INVESTIGATION INTO THE POTENTIAL OF TISSUE-SPECIFIC PROMOTERS FOR GENE SUPPLEMENTATION THERAPY.

ALISON H. TRAINER

Supervisor Dr R. J. Akhurst

Submitted for the degree of Doctor of Philosophy

University Of Glasgow

1999

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Für meine Mutti

Barbara Hedwig Herta

'Und das Schöne wird bleiben'

(R.M.Rilke)

Acknowledgements

I would like to sincerely thank all the people who helped me over the last few years. I am particularly indebted to Rosemary Akhurst for her invaluable scientific advice and encouragement in this work, as well as her seemingly limitless patience in proof reading this manuscript. The time that I spent in her group was both scientifically and socially very enjoyable.

Frances and Dennis, the demonic duo, kept me sane with their large collection of 'music to microinject to' cassettes as I spent long hours staring down a microscope. I benefited and learnt a great deal from their technical skill and help.

I would also like to thank Marion and Yvonne for all their help and friendship, both in the lab and out; Sarah, Stephen, Pete and Travis the dog for leading me astray when I took myself too seriously; Anne Theriault for those numerous administrative tasks that kept the lab running smoothly and Gordon Graham for resuscitating my computer on numerous occasions.

I owe a great deal to John, Rosemarie, Margo and David for their friendship and encouragement.

Lastly and most importantly, I would like to thank my dad and wee brother, Mike, for always being there when I need them.

This thesis is dedicated to the memory of my mother.

Abstract.

Ornithine transcarbamylase (OTC) is the second enzyme in the hepatic urea cycle. As such, it is important in the metabolism of neurotoxic waste products into a non--toxic, water-soluble compound, urea. In humans deficiency of ornithine transcarbamylase is an X-linked single gene disorