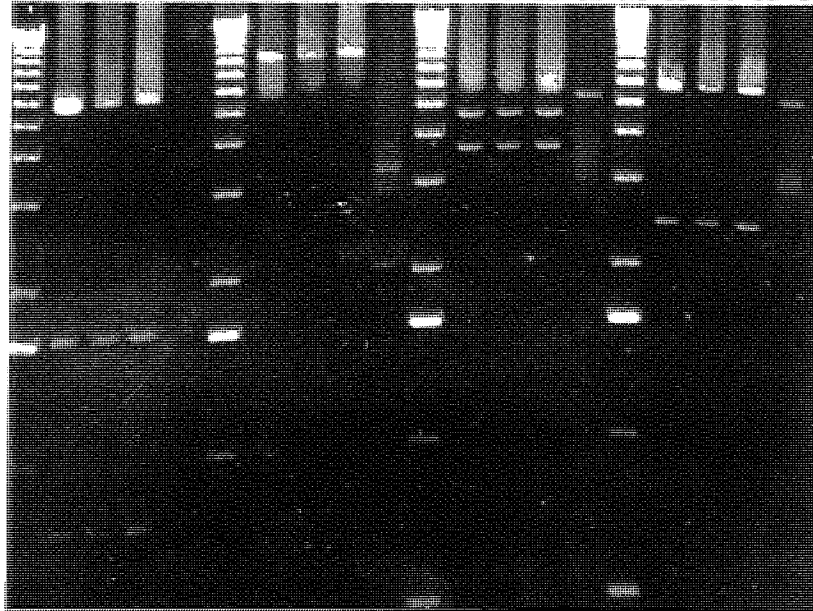
fourth clone is probably a contaminating colony. The four restriction enzyme digests were used to ensure that the correct plasmid had been obtained. Figure 3.4b shows digestion of clones obtained from the pCB7-hppI-1 ligation with the Hind III and Xba 1 restriction enzymes. This digest removes the insert from the vector hence yielding 2 bands of 6.0kb and 0.5kb. All of the 12 clones screened contained the hppI-1 insert. Figure 3.4c shows digestion, with the Hind III and Xba 1 restriction enzymes, of colonies obtained from the pCMV-hppI-1 ligation. Again the insert is cut out of positive plasmid in this digestion yielding the 5.5kb pCMV vector and the 0.5kb hppI-1 insert. 11 of the 12 colonies screened contained the correct plasmid. The fourth lane is probably a contaminating colony.

3.3.4 Maxipreparation of DNA

A positive clone for each plasmid was then grown up and a bulk preparation of DNA performed. The resultant DNA was analysed for quality by digesting with restriction enzymes and separating on an agarose gel alongside some of the uncut plasmid (figure 3.5a-c). Figure 3.5a shows the pCB7-PACE plasmid digested with Xba 1 in the first lane and Bam H1 in the second lane and the third lane shows uncut plasmid. 3.5b shows the pCB7-hppI-1 plasmid digested with Hind III and Xba 1 in the first lane and uncut in the second lane. 3.5c shows the pCMV-hppI-1 plasmid digested with Hind III and Xba 1 in the first lane and uncut in the second lane. In each case the uncut plasmid was well supercoiled without nicked DNA and there was little or no RNA contamination in the preparations. The digestions yielded the hppI-1 insert in figure 3.5b and 3.5c and the correct sized bands for the digestions of pCB7-PACE in figure 3.5a (as described in the miniprep digestions). The purified plasmid preparations were ready for use in transfection studies.

Maps of the constructed plasmids can be found in appendix 2.

Figure 3.5 Agarose gel electrophoresis for analysis of the purity of large scale preparations of the three constructed plasmids. 3.5a shows pCB7-PACE digested with Xba 1 in the first lane yielding bands of 6024bp and 2434bp, digested with Bam H1 in the second lane yielding bands of 6051bp, 1651bp and 780bp and uncut plasmid in the third lane. 3.5b shows pCB7-hppI-1 digested with Hind III and Xba 1 in the first lane yielding the 0.5kb hppI-1 insert and the 6.0kb vector and uncut plasmid in the second lane. 3.5c shows pCMV-hppI-1 digested with Hind III and Xba 1 in the first lane yielding the 0.5kb hppI-1 insert and the 5.5kb vector and uncut plasmid in the second lane.
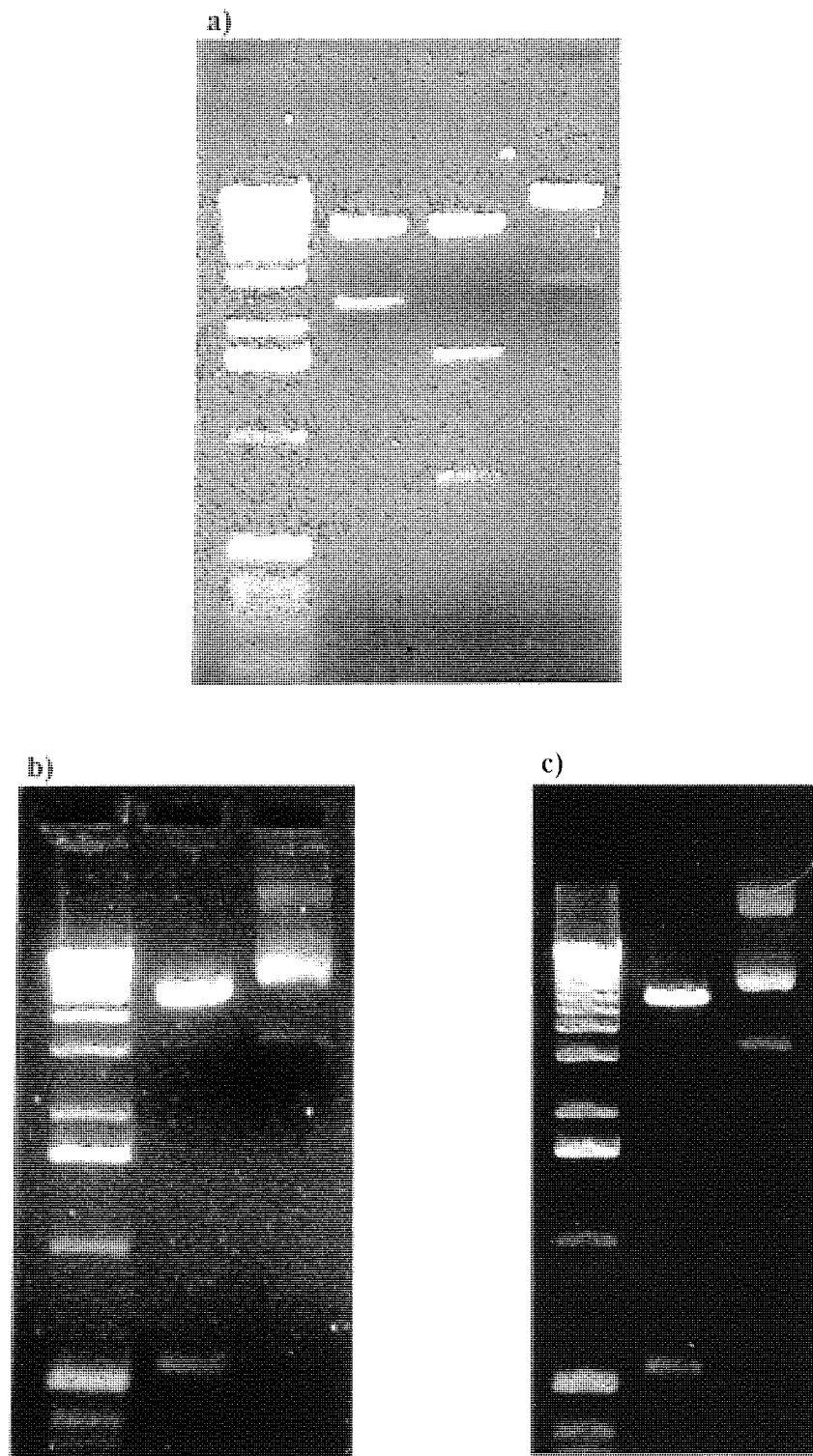
3.3.5 Oligonucleotide Site Directed Mutagenesis

DNA sequencing of the single stranded template preparation of M13K19hppI-1 was performed in order to determine which coding strand of DNA had been obtained. This was necessary to design the complementary mutant oligonucleotide primers. Figure 3.6 shows the sequence obtained from the sequencing reaction. The sequence is easily identifiable as the last section of the hppI-1 sequence with the characteristic A or T rich tail (the sequence of the hppI-1 cDNA can be found in appendix 4). In this case the tail is T rich indicates which strand has been prepared, allowing complementary oligonucleotide primers to be designed (see appendix 3). The T rich area causes the compression in the sequencing gel resulting in the poor band resolution. Following the mutagenesis reaction, the mixture was transformed into HB2154 *E.coli* yielding thousands of plaques in a lawn of HB2151 *E.coli*. Plaques were picked and single-stranded template DNA prepared from cultures of XL1 blue *E.coli* infected with single plaques. These templates were sequenced to determine whether the desired mutations had been introduced. However, difficulties in sequencing the template preparations were experienced and when a readable sequence was obtained, the sequence obtained (figure 3.7) did not correspond to any region of the published M13K19 or hppI-1 sequences. Template preparations carried out on uninfected wild type XL1 blue *E.coli* yielded phage DNA indicating a contaminating phage DNA was present in the wild type bacteria.

Figure 3.6 DNA sequencing of the M13K19hppI-1 template DNA to determine which strand had been obtained from the single stranded template preparation. The strand is easily identified from the T-rich region towards the end of the hppI-1 sequence. The poor resolution of the gel is due to a compression caused by the T-rich region.

Figure 3.7 DNA sequencing of the single-stranded template DNA prepared from plaques obtained by transformation with the site-directed oligonucleotide mutagenesis mixture. The sequence should show areas of the hppI-1 insert of M13K19hppI-1 mutated in the reaction. However, the sequence does not correspond to M13K19 or hppI-1.

3.3.6 PCR Mutagenesis

The PCR mutagenesis was carried out in 3 reactions to create 3 overlapping fragments containing the mutations. Following the PCR reactions the PCR products were electrophoresed on agarose gels stained with ethidium bromide to identify the correct band. This band was then excised from the gel and purified. The purified DNA was then electrophoresed to check the quality and quantity of the PCR fragment before use in the annealing reaction. Figure 3.8 shows each PCR fragment electrophoresed on agarose gels stained with ethidium bromide. 3.8a shows the 250 base pair fragment obtained from the PCR amplification of Sp64T-RhppI-1 using the Sp64T-R up primer (D5770) and Moli 3 (D4794) primers. 3.8b shows the 100 base pair fragment obtained from amplification of Sp64T-RhppI-1 with Cmoli 3 (D5936) and Moli 4 (D4795). 3.8c shows the 100 base pair fragment obtained from amplification of Sp64T-RhppI-1 with Cmoli 4 (D5937) and hppI-1 end (D6019). The gels show that the fragments are uncontaminated and of the expected size and that the DNA concentration of each fragment preparation is approximately the same. The 3 overlapping fragments were then annealed together and the annealed products amplified using the end most primers, the Sp64T-R up primer (D5770) in the Sp64T-R polylinker region and the hppI-1 end primer (D6019) at the end of the hppI-1 gene, to give a 450 base pair PCR product. Figure 3.9 shows the 3 annealing reactions carried out and the 3 separate fragments electrophoresed on an agarose gel stained with ethidium bromide. Lane 1 shows all 3 PCR fragments annealed and amplified, the topmost band is approximately 450 base pairs (compared to the Hind III λ markers in lane 4). The other bands in lane 1 appear to correspond to the bands obtained from annealing fragments 1 and 2 only in lane 3. These bands will correspond to the size of annealed fragments 1 and 2.

Figure 3.8 Agarose gel electrophoresis of the fragments obtained from the three PCR mutagenesis reactions. a) fragment one, the Sp64T-R up primer D5770 to the Moli 3 primer D4794 is 250 base pairs, b) fragment two, the Cmoli 3 primer D5936 to the Moli 4 primer D4795 is 100 base pairs, and c) fragment three, the Cmoli 4 primer D5937 to the hppl-1 end primer D6019 is also around 100 base pairs.
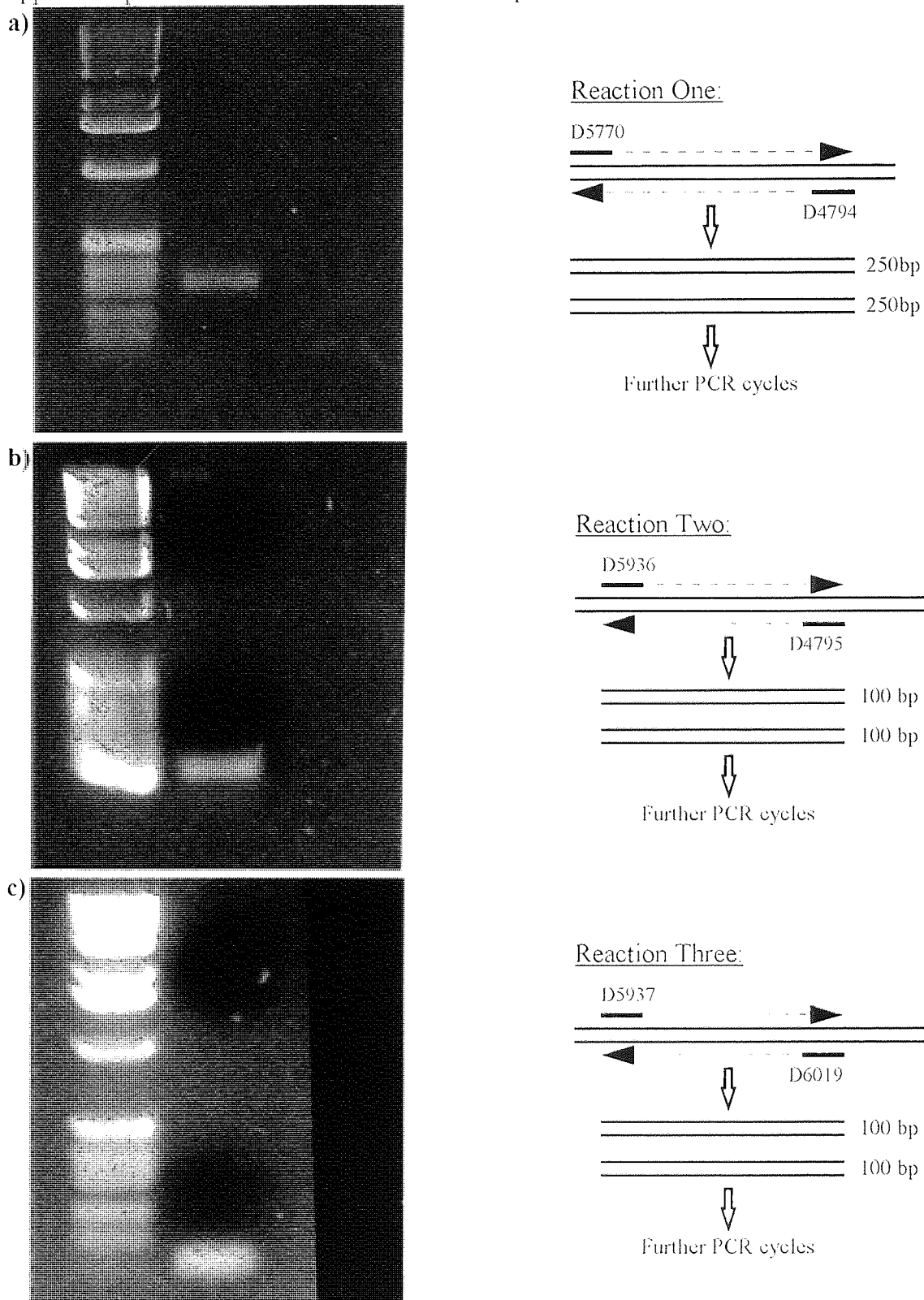
Figure 3.9 Agarose gel electrophoresis of aliquots from the amplified annealing reactions between the PCR mutagenesis fragments. Lane 1 shows PCR products obtained from the amplification of the annealing reaction between fragments 1, 2 and 3, lane 2 shows PCR products obtained from amplification of the annealing reaction between fragments 2 and 3, lane 3 shows PCR products from amplification of the annealing reaction of fragments 1 and 2, lane 4 shows a Hind III λ DNA marker ladder, lane 5 shows PCR mutagenesis fragment 3, lane 6 shows PCR mutagenesis fragment 2, and lane 7 shows PCR mutagenesis fragment 1. In lane 1 the top band corresponds to approximately 450 base pairs which is the size of the desired product.
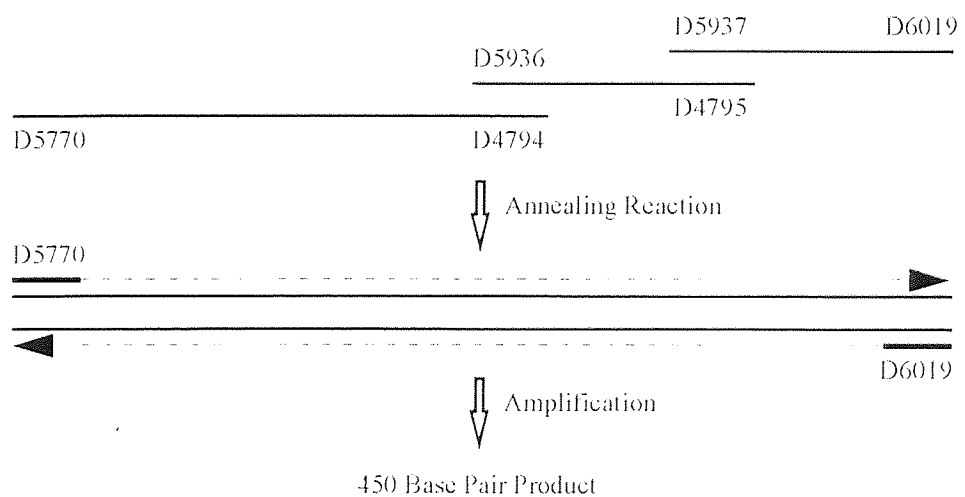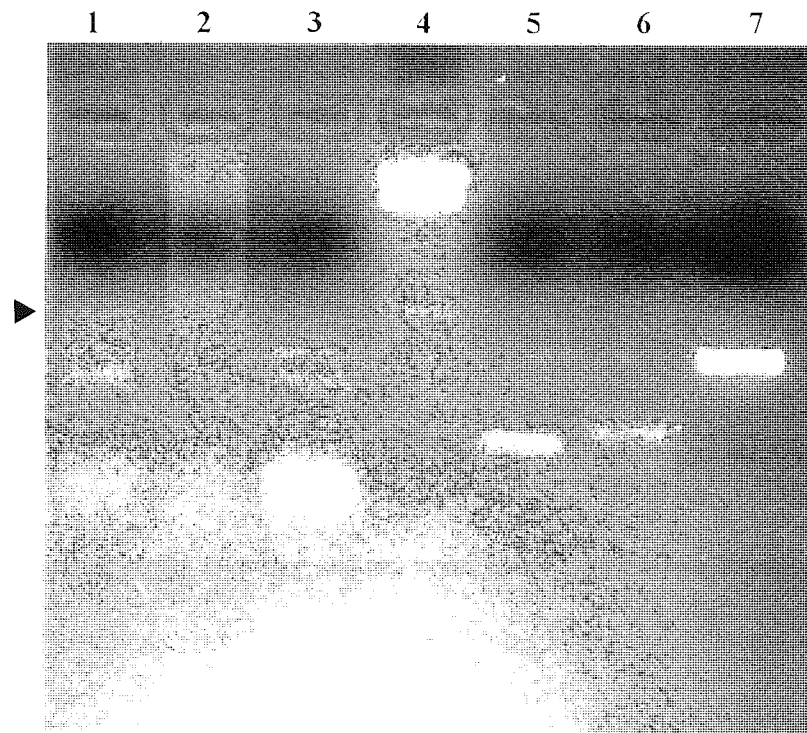
The annealing reaction between fragments 2 and 3 only (lane 2) does not appear to have amplified, probably due to an unfavourable annealing temperature in the PCR amplification cycle. However, the 450 base pair product that was required from the annealing reaction between all 3 overlapping PCR fragments was obtained and can now be excised from the gel and purified. The 450 base pair product was then digested with the EcoR1 restriction enzyme and gel purified. Figure 3.10 clearly shows the 450 base pair band just prior to being excised from the agarose gel for purification. The faint band in the marker ladder just above the purified PCR product band is approximately 500 base pairs. The purified EcoR1 fragment was then ligated into the EcoR1 site of the linearised Sp64T-R vector, to create the Sp64T-RhppI-4 plasmid (hppI-4 denoting it as the mutated hppI-1 cDNA), and sequenced to check for the desired mutations. Figures 3.11 shows the DNA sequences obtained from sequencing of the Sp64T-RhppI-4 plasmid. There are 2 intended mutations at the B chain/C-peptide site and 1 unwanted point mutation (figure 3.11a). The unwanted mutation is a T substituted for a C and occurs at the third base of a serine codon. The amino acid is therefore not changed and the serine codon remains a serine codon. This is a silent mutation which will not affect the action of insulin and is therefore ignored. There are 3 intended mutations at the C-peptide/A chain site (figure 3.11b) and no unwanted mismatches. The rest of the gene sequence was correct with no further unwanted mismatches.

Figure 3.10 Agarose gel electrophoresis of the annealed and amplified 450 base pair PCR product following digestion with the restriction enzyme EcoRI. The large band just below the 500 base pair band in the DNA marker ladder is seen just prior to being excised from the gel for purification. The purification is in preparation for subcloning into the Sp64T-R vector for DNA sequencing.
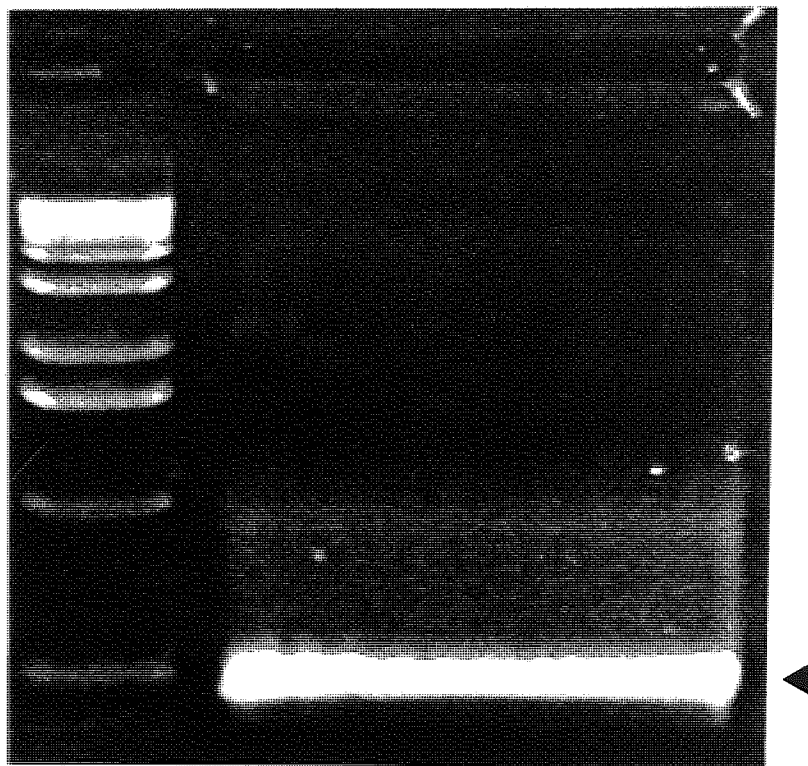
Figure 3.11a Sequence of the wild type and mutant C-peptide/A chain proinsulin cleavage site. Three bases are mutated, those marked * were mutated to change the proinsulin cleavage site to the consensus sequence for furin activity, the base change marked + is an unwanted point mutation that does not change the amino acid code and is therefore silent.

WILD TYPE

C T A G

Ser, Leu, Gln, Lys, Arg

MUTANT

C T A G

Ser, Arg, Arg, Lys, Arg
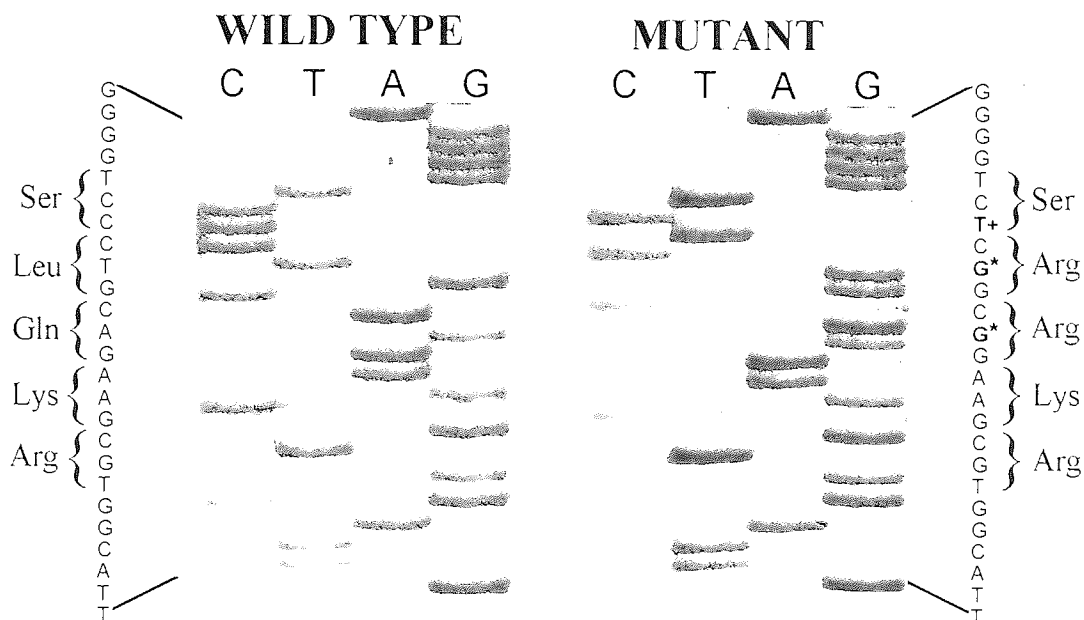
Figure 3.11b Sequence of the mutated B chain/C-peptide proinsulin cleavage site. There are three mutated bases marked with *.

WILD TYPE

Arg, Arg, Glu, Ala

MUTANT

C T A G

Arg, Arg, Lys, Arg

105
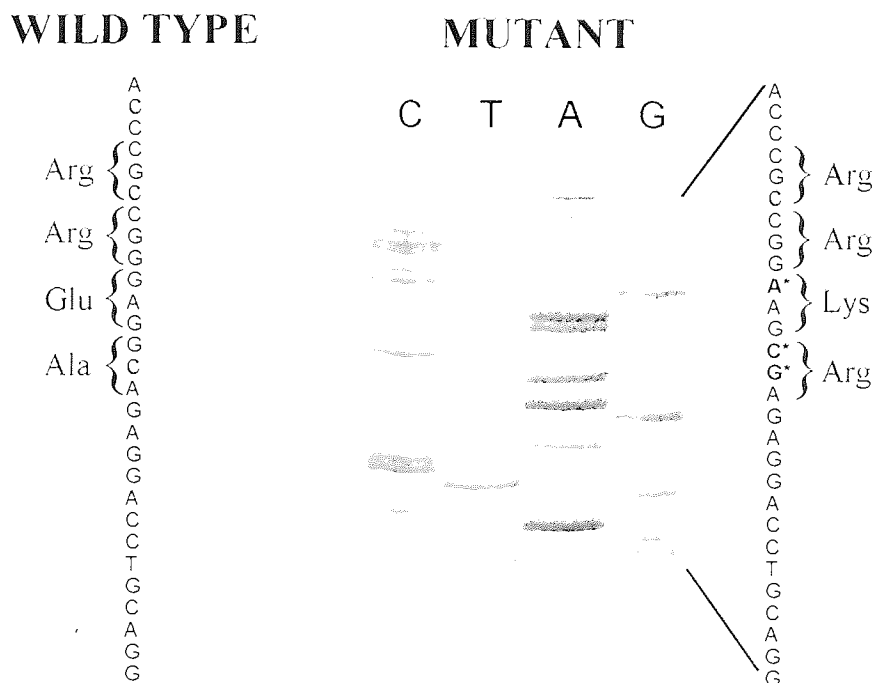
3.4 Discussion

Three plasmids were successfully constructed. Firstly pCB7-PACE, the human furin gene inserted into the polylinker region of the vector pCB7 at the Sst I and Sal I restriction sites. The pCB7 vector has a cytomegalovirus (CMV) promoter and ampicillin and hygromycin resistance genes. Secondly pCB7-hppI-1, was the wild type human preproinsulin gene inserted into the Hind III and Xba I sites of polylinker region of pCB7. Thirdly pCMV-hppI-1, the wild type human preproinsulin gene was inserted into the Hind III and Xba I restriction sites in the polylinker region of the pCMV vector. The pCMV vector also has a CMV promoter and the gene for ampicillin resistance, however, pCMV has the gene for neomycin (or G418 sulphate) resistance rather than hygromycin resistance. The hygromycin and neomycin resistance genes are both suitable for selection in eukaryotic cells using the antibiotics hygromycin B and G418 sulphate (neomycin) respectively. The concentration of the antibiotic required for selection in cultured cells varies from cell line to cell line and requires optimising using cell death studies. The vectors both contain the enhancer-promoter sequences from the immediate early gene of the human cytomegalovirus (CMV) for high level transcription of the inserted gene. In 1985 when this transcription enhancer was first identified it was found to be the strongest promoter so far described, stronger than enhancers from rous-sarcoma virus (RSV), herpes virus saimiri, and hepatitis B virus (Boshart *et al*, 1985). The other plasmid to be used in the transfection studies (chapter 5) was pLK444/GLUT2 (Brant *et al*, 1994). This plasmid has the human GLUT2 cDNA under the control of a β-actin promoter and has resistance genes for ampicillin and neomycin. Since β-actin is abundantly expressed in all non-muscle cells, the promoter region gives reliable high level transcription in many different cell types unlike some promoters which show significant variation of transcription among different cell types

(Gunning *et al*, 1987). It is important that high levels of transcription are achieved to ensure that resulting cell lines produce as much protein as possible, particularly insulin. Cell lines producing only small quantities of insulin would not be of any use even if the insulin was fully processed and its secretion was properly regulated by glucose. Cell lines would ideally need to secrete similar amounts of insulin to β cells, or very large numbers of cells would be needed to have an effect on plasma glucose concentrations, making the procedure unfeasible. The CMV promoter is one of the strongest promoters available in eukaryotic expression vectors and should give transcription levels higher than the fully induced mouse metallothionein promoter that was used in previous studies (Stewart *et al*, 1993), resulting in transfected cells with higher yields of insulin.

The oligonucleotide site-directed mutagenesis in the single stranded M13 phage resulted in sequences that could not be identified as M13K19 or the human preproinsulin gene. These aberrant sequences were only obtained after substantial difficulties in obtaining a readable DNA sequence with the M13 primers, and are probably due to a contaminant in the transformation procedure. The wild type XL1 blue *E.coli* may have become infected with a phage other than the mutated M13K19hppI-1. The failure to obtain the mutant with the site-directed mutagenesis method in M13 led to this method being abandoned in favour of a newer technique offering better mutant frequencies.

PCR mutagenesis offers a simple technique with expected mutant frequencies of 100%. The overlap extension technique as described in the introduction to this chapter was used to perform the mutagenesis of the proinsulin cleavage sites. As two separate regions were to be mutated, two overlaps rather than one were used, creating three overlapping fragments which were then annealed together by denaturing and renaturing the purified fragments. Extension with primers from the end of the human preproinsulin gene and the Sp64T vector

107

at the other end, amplified a fragment the correct size for the fully extended annealed mutant. The fragment was digested with the EcoRI restriction enzyme and subsequently purified. The EcoR1 mutant fragment was ligated into the EcoRI restriction site of the Sp64T-R vector to create the Sp64T-RhppI-4 plasmid. The plasmid was then sequenced to check for the desired mutations and to ensure that no unwanted mutations had occurred. The desired base changes were achieved, and one unwanted mutation occurred just before the B chain/C-peptide cleavage site. The base change was a T substituted for a C at the third base of a serine codon. This does not change the amino acid and is therefore regarded as a silent mutation that will not affect the transcription, translation or activity of the insulin gene. We know that DNA polymerases can make mistakes in nucleotide incorporation during polymerisation and indeed Taq DNA polymerase has a relatively high error rate. Base substitutions occur about one per 9000 nucleotides and frameshifts about one per 40000 nucleotides. While one error in 9000 base pairs of sequence seems insignificant, after 30 cycles of PCR it leads to an error every 300 base pairs of product (Kocher and Wilson, 1993). Misincorporated bases cannot be removed because Taq DNA polymerase has no 3' to 5' proof-reading exonuclease, but these misincorporations often result in termination of the extending DNA chain (Innis *et al*, 1988). The DNA polymerase used in this project was, however, Vent DNA polymerase (New England Biolabs). Tli or Vent DNA polymerase was obtained from a species of extreme thermophile *Thermococcus litoralis* (T.li) which was isolated from a submarine thermal vent and can be cultured up to 98°C (Perler *et al*, 1992). Vent DNA polymerase has an extension rate similar to that of Taq DNA polymerase (4000 nucleotides/minute). However, unlike Taq, Vent possesses 3' to 5' proof-reading exonuclease activity resulting in a much higher fidelity of base incorporation (Kong *et al*, 1993). Vent DNA polymerase may have a higher fidelity than Taq DNA

polymerase but it is still not perfect and sequencing of products is always recommended to exclude unwanted point mutations. The frequency of such mutations in PCR products accumulates linearly with the number of doublings of DNA copy number (Saiki *et al*, 1988), therefore, errors can be limited by starting with as much template DNA as possible and performing as few PCR cycles as are necessary to provide an adequate yield. Lower concentrations of dNTPs (50-200μM) may help to prevent misincorporation of bases, and primer annealing temperatures as high as possible are more likely to result in chain termination at misincorporations, meaning errors will not be propagated in subsequent PCR cycles (Higuchi, R., 1990). The final stages of the construction of the hppI-4 mutant were achieved with the help of Dr. H. Cragg, Dr. N.A. Taylor and A. Harte at the University of Aberdeen. The finished hppI-4 mutant was not obtained in time to be used in the transfection studies described in this thesis, however, the mutant will be very important in further transfection studies aimed at achieving processing of transfected proinsulin in non-endocrine cell lines.

Chapter five is further concerned with the processing of proinsulin to mature insulin in the constitutive secretory pathway of non-endocrine cell lines, concentrating on the co-expression of the wild type human preproinsulin gene with furin in a human skin fibroblast. Furin is already found in fibroblast cells, and it is possible that overexpression of the enzyme may allow some processing of the wild type proinsulin despite the cleavage sites not being the optimum consensus sequence for cleavage by furin. Yanigita *et al* (1992) have shown that wild type rat proinsulin I and furin co-expressed in COS-7 monkey kidney cells resulted in over 50% of the immunoreactive insulin (IRI) being processed to mature insulin. They also showed in 1993 that there is not enough furin endogenously expressed in a variety of cell lines for 100% processing of a rat proinsulin I gene with its cleavage sites mutated to the

consensus sequence for cleavage by furin. But, co-expression of this mutant with furin allowed 100% processing to mature insulin (Yanigita *et al*, 1993). Vollenweider *et al* (1995) have more recently shown that wild type human preproinsulin co-expressed with furin in COS (monkey kidney) cells processed around 60% of proinsulin to mature insulin. Whilst only 77% of their mutant (Human Insulin Arg^{62}) was processed to mature insulin when co-transfected with furin.

In the next chapter (chapter four) we are concerned with the creation of a glucose-sensitive insulin-secreting cell line. The use of the established AtT20 rat pituitary cell model (Stewart *et al*, 1993) ensures the secretion of mature processed insulin, but can co-transfection of the GLUT2 gene confer glucose-induced insulin secretion?

Chapter Four

Cellular Engineering of Glucose-Stimulated Insulin Secretion in the Murine Pituitary AtT20 Cell and Implantation Studies in Diabetic Nude (nu/nu) Mice.

Chapter Four : Cellular Engineering of Glucose-Stimulated Insulin Secretion in the Murine Pituitary AtT20 Cell and Implantation Studies in Diabetic Nude (nu/nu) Mice.

4.1 Introduction

The neuroendocrine cell line AtT20d16v is derived from the murine anterior pituitary (Richardson, 1978). The cell line synthesises an adrenocorticotropic hormone (ACTH) precursor, proteolytically processes it to mature ACTH, stores it in secretory granules and releases mature ACTH on stimulation with a secretagogue. The ability of these cells to proteolytically process prohormone precursors indicates that there is a well developed regulatory pathway and this led to the discovery that they can process proinsulin to mature insulin on transfection with the preproinsulin gene (Moore *et al*, 1983). Moore *et al* (1983) showed that insulin is packaged into secretory granules in the AtT20 cells and compared them to fibroblast cells transfected with the preproinsulin gene. The fibroblasts only secreted proinsulin and did not store it in secretory granules but transported newly synthesised proinsulin via the constitutive pathway to the cell surface for immediate secretion. Proinsulin has <10% of the biological potency of mature insulin (Revers *et al*, 1984), therefore, it is very important that proinsulin is correctly processed to insulin. As the AtT20 cell line possesses the requisite endopeptidases for processing prohormones, this cell line provides a useful model in which to express proinsulin (Moore *et al*, 1983; Taylor & Docherty, 1992).

Insulin secretion from transfected AtT20 cells is not significantly altered by raised concentrations of glucose (Stewart *et al*, 1993). Other cell types transfected with the human or rat proinsulin gene, such as fibroblasts, COS cells and CHO cells (Kawakami *et al*, 1992; Yanigita *et al*, 1993; Groskreutz *et al*, 1994; Vollenweider *et al*, 1995) exhibit the same lack

of response to increased glucose concentrations. To engineer surrogate β-cells it will be necessary to introduce glucose-stimulated insulin secretion to the cell line chosen. To achieve this we must consider the mechanism of glucose-stimulated insulin secretion in normal β-cells to decide which aspects are missing from the candidate cell line and need to be replaced. Figure 4.1 shows a schematic summary of the biochemical events involved in glucose-stimulated insulin-secretion. It is generally accepted that elevations in the concentration of glucose stimulates insulin secretion from the β-cells through its own metabolism (MacDonald, 1990). Glucose metabolism appears to trigger a number of electrochemical events, including inhibition of ATP-sensitive K^+ channels and activation of voltage gated Ca^{2+} channels (Rajan *et al*, 1990). Mitochondrial metabolism appears to play an important role in the glucose response, because glucose stimulates islet cell respiration and agents that block mitochondrial electron transport or oxidative phosphorylation also block glucose-stimulated insulin secretion (Meglasson & Matschinsky, 1986). The precise role of mitochondrial metabolites, however, is still unclear. The signalling pathway for the control of glucose-stimulated insulin secretion response requires further investigation, however, the secretory response does appear to be tightly linked to an increase in cytosolic free Ca^{2+}, much of which accumulates by influx through the voltage gated Ca^{2+} channel (Prentki & Matschinsky, 1987). Increases in Ca^{2+} have a range of effects in the β-cell including activation of mitochondrial enzymes and activation of protein kinases. A role for kinases, protein kinase C and members of the Ca^{2+}/calmodulin class in activation of insulin exocytosis has also been proposed (Ashcroft & Hughes, 1990). The magnitude of the insulin secretory response is proportional to the rate of glucose metabolism in the β-cell. Thus, proteins or enzymes that control the glucose flux in β-cells can be thought of as

Figure 4.1 Schematic summary of the biochemical events involved in glucose-stimulated insulin-secretion.

GLUCOSE

GLUT2

Glucokinase

Glucose-6-Phosphate

Glucose Metabolism

Signal(s)

ATP Sensitive
K^+ Channels

Depolarisation

Ca^{2+} Channels

Ca^{2+}-Dependent
Regulatory Proteins

Kinases

INSULIN SECRETION
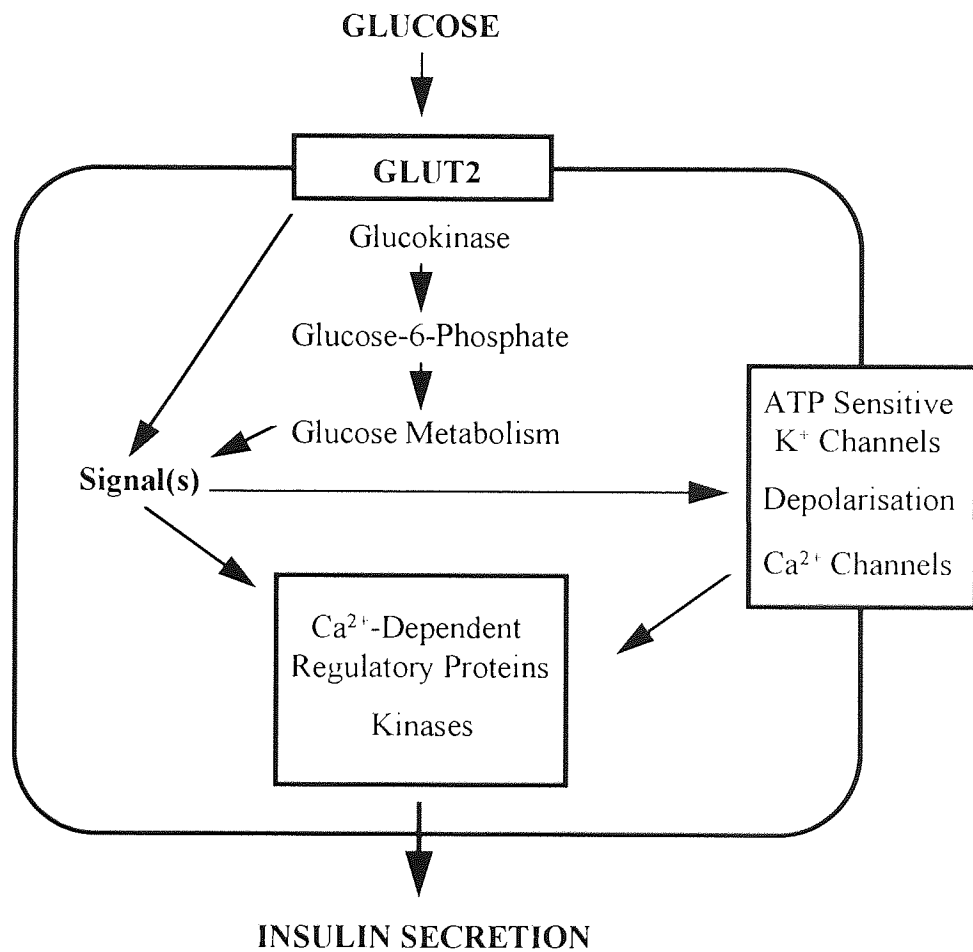
glucose sensors controlling insulin release in response to changes in extracellular glucose concentration. Two candidates for this glucose sensing role are the GLUT2-facilitated glucose transporter and the glucose phosphorylating enzyme glucokinase. Both glucokinase and GLUT2 have a higher Km (lower affinity) for glucose than other members of their respective gene families. GLUT2 is the major glucose transporter isoform expressed in β-cells (Johnson *et al*, 1990). The tissue distribution of both glucokinase and GLUT2 are limited, glucokinase is found in the β-cell and in liver and in some discrete cell populations such as the anterior pituitary corticotrophs (Hughes *et al*, 1991) and GLUT2 is also restricted to the β-cells and the liver. These considerations have led to molecular engineering of glucose-stimulated insulin secretion in cell lines that lack glucose sensing function centred on the GLUT2 glucose transporter and modulation of the glucokinase:hexokinase ratio (Newgard *et al*, 1993). Anterior pituitary AtT20 cells express the islet isoform of the glucokinase gene and show low levels of glucokinase activity (Hughes *et al*, 1991), but are completely lacking in GLUT2 expression (Hughes *et al*, 1992). In this chapter we investigate whether the transfection of the insulin and GLUT2 genes can confer glucose-stimulated insulin secretion to the AtT20 cell line.

Immunoincompetent athymic nude mice with streptozotocin induced diabetes provide a useful model in which to study the behaviour of the surrogate β-cell models. The animals cannot mount an immune response against the implanted cells allowing them to survive unhindered wherever they are implanted. In previous experiments implants of insulin secreting AtT20 cells were administered before streptozotocin treatment which resulted in a delay in development of hyperglycaemia compared to controls (Stewart *et al*, 1993). However, there is evidence to suggest that the cytotoxic effect of streptozotocin on neuroendocrine cell lines is dependent on the expression of GLUT2 by such cells (Schnedl *et*

al, 1994). Therefore GLUT2 expressing insulin secreting AtT20cells would have to be implanted after streptozotocin treatment to prevent their destruction along with the β-cells.

4.2 Methods

4.2.1 Double Transfection of AtT20d16v Cells

AtT20d16v cells were double transfected using the calcium phosphate co-precipitation method as described in chapter two. 15μl of pCB7-hppI-1 and 15μl of pLK444/GLUT2 at a concentration 1μg/μl were used in the precipitates which were added to each plate of cells to be transfected. Control precipitates were prepared without DNA and control plates received the same treatments with the control precipitates. Selection was performed using G418 sulphate at a concentration of 500μg/ml of complete medium. Resulting colonies of cells were picked using sterile cloning rings with trypsin and transferred to 6 well plates to grow up larger numbers of each clone to screen for expression of the genes of interest. When 2x10cm dishes of each clone were available, a sample of culture medium was collected for radioimmunoassay and the cells from the dish were used for mRNA preparation. The other dish of cells was cryopreserved ready to thaw and use should the clone be selected for further study.

4.2.2 Screening for hppI-1 and GLUT2 mRNA Expression in the Transfectants

Northern blots were performed using mRNA prepared from each clone. The blots were probed with ^{32}P labelled cDNA probes prepared from the cDNA inserts of the two plasmids used in the transfection. Therefore, a hppI-1 probe and a GLUT2 probe. Probings were carried out sequentially, stripping the filter in between with boiling 1% SDS. The final probe was for the 18S subunit of RNA as a control for loading variations on the Northern gel. Clones showing expression of mRNA for both GLUT2 and hppI-1 were selected to test for secretion of insulin into the culture medium by radioimmunoassay.

4.2.3 Differential Radioimmunoassay of the Culture Medium from AtTinsGLUT2.36

Six 75cm^3 flasks were seeded at 10^6 AtTinsGLUT2.36 cells per flask. The cells were allowed to settle to a monolayer overnight, then the medium was replaced with 7ml of serum-free DMEM. After 24 hours this medium was collected and stored at -20°C until required for radioimmunoassay. 3 flasks were assayed with the specific human insulin antibody and 3 with the non-specific insulin antibody. The differential assay determines that the AtTinsGLUT2.36 cells are producing fully processed mature insulin rather than proinsulin as expected. The method also allows the percentage of processed mature insulin that is secreted to be calculated.

4.2.4 Insulin Secretion in Response to Extracellular Glucose

The AtTinsGLUT2.36 clone was subcultured in 6 well plates and grown to 75% confluence. After a 1 hour incubation in serum-free medium without glucose, the cells were placed in increasing concentrations of glucose (0, 10μM, 25μM, 50μM, 75μM, 0.1mM, and 2.5mM) or secretagogues (20mM arginine, 0.5μg/ml forskolin with 5mM glucose or 0.5μg/ml forskolin alone) in 0.5ml serum-free DMEM per well. After 4 hours the culture medium was collected and stored at -20°C until analysed by insulin radioimmunoassay.

4.2.5 Insulin Secretion Following Preincubation in 2-Deoxyglucose

The AtTinsGLUT2.36 clone was subcultured in 6 well plates and grown to 75% confluence. Half of the plates were placed in glucose-free complete medium supplemented with 14mM 2-deoxyglucose and incubated overnight. The treated cells were gently rinsed in serum-free medium and all the plates were incubated for 1 hour in serum-free medium without glucose. The AtTinsGLUT2.36 cells were then placed in increasing concentrations

of glucose or fructose for 4 hours, the medium was then collected and stored at -20°C until analysed by insulin radioimmunoassay.

4.2.6 GLUT2 mRNA Expression in Response to Extracellular Glucose

10cm dishes of the AtTinsGLUT2.36 clone were incubated in increasing concentrations of glucose or secretagogues in 3ml of serum-free DMEM per dish for 4 hours. The medium was then removed and the cells used to prepare mRNA for Northern blotting. The Northern blot was probed with ^{32}P labelled GLUT2 cDNA and a control 18S cDNA probe to adjust for equal mRNA loading on the Northern gel. Autoradiographs were quantified using laser densitometry (2202 Ultroscan laser densitometer, LKB; Hewlett-Packard/LKB reporting integrator 3390A) to calculate the expression of GLUT2 mRNA for each glucose concentration or secretagogue.

4.2.7 Implantation of AtTinsGLUT2.36 into Diabetic Nude Mice

Diabetes was induced in immunoincompetent nude mice by administration of 200mg/kg streptozotocin by intraperitoneal injection (day 0). When the plasma glucose concentrations of the mice were >12mmol/L the mice were implanted with either 2×10^7 insulin-secreting AtTinsGLUT2.36 cells or 2×10^7 wild type AtT20d16v cells by intraperitoneal injection. Two implant experiments were carried out. Implant group one had 4 test mice and 4 control mice. Implant group two had 9 test mice and 6 control mice. Following implantation the mice were tail bled once or twice weekly to determine the plasma glucose concentration, and aliquots of plasma were stored at -20°C for insulin and ACTH immunoradiometric assays. Body weight and food intake were also monitored throughout

the experiment and animals with >25% reduction in body weight were killed by cervical

dislocation and examined by autopsy for evidence of cell growth in the peritoneum.

An oral glucose tolerance test was carried out on one test mouse from implant group two

and a normal non-diabetic nude mouse. The test mouse chosen had responded well to the

implant of AtTinsGLUT2.36 cells with lowered basal plasma glucose concentrations. At

time 0 a blood sample from the tail tip was taken from each mouse for plasma glucose

determination. A 2g/kg dose of 40% w/v glucose was then administered using an oral

gavage. Further blood samples for plasma glucose determination were obtained at 30, 60

and 90 minutes following the administration of the glucose load.

4.2.8 Histology and Immunohistochemistry

Tumours removed from mice at autopsy were preserved in 10% formaldehyde in PBS for at

least 24 hours, then processed for histological examination. Haematoxylin and eosin

staining was performed for general cell morphology and immunohistochemical staining was

performed for detection of insulin-secreting cells. These techniques were carried out as

described in chapter two.

4.3 Results

4.3.1 Double Transfection of AtT20d16v Cells

G418 selection at 500µg/ml killed all the control untransfected cells within 2 weeks. After 4 weeks, the cells which were transfected with the pLK444/GLUT2 and the pCB7-hppI-1 plasmids had formed discrete colonies of cells. Forty five colonies were picked aseptically from these plates and grown up in selection medium to be screened for insulin and GLUT2 expression.

4.3.2 Screening for hppI-1 and GLUT2 mRNA Expression in the Transfectants

Northern blots were performed with mRNA prepared from clones obtained by double transfection of the AtT20d16v pituitary cells with plasmids containing the genes for insulin and GLUT2 and conferring G418 resistance (AtTinsGLUT2 clones). mRNA from clones selected in G418 sulphate (500µg/ml) were screened for GLUT2 and insulin expression with ^{32}P labelled cDNA probes. A ^{32}P labelled cDNA probe to the 18S subunit of ribosomal RNA was used as a positive control to show that mRNA was equally loaded onto the Northern gel (figure 4.2). The GLUT2 mRNA has two signal transcripts at approximately 3.9 and 2.8kb, whilst the insulin mRNA has a single transcript at about 0.5kb. The untransfected wild type AtT20d16v pituitary cells do not show expression of either the GLUT2 or the insulin mRNA. All the transfected clones shown in the figure expressed the insulin mRNA, although in variable amounts when compared to the corresponding 18S mRNA bands. All the 18S mRNA bands are relatively constant implying that equal quantities of mRNA was loaded onto the gel for

Clone Number

WT 1 2 3 5 7 11 12 13 16 18 23 28 31 34 35 36 37 38 39 40 42 43 44 45

GLUT2

18S

INSULIN

18S

122

Figure 4.2 Northern blots of mRNA prepared from clones obtained by double transfection of AtT20d16v pituitary cells with plasmids containing genes for insulin and GLUT2 and conferring G418 resistance. Clones selected in G418 sulphate (500µg/ml) were screened for GLUT2 and insulin mRNA expression with oligolabelled cDNA probes. An oligolabelled cDNA probe to the 18S subunit of ribosomal RNA was used as a positive control to show that RNA was loaded equally on to the northern gel. GLUT2 has two transcript signals at approximately 3.9 and 2.8kb. Insulin has a single transcript at around 0.5kb. WT is mRNA prepared from wild type untransfected AtT20d16v pituitary cells. Clones 16, 28, 35 and 36 show strong expression of both GLUT2 and insulin mRNA.

each clone screened. Nearly all of the clones shown in figure 4.2 also express the GLUT2 mRNA with the exception of numbers 44 and 45. Number 3 shows very little expression of GLUT2 mRNA whilst clones 5, 11, 16, 28, 35 and 36 show very strong GLUT2 expression. Of these clones with strong GLUT2 expression numbers 16, 28, 35 and 36 also have strong expression of insulin mRNA, making them good choices for further study. Table 4.1 shows the results for all of the AtTinsGLUT2 clones screened by Northern blotting for GLUT2 and insulin expression. The expression of mRNA was scored by eye using the density of bands on the autoradiographs. Weak expression was scored as + and strong expression as +++, negative expression is denoted by - and nt means that the clone was not tested for expression of that particular mRNA. The table reiterates that clones 16, 28, 35 and 36 are the best double transfectants with +++ expression for both GLUT2 and insulin mRNA. Clone AtTinsGLUT2.36 was chosen for further study.

4.3.3 Differential Radioimmunoassay of Culture Medium from AtTinsGLUT2.36

Differential radioimmunoassay of culture medium from the AtTinsGLUT2.36 clone was performed in order to determine the quantity of insulin secretion from the cells and the extent of processing of proinsulin to mature insulin occurring in the cells. Insulin-like immunoreactivity in the culture medium, after culturing 1×10^6 cells for 24 hours in 7ml of serum free medium, was determined using both a non-specific insulin antiserum and an antiserum specific for mature human insulin (and partially specific (76%) for split des-64,65 proinsulin) in the RIA procedure. Figure 4.3 shows that $7.49ng/10^6$ cells/24 hours of insulin-like immunoreactivity was detected using the non-specific insulin

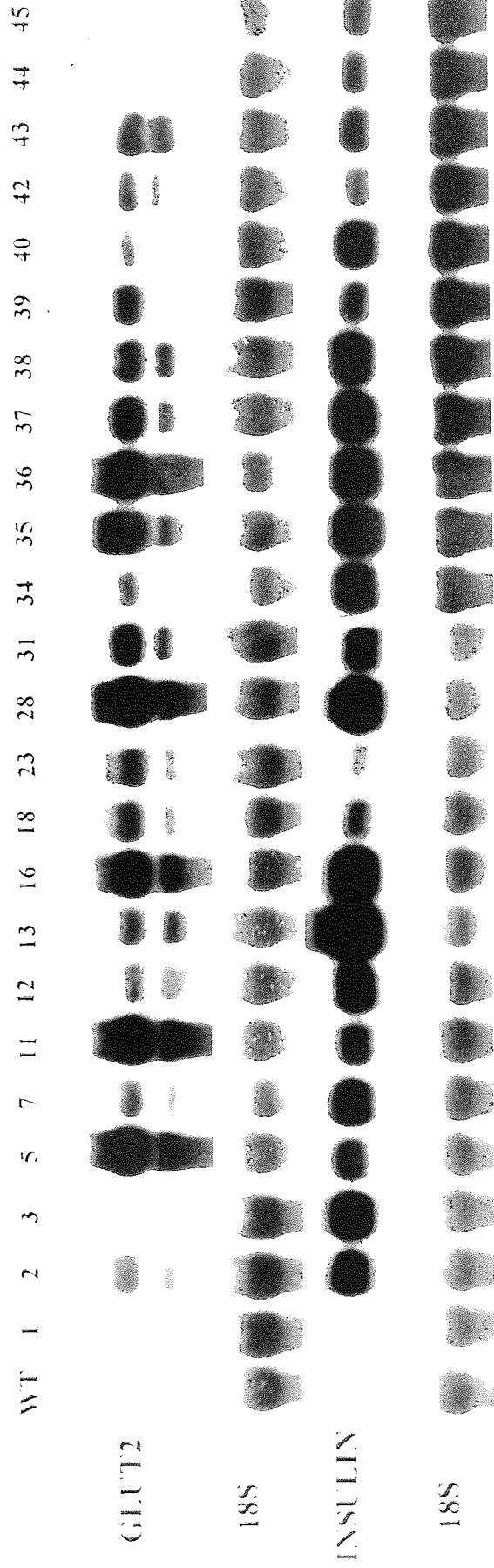**Table 4.1** Expression of GLUT2 and insulin mRNA scored using the density of bands on autoradiographs of Northern blots of mRNA prepared from clones obtained by double transfection of AtT20d16v pituitary cells with plasmids containing GLUT2 and insulin genes and conferring G418 resistance. Clones were selected in G418 sulphate (500µg/ml) and Northern blots screened using ^{32}P labelled cDNA probes. Expression was scored as + for weak expression, ++ for good expression, +++ for strong expression, - for no expression and nt when the clone was not tested for that particular mRNA. Clones 16, 28, 35 and 36 show strong expression of both GLUT2 and insulin mRNA.

Clone Number	GLUT2 mRNA	Insulin mRNA
1	+	-
2	++	++
3	+	+++
4	-	nt
5	+++	+
6	+	nt
7	++	++
8	-	nt
9	-	nt
10	-	nt
11	+++	+
12	++	++
13	++	+++
14	-	nt
16	+++	+++
17	+	nt
18	++	+
21	-	nt
22	-	nt
23	++	+
25	-	nt
26	-	nt
27	-	nt
28	+++	+++
29	++	nt
31	++	++
32	-	nt
33	-	nt
34	+	++
35	+++	+++
36	+++	+++
37	+++	++
38	+++	++
39	++	+
40	+	++
42	++	+
43	++	++
44	-	+
45	-	+

Figure 4.3 Insulin-like immunoreactivity in the medium after culturing 1×10^6 AtTinsGLUT2.36 cells for 24 hours in 7ml of serum free medium. Aliquots of the medium were assayed with a non-specific insulin antiserum to determine total insulin secretion and a specific human insulin antiserum to detect only processed mature insulin secreted into the medium. Values are means ∓ S.E.M where n=3. The graph indicates that the percentage of total insulin-like immunoreactivity that is secreted as mature human insulin is approximately 75%.
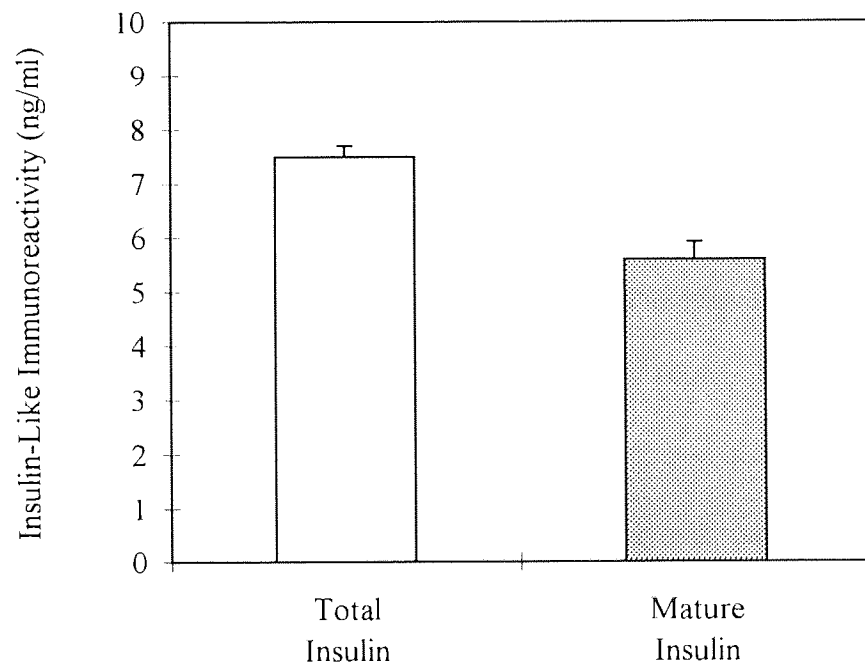
antiserum. This can be considered as the total insulin secretion from these cells. The specific insulin antiserum detected $5.6ng/10^6$ cells/24 hours of mature insulin-like immunoreactivity in the culture medium. These data indicate that approximately 75% of the insulin-like immunoreactivity secreted by the AtTinsGLUT2.36 clone is fully processed mature insulin.

4.3.4 Insulin Secretion in Response to Extracellular Glucose by AtTinsGLUT2.36

Culture medium from AtTinsGLUT2.36 cells incubated in various concentrations of glucose and the β-cell secretagogues forskolin and arginine was assayed for insulin-like immunoreactivity using the non-specific insulin antiserum. Figure 4.4 shows the insulin secretion from the cells as a percentage increase over basal secretion, where basal secretion was the insulin secreted by the AtTinsGLUT2.36 cells incubated in serum free medium without glucose. In all the glucose concentrations there is some increase in insulin secretion over basal. Insulin secretion increased from 10% over basal at 10μM glucose up to 52% over basal at 50μM glucose. At 75μM, 0.1mM and 2.5mM glucose insulin secretion was lower than at 50μM glucose although it was still increased over basal secretion and secretion at 10μM glucose. Forskolin (0.5 μg/ml) without glucose caused a 25% increase over basal insulin secretion from the AtTinsGLUT2.36 cells, whilst forskolin at the same concentration with 5mM glucose caused very little increase over basal insulin secretion. 20mM arginine caused a significant increase over basal secretion of insulin with around 85% more insulin secreted in the presence of 20mM arginine by the AtTinsGLUT2.36 cells. From the results, the cells appear to respond to increases in extracellular glucose though at subphysiological concentrations.

Figure 4.4 Percentage increase in insulin secretion over basal insulin secretion (serum free medium without glucose) from AtTinsGLUT2.36 cells incubated in increasing concentrations of glucose and with the β-cell secretagogues forskolin and arginine. Insulin secretion from the cells was determined by radioimmunoassay of the culture medium samples with a non-specific insulin antiserum. Insulin secretion is maximal at subphysiological glucose concentrations (50μM) and is increased by the presence of forskolin (0.5 μg/ml) or 20mM arginine in the culture medium.
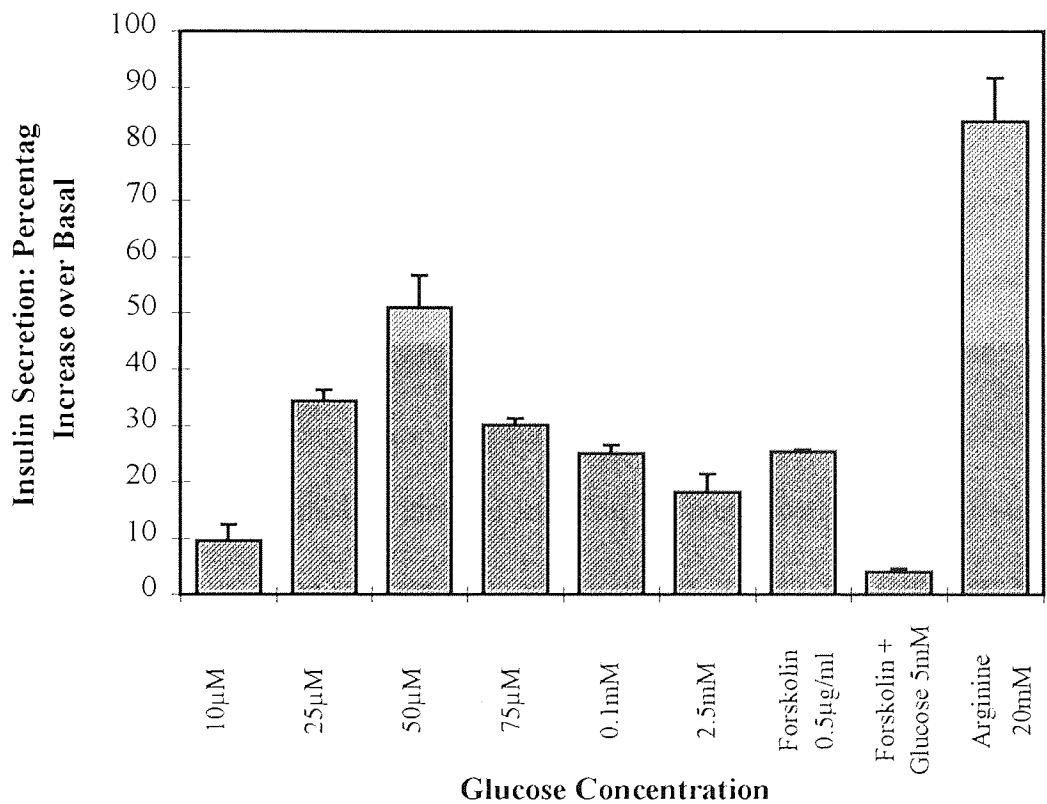
4.3.5 Insulin Secretion Following Preincubation in 2-Deoxyglucose

Culture medium from the AtTinsGLUT2.36 cells preincubated in 14mM 2-deoxyglucose overnight and control cells, subsequently treated with increasing concentrations of glucose or fructose, was assayed for insulin-like immunoreactivity using a non-specific insulin antiserum. Figure 4.5a shows the insulin released (ng/ml) in each of the increasing glucose concentrations for the control cells and the cells preincubated in 2-deoxyglucose. The control cells show a similar glucose response pattern to that shown in figure 4.4 with glucose increasing insulin secretion to $50\mu M$ glucose. The cells preincubated in 2-deoxyglucose all secreted more insulin during the experiment than the control cells, but showed no specific response to an increase in the glucose concentration. Figure 4.5b shows a similar phenomenon with cells preincubated in 2-deoxyglucose secreting much more insulin than the control cells. The response to high and low glucose of the preincubated cells was similar to that of the control cells with maximal stimulation at $50\mu M$ glucose. In high and low fructose the 2-deoxyglucose preincubated cells again produced much more insulin than control cells, but seemed to produce less than the preincubated cells incubated with glucose. There was some increase in insulin secretion at both high and low fructose concentrations, but secretion at 10mM fructose was similar to that at $50\mu M$ fructose with no increased response to the higher concentration. The $50\mu M$ fructose control sample was not assayed, but the control cells appeared to respond to fructose in a similar way to glucose at 10mM and may be expected to react to fructose in the same way as glucose. Preincubation in 2-deoxyglucose caused an increase in insulin secretion compared to control cells preincubated in complete culture medium (with glucose). However, AtTinsGLUT2.36 cells preincubated in 2-deoxyglucose did not respond to increases in glucose or fructose concentration.

Figure 4.5 Insulin-like immunoreactivity (ng/ml) in culture medium of AtTinsGLUT2.36 cells preincubated in 14mM 2-deoxyglucose overnight and of control cells, subsequently treated with a) increasing concentrations of glucose, or b) high and low concentrations of fructose, determined by radioimmunoassay with a non-specific insulin antiserum. Cells preincubated in 2-deoxyglucose secreted larger quantities of insulin than control cells but failed to show a response to increased glucose or fructose concentrations.

a)
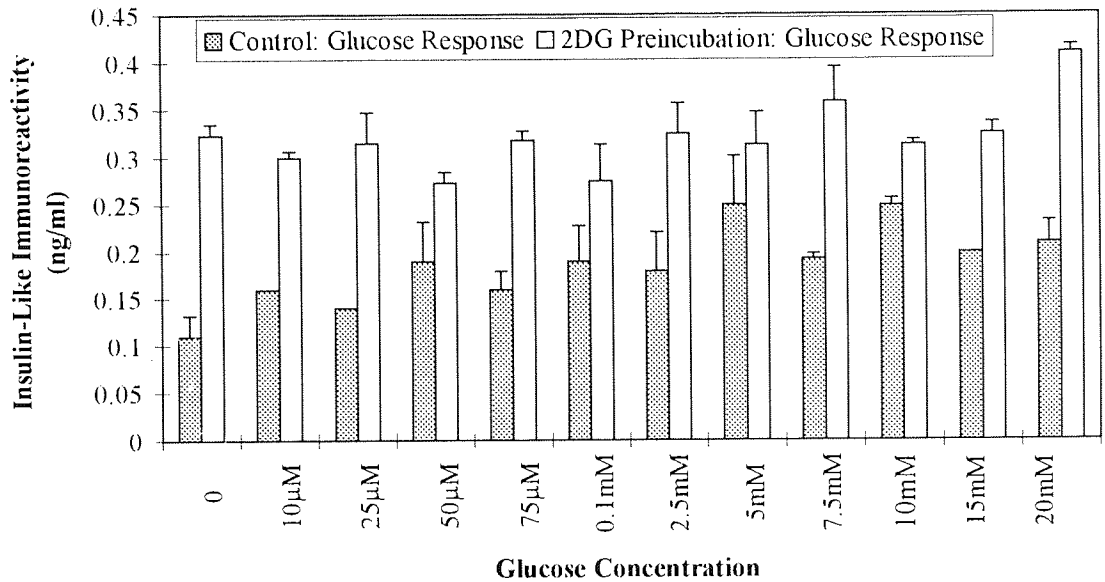
b)
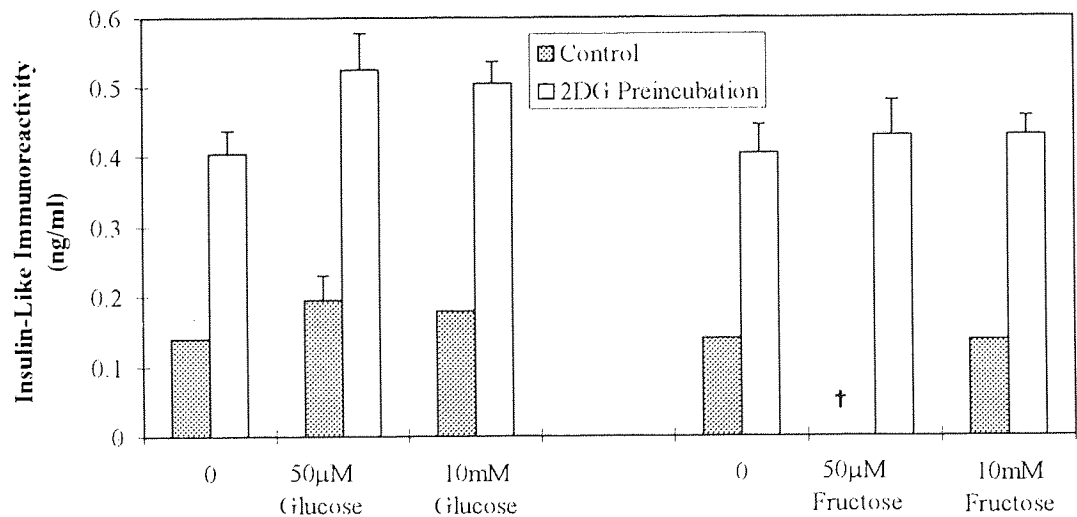
† =Sample Spoiled

4.3.6 GLUT2 Expression in Response to Extracellular Glucose in AtTinsGLUT2.36

Messenger RNA was prepared from AtTinsGLUT2.36 cells incubated for 4 hours in increasing concentrations of glucose and the β-cell secretagogues forskolin (0.5 μg/ml) and arginine (20mM). Northern blots with this mRNA were probed with a ^{32}P labelled GLUT2 cDNA probe to measure GLUT2 expression in treated AtTinsGLUT2.36 cells. Figure 4.6 shows the Northern blot probed with the ^{32}P labelled GLUT2 cDNA probe and the corresponding band for the 18S subunit of ribosomal RNA as a control for the quantity of RNA loaded onto the Northern gel. The 18S bands appear to be of a uniform density indicating that loading of RNA on to the Northern gel was relatively equal. The two transcript signals for GLUT2 at approximately 3.9 and 2.8kb increased in density as the glucose concentration increased from barely visible at 0, 10 and 25μM glucose to a substantial increase at 50μM glucose up to dense bands at 5 and 10mM glucose. The density of the bands on the autoradiograph were quantified using laser densitometry and the density of the GLUT2 bands adjusted using the density of the corresponding 18S band. Only the top 3.9kb GLUT2 band was used in the quantification, although both bands appeared to increase in density in response to glucose. Figure 4.7 shows the increase in density of the GLUT2 bands over the density of the 0 glucose band (basal) adjusted to account for the density of the corresponding 18S band. The densitometry supports the visual interpretation of the Northern blot with a 3.4 fold increase in density at 50μM glucose increasing up to 10 fold in density at 5mM glucose. Forskolin (0.5μg/ml) caused a 6 fold increase in density, though forskolin with 5mM glucose showed only an 8 fold increase in density compared to a 10 fold increase with 5mM glucose alone. Arginine (20mM) induced

Figure 4.6 Northern blot of mRNA prepared from AtTinsGLUT2.36 cells incubated for 4 hours with increasing concentrations of glucose and with the secretagogues forskolin (0.5µg/ml) and arginine (20mM). The blot was probed with a cDNA probe for GLUT2 mRNA and with an 18S cDNA probe to assess the loading of RNA on to the Northern gel. The GLUT2 probe gave two transcript signals (3.9 and 2.8kb) and the blot showed the bands increasing in density with increasing glucose concentration. Forskolin 0.5µg/ml (F), forskolin (0.5µg/ml) and 5mM glucose (F+G) and arginine 20mM (A) treatments also resulted in an increase in GLUT2 expression (density) compared to the density of the 0 glucose (basal expression) band.
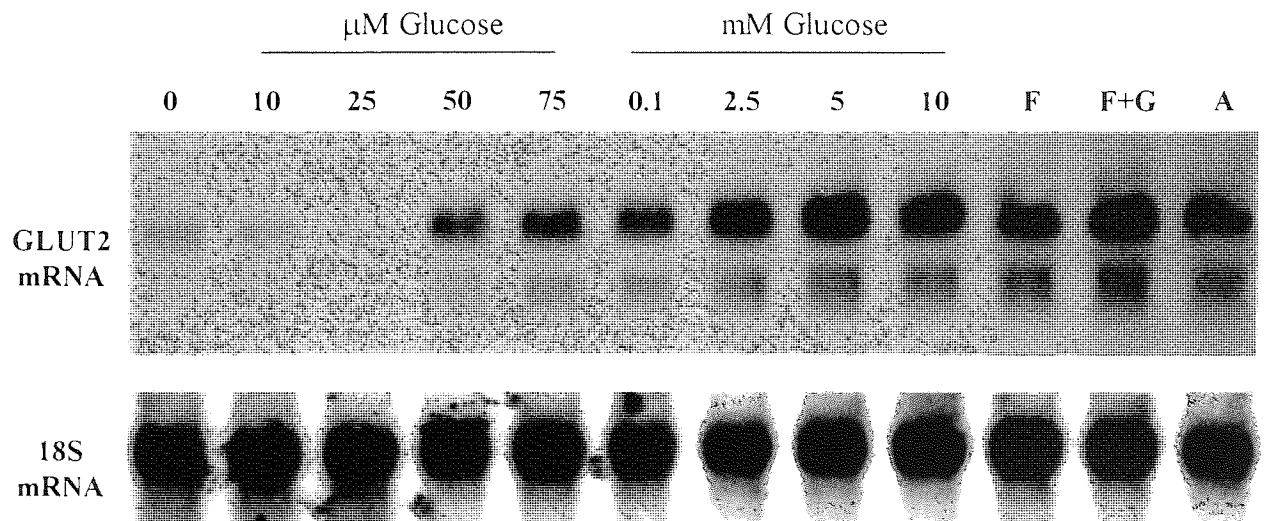
Figure 4.7 Increase in density over basal density (0 glucose) of GLUT2 mRNA bands determined by laser densitometry of the autoradiograph bands from Northern blots of mRNA prepared from AtTinsGLUT2.36 cells. The AtTinsGLUT2.36 cells were preincubated in increasing concentrations of glucose and with the secretagogues forskolin ($0.5\mu g/ml$) and arginine (20mM). The top 3.9kb GLUT2 band was quantified and density adjusted according to the density of the corresponding 18S band. GLUT2 expression (density) increased in a glucose-dependent manner up to 5mM glucose and was increased by the secretagogues forskolin and arginine.
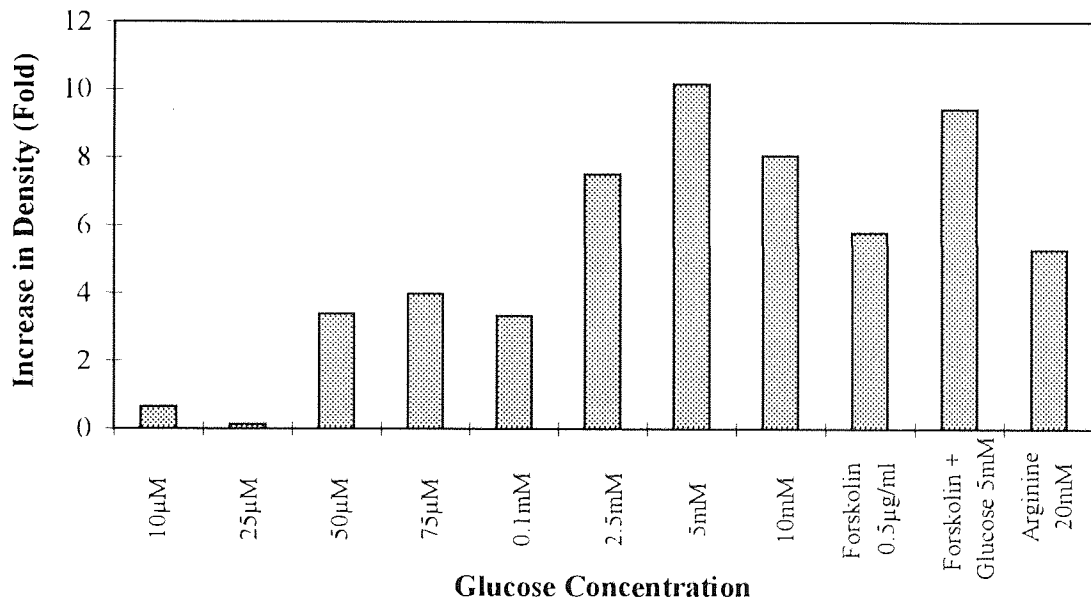
a 5 fold increase in density over basal. Thus, expression of the transfected GLUT2 gene in AtTinsGLUT2.36 cells appears to be regulated by glucose, with an increased glucose concentration leading to an increase in the expression of the GLUT2 glucose transporter. The secretagogues forskolin and arginine also increased the expression of the GLUT2 gene.

4.3.7 Implantation of AtTinsGLUT2.36 cells into Diabetic Nude Mice

Experimental diabetes was induced in nude (nu/nu) mice by administration of 200mg/kg streptozotocin by intraperitoneal injection on day 0. When plasma glucose, determined using blood samples obtained from the tail tips of the mice, was greater than 10-12mmol/l, mice were given 2×10^7 AtTinsGLUT2.36 or 2×10^7 wild type AtT20d16v cells by intraperitoneal injection. Mice were then monitored for plasma glucose, body weight and food intake once or twice weekly. Blood samples were also taken to determine plasma human insulin and plasma ACTH levels during the experiment.

Plasma Glucose

Plasma glucose of all the experimental nude (nu/nu) mice was determined once or twice weekly by blood sampling from the tail tip and separation of the plasma by centrifugation. The glucose concentration in plasma was then assayed by an automated glucose oxidase procedure using a Beckman glucose analyser. Figure 4.8a shows the plasma glucose concentrations of the streptozotocin diabetic nude (nu/nu) mice in implant group one. The 4 mice intraperitoneally implanted with 2×10^7 wild type AtT20d16v pituitary cells initially showed a gradual increase in plasma glucose concentrations, with a rapid increase between day 12 and day 18 after which this group was terminated due to the severity of the hyperglycaemia. The 4 mice intraperitoneally implanted with 2×10^7 insulin-secreting

AtTinsGLUT2.36 cells showed a gradual increase in plasma glucose concentration up to day 11 after plasma glucose concentrations declined slightly. This decrease continued to day 18 when plasma glucose began to rise until the mice reached lethal hyperglycaemia or were killed due to excessive weight loss (1 mouse day 22, 1 mouse day 24 and 1 mouse day 27). On day 18 the mice implanted with the wild type AtT20d16v cells had significantly higher plasma glucose concentrations ($p < 0.012$) than the mice implanted with the insulin-secreting AtTinsGLUT2.36 cells.

Implant group two (figure 4.8b) showed a similar plasma glucose concentration profile to implant group one. However the mice in this group took much longer to become diabetic and were less diabetic on implantation (day 44) than group one. Within group two, the 9 nude (nu/nu) mice with higher plasma glucose concentrations were implanted with 2×10^7 of the insulin secreting AtTinsGLUT2.36 cells. The 6 slightly less diabetic nude (nu/nu) mice were implanted with 2×10^7 of the wild type AtT20d16v cells. On day 50 the plasma glucose concentrations of the mice began to rise rapidly and by day 57 all the mice had plasma glucose concentrations around 26mmol/l. On day 66 the plasma glucose concentrations of the mice implanted with the AtTinsGLUT2.36 cells had fallen to around 20mmol/l, significantly lower than the mice receiving the wild type AtT20d16v cells ($p < 0.02$) which remained around 26mmol/l. The plasma glucose concentrations of the mice implanted with the AtTinsGLUT2.36 cells continued to fall to day 72, however, by this time most of the mice had sustained substantial weight loss and the study was terminated. However, one mouse maintained body weight and exhibited plasma glucose concentrations between 15-22mmol/l since day 66 despite having reached nearly 30mmol/l on day 57. The glycaemia of this mouse remained between 15-22mmol/l and the mouse remained in good health. This mouse was given an oral glucose challenge on day 100. Two of the mice implanted with the

Figure 4.8a Implant group one: Plasma glucose concentrations of streptozotocin diabetic nude (nu/nu) mice (Streptozotocin 200mg/kg given day 0 by intraperitoneal injection) implanted intraperitoneally on day 6 with 2×10^7 AtTinsGLUT2.36 cells or wild type AtT20d16v pituitary cells. Values are means ± S.E.M for 4 observations. The mice receiving the AtT20d16v cells were terminated at day 18 due to excessive hyperglycaemia. On day 18 the AtT20d16v implanted mice had significantly higher plasma glucose concentrations than the mice receiving the AtTinsGLUT2.36 cells (p<0.012). Amongst the mice receiving the AtTinsGLUT2.36 cells three were lost between day 22 and day 27 due to hyperglycaemia or weight loss. The other mice survived to day 34 when the experiment was terminated.
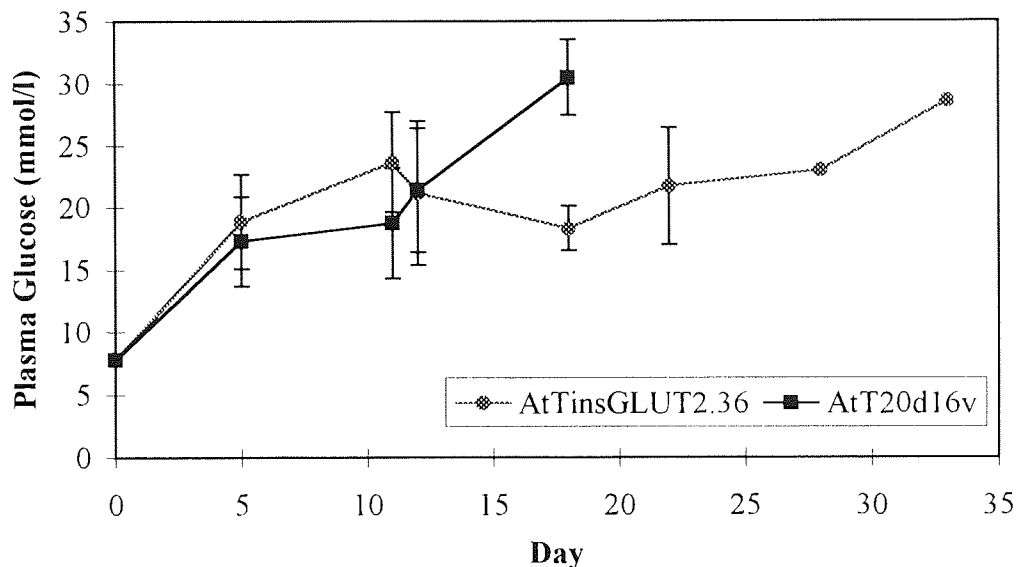
Figure 4.8b Implant group two: Plasma glucose concentrations of streptozotocin diabetic nude (nu/nu) mice (Streptozotocin 200mg/kg given day 0 by intraperitoneal injection) implanted intraperitoneally on day 44 with 2×10^7 AtTinsGLUT2.36 cells or wild type AtT20d16v pituitary cells. Values are means ± S.E.M for 9 AtTinsGLUT2.36 implanted mice and 6 AtT20d16v implanted mice. Two of the mice receiving AtT20d16v cells were culled due excessive weight loss on day 66, the remaining mice survived to day 72.
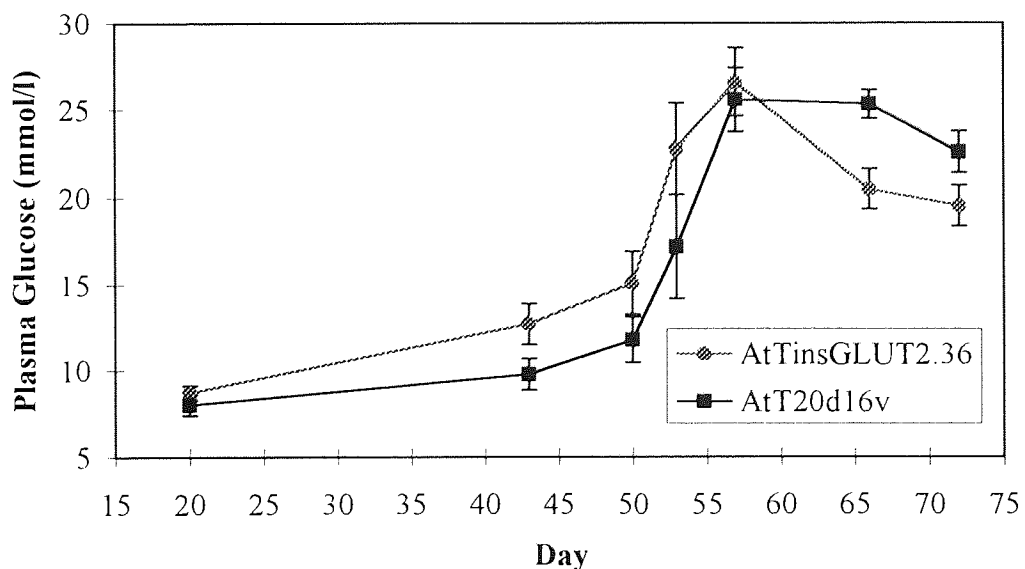
wild type AtT20d16v cells were culled on day 66 due to severe hyperglycaemia and the others were culled on day 72 due to weight loss, polydipsia and polyuria, all symptoms of the severe hyperglycaemia. The insulin secreting AtTinsGLUT2.36 cells showed a transient improvement in the glycaemia of streptozotocin diabetic nude (nu/nu) mice, and one showed a lasting improvement.

Body weight

Body weight of the nude (nu/nu) mice was monitored once or twice weekly throughout the implant experiments. In implant group one (figure 4.9a) the mice receiving the insulin-secreting AtTinsGLUT2.36 cells appear to lose weight at the same rate as the mice receiving the wild type AtT20d16v cells up to day 18. However the control mice lost slightly more weight than the AtTinsGLUT2.36 implanted mice between day 11 and day 18. The surviving AtTinsGLUT2.36 implanted mice maintained their body weight until day 22 after which they lost weight more rapidly, associated with the rise in plasma glucose concentration. In implant group two (figure 4.9b), mice implanted with AtTinsGLUT2.36 or wild type AtT20d16v cells lost weight rapidly between days 53 and 66. Between day 66 and 72 the mice implanted with AtTinsGLUT2.36 cells lost less weight than the mice implanted with the wild type AtT20d16v cells, corresponding to their improved plasma glucose status during this time. In both implant groups the loss of body weight was closely correlated with rise in plasma glucose concentrations.

Food Intake

Food intake in grams consumed per day (g/day) of the streptozotocin diabetic nude (nu/nu) mice implanted with insulin-secreting AtTinsGLUT2.36 cells and wild type AtT20d16v cells

Figure 4.9 Body weight in grams (g) of streptozotocin diabetic nude nu/nu) mice implanted with 2×10^7 insulin-secreting AtTinsGLUT2.36 cells or 2×10^7 wild type AtT20d16v pituitary cells. Streptozotocin (200mg/kg) was administered by intraperitoneal injection on day 0 and the cells were implanted by intraperitoneal injection on day 6 in implant group one a) and day 44 in implant group two b). The body weights fell throughout the experiments, but were better maintained during periods of improved glycaemia, day 18-22 in a) and day 66-72 in b).

a)

b)

137
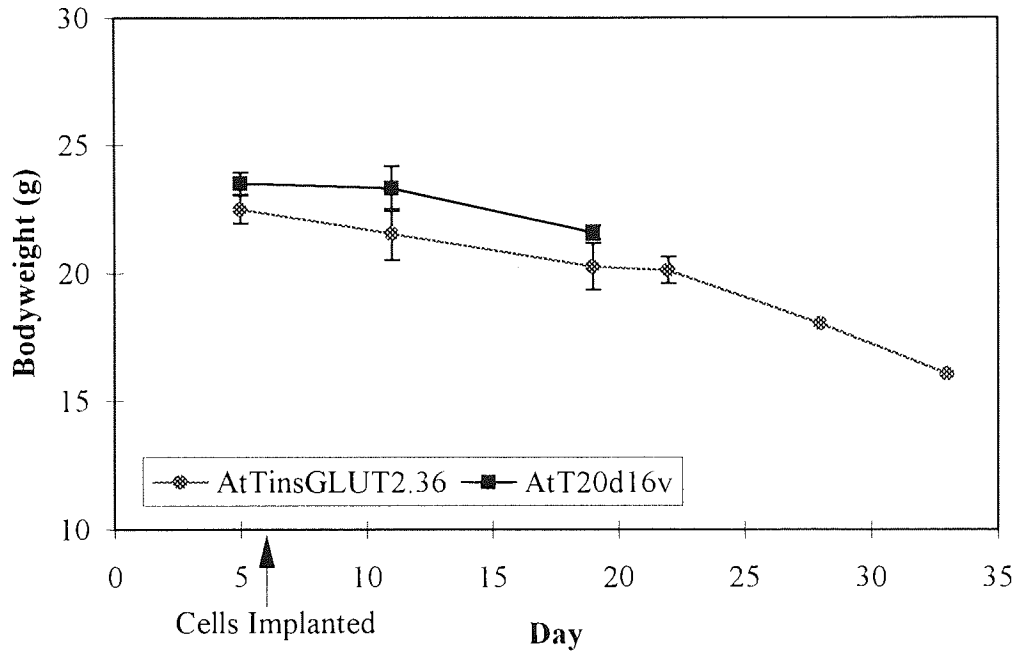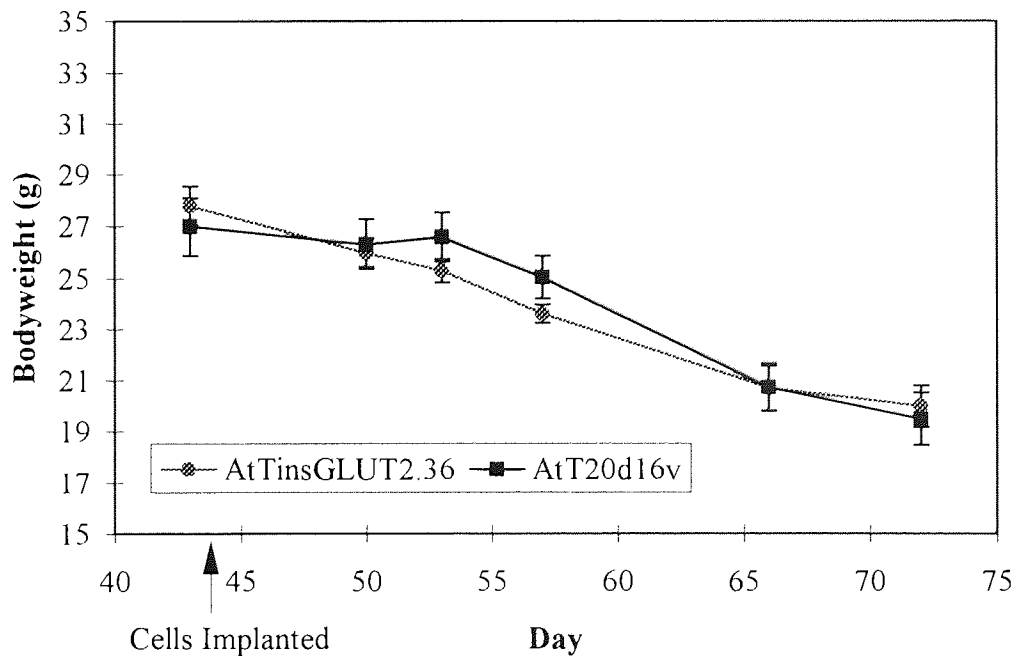
was measured throughout the implantation experiments. Food intake was monitored once or twice weekly and amount eaten (g) was divided by the number of days since the food was weighed last. In implant group one (figure 4.10a) the food intake of the mice implanted with insulin-secreting AtTinsGLUT2.36 cells increased gradually to day 18, whilst mice receiving the wild type AtT20d16v cells increased their food intake more rapidly. The AtTinsGLUT2.36 implanted mice slightly decreased their food intake between day 18 and 22 corresponding with the improvement in glycaemia of these mice during that time. After day 22 food intake rapidly increases corresponding to the rise in plasma glucose concentration after day 22. In implant group two (figure 4.10b) the mice implanted with the AtTinsGLUT2.36 cells increased their food intake rapidly between day 50 and 57 then gradually decreased food intake to day 72. This correlates well with the rise and fall of plasma glucose concentrations in these animals. The mice implanted with the wild type AtT20d16v cells increased food intake between days 50 and 53, then decreased food intake to day 57, increasing intake to day 66. The mice then decreased food intake to day 72. This decrease and the decrease between days 53 and 57 is likely to be due to severely hyperglycaemic animals being unable to eat properly. The hyperglycaemia is then reduced slightly although severe weight loss continues. Overall, food intake is also closely correlated to plasma glucose concentrations of the mice, with increases in plasma glucose causing hyperphagia and improved glycaemia resulting in a decrease in food intake.

Oral Glucose Tolerance Test

A streptozotocin diabetic mouse from implant group two, implanted with 2×10^7 insulin-secreting AtTinsGLUT2.36 cells, was chosen for an oral glucose challenge due to the prolonged improvement in glycaemia following implantation. The mouse had a plasma

Figure 4.10 Food intake (g/day) of streptozotocin diabetic nude (nu/nu) mice implanted with 2×10^7 insulin-secreting AtTinsGLUT2.36 cells or 2×10^7 wild type AtT20d16v cells. Streptozotocin (200mg/kg) was administered by intraperitoneal injection on day 0 and cells were implanted by intraperitoneal injection on day 6 in implant group one a) and on day 44 in implant group two b). Food intake increased with increasing plasma glucose concentration and decreased during periods of improved glycaemia, day 18-22 in group one a) and day 57-72 in group two b).

a)
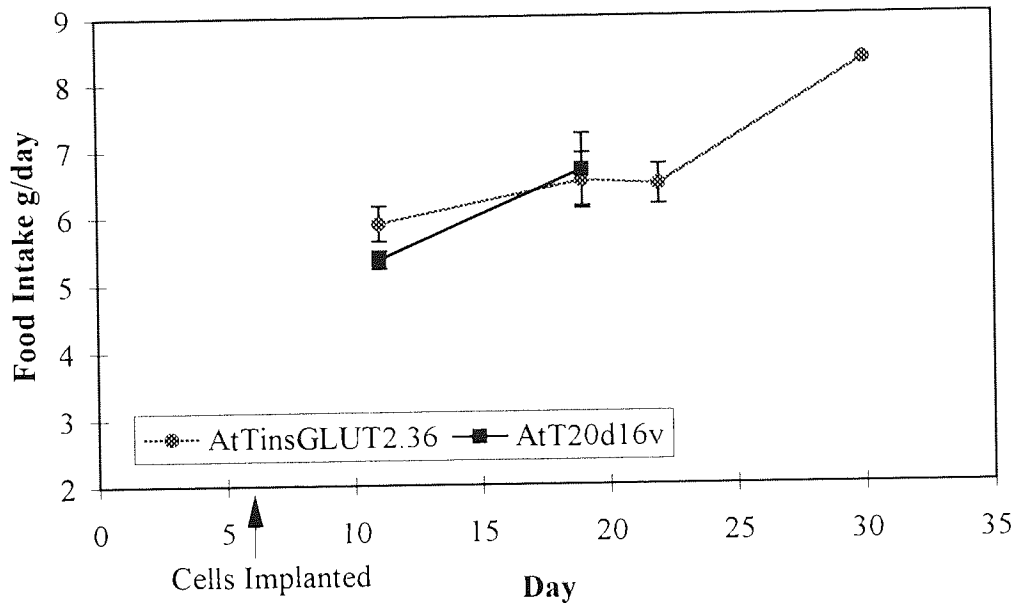
b)
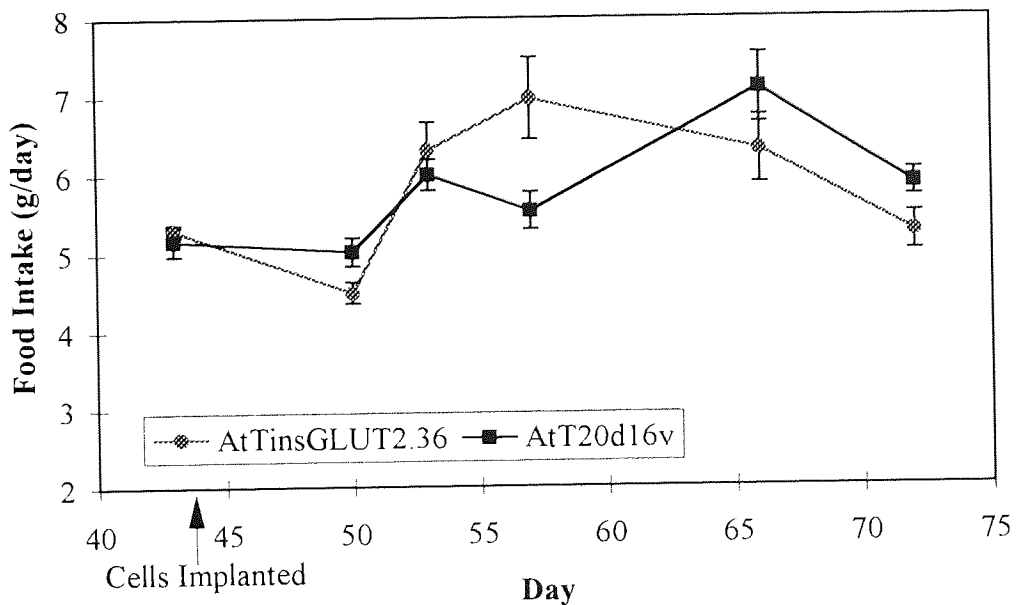
glucose concentration of nearly 30mmol/l on day 57 of the implant experiment and thereafter maintained a plasma glucose concentration of between 15 and 22 mmol/l. The mouse maintained body weight and food intake was increased compared to normal but the mouse was not significantly hyperphagic. There was no evidence of polydipsia or polyuria. The oral glucose tolerance test was carried out on day 100 (56 days post implantation) after a 5 hour fast. A non-diabetic non-implanted nude (nu/nu) mouse was used as a control. Plasma glucose was determined by obtaining blood samples from the tail tips before and after glucose administration (2g/kg by oral gavage) and plasma was separated and assayed in a Beckman glucose analyser. The mouse from implant group two had a plasma glucose concentration of 17.3mmol/l while the control mouse was 10.9mmol/l (figure 4.11). 30 minutes after the glucose challenge the control mouse had a raised plasma glucose concentration of 20mmol/l and the test mouse had risen to 25.2mmol/l. 60 minutes after the challenge the control mouse had returned to close to the pre-challenge plasma glucose concentration of 11.8mmol/l, however, the test mouse showed only a modest lowering of its plasma glucose concentration to 21.1mmol/l. After 90 minutes the control mouse showed an overcompensatory fall in plasma glucose concentration of 9.5mmol/l, slightly lower than the starting concentration. By 90 minutes the test mouse had almost reached its starting plasma glucose concentration of 17.3mmol/l. The response to the glucose challenge of the mouse from implant group two was not like that of the control mouse, but it was not severely and uncontrollably raised as seen in untreated diabetic mice (Shafrir, 1990). The implant of AtTinsGLUT2.36 cells does appear to help lower plasma glucose concentrations, although the implanted cells cannot respond with the rapidity or with the amount of insulin secretion shown by normal pancreatic β-cells. Thus the implant was not able to secrete enough insulin to enable the mouse to achieve normoglycaemia, but, the implant allowed the

Figure 4.11 Plasma glucose concentrations (mmol/l) of a streptozotocin diabetic mouse from implant group two implanted with 2×10^{7} AtTinsGLUT2.36 cells (streptozotocin 200mg/kg was administered by intraperitoneal injection on day 0 and cells implanted intraperitoneally on day 44, the challenge was carried out on day 100) and a normal non-implanted non-diabetic nude (nu/nu) mouse. The mice were given an oral glucose challenge of 2g/kg by oral gavage at time 0. Blood samples from the tail tip were obtained to assay for plasma glucose concentration using a Beckman glucose analyser at 30, 60 and 90 minutes. The plasma glucose concentration of the mouse from implant group two did not return its to the starting concentration as quickly as the normal mouse, taking 90 minutes as opposed to 30 minutes in the normal mouse.
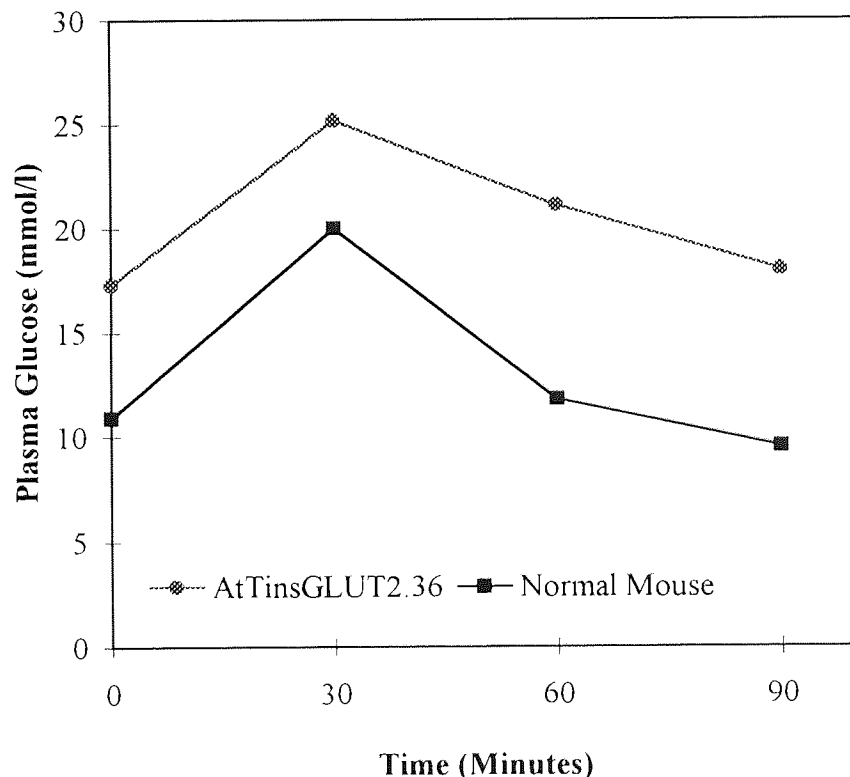
mouse to maintain basal plasma glucose concentrations around 15-22mmol/l. The mouse implanted with the AtTinsGLUT2.36 cells was maintained for 106 days when evidence of weight loss, polydipsia and polyuria indicated that severe hyperglycaemia had occurred and that the AtTinsGLUT2.36 cell implant was failing to prevent lethal hyperglycaemia.

Plasma Insulin

A specific human insulin immunoradiometric assay (IRMA) was used to assay plasma samples from the streptozotocin diabetic nude (nu/nu) mice implanted with 2×10^7 insulin-secreting AtTinsGLUT2.36 cells or 2×10^7 wild type AtT20d16v cells. The samples were aliquots from the plasma obtained for plasma glucose determination and preserved at -20°C. Mature human insulin was detected in some of the samples from the mice implanted with AtTinsGLUT2.36 cells, however, levels were very low and in many samples at the threshold level for the sensitivity of the assay. Thus the data are not presented for quantitative analysis since they offer only a semi-quantitative indication that mature human insulin was indeed released into the circulation by the implants. No mature human insulin was detected in the samples from mice implanted with the wild type AtT20d16v pituitary cells.

Plasma ACTH

Aliquots of plasma samples which were obtained once or twice weekly for plasma glucose determination were also preserved at -20°C and assayed using an ACTH immunoradiometric assay (IRMA). Two sets of samples were assayed: samples from streptozotocin diabetic nude (nu/nu) mice of implant group two implanted with wild type AtT20d16v pituitary cells, and samples from non-implanted non-diabetic nude (nu/nu) mice

142

taken at corresponding intervals to those of implant group two. Plasma samples from the mice during the implant experiments were limited due to the small quantities of blood available from each mouse (around 100µl per sample). Because the wild type AtT20d16v cells should secrete the same amount of ACTH as the transfected AtTinsGLUT2.36 cells, the implanted cells should have the same effect on plasma ACTH levels in the control mice as in the mice implanted with the transfected AtTinsGLUT2.36 cells. Therefore, samples from the mice implanted with wild type AtT20d16v cells were assayed for ACTH concentration while samples from the mice implanted with the transfected AtTinsGLUT2.36 cells were assayed for insulin. A few samples from the control mice were assayed for insulin concentration to show that there was no human insulin present. Figure 4.12 shows the plasma ACTH concentration (pg/ml) for the streptozotocin diabetic nude (nu/nu) mice implanted with AtT20d16v cells and non-implanted non-diabetic mice. The control mice maintained plasma ACTH levels between 5 and 70pg/ml throughout the experiment. The mice implanted with the AtT20d16v cells on day 44 gradually increased their plasma ACTH levels to around 150pg/ml at day 57 and 320pg/ml at day 66. Levels of plasma ACTH decreased slightly after day 66, supporting the view that the cells ceased to function properly after this period of implantation. Overall the implantation of mice with ACTH secreting AtT20d16v pituitary cells substantially increases their plasma ACTH concentrations above normal levels, raising concerns about the suitability of such a cell line for more detailed studies on the efficacy of glycaemic control using this source of insulin delivery.

4.3.8 Histology and Immunohistochemistry

At the end of the implant experiments the mice were examined by autopsy for evidence of the implanted cells. Nearly all of the mice implanted with AtT20 cells, wild type or

Figure 4.12 Plasma ACTH concentration (pg/ml) of streptozotocin diabetic nude (nu/nu) mice implanted with AtT20d16v pituitary cells and non-diabetic nude (nu/nu) mice without implants. Streptozotocin 200mg/kg was administered by intraperitoneal injection on day 0 and the cells were implanted by intraperitoneal injection with 2×10^{7} cells on day 44. Plasma ACTH was determined using an ACTH immunoradiometric assay. Mice implanted with AtT20d16v cells showed a substantial increase in plasma ACTH concentration during the experiment compared to the control non-implanted animals.

144
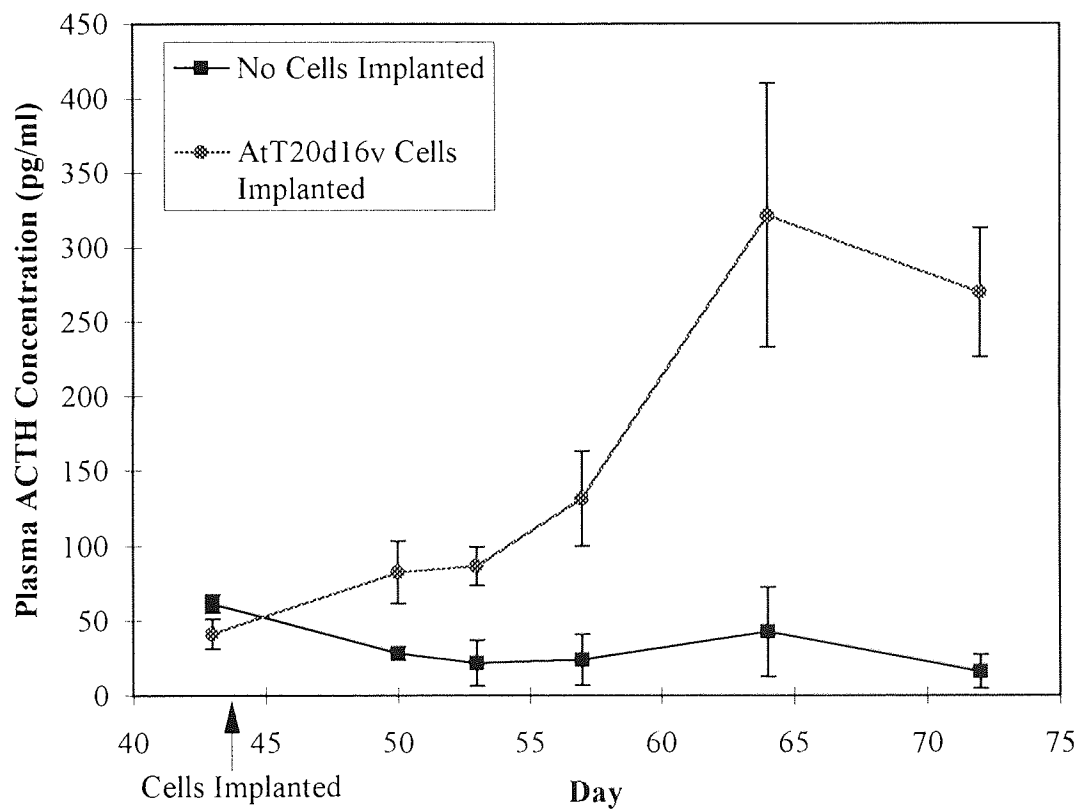
AtTinsGLUT2.36 cells, formed tumour like aggregations. Most of these tumours were found around the site of the intraperitoneal injection, growing into the muscle wall. Some tumours were localised to the liver or kidney and in some mice aggregations which appeared to be secondary tumours were found adhered to the diaphragm or in the liver. All tumours were well vascularised and varied in size from between 0.5mm to 8mm in diameter. Tumours were excised and preserved in 10% formaldehyde in PBS. They were then histologically processed as described in chapter 2 and stained with haematoxylin and eosin for general cell morphology or immunohistochemically treated for insulin presence. Figure 4.13a and 4.13b show sections from tumours, removed from mice implanted with AtTinsGLUT2.36 cells, stained with haematoxylin and eosin. The tumours have normal healthy cells around the periphery and appear to be necrotic in the centre. Figure 4.13b shows the tumour (darker tissue) attached to part of the peritoneal muscle. Figure 4.14a and 4.14b show the same tumours stained using immunohistochemistry for insulin detection. The insulin appears as a dark brown stain and positive cells were mostly found around the periphery of the tumours where the healthy tissue was found. The lighter brown stain in the necrotic area of the tumour in figure 4.14a is thought to be degrading insulin remaining from the necrosed cells. No correlation was evident between the size of the tumours at autopsy and previous glycaemic control. While it is possible that not all of the implants were retrieved, the sparcity and variability in the number and intensity of insulin positive staining cells suggests that a) the size of the tumours was not directly associated with the number and intensity of staining of insulin positive cells; and b) by the time of autopsy the level of hyperglycaemia indicates that implants were failing, so any associations with previous glycaemic control would be unlikely.

Figure 4.13 Sections from tumours removed from diabetic nude (nu/nu) mice implanted with AtTinsGLUT2.36 cells after 28 days growth *in vivo*. The tumours are stained with haematoxylin and eosin for general cell morphology. 4.13a shows the healthy periphery of the tumour with a necrotic core. 4.13b shows a tumour (darker tissue) still attached to the peritoneal muscle.

a) Tumour from a mouse in implant group one (magnification x120).
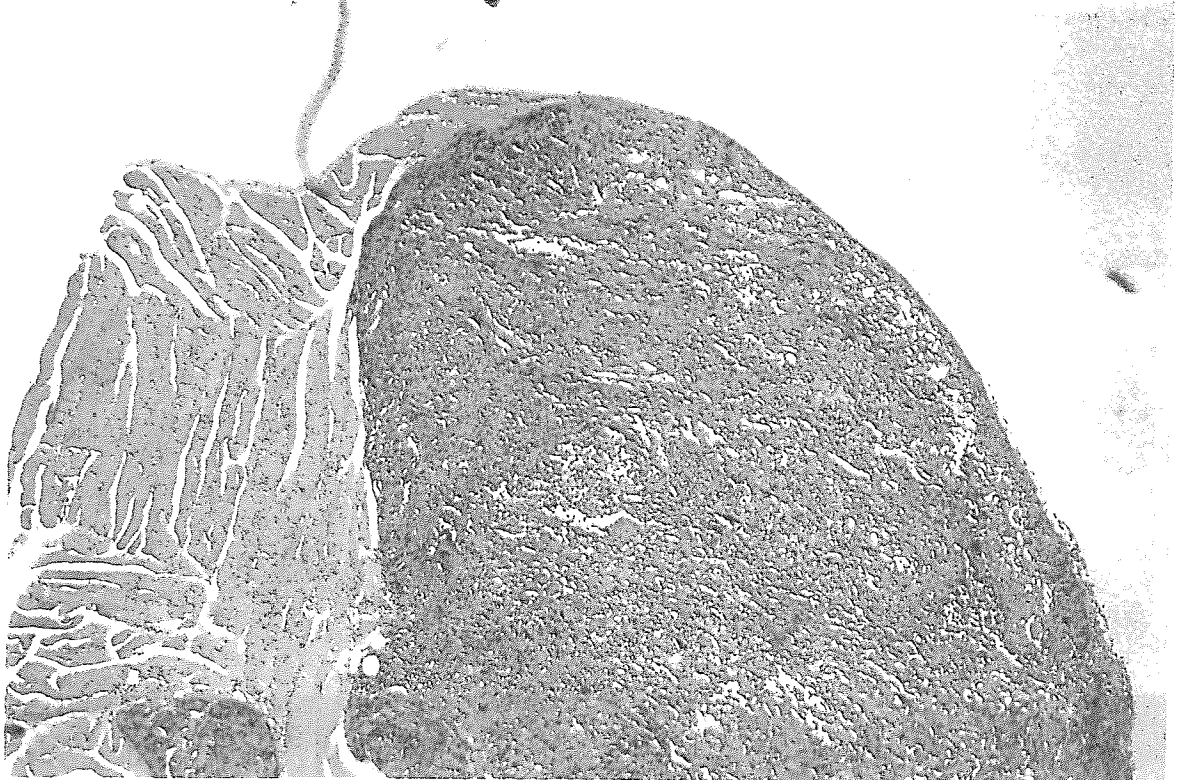
b) Tumour from a mouse in implant group two (magnification x48).
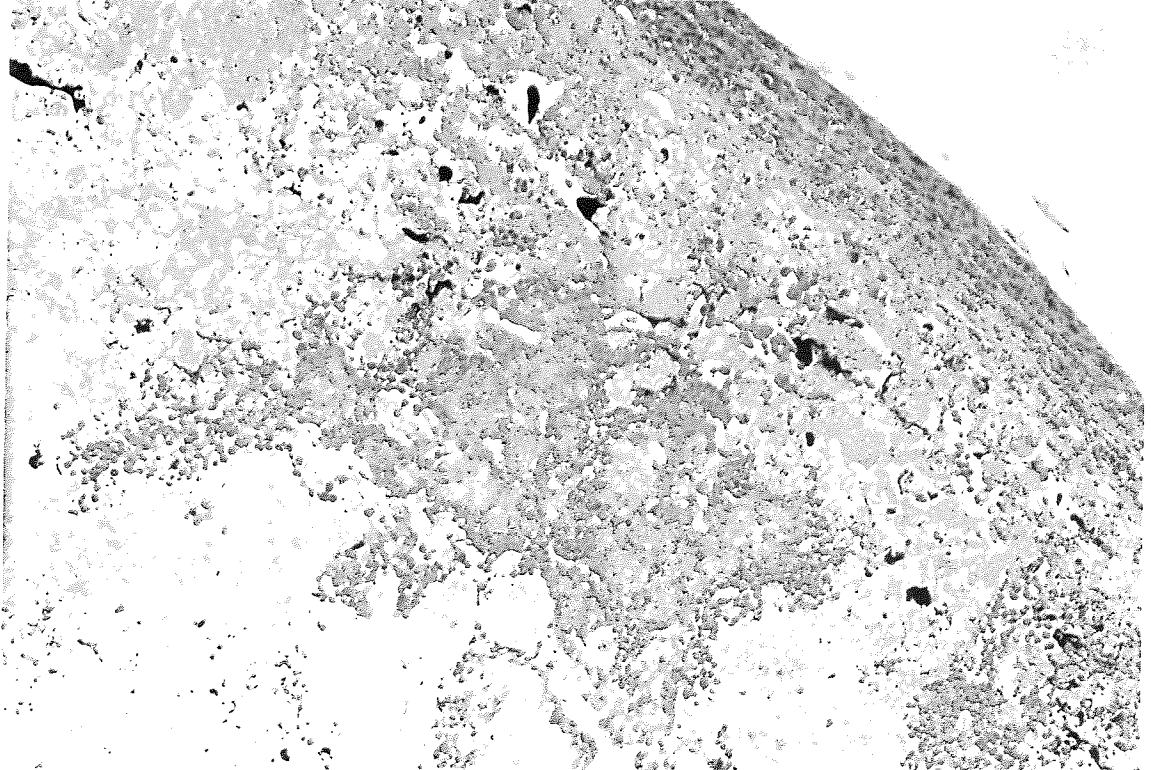
Figure 4.14 Sections from tumours removed from streptozotocin diabetic nude (nu/nu) mice implanted with AtTinsGLUT2.36 cells after 28 days *in vivo*. The sections are immunohistochemically stained for insulin. Cells positive for insulin secretion are stained dark brown and were mostly found near the periphery of the tumours.

a) Tumour from a mouse in implant group one (magnification x480).
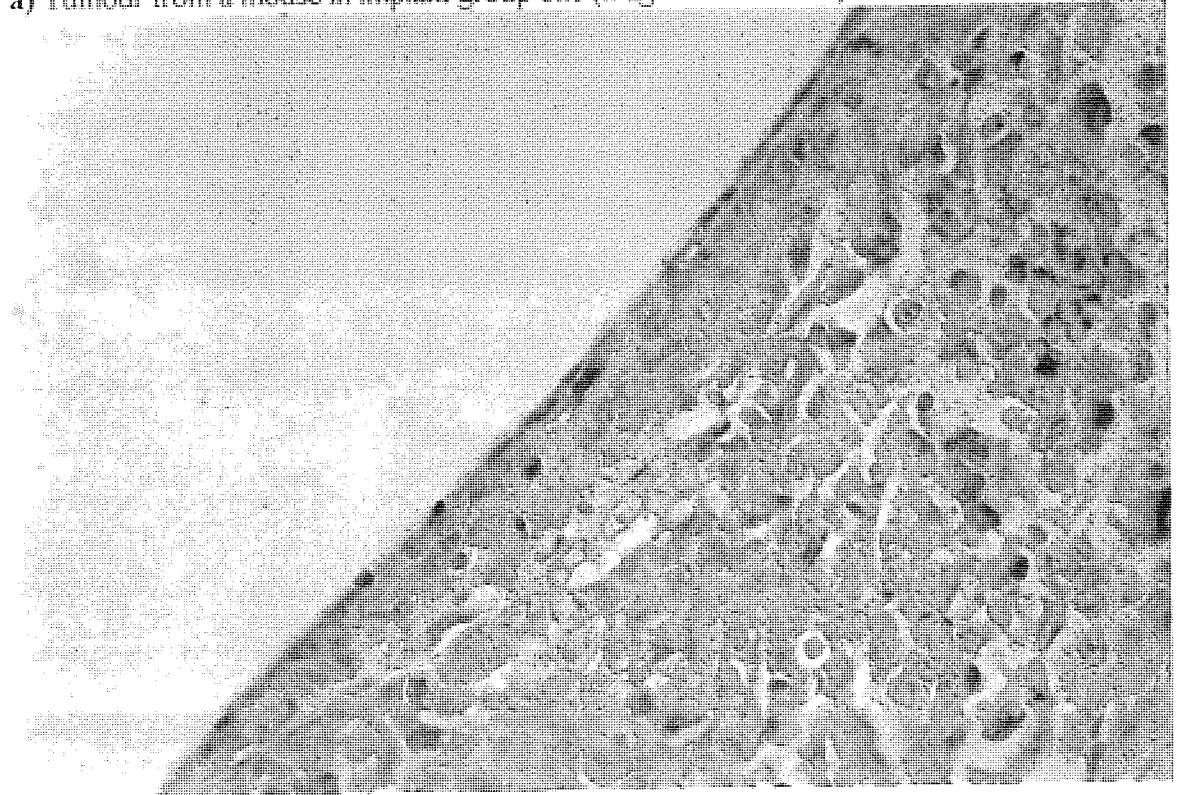
b) Tumour from a mouse in implant group two (magnification x480).
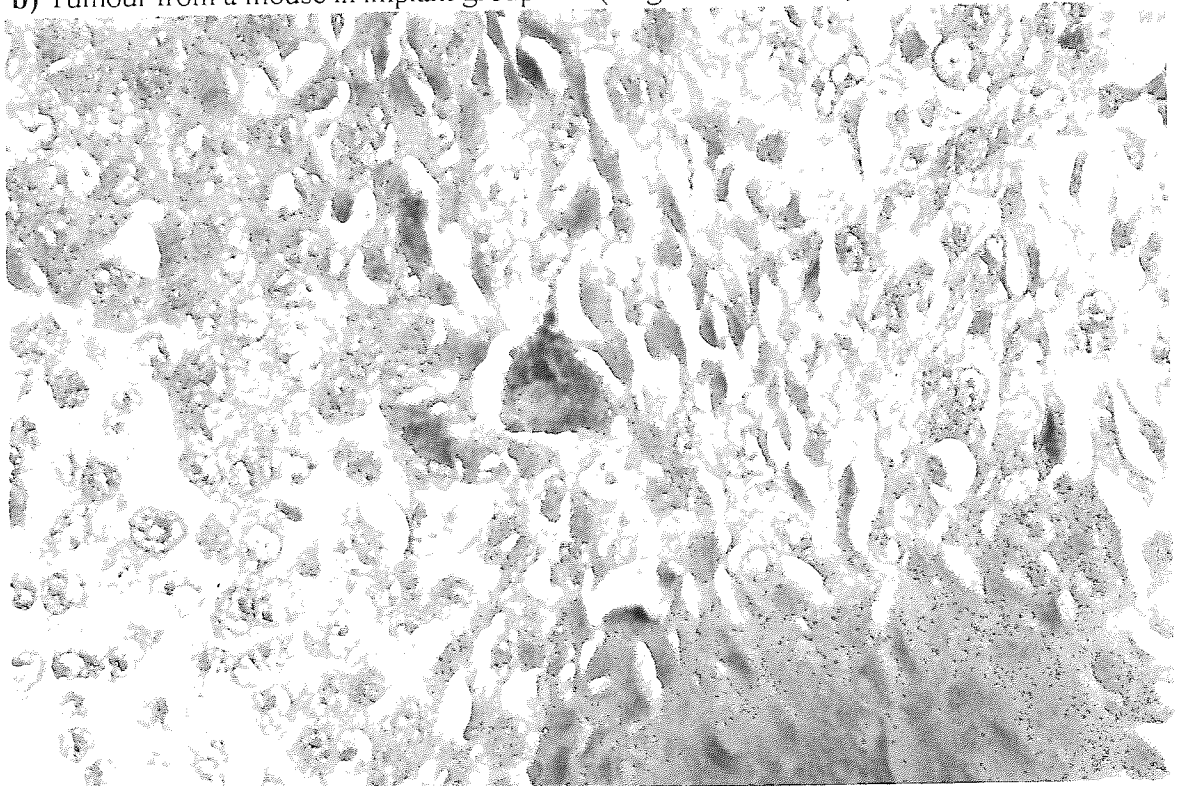
The fact that insulin secreting cells were visualised by immunohistochemistry supports the data shown in the implant experiments where implants of insulin secreting cells reduce plasma glucose concentrations and cause transient improvements in the experimental diabetes of the mice.

4.4 Discussion

AtT20d16v pituitary cells were double transfected with genes for the GLUT2 glucose transporter and for insulin using the calcium phosphate coprecipitation method. Transfectants were selected in medium containing G418 sulphate (500μg/ml) and resistant colonies of transfected cells screened for expression of the GLUT2 and insulin genes by Northern blotting. Clone AtTinsGLUT2.36 was found to strongly express both insulin and GLUT2 mRNA, and radioimmunoassay for insulin-like immunoreactivity with a non-specific insulin antiserum showed the cells to be producing $7.5ng/10^6$ cells/24 hours of total insulin-like material. Further radioimmunoassay of an aliquot of the same sample with a specific human insulin antiserum (crossreactivity with mature human insulin 100%, split des-64,65 proinsulin 76%, proinsulin/split des-31,32 proinsulin <0.2% and rat insulin <0.2%), showed that 75% of the total insulin-like material secreted from the cells was mature human insulin (or split des-64,65 proinsulin). It has previously been shown that pituitary AtT20 cells can process prohormone precursors using endogenous endopeptidases. The AtT20 cell line is derived from the murine anterior pituitary and expresses the proopiomelanocortin (POMC) precursor. The POMC prohormone can be split endoproteolytically at dibasic amino acid pair sites to form a variety of products. The precise products formed depends on the tissue in which POMC is expressed (Hadley, 1992). The AtT20 cell line processes the POMC precursor to release the N-terminal fragment, ACTH, β-lipotropin and some β-endorphin (Eipper & Mains, 1980). The prohormone processing enzymes contained by the pituitary cells are PC1 and PC2, which are homologues of the yeast Kex2 endopeptidase (Bloomquist et al, 1991). In the anterior pituitary, it is mostly PC 1 which is expressed, and this expression is inducible by corticosteroids (Day et al, 1992). The AtT20 cell lines have been shown to process a variety of precursors, following transfection of exogenous genes

149

including proinsulin, proenkephalin, prosomatostatin and procholecystokinin (Dickerson & Noel, 1991). The production of mature peptide from each of these precursors requires cleavage at Lys-Arg and Arg-Arg dibasic sites, which are also the sites cleaved during the processing of endogenous POMC. The production of correctly processed insulin by these cells has been shown to be via the regulated pathway, one of two secretory pathways found in secretory cells. The constitutive pathway comprises the export of proteins to the cell surface directly after synthesis by a bulk flow process. The regulated pathway actively sorts peptides into secretory granules for storage prior to release (Kelly, 1985). Secretagogues can only stimulate the release of those proteins packaged in secretory vesicles in the regulated pathway; proteins in the constitutive pathway are unaffected (Moore *et al*, 1983b). In the AtT20 cell line, POMC is cleaved within the secretory granules and mature hormone products are secreted by the regulated pathway on stimulation. Over 80% of ACTH is released by this pathway (Gumbiner & Kelly, 1981). In contrast the constitutive pathway releases mostly unprocessed POMC (Gumbiner & Kelly, 1982). The endogenous sorting of proinsulin in normal β-cells is very efficient with less than 1% of unprocessed proinsulin secreted constitutively (Rhodes & Halban, 1987). In the AtT20 cells transfected with the preproinsulin gene, it has been reported that mature processed insulin and ACTH are present within the same secretory granules (Orci *et al*, 1987; Powell *et al*, 1988) and are therefore both secreted via the regulated pathway, although the amount of insulin secreted is much less than that of ACTH (Moore *et al*, 1983).

The double transfected AtTinsGLUT2.36 clone exhibited glucose stimulated insulin secretion, with maximal secretion at around 50µM glucose, when incubated with increasing concentrations of glucose *in vitro*. Normal islets do not respond to glucose concentrations of less than 4-5mM, the AtTinsGLUT2.36 cells therefore show an increased sensitivity to

glucose compared to normal islets. The fact that these cells respond to subphysiological levels of glucose, despite the expression of GLUT2 and glucokinase (endogenously expressed by AtT20 cells), suggests that the regulatory function of the high Km components is overridden by another metabolic determinant. With increased rates of glucose transport, even at a low glucose concentration, glucose phosphorylation is likely to become the rate limiting step for the generation of glucose-related signals. Although AtT20 cells express the mature islet glucokinase transcript, their predominant glucose phosphorylating enzyme is hexokinase which has a Km for glucose of 10-50 μM (Hughes *et al*, 1992). Support for this model where hexokinase can exert a dominant effect is provided by a study where normal islets transfected with hexokinase results in a shift of the glucose-dose response curve such that transfected cells are maximally responsive to glucose at subphysiological levels (German & Rutter, 1991). Further support for a role for hexokinase in mediating the change in glucose dose response was achieved by preincubation of hexokinase transfected islets with 2-deoxyglucose, which accumulates as 2-deoxyglucose-6-phosphate and inhibits hexokinase activity (Randle, 1993), resulting in a return to the threshold for glucose stimulation (of insulin release) within the physiological range (German, 1993). Further evidence for a limiting regulatory role of hexokinase derives from an insulinoma cell line, which exhibits glucose-stimulated insulin secretion over the physiological range of glucose concentrations. The cell line is derived from transgenic animals with insulin promoter-directed T antigen expression in their islets. At the time of isolation, the glucokinase:hexokinase ratio of these cells was found to be similar to that of normal islets, however, with time in culture hexokinase activity was markedly increased resulting in a reduction of the glucose concentration threshold for insulin secretion (Efrat *et al*, 1993).

The observation by German (1993) that islets transfected with hexokinase showed a reduction in the glucose concentration threshold for insulin secretion that could be reversed by preincubation with 2-deoxyglucose, led to the experiment with the AtTinsGLUT2.36 cells to see if the hexokinase activity of these cells could be inhibited. 2-deoxyglucose is phosphorylated to 2-deoxyglucose-6-phosphate by the cell but is then not metabolised further, 2-deoxyglucose-6-phosphate accumulates in the cells and inhibits hexokinase activity but does not inhibit glucokinase activity (Randle, 1993). It was also observed that hexokinase expressing cells responded to increases in concentrations of fructose. Glucokinase is highly specific for glucose, whereas hexokinase efficiently phosphorylates most hexoses. Therefore the AtTinsGLUT2.36 cells preincubated in 2-deoxyglucose should not respond to fructose as hexokinase activity should be inhibited by accumulation of 2-deoxyglucose-6-phosphate.

The results of the preincubation experiments with 2-deoxyglucose show that the AtTinsGLUT2.36 cells show a small response to 50μM glucose after preincubation with 14mM 2-deoxyglucose overnight, but the response is less than that to 50μM fructose, and the production of insulin is lower when the cells are incubated in fructose. This result suggests that some inhibition of hexokinase has occurred, and as the glucokinase will not phosphorylate fructose, the overall rate of phosphorylation is reduced in the cells resulting in less insulin secretion. With the 2-deoxyglucose (14mM) preincubated cells incubated with glucose, glucokinase will phosphorylate glucose bringing the rate of phosphorylation back up to normal. The fact that the cells respond to fructose at all shows that hexokinase activity is not totally inhibited although it does appear to be reduced. This explains the inconclusive nature of the detailed glucose response experiment. With hexokinase activity present, even if reduced, the glucose-dose response curve will not be shifted towards the

physiological range. The experiment suggests overall that although some glucokinase activity is present in the AtTinsGLUT2.36 cells, the glucokinase:hexokinase ratio is still in favour of hexokinase, even when the enzyme is partially inhibited. This indicates the dominance of the hexokinase enzyme in these cells. German (1993) found that 14mM 2-deoxyglucose was sufficient to totally inhibit hexokinase in isolated islet β-cells, however in AtTinsGLUT2.36 cells it is not sufficient. To obtain a shift of the glucose-dose response curve to the physiological range more extreme measures would need to be taken to inhibit hexokinase or increase glucokinase activity in these cells.

Ferber *et al* (1994) showed that preincubation of GLUT2 expressing intermediate passage RIN (rat insulinoma) cells with 50mM 2-deoxyglucose for 30 minutes resulted in a glucose-stimulated insulin secretion response that was shifted toward the physiological range. RIN cells in culture lose glucose-stimulated insulin secretion, and this is correlated with reduced expression of GLUT2 and glucokinase. Ferber *et al* (1994) showed that restoration of GLUT2 expression in the cells conferred glucose-stimulated insulin secretion, albeit at subphysiological glucose concentrations ($10-50\mu M$). Preincubation with 50mM 2-deoxyglucose shifted the glucose dose response back to the physiological range by inhibiting the increased hexokinase activity that the cells had acquired as a function of time in culture. 14mM 2-deoxyglucose may be sufficient to inhibit hexokinase activity in β-cells but 3.5 times this concentration is required in RIN cell lines of intermediate passage number.

AtTinsGLUT2.36 cells show many similarities to the RIN cells rather than to freshly isolated islets in culture. With the benefit of hindsight from the experience of Ferber *et al* (1994) one might anticipate that AtTinsGLUT2.36 cells would probably show a similar shift of glucose-stimulated insulin secretion to the physiological range with 50mM 2-deoxyglucose,

especially as 14mM 2-deoxyglucose resulted in some inhibition of hexokinase in the experiments described.

A long term increase in glucokinase activity relative to hexokinase activity might be achieved by co-expressing the glucokinase gene with insulin and GLUT2 in the AtT20d16v pituitary cells to increase glucokinase expression or even over-express the gene, hopefully pushing the glucokinase:hexokinase ratio sufficiently in favour of glucokinase to raise the glucose sensitivity into the physiological range. Another option would be to knock out the expression of hexokinase by homologous recombination or to stably express antisense hexokinase transcripts in the cells (Newgard et al, 1993; Newgard, 1994). Initial attempts at expressing antisense hexokinase cDNA by Newgard (1994) resulted in a reduction of approximately 75% in immunodetectable hexokinase protein and shifted the glucose-dose response curve into the physiological range.

The second major observation in the 2-deoxyglucose preincubation experiment is that cells preincubated in 2-deoxyglucose secrete more insulin than cells preincubated in standard culture conditions. This is an unexpected result and also occurs in the control cells where no glucose or fructose is added to the medium for the 4 hour response experiment. The cells preincubated in 2-deoxyglucose have effectively been starved of glucose for around 16 hours prior to the experiment they did not appear to be as healthy as the control cells during visual inspection under the microscope. There was more rounded-up cells and some detached cells. Two possible explanations for the increase in insulin secretion by cells deprived of their usual energy source may be suggested. Firstly, some of the cells are dying, and lysis of the cell membranes may cause release of stored insulin from the cells into the culture medium. Alternatively the cells may utilise an alternative energy source in the medium leading to anaerobic glycolysis and accumulation of metabolites which may

stimulate insulin secretion indirectly by activating mitochondrial oxidation or indirectly by forming intracellular signalling messengers. The involvement of such metabolites or intracellular signalling has yet to be elucidated (German, 1993).

Forskolin increases intracellular cAMP concentrations by stimulating cAMP production. cAMP is an important second messenger in many cell types and activates protein kinase A. cAMP potentiates insulin secretion by sensitisation of the cell to the stimulatory action of calcium. Sensitisation may occur by activation of enzymes, such as protein kinase A, which influence the function of microtubules and microfilaments. Calcium then facilitates the movement of secretory granules within the cell and assists exocytosis by decreasing the electrostatic repulsion between the negative surface charges on the granules and on the plasma membranes (Flatt & Bailey, 1991). Secretagogues such as forskolin only increase insulin secretion from cells where the insulin is stored in secretory granules of the regulated pathway. The AtTinsGLUT2.36 cells do appear to increase their insulin secretion over basal in the presence of forskolin, as would be expected, since AtT20 cells have previously been shown to co-localise insulin and ACTH to the same secretory granules in the regulatory pathway (Moore *et al*, 1983).

Arginine is known to stimulate the secretion of insulin from islets by causing membrane depolarisation (Blachier *et al*, 1989). This effect is thought to be mediated by nitric oxide, which is produced by the action of nitric oxide synthase on L-arginine (Schmidt *et al*, 1992). Nitric oxide causes an increase in intracellular cGMP by activation of guanylyl cyclase which converts GTP to cGMP (Feelisch & Noack, 1987). cGMP can exert several effects including effects on ion channels, stimulation of cGMP-dependent protein kinases and both increases or decreases in cAMP. As we have already discussed, protein kinases can stimulate insulin secretion and this is the most likely way in which arginine mediates

stimulation of insulin secretion in the AtTinsGLUT2.36 cells. The AtTinsGLUT2.36 cells show a marked 85% increase in insulin secretion in the presence of 20mM arginine.

The pLK444/GLUT2 plasmid, used to confer GLUT2 expression to the AtT20d16v cells and create the AtTinsGLUT2.36 cell line when co-expressed with the pCB7-hppI-1 plasmid, had the GLUT2 cDNA under the control of a β-actin promoter. The β-actin promoter is a strong mammalian promoter and expression of GLUT2 mRNA by the AtTinsGLUT2.36 clone is at a high level. In pancreatic β-cells GLUT2 mRNA expression is regulated by glucose (Chen *et al*, 1990), however, little information is available concerning the mechanisms instrumental in this regulation. It is thought that signals from sugar metabolism affect production of GLUT2 at a transcriptional level (Ferrer *et al*, 1993). The glucose dependent expression of GLUT2 mRNA in the AtTinsGLUT2.36 cells was unexpected as the β-actin promoter was not known to have a glucose stimulatory effect. The Northern blot performed with RNA prepared from cells incubated in increasing glucose concentrations should show no difference between the expression of GLUT2 mRNA at any of the glucose concentrations with the β-actin promoter promoting transcription of the gene independently of the intracellular or extracellular conditions. One explanation for the unexpected stimulatory effect of glucose is that the β-actin promoter, or sequences in the pLK444 vector have an glucose response element that enhances transcription of the GLUT2 gene at higher glucose concentrations. It has been shown that actin mRNA concentrations are regulated by cAMP and by Ca^{2+}-dependent processes (Phillips *et al*, 1988) making it entirely possible that the β-actin promoter is regulated by glucose. Hence the use of the β-actin promoter may confer glucose regulated transcription of the gene it controls, due to a glucose response element in the promoter region. An example of a similar case is given by

156

Hughes *et al* (1992), where despite insulin gene expression in their AtT20ins cells being directed by a Rous Sarcoma Virus long terminal repeat sequence, transfection of the cells with GLUT2 caused dramatic increases in intracellular insulin content compared to untransfected cells and conferred glucose regulation of insulin biosynthesis. The group postulated that GLUT2 transfection of the AtT20ins cells unmasks post-transcriptional control mechanisms of insulin biosynthesis similar to those operative in islet β-cells. The same post-transcriptional control mechanisms could explain the increased accumulation of GLUT2 mRNA transcripts at higher glucose concentrations in the AtTinsGLUT2.36 cells.

The animal model chosen for implantation of the insulin secreting cells was the streptozotocin diabetic athymic nude (nu/nu) mouse. Nude mice have been widely used as recipients for transplanted allogeneic and xenogeneic tissues (Ziedler *et al*, 1982). In the absence of the thymus, nude mice display lymphopenia and a decline in cell-mediated immune reactions. They are therefore immunoincompetent and a transplant of foreign tissue is not rejected. Nude mice also exhibit certain endocrine abnormalities in thyroid, adrenal, gonadal, and pancreatic function (Shafrir, 1990). The effects on endogenous β-cell function of these abnormalities result in modest hyperglycaemia, impaired glucose tolerance, low basal and low glucose-stimulated plasma insulin levels, and an elevated glycohaemoglobin when compared to heterologous litter mates. These effects are mostly observed in male nude mice between the ages of 6 and 12 weeks (Ziedler *et al*, 1982; Ziedler *et al*, 1984). The fasting plasma glucose concentrations of the nude mice were found to be between 6 and 11mmol/l, higher than in normal mice which would be expected to be around 5mmol/l after fasting. It was originally reported that nude mice were resistant to streptozotocin induced diabetes due to a dependence of streptozotocin action on thymus function (Buschard & Rygaard, 1978). However subsequent workers found that intraperitoneal administration of

a large dose of streptozotocin (200mg/kg) resulted in the development of a rapid and severe hyperglycaemia (Paik *et al*, 1980). Male mice were found to be more susceptible to streptozotocin than females. Thus male nude (nu/nu) mice were used in this study and diabetes was induced by a single intraperitoneal injection of 200mg/kg streptozotocin. Some variability in activity between batches of the streptozotocin were observed during the experiments with implant group two taking much longer to show signs of hyperglycaemia. Consequently these mice received implants at an earlier stage in the development of diabetes than implant group one. A few animals in both groups did not develop hyperglycaemia at all and were excluded from the implant studies.

Due to the expression of GLUT2 by the AtTinsGLUT2.36 cells, implantation had to follow streptozotocin administration. This is because streptozotocin is reported to be transported only by the glucose transporter GLUT2 and not GLUT1; thus killing only neuroendocrine cells expressing GLUT2 (Schnedl *et al*, 1994). As the expression of GLUT2 is limited to β-cells and some liver cells the cytotoxic effect of streptozotocin is mainly diabetogenic. As a neuroendocrine cell line expressing GLUT2, the AtTinsGLUT2.36 cells would undoubtedly be destroyed by streptozotocin treatment, so implantation was performed following streptozotocin treatment.

In both implant groups the immediate effect of implantation of the AtT20d16v wild type or AtTinsGLUT2.36 cells was an increase in plasma glucose. However, mice receiving the insulin secreting AtTinsGLUT2.36 cells had significantly lower plasma glucose concentrations 12 days after implantation in implant group one, and 22 days after implantation in implant group two, than mice receiving the wild type AtT20d16v cells. The mice implanted with AtTinsGLUT2.36 cells in implant group one resisted lethal hyperglycaemia for a further 16 days after the significant difference in plasma glucose

between them and the control mice was observed 12 days after implantation. With implant group two the plasma glucose concentrations of the mice receiving the AtTinsGLUT2.36 cells were still falling 6 days after the significant difference was observed between them and the control mice on day 66. However the experiment was terminated at this point due to the overall weight loss of all the animals in the experiment. In both implant groups, although changes in body weight were closely correlated to changes in plasma glucose, there was no significant difference in the body weights of test or control mice. The same was true with food intake during the experiment. Although food intake was reduced in animals showing improvements in glycaemia, there were no significant differences observed between test and control mice.

As discussed the AtTinsGLUT2.36 and wild type AtT20d16v cells secrete large quantities of pituitary peptides as well as the transfected insulin. *In vivo,* ACTH stimulates the synthesis and release of glucocorticoids from adrenal tissue (especially cortisol in humans, corticosterone in rodents)(Hadley, 1992). Glucocorticoids produce metabolic effects antagonistic to those of insulin and a prolonged excess can cause steroid-induced diabetes. Administration of glucocorticoids causes prolonged hyperglycaemia (mediated by increased gluconeogenesis, potentiation of glucagon and catecholamine action, insulin resistance and impaired peripheral glucose utilisation), an impaired glucose tolerance, a mild insulinogenic effect and β-cell cytotropic effect (Lenzen & Bailey, 1984). AtT20 cells also secrete β-endorphin which binds to opiate receptors and causes increased plasma glucose concentrations (Bailey & Flatt, 1987). It appears that implantation of ACTH/ β-endorphin secreting cells will have deleterious effects on glucose homeostasis, causing an increase in hyperglycaemia and insulin resistance. In animals already treated with streptozotocin the effect is seen soon after implantation. Implant group two particularly show a large response

to implantation of the cells with plasma glucose concentrations rising from around 12mmol/l to 26mmol/l in 10 days. ACTH concentrations in the plasma of the implanted mice rose from around 50pg/ml to over 300pg/ml in the same time period. The effects of ACTH and β-endorphin may also explain the failure of the implant to reduce plasma glucose concentrations to normoglycaemia and may provide the reason for the implant failing to sustain functional viability after a period of time *in vivo*. The insulin resistance induced by the corticosterone combined with further insulin resistance induced by the streptozotocin treatment, (streptozotocin treated animals are known to display insulin resistance; Nishimura *et al*, 1989), would conspire to reduce the efficacy of insulin released from the implanted cells. As it appears that the implanted cells became less efficient as a function of time *in vivo* and the animals became progressively more insulin resistant, it is perhaps not surprising that lethal hyperglycaemia eventually ensued. Overall the ability of the implanted cells to reduce plasma glucose concentrations at all, let alone to produce a significant decrease in glycaemia for up to 16 days, is an indication that the implanted cells are indeed functioning well *in vivo*, and producing bioactive insulin despite very difficult physiological conditions.

Tumours removed from the animals at autopsy were mostly located near the site of intraperitoneal injection of the cells. They were usually attached to the muscle wall and sometimes transversed the peritoneum at the injection site. The tumours were very well vascularised and grew quickly. Histological staining showed that the tumours were necrotic at the centre with a peripheral covering of healthy cells. Immunohistochemistry for insulin detection revealed insulin secreting cells around the healthy periphery of the tumours. The core of the tumours often showed faint traces of insulin staining which could be the remains of degrading insulin from necrosing cells. The general necrosis of cells could be one of the reasons for the failure of the implants to function for longer periods *in vivo*. Another

explanation may be provided by the fact that the implanted cells are no longer under G418 selection *in vivo*, the lack of selection leading to the transfected cells losing or inactivating the selected genes. The transfected cells could also have been outcompeted for nutrients and space in the tumour by infiltrating fibroblasts or other endogenous cell types that divide faster than the implanted cells. The transfected cells may divide more slowly than their wild type equivalent due to the extra time needed to replicate this exogenous DNA. Possible ways to contain tumour growth and prevent necrosis are considered in chapter 6.

The work described in this chapter has shown that AtT20 pituitary cells provide a good model *in vitro* in which to develop glucose mediated insulin secretion. This is due to the ability of these neuroendocrine cells to process proinsulin to mature insulin and secrete the peptide via a regulated pathway. When implanted into streptozotocin diabetic nude (nu/nu) mice, the cells can cause a transient reduction in hyperglycaemia, however, co-secretion of ACTH and opiates by these cells is antagonistic to the insulin secreted and eventually results in insulin resistance and lethal hyperglycaemia. The use of AtT20 pituitary cells may therefore have limitations for further implantation investigations unless some way of suppressing the synthesis and secretion of ACTH and the other products of POMC could be achieved. However, further investigations into the mechanism of glucose stimulated insulin secretion and the engineering of a cell line with glucose-stimulated insulin secretion in the physiological range may well utilise this useful cell line.

In the next chapter we consider a fibroblast cell line as a candidate for insulin delivery and address the problem of achieving processing of proinsulin in such a cell line.

Chapter Five

Proinsulin Processing in a Human Fibroblast Cell Line (1BR.3.G) and Implantation Studies in Diabetic Nude (nu/nu) Mice.

Chapter Five : Proinsulin processing in a Human Fibroblast Cell Line (1BR.3.G) and Implantation Studies in Diabetic Nude (nu/nu) Mice.

5.1 Introduction

Skin fibroblasts provide a convenient cell type when considering somatic cell gene therapy. They are easily accessible from the donor, robust and plentiful. Reimplantation could be performed via a skin graft procedure which is already clinically successful. Fibroblast cells have a very different physiology to pancreatic β-cells and present a difficult challenge when trying to engineer the glucose-mediated insulin secretion required of a surrogate β-cell. Fibroblast cells, amongst other non-endocrine cell types, contain only the constitutive pathway for secretion. β-cells are a highly specialised secretory cell type and contain both the constitutive and the regulatory pathway of secretion. Other neuroendocrine cell types such as the AtT20 cell also use the regulatory pathway for secretion of proteins.

In the β-cells of healthy individuals newly synthesised proinsulin molecules are targeted to the granules of the regulatory pathway (Halban, 1990). It is here in the granules that proinsulin is processed into mature insulin by the requisite processing enzymes (figure 5.1). During processing, the C-peptide, which resides between the B and A peptide chains in proinsulin, is excised by the enzymes that make two separate cleavages: one at the B-C junction (Arg-Arg dibasic site), and one at the C-A junction (Lys-Arg dibasic site) (Groskreutz et al, 1994). The endopeptidase which cleaves at the B-C junction (Arg^{31}, Arg^{32}) is known as PC3, and PC2 cleaves at the C-A junction (Lys^{64}, Arg^{65}). Following endoproteolytic cleavage the newly exposed COOH-terminal basic amino acids are specifically removed by the rapid action of an exopeptidase, carboxypeptidase-H, to yield the products insulin and C-peptide. Cleavage at the Arg^{31}, Arg^{32} site but not the Lys^{64},

Figure 5.1   The two possible routes for proinsulin processing via the conversion intermediates, des-31,32 proinsulin and des-64,65 proinsulin. Cleavage is achieved by the combined action of an endoprotease and a carboxypeptidase to cleave the site and remove the C-terminal basic amino acids. The dominant route for human proinsulin processing is via the des-31,32 proinsulin intermediate.
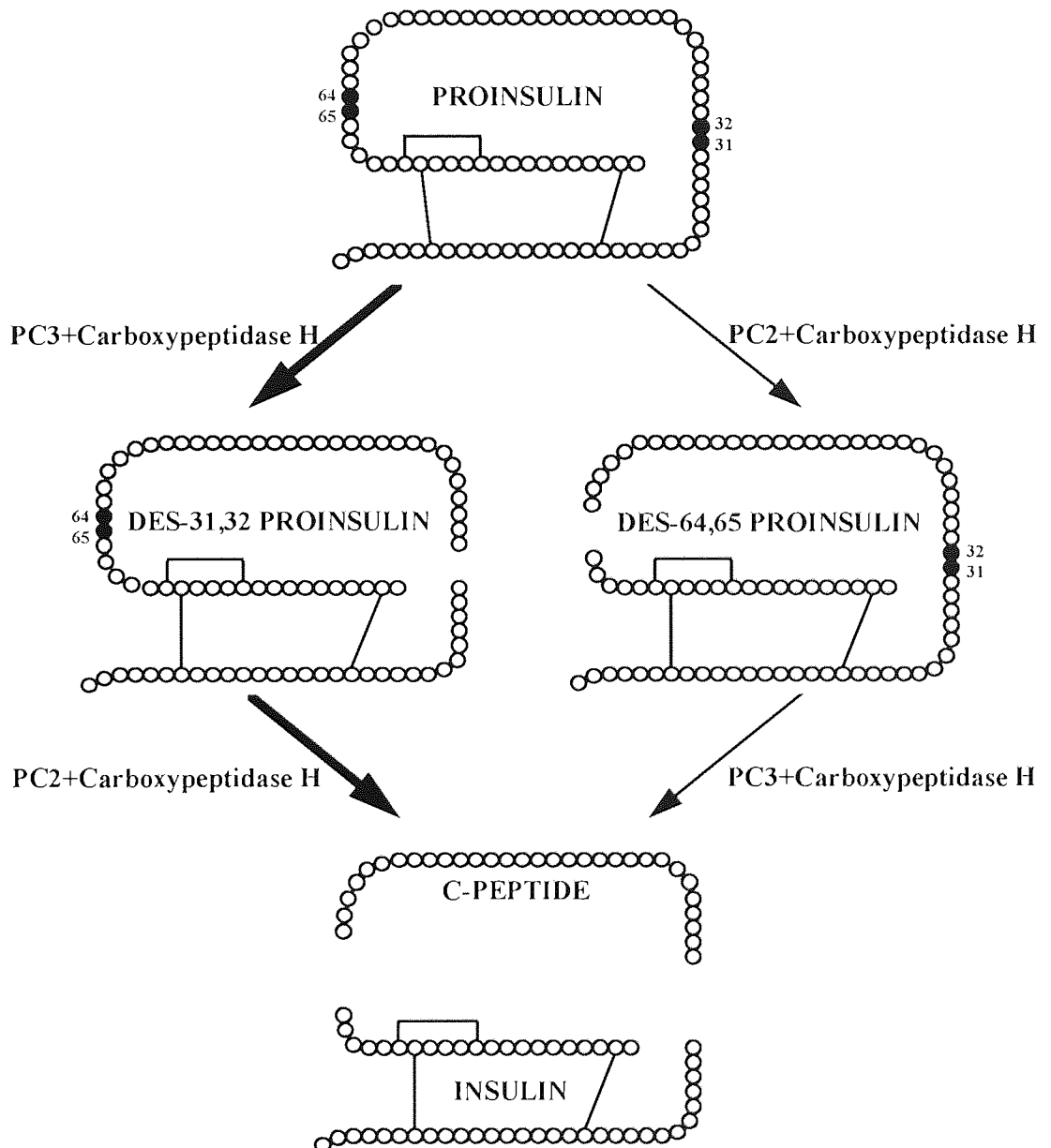
Arg^{65} site creates the conversion intermediate des-31,32 proinsulin. Conversely, cleavage at the Lys^{64}, Arg^{65} site but not the Arg^{31}, Arg^{32} site creates the des-64,65 proinsulin conversion intermediate. In theory, either site could be processed first, giving equal quantities of the conversion intermediates (figure 5.1). However, there is increasing evidence to suggest that human proinsulin conversion has a preferred sequential route via des-31,32 proinsulin. Firstly des-31,32 proinsulin is present in higher quantities both in the circulation and in pancreatic extracts, whereas des-64,65 proinsulin is low. In NIDDM, hyperproinsulinaemia consists of elevated intact proinsulin and des-31,32 proinsulin levels; des-64,65 proinsulin levels are difficult to measure and most likely do not contribute to increased levels of proinsulin-like molecules (Sobey *et al*, 1989). Secondly, kinetic studies of proinsulin conversion in isolated human pancreatic islets have shown that des-31,32 proinsulin appears as a transient conversion intermediate at a greater than seven-fold higher level than des-64,65 proinsulin (Sizonenko *et al*, 1993). Thirdly, an Arg^{31}, Arg^{32} to Arg^{31}, Gly^{32} proinsulin mutation was processed inefficiently by PC2 activity at Lys^{64}, Arg^{65}, even though this site was present in the Gly^{32}-proinsulin variant (Docherty *et al*, 1989). This finding led to the idea that some degree of secondary structure, as well dibasic specificity, is necessary for efficient PC2 cleavage of proinsulin. Finally, it has been demonstrated that PC2 activity has a much greater preference for des-31,32 proinsulin than intact proinsulin (Rhodes *et al*, 1992). These lines of evidence indicate that the route via des-31,32 proinsulin is the predominant pathway for proinsulin to insulin conversion in human β-cells (Rhodes & Alarcon, 1994).

Many non-endocrine cells, including fibroblasts, hepatocytes and lymphocytes, produce biologically inactive propeptides and convert them to biologically active peptides by cleaving a consensus sequence. Proteolytic cleavage at the consensus sequence, Arg^{-4}, Xaa^{-3},

Lys/Arg^{-2}, Arg^{-1}, ↓, Xaa^{+1} is catalysed by a subtilisin-like serine protease, furin, which has structural homology to the yeast Kex2 gene (Hosaka *et al*, 1991). Furin has been demonstrated to be present in virtually all non-endocrine cells, including fibroblasts, epithelial cells and lymphocytes (Hatsuzawa *et al*, 1990), and is believed to be implicated in proprotein processing in the constitutive secretory pathway (Halban & Irminger, 1994). In the constitutive pathway there are no granular storage compartments and proteins or their conversion products are released from the cells within some 60 minutes of their synthesis (Vollenweider *et al*, 1993). Transfection of non-endocrine cells with proinsulin has shown that the proinsulin is not converted to mature insulin to any great extent (Moore *et al*, 1983). However, rat proinsulins have been found to be processed to a greater extent than human proinsulin in non-endocrine cells. This is probably due to the inter-species variation in the amino acid sequence at the cleavage sites giving homology to the consensus cleavage sequence for the endogenous protease, furin, in rat proinsulins (Yanigita *et al*, 1992).

There have been two approaches to achieving better processing of human proinsulin in non-endocrine cells. Firstly, site-directed mutagenesis of the prohormone cleavage sites to the consensus cleavage sequence for furin and subsequent transfection of the mutant proinsulin into non-endocrine cells. This method relies on the proteolytic activity of endogenous furin. Secondly, the co-expression of wild type proinsulin with exogenous proteases by double transfection. It has been suggested that in terms of gene therapy, the expression of mutant genes in humans would not be readily acceptable, making the second approach more desirable (Vollenweider *et al*, 1995). Vollenweider *et al* (1995) transiently transfected monkey kidney COS 7 cells with wild type proinsulin and a variety of proteases; PC2, PC3 and furin. They found that despite PC3 requiring a specific intracellular environment for processing in β-cells, PC3 processed over 80% of proinsulin to insulin in transfected COS 7

166

cells. When co-transfected with proinsulin, PC2 was found to be ineffective in processing the proinsulin to mature insulin, and furin showed over 60% conversion when co-transfected with proinsulin in the COS 7 cells.

In this study we aimed to co-transfect human fibroblast (1BR.3.G) cells with wild type human preproinsulin and furin. The transfectants are selected using antibiotic resistance conferred by the plasmids, to create stable cell lines. One of these cell lines with the ability to secrete fully processed mature insulin would then be selected for further *in vitro* study, and *in vivo* study after implantation into a streptozotocin diabetic nude (nu/nu) mouse model.

5.2 Methods

5.2.1 Double Transfection of 1BR.3.G Cells

1BR.3.G cells were double transfected using the calcium phosphate co-precipitation method as described in chapter two. 15μl of pCB7-hppI-1 and 15μl of pCB7-PACE at a concentration of 1μg/μl were used in the precipitates which were added to each plate of cells to be transfected. Control precipitates were prepared without DNA and control plates received the same treatments as the transfection plates with the control precipitates. Selection was performed using hygromycin B at a concentration of 125μg/ml of complete medium. Resulting colonies of cells were picked using sterile cloning rings with trypsin and transferred to 6 well plates to grow up larger numbers of each clone to screen for expression of the genes of interest. When 2x10cm confluent dishes of each clone were available, a sample of culture medium was collected for differential radioimmunoassay of insulin, and the cells from the dish were used for mRNA preparation. The other dish of cells was cryopreserved ready to thaw and use should the clone be selected for further study.

5.2.2 Screening for hppI-1 and Furin mRNA Expression in the Transfectants

Northern blots were performed using mRNA prepared from each clone. The blots were probed with ^{32}P labelled cDNA probes prepared from the cDNA inserts of the two plasmids used in the transfection, an hppl-1 probe and a furin probe. Probings were carried out sequentially, stripping the filter in between with boiling 1% (w/v) SDS. The final probe was for the 18S subunit of ribosomal RNA as a control for the amount of RNA loaded onto the Northern gels.

5.2.3 Screening for Insulin by Differential Radioimmunoassay of the Transfectants

Aliquots of medium, collected as described above, were assayed with a non-specific insulin antibody and with a specific human insulin antibody. The non-specific antiserum detected any insulin-like immunoreactivity in the sample giving a value for the total amount of insulin secreted by the cells. The specific human insulin antiserum detected only mature human insulin in the sample (and 76% of any split des-31,32 proinsulin present in the sample due to crossreactivity of the antiserum). Therefore the differential assay determined the proportion of insulin produced by the 1BR.3.GinsPACE clones that was fully processed mature insulin. The method allows calculation of the percentage of processed mature insulin that is secreted.

5.2.4 Insulin Secretion in Response to Secretagogues by 1BR.3.GinsPACE14d

The 1BR.3.GinsPACE14d clone was subcultured in 6 well plates and grown to 75% confluence. The cell monolayer was washed with serum-free medium and the cells were placed in high (25mM) glucose, no glucose, or the secretagogues arginine (20mM), forskolin (0.5µg/ml), K^+ (15mM) or Ca^{2+} (7.6mM) in 0.5ml serum-free DMEM per well. After 4 hours the culture medium was collected and stored at -20°C until analysed by insulin radioimmunoassay.

5.2.5 Implantation of 1BR.3GinsPACE14d into Diabetic Nude Mice

Diabetes was induced in immunoincompetent nude mice by administration of 200mg/kg streptozotocin by intraperitoneal injection (day 0). On day 7, 10 mice were implanted with 1×10^7 insulin-secreting 1BR.3.GinsPACE14d cells and 7 with 1×10^7 wild type 1BR.3.G cells by intraperitoneal injection. Following implantation the mice were bled from the tail

once or twice weekly to determine plasma glucose concentration, and aliquots of plasma were stored at -20°C for insulin immunoradiometric assay. Body weight and food intake were also monitored throughout the experiment. A further implant of cells was given on day 28, 3×10^7 insulin secreting 1BR.3.GinsPACE14d and 1.8×10^7 wild type 1BR.3.G cells to the respective animals via intraperitoneal injection. The experiment was terminated on day 43 and the mice examined by autopsy for evidence of cell growth in the peritoneum.

5.2.6 Histology and Immunohistochemistry

Tumours/cell aggregations removed from mice at autopsy were preserved in 10% (v/v) formaldehyde in PBS for at least 24 hours, then processed for histological examination. Haematoxylin and eosin staining was performed for general cell morphology and immunohistochemical staining for detection of insulin-secreting cells. The staining was carried out as described in chapter two.

5.3 Results

5.3.1 Double Transfection of 1BR.3.G Cells

Selection with hygromycin B at 125µg/ml killed all of the control untransfected cells within 2 weeks. After 4 weeks, cells double transfected with pCB7-PACE and pCB7-hppI-1 had formed discrete colonies. 72 colonies were picked aseptically from these plates and grown up in selection medium. However, only 59 of the 72 colonies grew sufficiently to be screened for insulin and furin mRNA expression and to measure the secretion of mature insulin.

5.3.2 Screening for hppI-1 and Furin mRNA Expression in the Transfectants

Northern blots were performed with mRNA prepared from clones obtained by double transfection of the 1BR.3.G fibroblast cells with plasmids containing the genes for insulin and furin and conferring hygromycin B resistance (1BR.3.GinsPACE clones). mRNA from clones selected in hygromycin B (125µg/ml) were screened for insulin and furin expression with ^{32}P labelled cDNA probes. A ^{32}P labelled cDNA probe to the 18S subunit of ribosomal RNA was used as a positive control to show that mRNA was equally loaded onto the Northern gel (figure 5.2). The furin mRNA has a single transcript at approximately 4.0kb, the insulin mRNA also has a single transcript at about 0.5kb. The untransfected wild type 1BR.3.G fibroblast cells do not show expression of either the furin or the insulin mRNA. Although furin is endogenously expressed as an approximately 5.0kb transcript in the fibroblasts, the sensitivity of the Northern blot only detects the transfected transcript. In clone 10b and 11d the furin mRNA transcript appeared to be larger than 4.0kb: these clones were discarded in case the transcript was incorrectly spliced. 8 of the transfected clones shown in figure 5.2

Clone Number

Figure 5.2 Northern blots of mRNA prepared from clones obtained by double transfection of 1BR.3.G fibroblast cells with plasmids containing genes for insulin and furin and conferring hygromycin B resistance. Clones selected in hygromycin B ($125 \mu g/ml$) were screened for insulin and furin mRNA expression with oligolabelled cDNA probes. An oligolabelled cDNA probe to the 18S subunit of ribosomal RNA was used as a positive control to show that RNA was loaded equally on to the northern gel. Insulin has a single transcript signal at approximately 0.5kb. Furin was mostly found as a single transcript at around 4.0kb. WT was mRNA prepared from wild type untransfected 1BR.3.G fibroblast cells. Clone 21d had very strong insulin expression but did not express furin. Clones 6d, 7d, and 14d showed strong expression of both insulin and furin mRNA.

172

expressed the insulin mRNA, although in variable amounts when compared with the corresponding 18S mRNA bands. Clone 13d and 15d expressed the gene very weakly while clones 10c, 6d, 7d, 14d and 21d expressed the gene strongly. All the 18S mRNA bands were relatively constant, implying that equal quantities of mRNA were loaded onto the gel for each clone screened. 11 of the clones shown in the figure also expressed the furin mRNA. 23d showed very little expression of furin mRNA whilst clones 6d, 7d, 13d, 14d and 16d showed strong furin expression. Of these clones with strong furin expression numbers 6d and 14d also showed good expression of insulin mRNA, making them good choices for further study. However the strongest expression of both insulin and furin was in clone 1BR.3.GinsPACE14d. Table 5.1 shows the results for all of the 1BR.3GinsPACE clones screened by Northern blotting for insulin and furin mRNA expression. The expression of mRNA was scored by eye using the density of bands on the autoradiographs. Weak expression was scored as + and strong expression as +++, negative expression is denoted by -. The table reiterates that clone 1BR.3.GinsPACE14d was the best double transfectant with +++ expression for both insulin and furin mRNA.

5.3.3 Screening for Insulin by Differential Radioimmunoassay of the Transfectants

Differential radioimmunoassay of culture medium from the 1BR.3.GinsPACE clones was performed in order to determine the quantity of insulin secreted by the cells and the extent of processing of proinsulin to mature insulin occurring in the cells. Insulin-like immunoreactivity in the culture medium was determined using both a non-specific insulin antiserum and an antiserum specific for mature human insulin (and partially specific (76%) for split des-64,65 proinsulin) in the RIA procedure. Table 5.2

173

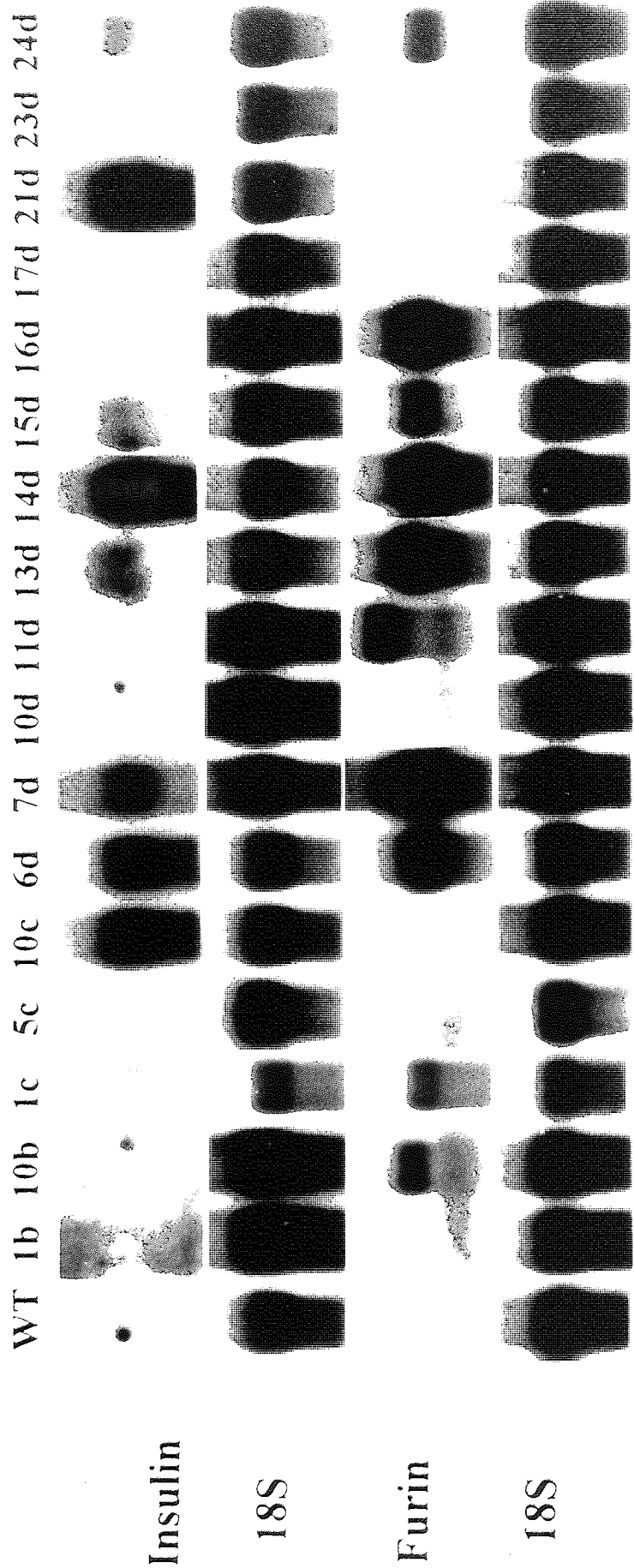**Table 5.1** Expression of insulin and furin mRNA scored using the density of bands on autoradiographs of Northern blots of mRNA prepared from clones obtained by double transfection of 1BR.3.G fibroblast cells with plasmids containing insulin and furin genes and conferring hygromycin B resistance. Clones were selected in hygromycin B (125μg/ml) and Northern blots screened using ^{32}P labelled cDNA probes. Expression was scored as +/- for very weak expression, + for weak expression, ++ for good expression, +++ for strong expression, and - for no expression. Clones 14d showed strong expression of both insulin and furin mRNA.

Clone No.	Insulin mRNA	Furin mRNA	Clone No.	Insulin mRNA	Furin mRNA
3a	-	-	1c	+	++
4a	-	-	2c	-	-
5a	-	-	3c	-	-
6a	-	-	4c	-	-
8a	+	+++	5c	-	-
9a	-	-	10c	++	-
11a	-	-	11c	-	+
12a	-	-	12c	-	-
14a	-	-			
17a	-	-	1d	-	-
18a	-	-	3d	-	-
20a	-	-	4d	-	-
21a	-	++	5d	+	+
22a	-	-	6d	++	++
23a	+	-	7d	+	+++
24a	-	-	8d	+	-
			9d	+	-
			10d	-	-
1b	+/-	-	11d	-	+
2b	-	-	12d	-	-
3b	++	-	13d	+	++
4b	+	-	14d	+++	+++
5b	-	-	15d	+	+
6b	-	-	16d	+/-	++
7b	-	-	17d	-	-
8b	-	-	18d	-	-
9b	-	-	19d	++	+
10b	-	+	20d	-	+++
11b	++	-	21d	+++	-
12b	-	-	22d	+	+
			23d	-	+
			24d	+	+

Table 5.2 Secretion of total insulin and mature insulin (ng/ml) by the 1BR.3.GinsPACE clones. Culture medium from the clones, incubated for 4 hours without serum, was removed and assayed for insulin using 2 different insulin antisera. A non-specific insulin antiserum which detects all insulin-like immunoreactivity in the sample, and a specific human insulin antiserum which detects only mature human insulin (and some split des-64,65 proinsulin). 11 clones secreted processed mature insulin, clone 14d secreting more than 10x that of any other clone.

Clone No.	Total Insulin (ng/ml)	Mature Insulin (ng/ml)	Clone No.	Total Insulin (ng/ml)	Mature Insulin (ng/ml)
3a	0	0	1c	1.25	1.1
4a	0	0	2c	0	0
5a	0	0	3c	0	0
6a	0	0	4c	0	0
8a	0	0	5c	0	0
9a	0	0	10c	2.5	0.15
11a	0	0	11c	0	0
12a	0	0	12c	0	0
14a	0	0			
17a	0	0	1d	0	0
18a	0	0	3d	0	0
20a	0	0	4d	0	0
21a	0	0	5d	2.0	0.76
22a	0	0	6d	1.45	0
23a	0	0	7d	1.3	0.94
24a	0	0	8d	6.0	0.51
			9d	2.65	0
			10d	0	0
1b	0	0	11d	0	0
2b	0	0	12d	0	0
3b	0.7	0	13d	4.1	0.71
4b	0.2	0	14d	27.0	14.0
5b	0	0	15d	3.2	0.59
6b	0	0	16d	2.6	0
7b	0	0	17d	0	0
8b	0	0	18d	0	0
9b	0	0	19d	4.2	0.1
10b	0	0	20d	0	0
11b	3.5	0	21d	14.0	0.56
12b	0	0	22d	6.0	0.84
			23d	0	0
			24d	6.1	0

shows total insulin-like immunoreactivity (total insulin), detected using the non-specific insulin antiserum, and the mature insulin-like immunoreactivity (mature insulin), detected using the antiserum specific for mature human insulin, in the culture medium of each clone. Of the 59 clones screened insulin-like immunoreactivity was detected in the culture medium of just 18. Of these 18 clones 11 were secreting a proportion of the insulin as mature insulin. Clones 10c, 8d and 21d secreted some mature insulin despite not expressing the furin mRNA, these clones secreted relatively large amounts of total insulin indicating that a basal amount of insulin is processed endogenously by these cells, but the proportion of insulin processed to mature insulin is too low to detect in clones secreting small quantities of total insulins. Figure 5.3 shows the percentage insulin-like immunoreactivity that is secreted as fully processed mature insulin in the clones where insulin was detected in the culture medium. The graph shows that clones 1c and 7d processed >70% percent of their total insulin to mature insulin, consistent with strong expression of furin in these clones. However, the total quantity of insulin secreted by clones 1c and 7d is very small (<1.5ng/ml) and a clone with stronger expression of insulin would be more desirable. 1BR.3.GinsPACE14d had the best processing ability of the rest of the clones, along with strong insulin expression and secretion. Clone 1BR.3.GinsPACE14d was therefore chosen for further study. Actual secretion of insulin from the 14d clone was found to be 9.24ng/10^6 cells/24 hours of total insulins and 5.32ng/10^6 cells/24 hours of mature insulin, representing 58% conversion.

Figure 5.3 Percentage insulin-like immunoreactivity secreted as mature insulin by the 18 1BR.3.GinsPACE clones found to be secreting insulin-like immunoreactivity into the culture medium. Medium from each clone which had been incubated for 4 hours in serum-free medium was assayed using 2 different insulin antisera. A non-specific insulin antiserum which detects all insulin-like immunoreactivity in the sample (total insulin secretion), and a specific human insulin antiserum which detects only mature (human) insulin (and some split des-64,65 proinsulin). The amount of mature insulin secreted divided by the total insulin secretion for each clone, gives the percentage of total insulin secreted as mature insulin for that clone. Clones 1c, 7d, and 14d have the greatest percentage of mature insulin, although clones 1c and 7d secreted only small quantities of insulin compared to clone 14d.
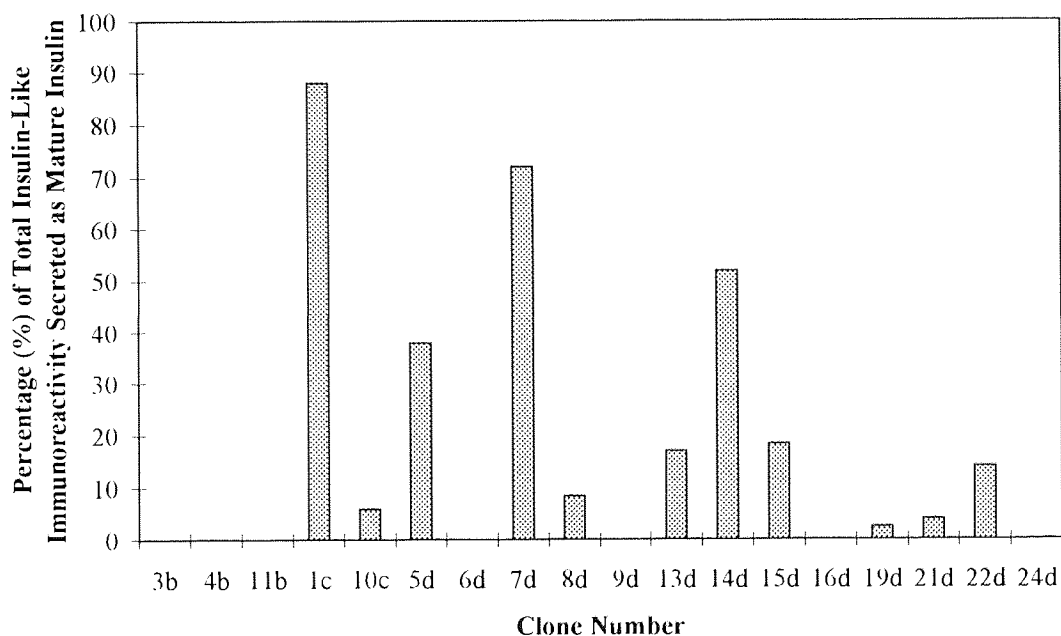
5.3.4 Insulin Secretion in Response to Secretagogues by 1BR.3.GinsPACE14d

Culture medium from 1BR.3.GinsPACE14d cells incubated with high (25mM) and low (0) concentrations of glucose and the β-cell secretagogues forskolin (0.5µg/ml), arginine (20mM), K^+ (15mM) and Ca^{2+} (7.6mM) was assayed for insulin-like immunoreactivity using the non-specific insulin antiserum. Figure 5.4 shows the insulin secretion from the cells in ng/ml. The cells incubated without glucose secreted less insulin than those incubated with high glucose. This is probably due to a down regulation in cell function in the absence of their usual energy source. The cells incubated with arginine, forskolin and K^+ secreted slightly more insulin than the cells incubated without glucose, although they still secreted less than the cells incubated in high glucose. As they were exposed to secretagogues but no glucose these cells may also be down regulated in their responsiveness to stimuli, due to the absence of glucose. The cells incubated with high extracellular Ca^{2+} secreted much less insulin than any of the cells receiving other treatments indicating that insulin secretion was inhibited by 7.6mM Ca^{2+} in the 1BR.3.GinsPACE14d cells. Thus, the 1BR.3.GinsPACE14d cells did not seem to respond to the secretagogues with an increase in insulin secretion in the same way as seen with the AtTinsGLUT2.36 cells in chapter 4, or like normal pancreatic β-cells.

5.3.5 Implantation of 1BR.3GinsPACE14d into Diabetic Nude Mice

Experimental diabetes was induced in nude (nu/nu) mice by administration of 200mg/kg streptozotocin by intraperitoneal injection on day 0. On day 7 the mice were given 1×10^7 insulin-secreting 1BR.3.GinsPACE14d or 1×10^7 wild type 1BR.3.G cells by intraperitoneal injection. Mice were then monitored for plasma glucose, body weight and food intake once or twice weekly. Blood samples were also taken to determine plasma human insulin levels

Figure 5.4 Insulin secretion (ng/ml) by 1BR.3.GinsPACE14d cells incubated in high (25mM) and zero glucose, 20mM arginine, 0.5µg/ml forskolin, 15mM K^+, and 7.6mM Ca^{2+} for 4 hours without serum. Insulin was measured by radioimmunoassay of the culture medium with a non-specific insulin antiserum. The β-cell secretagogues arginine, forskolin and K^+ exerted have little or no effect on insulin secretion, whilst high Ca^{2+} inhibited insulin secretion from the 1BRinsPACE14d fibroblast cells.
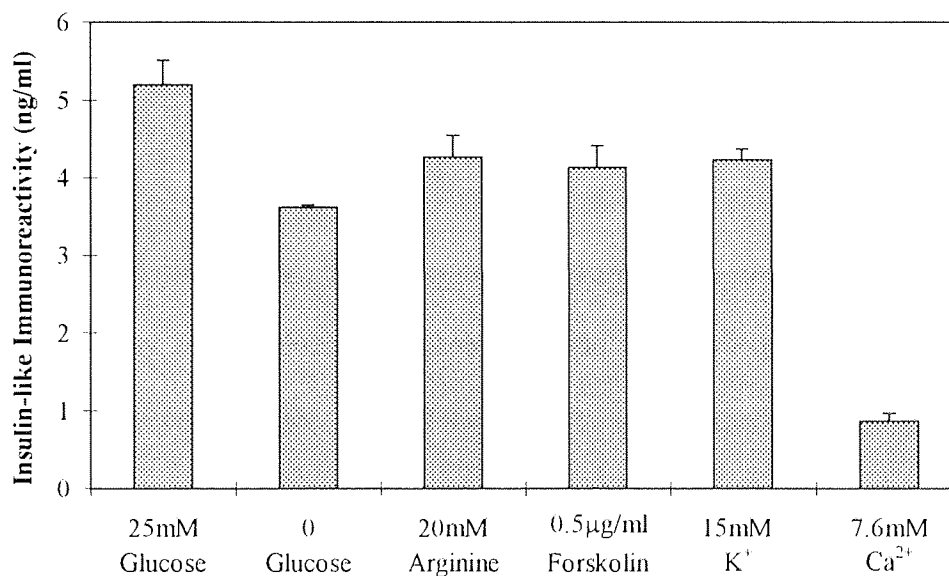
during the experiment. A further implant of 3×10^7 insulin-secreting 1BR.3.GinsPACE14d or 1.8×10^7 wild type 1BR.3.G cells was given by intraperitoneal injection on day 28.

Plasma Glucose

Plasma glucose of all the experimental nude (nu/nu) mice was determined once or twice weekly by blood sampling from the tail tip and separation of the plasma by centrifugation. The glucose concentration in the plasma was then assayed by an automated glucose oxidase procedure using a Beckman glucose analyser. Figure 5.5 shows the plasma glucose concentrations of the streptozotocin diabetic nude (nu/nu) mice implanted intraperitoneally with 2×10^7 wild type 1BR.3.G cells or 2×10^7 insulin-secreting 1BR.3.GinsPACE14d cells. The mice receiving the insulin-secreting 1BR.3.GinsPACE 14d cells had slightly higher plasma glucose concentrations than the control mice on implantation on day 7 and this remained the case throughout the experiment. However, 2 days after the second implantation on day 28 the glucose concentrations did fall more quickly than the small decrease seen in the control mice. Overall the mice developed experimental streptozotocin diabetes much more slowly than previously (chapter 4, implant group one) with only very mild fasting hyperglycaemia.

Body weight

Body weight of the nude (nu/nu) mice was monitored once or twice weekly throughout the implant experiments. Figure 5.6 shows the body weights of the mice throughout the implantation period. The mice receiving the insulin secreting 1BR.3.GinsPACE14d cells were slightly heavier than the control mice when implanted and this continued throughout

Figure 5.5 Plasma glucose concentrations of streptozotocin treated nude (nu/nu) mice (Streptozotocin 200mg/kg given day 0 by intraperitoneal injection) implanted intraperitoneally on day 7 with 1×10^7 1BR.3.GinsPACE14d cells or wild type 1BR.3.G fibroblast cells and again on day 28 with a further 3×10^7 1BR.3.GinsPACE14d cells or 1.8×10^7 wild type 1BR.3.G fibroblast cells. Values are means ± S.E.M for 10 and 7 observations respectively. The plasma glucose concentrations of mice in both groups steadily increased over the implantation period. The mice receiving the 1BR.3.GinsPACE14d cells had slightly higher plasma glucose levels than the control mice when implanted and stayed higher throughout the experiment.

181
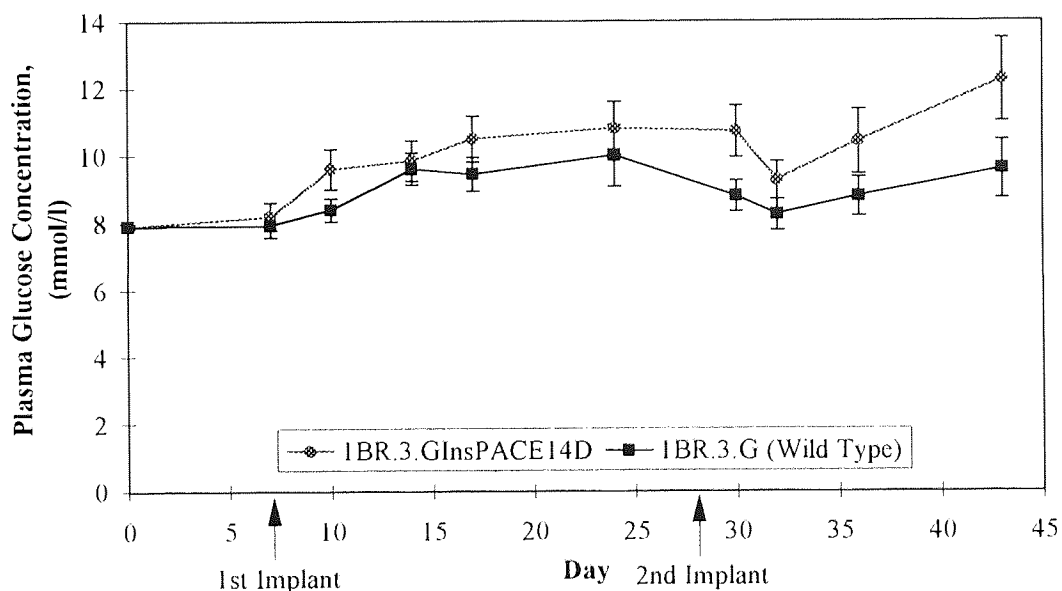
Figure 5.6 Body weight in grams (g) of streptozotocin treated nude (nu/nu) mice implanted intraperitoneally with 1×10^{7} insulin-secreting 1BR.3.GinsPACE14d cells or 1×10^{7} wild type 1BR.3.G fibroblast cells on day 7 and with a further 3×10^{7} 1BR.3.GinsPACE14d cells or 1.8×10^{7} wild type 1BR.3.G cells. Streptozotocin (200mg/kg) was administered by intraperitoneal injection on day 0. The body weights rise slowly throughout the experiment in both the test and control mice with no difference seen between the two groups.
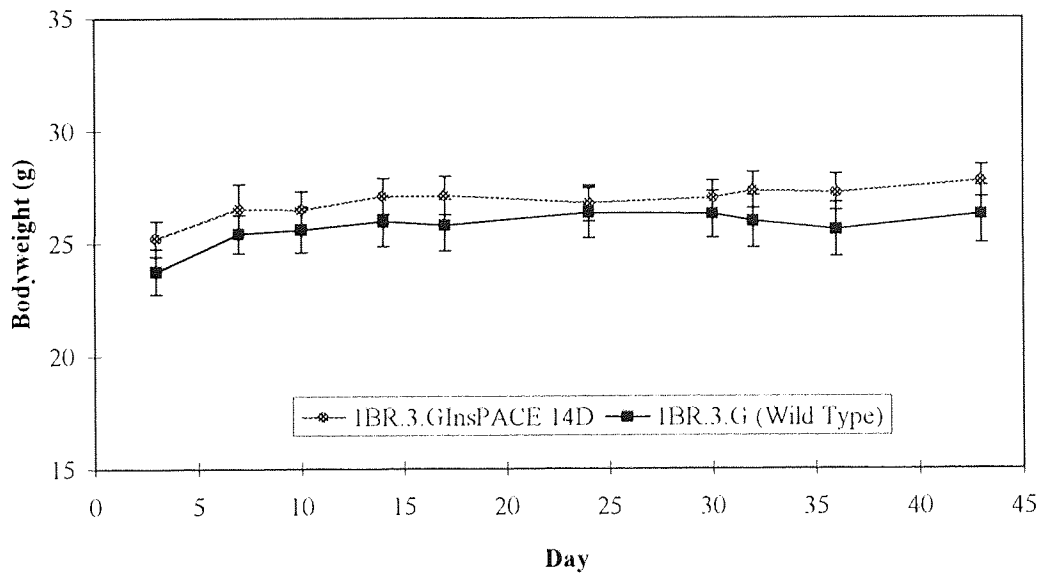
the implant period. There was no difference in weight gain or loss between the test and control mice during the experiment.

Food Intake

Food intake in grams consumed per day (g/day) of the streptozotocin diabetic nude (nu/nu) mice implanted with insulin-secreting 1BR.3.GinsPACE14d cells or wild type 1BR.3.G cells was measured throughout the implantation experiment. Food was weighed once or twice weekly and the number of grams eaten (g) was divided by the number of days since the food was previously weighed. Figure 5.7 shows the food intake of the mice during the implant experiment. Food intake of all mice was characteristically increased in the first few days after streptozotocin treatment, but remained fairly constant thereafter. There was a decrease in the intake of the control mice after the second implant of cells, however their food intake is still not significantly lower than that of the test mice.

Plasma Insulin

A specific human insulin immunoradiometric assay (IRMA) was used to assay plasma samples from the streptozotocin diabetic nude (nu/nu) mice implanted with 2×10^7 insulin-secreting 1BR.3.GinsPACE14d cells or 2×10^7 wild type 1BR.3.G cells. The samples were aliquots from the plasma obtained for plasma glucose determination and were preserved at -20°C. Mature human insulin was detected in some of the samples from the mice implanted with the insulin secreting 1BR.3.GinsPACE14d cells, however, levels were very low and at the threshold level for the sensitivity of the assay in most samples. No mature human insulin was detected in the samples from mice implanted with the wild type 1BR.3.G fibroblast cells.

Figure 5.7 Food intake (g/day) of streptozotocin treated nude (nu/nu) mice implanted intraperitoneally on day 7 with 1×10^7 insulin-secreting 1BR.3.GinsPACE14d cells or 1×10^7 wild type 1BR.3.G cells. A second implant of 3×10^7 and 1.8×10^7 cells respectively was administered on day 28. Streptozotocin (200mg/kg) was administered by intraperitoneal injection on day 0. Food intake increased in the first few days after streptozotocin treatment, then remained fairly constant. There were no significant differences between the food intake of the test and control mice.
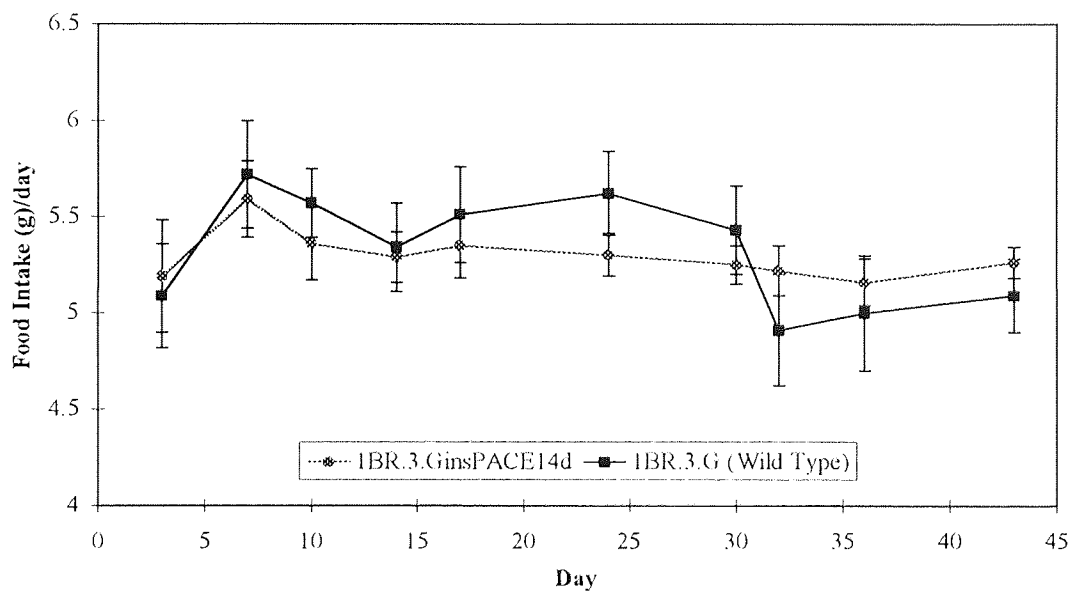
5.3.6 Histology and Immunohistochemistry

At the end of the implant experiments the mice were examined by autopsy for evidence of the implanted cells. Nearly all of the mice implanted with the fibroblast cells, wild type or transfected cells, had formed aggregations of cells that were found in the peritoneal cavity. The cell aggregations were between 0.5mm and 3mm in diameter and were not attached to any organs or tissues. They were not vascularised and had a whitish appearance similar to that of the cultured 1BR.3.G cells pelleted by centrifugation. Cell aggregations were removed and preserved in 10% (v/v) formaldehyde in PBS. They were then histologically processed as described in chapter 2 and stained with haematoxylin and eosin for general cell morphology or immunohistochemically stained for insulin. Figure 5.8a and 5.8b show sections from cell aggregations, removed from mice implanted with 1BR.3.GinsPACE14d cells, stained with haematoxylin and eosin. In figure 5.8a the cell aggregation typically shows normal healthy cells around the periphery and necrosis in the centre. The aggregation in figure 5.8b has fewer healthy cells in the periphery and more necrosis. Figure 5.9a and 5.9b show the same cell aggregations stained for insulin detection by immunohistochemistry. The insulin appears as a dark brown stain and positive cells were mostly found around the periphery of the tumours where the healthy tissue was found. The lighter brown stain in the necrotic area of the tumour in figure 5.9a is thought to be degrading insulin remaining from the necrosed cells. The fact that insulin secreting cells were visualised indicates that the cells did secrete insulin *in vivo*, despite the fact that plasma glucose concentrations were unchanged. However, relatively few insulin secreting cells were found in the cell aggregations when removed at the end of the experiments, suggesting that the implants lost insulin secreting cells after a period of implantation.

Figure 5.8 Sections from tumours removed from streptozotocin treated nude (nu/nu) mice implanted with 1BR.3.GinsPACE14d cells after 15 or 36 days growth *in vivo*. The tumours are stained with haematoxylin and eosin for general cell morphology. 5.7a shows a typical cell aggregation with a healthy periphery of normal cells and a necrotic core. 5.7b shows a cell aggregation with fewer healthy cells in the periphery.

a) Magnification x48
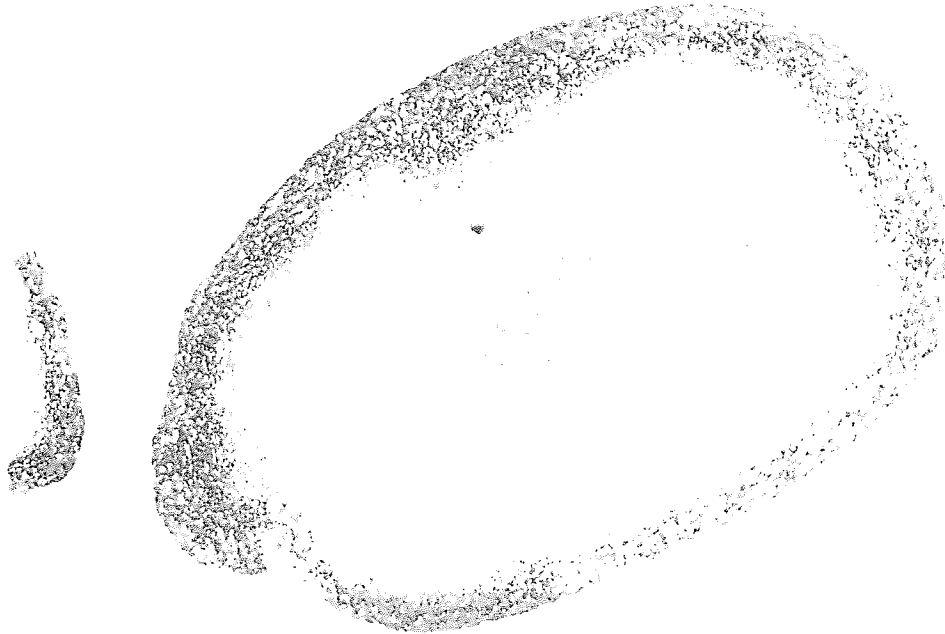
b) Magnification x48
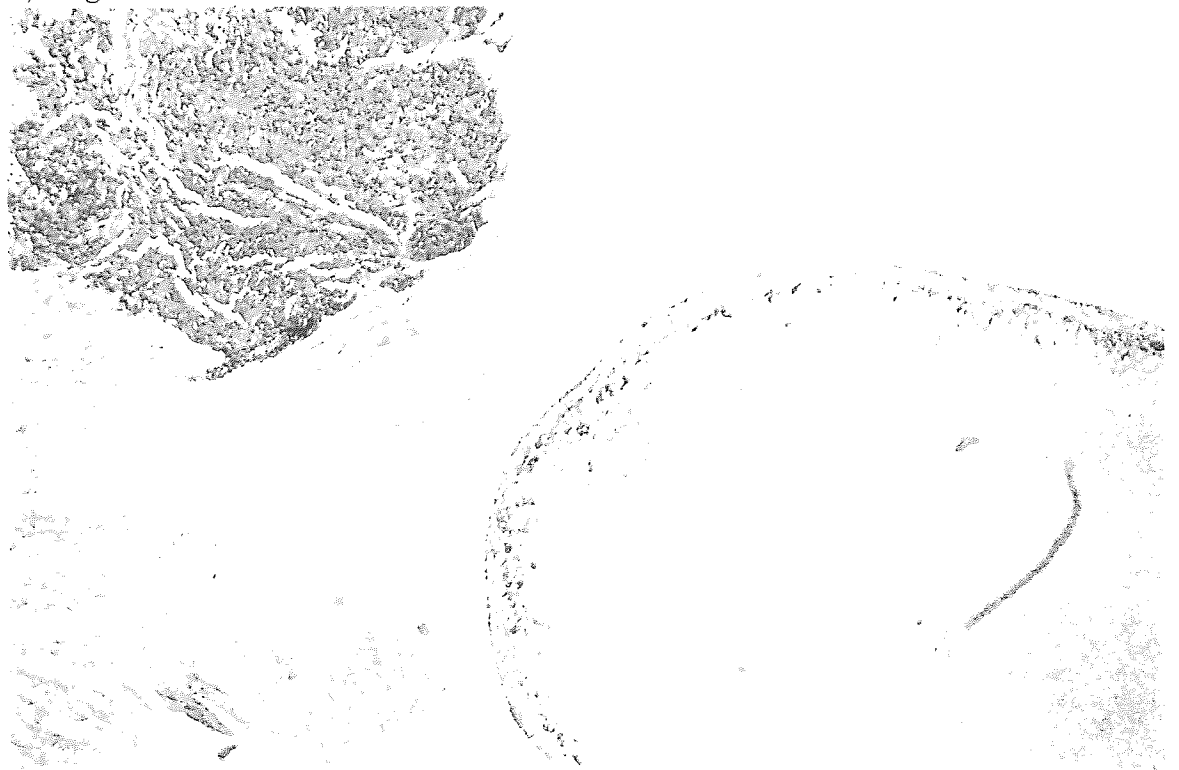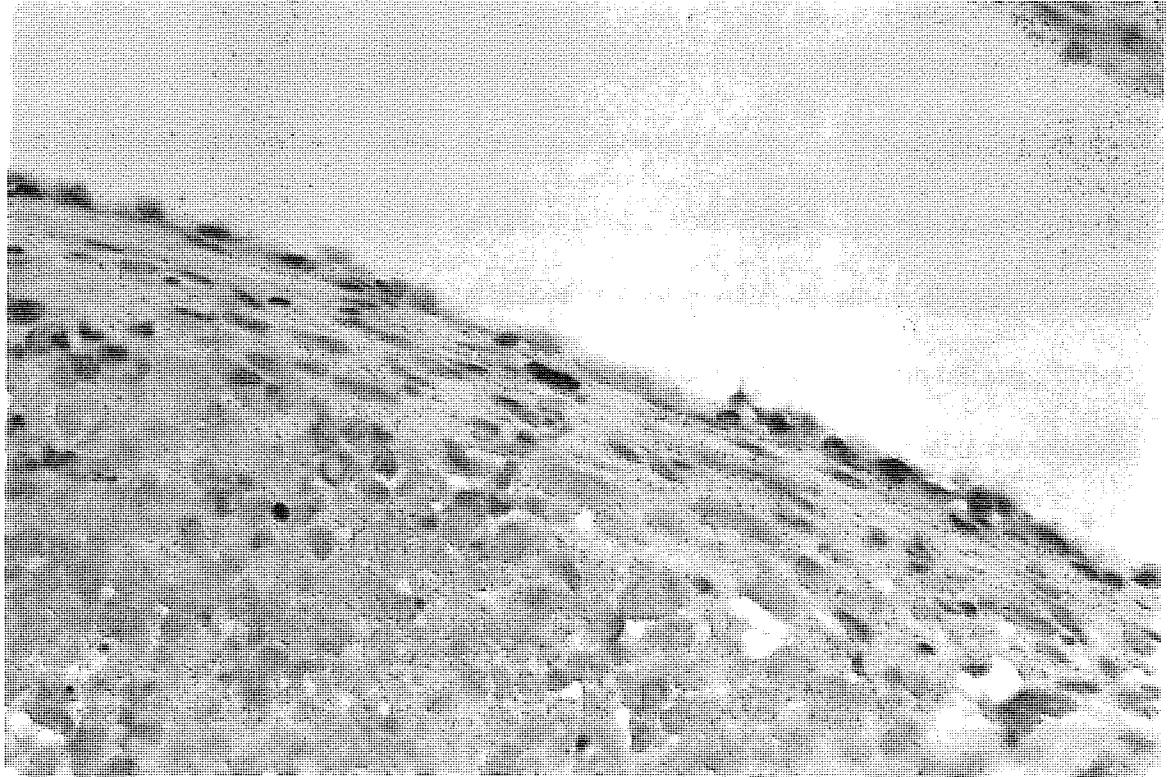
Figure 5.9 Sections from tumours removed from streptozotocin treated nude (nu/nu) mice implanted with 1BR.3.GinsPACE14d cells after 28 days *in vivo*. The sections are immunohistochemically stained for insulin. Cells positive for insulin secretion are stained dark brown and were mostly found near the periphery of the tumours.

a) Magnification x480

b) Magnification x480
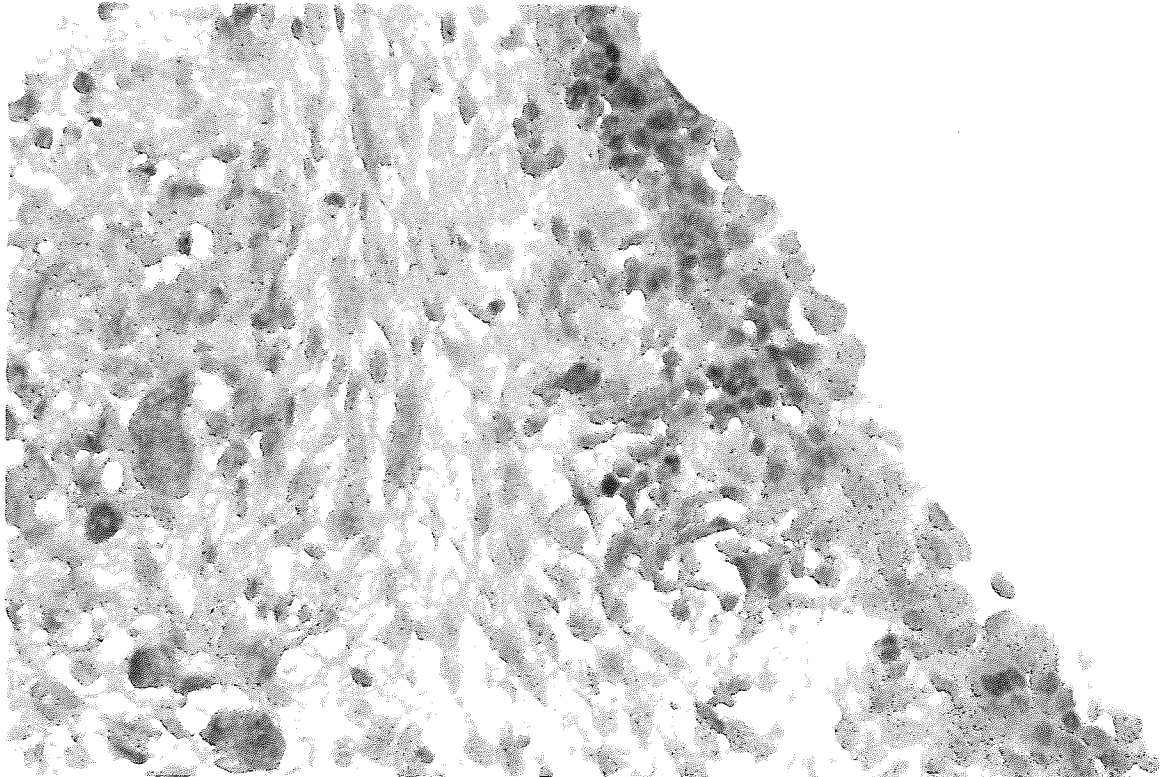
5.4 Discussion

1BR.3.G fibroblast cells were double transfected with genes for human preproinsulin and furin. Transfectants were selected in medium containing hygromycin B (125μg/ml) and resistant colonies of transfected cells screened for expression of the insulin and furin by Northern blotting. The colonies were also tested for the presence of insulin-like immunoreactivity secreted into the culture medium, and, using a specific human insulin antiserum, for the presence of fully processed mature insulin. Northern blotting showed clone 1BR.3.GinsPACE14d to be strongly expressing both insulin and furin mRNA. Other clones showed strong expression of insulin or furin alone, but all other double transfected clones had weaker expression of one or the other of the genes than clone 14d.

In this study we needed as much insulin as possible to be produced from the cells and, to obtain as much processing as possible, the furin needed to be overexpressed. The differential radioimmunoassay showed that clones 1c and 7d were processing 88% and 72% of their total insulins to mature insulin respectively. However as these clones were only producing 1.25ng/ml and 1.3ng/ml of total insulin-like immunoreactivity per confluent 10cm dish of cells, they were not an ideal choice for further study. Clone 14d, which secreted more than 10ng/ml of total insulin-like immunoreactivity per confluent 10cm dish of cells, processed around 55% of this insulin-like material to mature insulin making it a much better clone for further study.

It is interesting to note that clones with weak expression of insulin mRNA and strong expression of furin mRNA secreted a higher proportion of processed insulin to the culture medium when compared to clone 14d, which had strong expression of both insulin and furin. This is perhaps due to the relatively short time from gene transcription to protein secretion in the constitutive pathway, and the ratio of substrate to enzyme. In clone 14d the

ratio of insulin to furin is 1:1, whereas in clones 1c and 7d the ratio is more like 1:3. With such an excess of enzyme the insulin is more likely to encounter the furin during the constitutive pathway to release. Although ideally a 1:3 ratio would give a better degree of processing to mature insulin in the 1BR.3.G fibroblasts, it is unlikely that a clone would present with strong enough insulin mRNA expression for large quantities of insulin secretion to the culture medium and furin mRNA expression 3 times stronger. Vollenweider *et al* (1995) showed approximately the same percentage conversion in an experiment with monkey kidney COS7 cells transiently transfected with human preproinsulin and furin. However, there are no data to suggest the ratio of the expression of insulin and furin in these cells.

The specific human insulin antiserum used to detect mature processed insulin has 76% crossreactivity to split des-64,65 proinsulin. The antibody does not cross react with intact proinsulin or des-31,32 proinsulin. Therefore we must consider whether the 1BR.3.GinsPACE14d cells may be secreting des-64,65 proinsulin rather than insulin, or as part of the mature insulin-like material secreted. High performance liquid chromatography could be used to confirm the precise percentages of insulin and conversion intermediates secreted by the 1BR.3.GinsPACE14d cells. Since this technique was not available we must evaluate whether these cells secrete fully processed mature insulin.

As discussed earlier in this chapter human proinsulin is sequentially processed via the des-31,32 proinsulin intermediate in β-cells with the des-64,65 proinsulin intermediate undetectable in the circulation of healthy individuals or those with hyperproinsulinemia (Rhodes & Alarcon, 1994). There is also evidence that the secondary structure of proinsulin prevents cleavage of the $Lys^{64}Arg^{65}$ site until the Arg^{31}, Arg^{32} site has been processed (Docherty *et al*, 1989). Unless the secondary structure is changed when the insulin gene is

transfected into a non-endocrine cell line, the Arg^{31}, Arg^{32} site should still be processed first otherwise mostly intact proinsulin would be secreted by the cells.

Further evidence that the 1BR.3.GinsPACE14d cells are most likely to be secreting processed insulin is provided by Vollenweider *et al* (1995). HPLC of culture medium collected from COS7 cells co-transfected with insulin and furin showed that 60% of the insulin-like immunoreactivity was mature insulin, 21.8% was des-31,32 proinsulin, 11.7% was intact proinsulin and just 6% was des-64,65 proinsulin. If similar processing occurs in the 1BR.3.G fibroblasts then around 4.5% (76% of 6%) of the immunoreactivity detected using the specific insulin antiserum would be des-64,65 proinsulin. This would not affect the significance of the percentage of insulin-like immunoreactivity processed to mature insulin in any of the 1BR.3.GinsPACE clones.

We have shown that non-endocrine cells transfected with preproinsulin can be manipulated to process the prohormone to insulin in the constitutive pathway by co-expression with furin. Endogenous furin is not sufficient to allow processing to occur, but when furin is over-expressed, and especially when present in larger quantities than proinsulin, efficient processing will occur. However, the conversion to insulin in these cells is not as efficient as in β-cells. Several groups have investigated mutagenesis of the proinsulin dibasic cleavage sites to the consensus furin cleavage sequence, to achieve full processing to mature insulin in the constitutive pathway of non-endocrine cells (Yanigita *et al*, 1992; Yanigita *et al*, 1993; Groskreutz *et al*, 1994). These experiments showed that mutant proinsulin was processed to insulin in the constitutive pathway of non-endocrine cells using endogenous endoproteases. However, only 60% conversion was achieved by the endogenous enzymes and co-transfection with furin was required to achieve 100% conversion to mature insulin. As previously mentioned, the use of mutated genes for insulin gene replacement therapy in

humans may not be readily acceptable, making the over-expression of furin a better prospect for proinsulin processing in non-endocrine cells.

When the 1BR.3.GinsPACE cells were incubated with β-cell secretagogues such as glucose, K^+, arginine and forskolin, there was little or no effect upon insulin secretion. This confirmed that the human fibroblasts were not using a regulated pathway for secretion, and that the constitutive pathway was unaffected by the secretagogues. The small increase in insulin secretion between 0 glucose and 25mM glucose is probably attributable to down regulation of the cells in 0 glucose when they are deprived of their usual energy source. Interestingly, a raised extracellular Ca^{2+} (7.6mM) inhibited insulin secretion by the 1BR.3.GinsPACE14d cells. Calcium plays an important role in insulin secretion in β-cells, increasing secretion when the cytosolic concentration rises. Ca^{2+} has been shown to play a significant role in DNA synthesis and cell proliferation in 1BR human fibroblasts (Batra *et al*, 1991) although at lower concentrations than 7.6mM. It is possible that this concentration of Ca^{2+} is toxic to the fibroblast cells, inhibiting DNA synthesis and hence reducing insulin secretion. This is supported by the microscopic observation that the cells were rounded up and detaching from the monolayer.

Streptozotocin treatment given to the nude mice in the implant experiment initially appeared to produce a small increase in plasma glucose concentrations, however further increase was very slow and occurred over a prolonged period. Hence, when implanted with the 1BR.3.G or 1BR.3.GinsPACE14d cells the animals were only borderline diabetic. Unlike the AtT20 cells, which cause an increase in plasma glucose concentrations due to the secretion of ACTH, the fibroblast cells have no effect on the progression of the experimental diabetes. During the first implant of cells the mice were not frankly diabetic and any effect of the insulin secreted by the 1BR.3.GinsPACE14d cells *in vivo* would be counter balanced by the

pancreatic β-cells of the mice, hence there would be no overall change in plasma glucose concentration. Following the second, larger, implant of cells there was a small decrease in the plasma glucose concentration which was not observed to the same extent in the control animals. This may have been a small response to the cells now that the animals were very mildly diabetic. However, the plasma glucose concentrations rose again after a few days and the mice continued in the slow progression to diabetes. The batch of streptozotocin used for treatment of this group of mice appeared to have induced a chronic experimental diabetes instead of the desired acute disease. Body weight and food intake both increased in the animals in the few days following streptozotocin treatment, but were then relatively constant throughout the implant period.

At autopsy all the mice receiving fibroblasts, insulin-secreting or wild type, were found to contain whitish aggregations in the peritoneal cavity. These aggregations of cells were not attached to any organs or blood supply and were found floating between other visceral organs. The cell aggregations resembled the fibroblast cells following pelleting by centrifugation during culture *in vitro*. Histology showed the aggregations to be tumour-like, with a necrotic core and a peripheral covering of healthy cells. Immunohistochemistry for insulin revealed insulin-secreting cells in the periphery of the aggregations and traces of staining in the necrotic area was probably insulin released from necrosed cells. That the fibroblasts failed to form vascularised tumours, like those formed by the AtT20 cells, could explain the lack of effect on plasma glucose concentrations of the insulin-secreting 1BR.3.GinsPACE14d implants. If insulin was secreted only into the peritoneal cavity, instead of directly into the bloodstream some would undoubtedly be degraded before it could be absorbed into the bloodstream. Thus more insulin would be required to have an effect. Also, should the 1BR.3.GinsPACE14d cells be secreting des-64,65 proinsulin, which

has half the biologic activity of mature insulin (Gabbay, 1980), then twice as many cells would be required to have an effect on plasma glucose concentration. However, as it is unlikely that the cells were producing only des-64,65 proinsulin, it is likely that a combination of the failure of the fibroblast cells to vascularise *in vivo*, and the non-diabetic state of the animals caused the failure of the insulin-secreting implant to significantly reduce plasma glucose concentrations in the nude mice.

In conclusion, non-endocrine human fibroblasts can process transfected proinsulin to mature insulin in the constitutive pathway when co-transfected with furin, and the insulin is secreted into the culture medium. *In vivo* the 1BR.3.G cells failed to form vascularised tumour-like structures, but aggregated and remained independent in the peritoneal cavity. The insulin-secreting 1BR.3.GinsPACE14d cells failed to cause significant changes in plasma glucose concentrations of very mildly diabetic nude (nu/nu) mice. However, further implantation experiments are required to investigate the suitability of the 1BR.3.GinsPACE14d cells to deliver bioactive insulin *in vivo*.

This particular approach to the cellular engineering of a surrogate β-cell circumvents the less ethically acceptable mutagenic manipulation of the human preproinsulin gene to achieve processing of proinsulin in non-endocrine cells. However, the mutagenic manipulation would certainly improve the processing ability of non-endocrine cells when co-expressed with furin.

Non-endocrine cells, manipulated to secrete mature insulin, do not exhibit glucose-stimulated insulin secretion. Further genetic manipulation of the cells would be required to rectify this problem. We know that GLUT2 and glucokinase are required for glucose-stimulated insulin secretion and these elements would need to be incorporated into the cells.

However, whether a non-endocrine cell could incorporate so many new genetic elements or orchestrate glucose-stimulated insulin secretion remains to be determined.

Chapter Six

General Discussion

Chapter Six : General Discussion

6.1 Introduction

The current conventional treatment of IDDM by single and multiple daily subcutaneous insulin injections, although life-saving, can be considered inadequate. The diabetic control and complications trial has shown that the progression of the complications associated with IDDM can be slowed with intensive insulin treatment regimens (DCCT research group, 1993). However, such regimens still do not reinstate an entirely normal physiological control of glucose homeostasis. They are also costly in terms of the amount of healthcare resources required, and require a high level of patient compliance.

The cost to the NHS in the UK of treating diabetes and its complications constitutes up to 5% of the total annual healthcare budget (BDA report, 1995). The indirect costs of the disease, such as loss of productive output due to sickness absence, early retirement and premature mortality, further increase the cost of the disease to society.

A method of delivering insulin to re-create the normal physiological contribution of the pancreatic β-cells needs to be developed. As discussed in chapter one, pancreas and islet transplantation have met with considerable problems. In particular, the lack of human donor tissue and the need for immunosuppressive regimens precludes the extensive use of pancreas/islet allografts. Xenografting of pig islets, encapsulated to prevent immune destruction, and the use of transgenic pig islets with reduced immunogenicity, await further development before their feasibility can be realistically assessed. Concerns about interspecies cross contamination and the need for more effective encapsulation devices pose substantial additional problems. The same problems exist with xenotransplantation of engineered rodent insulinoma cell lines in immunoisolating devices.

A major complication of all implantation techniques is the need to contain and separate the transplanted cells from the immune system without compromising the ability of the cells to function adequately *in vivo*. With somatic cell gene therapy, engineered autologous cells would still need to be contained, to prevent their spread around the body and to guard against over-proliferation and hypoglycaemia. However, the containment would not need to isolate the cells from the host immune system. Such containment would be technically far simpler than encapsulation of xenografts, and the existing technology in this area (although inadequate for immunoisolation), may already be suitable for containing autografts of engineered cells.

Gene therapy is a rapidly growing area in scientific and clinical research. Clinical trials have already been completed in some fields. The preliminary results of human trials have proved disappointing, with inefficient gene transfer reported as the most common reason for reduced efficacy of the procedures. *In vivo* somatic cell gene therapy, especially, has been plagued by inefficient gene transfer in clinical trials compared with the results of *in vitro* experiments and animal studies. *Ex vivo* therapy has shown that reimplanted cells have problems reintegrating into the host tissue and many are destroyed in the procedure. These problems offer a severe challenge to the innovation of gene therapy, but research into more efficient gene transfer continues to make advances and will no doubt bear fruit in the long-term.

6.2 Gene Therapy for IDDM

IDDM is an extremely challenging target disease for treatment by gene therapy, for a number of reasons. In some gene therapy protocols the expression of the therapeutic gene can be achieved in the native tissue. However in IDDM the pancreatic β-cells are the

subject of autoimmune destruction and are therefore not available to be used in subsequent gene therapy treatment. Consequently a new target cell population must be chosen. Another problem not experienced so-far with other gene therapy protocols is that insulin is produced as a prohormone and must be processed to mature insulin. The target cells must therefore be capable of processing prohormones, or must be engineered to do so. The biological activity of proinsulin is <10% of mature insulin, underlining the need for efficient processing.

As previously considered, the plasma concentration of insulin is critical. Thus unlike other therapeutic proteins the expression of the insulin gene and the secretion of insulin must be tightly controlled to give plasma levels within 50-200% of normal. Conversely expression of the ADA gene can vary between 5-5000% of normal and be effective and safe (Steinberg, 1991). Moreover, the regulation of insulin biosynthesis and secretion must be rapidly adjusted in concert with plasma glucose concentrations, if the therapy is to be a truly effective treatment for diabetes.

The selection of a suitable cell type for gene therapy of IDDM is fundamental. Although neuroendocrine cells with their processing abilities would be useful, the AtT20 cells have demonstrated the problems associated with the co-release of ACTH and β-endorphins together with insulin. Also the collection of pituitary cells or indeed other neuroendocrine cells for primary culture is unlikely to be acceptable. Fibroblasts, keratinocytes and myoblasts are manipulable *in vitro*, amenable to genetic modification and could be harvested for primary culture less invasively than neuroendocrine cells. However, these cell types have a limited secretory capacity and will not process proinsulin to insulin (Moore *et al*, 1983). Fibroblasts, keratinocytes and myoblasts do contain the serine protease, furin, which is a Kex2 homologue (Hatsuzawa *et al*, 1990). Furin has been shown to process proinsulin

198

when the dibasic cleavage sites were mutated to the consensus cleavage sites for furin activity in kidney cell lines (Groskreutz *et al*, 1994). Other cell types such as hepatocytes may be useful in creating a surrogate β-cell with less manipulation than fibroblasts. However, a more invasive biopsy would be required to obtain autologous liver cells. The site of reimplantation and the type of containment used are also of extreme importance. Hepatocytes are mostly of interest in gene therapy of IDDM because portally delivered insulin would be physiologically desirable. While it may not be possible to contain insulin-secreting cells adequately in a location that drains into the portal circulation, a splenic or intraperitoneal implantation site would deliver insulin via the physiological route e.g. via the liver (Stewart *et al*, 1993).

Containment of the implanted cells could further delay the insulin response to glucose compared to the normal response to increases in plasma glucose concentrations. This is a problem with any insulin-secreting cell lacking the normal nervous innervation as the normal vascular supply and drainage of the islets. Where islets have been encapsulated and implanted in the peritoneum the response may be especially slow (DeVos *et al*, 1996). Implantation into a non-invasive site, such as subcutaneously, has the merit of making the implant easy to implant and remove. However, the response to glucose may be even slower than in the peritoneum.

6.3 Overview of Results

The selection of a vector is very important in any gene therapy protocol. In this study, where *ex vivo* was the choice of protocol, plasmids were constructed for use in *in vitro* chemical transfection. The plasmid constructs described in chapter 3 contained the human preproinsulin gene under the control of the cytomegalovirus (CMV) promoter. This

promoter is one of the strongest promoters available and should direct strong expression of the engineered gene. Furin was also promoted by the CMV promoter.

The plasmids were selected for their antibiotic resistance genes as well as their strong promoters. Neomycin resistance and hygromycin B resistance allowed selection of the transformed cells in tissue culture conditions by addition of the relevant antibiotic to the medium.

Standard molecular cloning techniques were used to construct the plasmids, and maps can be found in appendix 2. pLK444/GLUT2 was a gift of Dr. G. Gould, Department of Biochemistry, University of Glasgow. The human GLUT2 gene was controlled by a β-actin promoter, another strong promoter, and the plasmid conferred neomycin resistance (Brant *et al*, 1994).

The PCR mutagenesis eventually resulted in successful mutagenesis of the two dibasic cleavage sites of proinsulin to the consensus cleavage sequence for furin activity. The resulting preproinsulin, termed hppl-4, had an unwanted point mutation as a result of polymerase infidelity, but the mutation did not result in an amino acid substitution and should not have affected the biological activity of the protein. hppl-4 should be more effectively processed by furin in conditions where the enzyme:substrate ratio is low.

In some non-endocrine cells the concentration of endogenous furin was found to be too low to result in 100% processing of mutated rat proinsulin I, but co-expression with exogenous furin with the mutant insulin resulted in complete processing (Yanigita *et al*, 1993).

In chapter 4, murine pituitary AtT20 cells were co-transfected with hppl-1 and GLUT2. A clone expressing good quantities of insulin and GLUT2 mRNA was chosen for further study, namely AtTinsGLUT2.36.

AtTinsGLUT2.36 cells were shown to secrete around 5.6ng of mature insulin/10^6 cells/24 hours. 75% of the insulin secreted was shown to be mature insulin as would be expected with this neuroendocrine cell line. When incubated with increasing concentrations of glucose, insulin secretion from AtTinsGLUT2.36 cells was found to be maximal at 50μM glucose, with a 50% increase over basal secretion. This concentration of glucose (50μM) corresponds to the Km for hexokinase which has been shown to be the predominant glucose phosphorylating enzyme in this cell type (Hughes *et al*, 1992). In an attempt to shift the glucose-dose response of the cells into the physiological range, the AtTinsGLUT2.36 cells were preincubated in 2-deoxyglucose, 2-deoxyglucose is phosphorylated to 2-deoxyglucose-6-phosphate and accumulates in this form inhibiting hexokinase but not glucokinase activity (Randle, 1993). The AtTinsGLUT2.36 cells showed a small inhibition of hexokinase activity by an overall down-regulation of glucose phosphorylation, and hence a decrease in insulin secretion, in cells incubated in fructose following preincubation in 14mM 2-deoxyglucose. The dominance of hexokinase in AtT20 cells means that 14mM 2-deoxyglucose preincubation is insufficient to inhibit hexokinase activity in these cells, despite being sufficient to completely inhibit hexokinase in isolated islet cells. It was recently shown that 50mM 2-deoxyglucose was required to inhibit hexokinase activity in cultured rat insulinoma cell lines (Ferber *et al*, 1994). As AtT20 cells are more likely to behave like cultured insulinoma cell lines (hexokinase activity increases as a function of time in culture) than isolated islet cells, this concentration (50mM) of 2-deoxyglucose is likely to be required to inhibit their hexokinase activity.

The response to glucose exhibited by the expression of GLUT2 mRNA was unexpected. Although GLUT2 mRNA expression is regulated by glucose in pancreatic β-cells (Chen *et al*, 1990), the β-actin promoter was not thought to be regulated in the same way. However,

the β-actin gene has been shown to be regulated by cAMP and Ca^{2+}-dependent processes (Phillips *et al*, 1988) and it is therefore entirely possible that it could be regulated by glucose. Hence the use of the β-actin promoter may confer glucose regulated transcription of the gene it controls, due to a glucose response element in the promoter region. An alternative explanation to the glucose regulation of the β-actin driven GLUT2 gene, is that a post transcriptional control mechanism is in operation. The regulation of both insulin secretion, and GLUT2 expression by glucose in the AtTinsGLUT2.36 cells *in vitro* showed the cells behaving in a similar manner to normal β-cells. Despite glucose induced insulin secretion being maximal at subphysiological glucose concentrations, which may be corrected by modulation of the glucokinase:hexokinase ratio in these cells, they are a good surrogate β-cell model *in vitro*. Down regulation or knockout of hexokinase by the expression of antisense hexokinase transcripts offers the possibility of further improving this *in vitro* model.

The implantation of AtTinsGLUT2.36 cells into streptozotocin diabetic nude mice resulted in a transient reduction in plasma glucose concentrations. Body weight and food intake of the mice were closely associated with plasma glucose concentrations throughout the experiments.

An oral glucose tolerance test carried out on one mouse implanted with functioning AtTinsGLUT2.36 cells for over 3 months showed that the implanted diabetic mouse could respond to an oral glucose challenge, although, the response was much slower than that of a normal non-diabetic nude mouse.

The ACTH concentrations of plasma from mice implanted with AtT20 pituitary cells were raised to up to 7 times that of normal. These levels of ACTH undoubtedly will have

impaired glucose homeostasis due to a chronic increase in glucocorticoid production, which may explain the transience of the decrease in plasma glucose caused by the implantation of the insulin-secreting cells.

Tumours were removed from the nude mice implanted with AtTinsGLUT2.36 cells 28 days after implantation. The tumours were necrotic in the centre with a mantle of normal healthy cells. The core necrosis of the tumours and failure of the implants to sustain the decreased plasma glucose concentrations in the mice, indicates that the cells need to be contained in some way that would prevent tumour formation. It seems that tumour formation could be detrimental to cell survival, perhaps preventing delivery of nutrients and oxygen to the central cells. A containment device where the cells could grow as a monolayer, as in culture, may be the answer. However, development of such a device is awaited.

Although the AtTinsGLUT2.36 neuroendocrine surrogate β-cell model offers *in vitro* responses similar to those of normal β-cells, the co-secretion of ACTH and β-endorphins and the difficulties that would be involved in obtaining a primary culture, preclude the use of this and many other neuroendocrine cells for human gene therapy. The manipulation of an easily accessible non-endocrine cell line to a surrogate β-cell would be desirable. In non-endocrine cells the added complication of achieving proinsulin processing and secretion from a constitutive pathway, as well as the lack of a glucose induced response are encountered. Further manipulations with enzymes such as furin, or to the sequence of the preproinsulin gene itself would be required to process proinsulin in non-endocrine cells.

In chapter 5, human fibroblasts (1BR.3.G) were co-transfected with the wild-type preproinsulin gene and the furin gene. The clones obtained were screened for insulin and furin mRNA expression by Northern blotting, and for the secretion of mature insulin relative to total insulins by differential radioimmunoassay. 11 of the clones were found to secrete

some mature insulin. Of these clones, the ones with strong expression of furin mRNA showed the greatest percentage of mature insulin secretion. Clones with strong expression of furin but weak expression and secretion of insulin, showed the greatest degree of processing. This suggests that the enzyme:substrate ratio is important for cleavage of proinsulin in the constitutive pathway of these cells. Clone 14d (1BR.3.GinsPACE14d) released 5.32ng of mature insulin/10^6 cells/24 hours, which was 58% of the total insulin-like material secreted. This clone produced the most insulin overall and was selected for further study. The level of insulin secretion of these and most cells engineered to secrete insulin is markedly lower than that of normal β-cells, and further manipulations to increase insulin secretion from engineered cells may be necessary.

As discussed in chapter 5, although there is some cross reactivity to des-64,65 proinsulin with the specific human insulin antibody used to detect mature processed insulin, it is highly unlikely that more than a tiny percentage of the insulin detected with this antibody is this particular split proinsulin.

Insulin secretion from the 1BR.3.GinsPACE14d cells was shown not to be stimulated by glucose or other β-cell secretagogues, indicating that only a constitutive pathway of secretion was present.

Implantation of the insulin-secreting 1BR.3.GinsPACE14d fibroblasts into streptozotocin diabetic nude mice failed to show any conclusive decrease in plasma glucose. There are several possible reasons for this. Firstly, the streptozotocin treatment failed to cause a frank hyperglycaemia, but resulted in a borderline diabetic state. Thus, insulin-secreting implants would be countered by down-regulation of functional pancreatic β-cells. Secondly, upon autopsy tumour-like aggregations that had failed to vascularise in the peritoneum were removed from the mice. Histology showed these aggregations to have necrotic cores with a

peripheral covering of normal healthy cells. Inefficient transfer of insulin to the circulation may result from the inability of the cells to vascularise in the peritoneum. It may be that the human skin-type fibroblasts found conditions in the mouse peritoneum unfavourable, and this decreased insulin secretion. Insulin-secreting cells were detected in the healthy periphery of the tumour-like aggregations, although quantification of secretion was not possible.

Again, containment of the fibroblasts in a collagen lattice, or in a device where they could grow as a monolayer, may help them to survive and function better *in vivo*.

6.4 Concluding Remarks

Overall, steps towards the construction of a non-endocrine surrogate β-cell have been examined in this thesis. The endocrine AtT20 model was studied first, followed by the 1BR.3.G human fibroblasts.

It is known that a human fibroblast can secrete mature processed insulin following manipulation with the furin gene. The efficiency of this processing reaction is likely to be improved from 58% to 100% using the mutated hppl-4 gene with co-transfected furin in these cells.

Experiments described in chapter 4 have shown that glucose-stimulated insulin secretion can be conferred by manipulation with the GLUT2 gene, although further manipulations to bring the glucose-dose response into the physiological range are required. This will involve increasing the ratio of glucokinase to hexokinase, or knocking out hexokinase activity completely e.g. using antisense cDNA technology.

The next step in manipulating the fibroblasts would be to try to confer glucose-stimulated insulin secretion to these cells by transfection with GLUT2 and glucokinase. It is not clear

whether such non-endocrine cells will be able to orchestrate glucose-stimulated insulin secretion, but it remains a possibility. Certainly increased glucose metabolism is known to promote insulin biosynthesis at both the transcriptional and translational levels in the β-cell, which would increase the constitutive pathway leading to insulin release.

Following construction of a suitable fibroblast, or other such surrogate β-cell, implantation studies could be performed using autologous fibroblasts from BB rats or NOD mice. These animal models of type I diabetes are spontaneously occurring and provide a good model, since the animals require daily insulin injections, and suffer from similar complications to patients with IDDM. The harvested primary cells would be manipulated *ex vivo* and reimplanted following rigorous testing *in vitro*. Candidate containment devices could then be tested in this model.

Somatic cell gene therapy for insulin delivery in IDDM patients remains an attractive possibility. The need for immunosuppression or development of immunoisolating devices for pancreas/islet allografting and xenografting makes these options at least as distant a possibility as somatic cell gene therapy. Somatic cell gene therapy would not require immunoisolation of implanted cells, making development of a suitable containment device a much simpler procedure. The construction of a suitable non-endocrine surrogate β-cell, with tightly regulated insulin secretion in response to glucose, is likely to be the biggest hurdle.

In this thesis, the studies with insulin-secreting AtT20 cells have shown that inclusion of only the first step towards regulating glucose metabolism in concert with glycaemia, namely the expression of GLUT2, has conferred a degree of glucose sensitivity. The improvement of this model by up-regulation of glucokinase activity or the removal of hexokinase activity is an encouraging prospect. Secretion of mature insulin by a human fibroblast is an important

step toward engineering a non-endocrine surrogate β-cell and inclusion of the GLUT2 and

glucokinase genes into this model may be able to confer a degree of glucose sensitivity.

There is realistic optimism that gene therapy will become a practical option for the treatment

of several diseases in the future, and there is no reason to suggest that diabetes mellitus

should not be one of them.

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211

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221

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Appendices

Appendix 1: Solutions, Buffers and Media

Solutions and Buffers

|	
TE ph 8.0	10mM Tris HCL ph 8.0, 1mM EDTA.
5xTBE	54g/l Tris base, 27.5g/l boric acid, 20ml 0.5M EDTA ph8.0.
Nick Translation Buffer	50mM Tris-HCl pH 7.2, 10mM $MgSO_4$, 0.1mM DTT.
Solution D	4M Guanidinium Thiocyanate, 25mM Sodium Citrate pH7.0, 0.5% Sarcosyl, 0.1M 2-Mercaptoethanol.
MOPS Buffer	0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA.
20xSSC	175.5g/l sodium chloride, 88.2g/l sodium citrate pH 7.0.
FSB	10mM potassium acetate, 100mM potassium chloride, 45mM magnesium chloride, 10mM calcium chloride, 3mM hexamincoboltic chloride, 10% glycerol, solution must be pH 6.4 exactly.
PBS	137mM Sodium Chloride, 2.7mM Potassium Chloride, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4, prepared by dissolving 1 PBS tablet (Mg^{2+} free, Ca^{2+} free) in 100ml of distilled water and autoclaving to sterilise. PBS was then stored at 4^oC.
Trypsin/EDTA	1mM EDTA in PBS was prepared by adding 200µl of a sterile 0.5M solution of EDTA to 200mls of sterile PBS (see above). 9mls of this solution was then added to 1ml of a 2.5% trypsin solution in a sterile 25ml universal tube. The final concentration of trypsin was 0.25%.
Citrate Buffer	0.1M $C_6H_8O_7.H_2O$ 2 parts, 0.1M $C_6H_5O_7Na_3.2H_2O$ 3 parts, pH4.8.
Radioimmunoassay Buffer	5g/l BSA made up in PBS (see above).
Peroxidase Blocking Solution	Methanol 97ml, hydrogen peroxide 3ml (prepare just before use).

228

| Copper Sulphate Solution	$CuSO_4$ 4g/L, NaCl 7.2g/L in distilled water.
Citrate Buffer (microwave antigen retrieval)	2.1g/L citric acid monohydrate in distilled water adjusted to pH 6.0.

Bacterial Culture Media

|	
L Broth	1% tryptone, 1% sodium chloride, 0.5% yeast extract pH 7.0.
2xTY	1.6% tryptone, 1% yeast extract, 0.5% sodium chloride pH 7.0.
SOB	2% tryptone, 0.5% yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate.
Agar Plates	For agar plates add 15g/l bactoagar to the relevant medium.
H- Agar	1% tryptone, 0.8% sodium chloride, 15g/l bactoagar
H-Top Agar	1% tryptone, 0.8% sodium chloride, 8g/l bactoagar
GMM Agar	1.5g agar in 90ml of water, 10ml 10xM9 salts, 0.1ml 1M magnesium sulphate, 0.1ml 0.1M calcium chloride, 0.1ml 1M thiamine HCL, 1ml 20% glucose.
10xM9 Salts	6% Na_2HPO_4, 3% KH_2PO_4, 1% NH_2CL, 0.5% sodium chloride.

Appendix 2: Plasmid Maps

Appendix 2.1 Vector pCB7 (6.0kb) with a CMV promoter, hygromycin B and ampicillin resistance genes. There are 9 unique restriction enzyme sites in the polylinker region. The furin (PACE) and hppI-1 genes were both cloned into this vector to create the plasmids pCB7-PACE and pCB7-hppI-1.
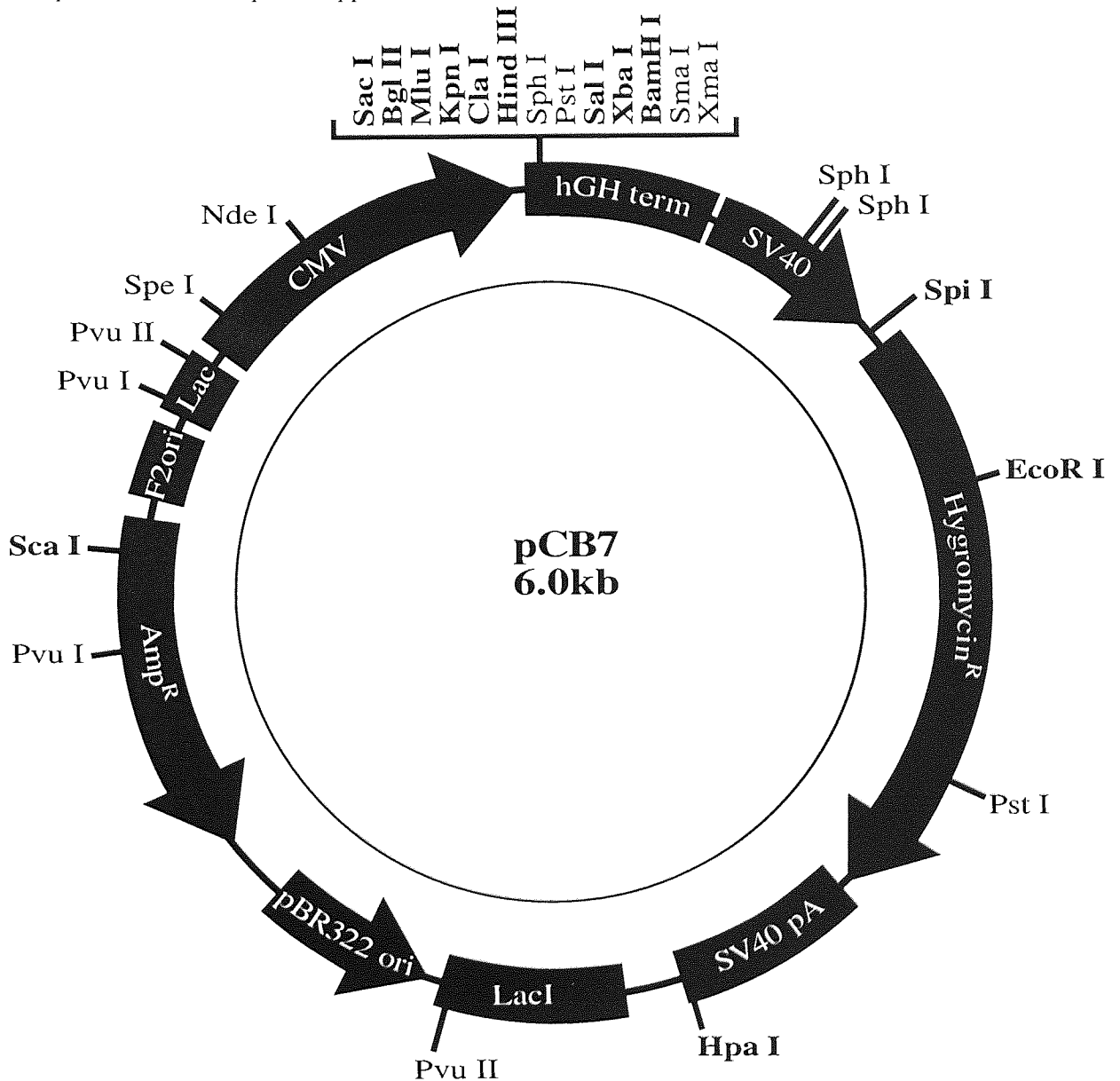
Appendix 2.2 Vector pCMV (5.5kb) with a CMV promoter, neomycin and ampicillin resistance genes. There are 6 unique restriction enzyme sites in the polylinker region. The hppI-1 gene was cloned into the Hind III and Xba I restriction site to create the pCB7-hppI-1 plasmid.
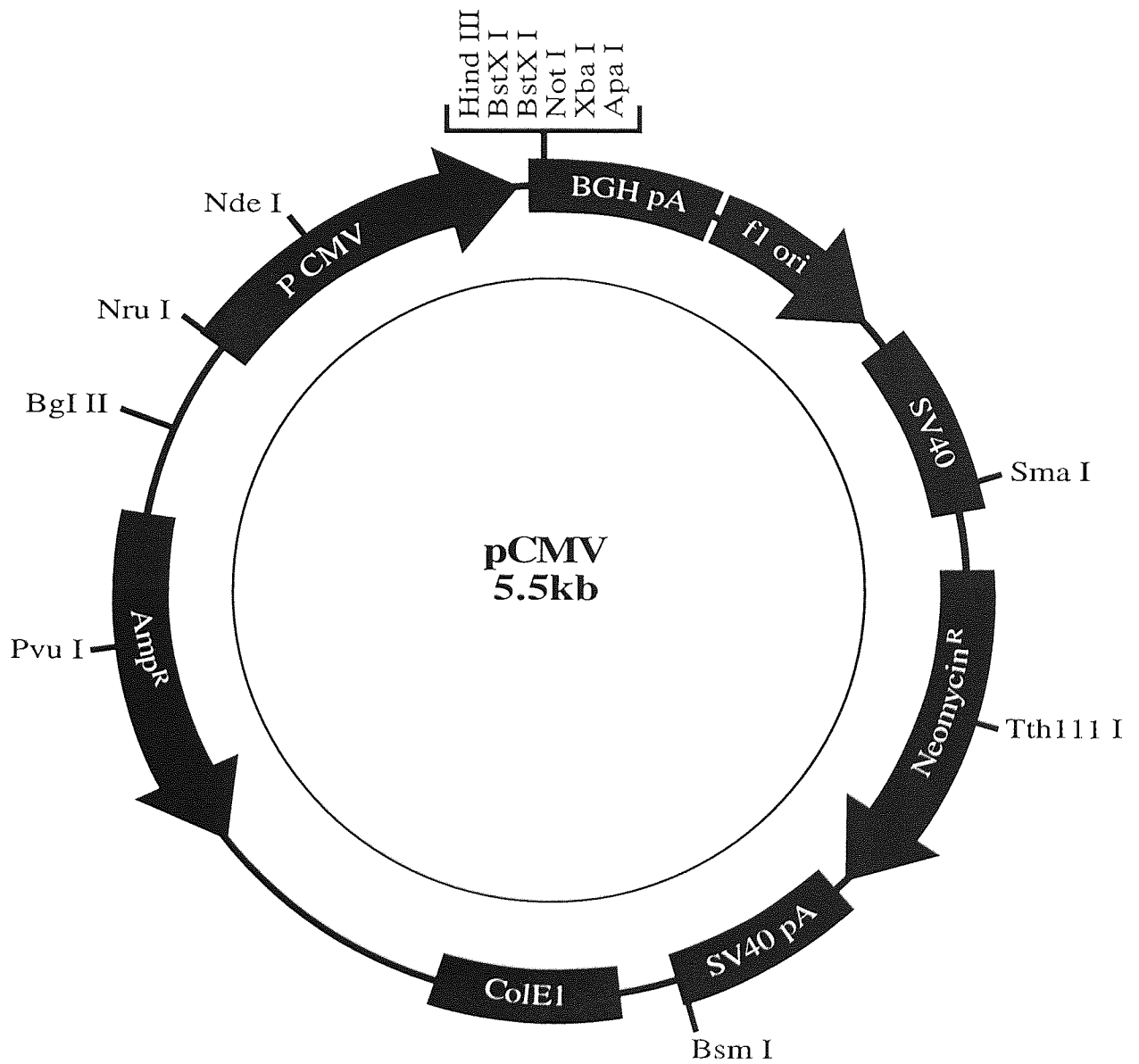
Appendix 2.3 The pCB7-PACE plasmid (8.5kb) with the furin gene in the Sac I (Sst I) and Sal I sites under the control of the CMV promoter. The plasmid can be selected in mammalian cell cultures using the hygromycin B antibiotic resistance gene with hygromycin B added to the culture medium.
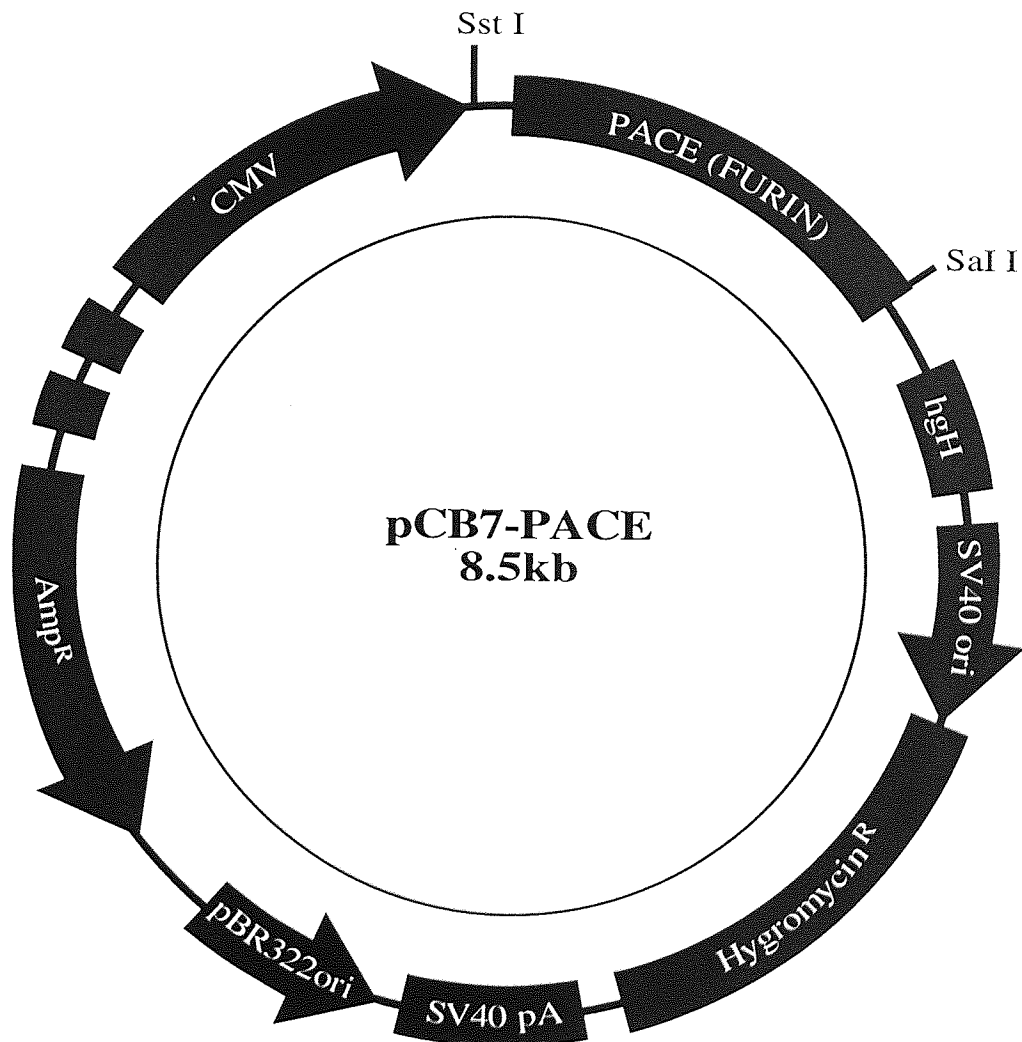
Appendix 2.4 The pCB7-hppI-1 plasmid (6.6kb) with the human preproinsulin gene in the Sac I (Sst I) and Sal I sites under the control of the CMV promoter. The plasmid can be selected in mammalian cell cultures using the hygromycin B antibiotic resistance gene with hygromycin B added to the culture medium.
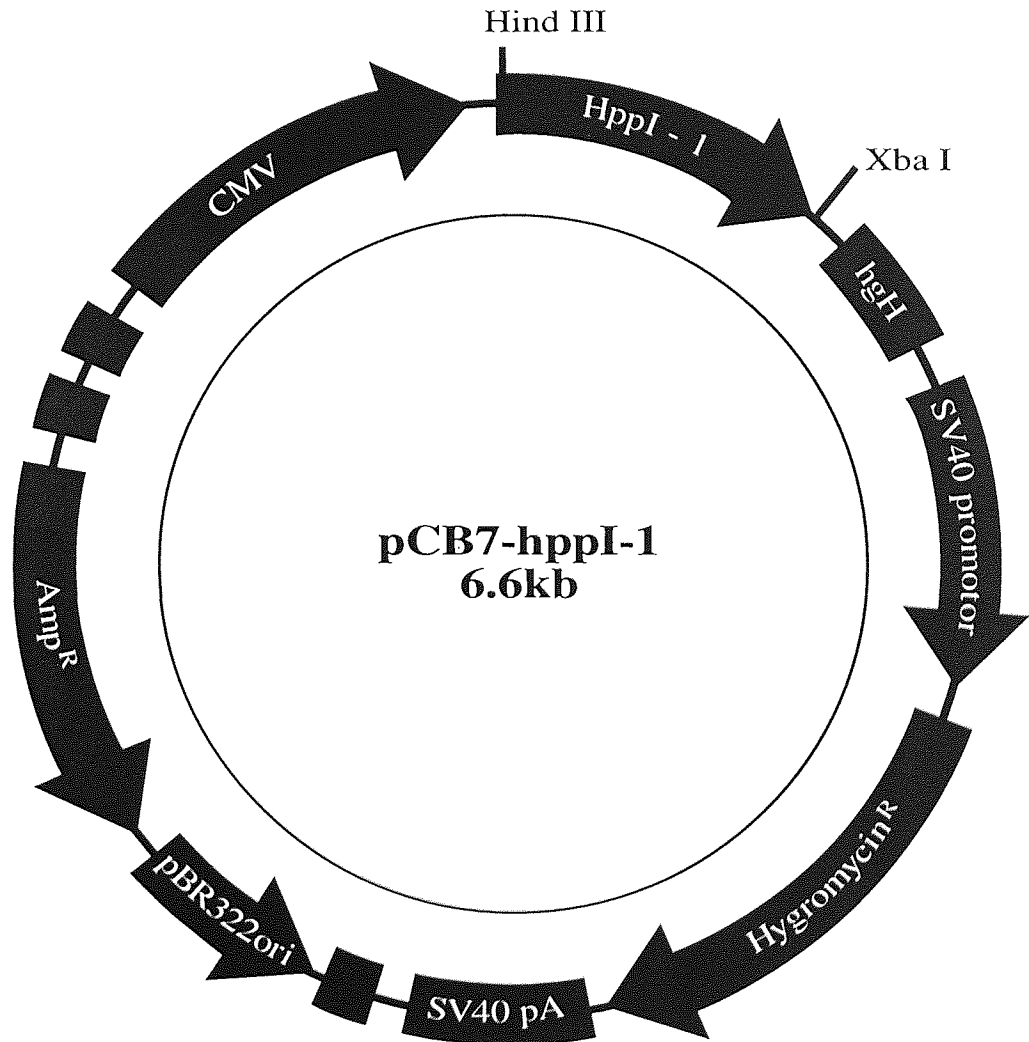
Appendix 2.5 The pCMV-hppI-1 plasmid (6.0kb) with the human preproinsulin gene in the Hind III and Xba I sites under the control of the CMV promoter. The plasmid can be selected in mammalian cell cultures using the neomycin antibiotic resistance gene with G418 sulphate (geneticin) added to the culture medium.
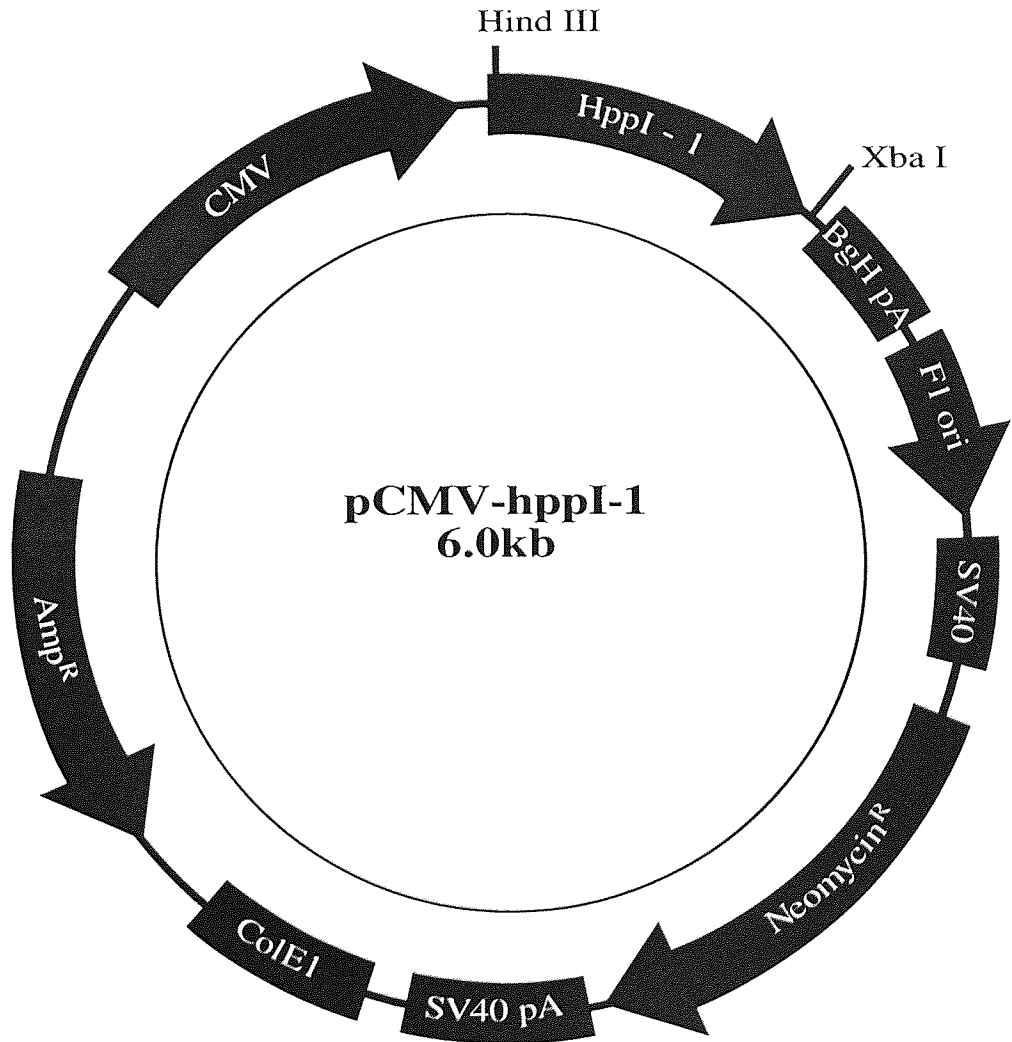
Appendix 2.6 The pLK444/GLUT2 (11.8kb) with the human GLUT2 gene in the Bam HI/Bgl II restriction site. The GLUT2 gene is under the control of a β-actin promoter. The plasmid can be selected in mammalian cell cultures using the neomycin antibiotic resistance gene with G418 sulphate (geneticin) added to the culture medium. The plasmid was the kind gift of Dr. G. Gould of the University of Glasgow (Brant *et al*, 1994).
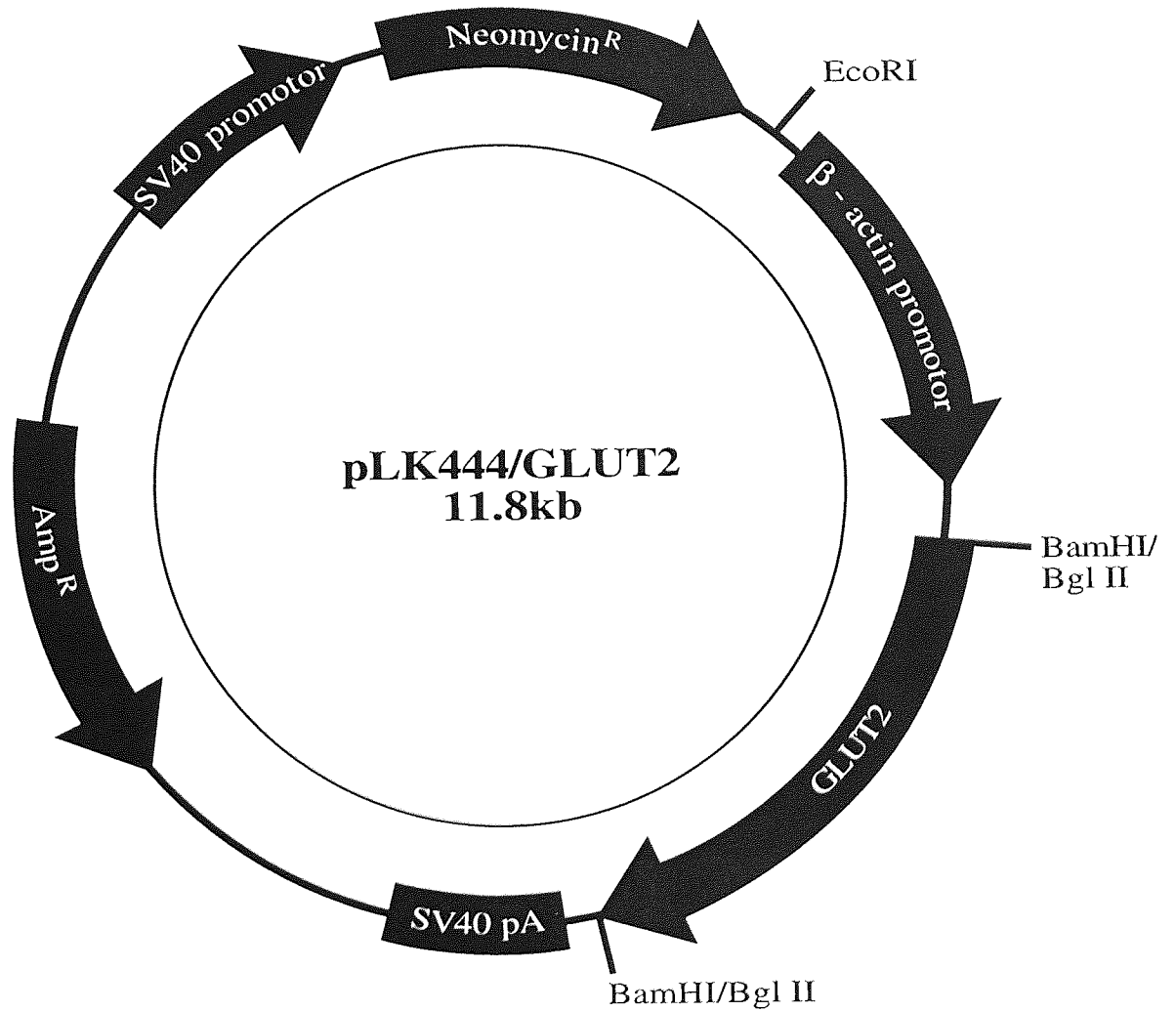
Appendix 3: Oligonucleotide Primer Sequences

Appendix 3.1 Oligonucleotide Site Directed Mutagenesis Primers

Sel2 : D144 Selection oligonucleotide primer

--GGA--GCT--ACT--GTA--AGA--TCA--

18 Bases

Melting Temperature 50°C

Moli1 : D3540 Mutant oligonucleotide primer 1 (B Chain/C-Peptide)

--GGT--CCT--CTC--GCT--TCC--GGC--GGG--TCT--

24 Bases

Melting Temperature 69°C

Moli2 : D3541 Mutant oligonucleotide primer 2 (C-Peptide/A Chain)

--GCC--ACG--CTT--CCG--CCG--GGA--CCC--CTC--

24 Bases

Melting Temperature 72°C

Appendix 3.2 PCR Mutagenesis Primers

Moli 3 : D4794 Mutant oligonucleotide primer 3 (B Chain/C-Peptide)

--CCT--GCA--GGT--CCT--CTC--GCT--TCC--GGC--GGG--TCT--T-

31 Bases

Melting Temperature 73°C

CMoli 3 : D5936 Complementary mutant oligonucleotide primer 3 (B Chain/C-Peptide)

--GGA--AGC--GAG--AGG--ACC--TGC--AGG--

21 Bases

Melting Temperature 63°C

Moli 4 : D4795 Mutant oligonucleotide primer 4 (C-Peptide/A Chain)

--CAC--AAT--GCC--ACG--CTT--CCG--CCG--GGA--CCC--CTC--CAG--

33 Bases

Melting Temperature 75°C

CMoli 4 : D5937 Complementary mutant oligonucleotide primer 4 (C-Peptide/A Chain)

--CGG--CGG--AAG--CGT--GGC--ATT--GTG--

21 Bases

Melting Temperature 63°C

(Mutant bases in bold)

Sp6 up primer : D5770 Primer to a region in the Sp6 promoter

--CAT--ACG--ATT--TAG--GTG--ACA--CTA--TAG--

24 Bases

Melting Temperature 55°C

hppI-1 end : D6019 Primer to a region at the end of the hppI-1 gene

--GCG--GAA--TTC--GTC--TAG--TTG--CAG--TAG--

24 Bases

Melting Temperature 60°C

Appendix 4: Sequences

Appendix 4.1: Sequence of the human preproinsulin cDNA.

```
                                -24             -20
                                MetAlaLeuTrpMetArgLeuLeuProLeuLeuAlaLeu
GAATTCCGGGGGTCCTTCTGCCATGGCCCTGTGGATGCGCCTCCTGCCCCTGCTGGCGCTG 60
CTTAAGGCCCCCAGGAAGACGGTACCGGGACACCTACGCGGAGGACGGGGACGACCGCGAC
  EcoR1                      NcoI


     -10                                        -1 B1
 LeuAlaLeuTrpGlyProAspProAlaAlaAlaPheValAsnGlnHisLeuCysGlySer
 CTGGCCCTCTGGGGACCTGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGCTCA 120
 GACCGGGAGACCCCTGGACTGGGTCGGCGTCGGAAACACTTGGTTGTGGACACGCCGAGT


 B10                                         B20
 HisLeuValGluAlaLeuTyrLeuValCysGlyGluArgGlyPhePheTyrThrProLys
 CACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCCAAG 180
 GTGGACCACCTTCGAGAGATGGATCACACGCCCCTTGCTCCGAAGAAGATGTGTGGGTTC


 B30 *   *
 ThrArgArgGluAlaGluAspLeuGlnValGlyGlnValGluLeuGlyGlyGlyProGly
 ACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGT 240
 TGGGCGGCCCTCCGTCTCCTGGACGTCCACCCCGTCCACCTCGACCCGCCCCGGGACCA
                         PstI


                                        *   * A1
 AlaGlySerLeuGlnProLeuAlaLeuGluGlySerLeuGlnLysArgGlyIleValGlu
 GCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAA 300
 CGTCCGTCGGACGTCGGGAACCGGGACCTCCCCAGGGACGTCTTCGCACCGTAACACCTT
             PstI                        PstI


             A10                            A20
 GlnCysCysThrSerIleCysSerLeuTyrGlnLeuGluAsnTyrCysAsnEND
 CAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAGACGCAG 360
 GTTACGACATGGTCGTAGACGAGGGAGATGGTCGACCTCTTGATGACGTTGATCTGCGTC
                        PvuII


 CCCGCAGGCAGCCCCCCACCCGCCGCCTCCTGCACCGAGAGAGATGGAATAAAGCCCTTG 420
 GGGCGTCCGTCGGGGGGTGGGCGGCGGAGGACGTGGCTCTCTCTACCTTATTTCGGGAAC


 AACCAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACCCCCCCCCCC 480
 TTGGTCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGGGGGGGGG


 CCCCTGCAGCAATGGCAACAACGTTGCGGAATTCC 3'   511
 GGGGACGTCGTTACCGTTGTTGCAACGCCTTAAGG 5'
     PstI                      EcoR1
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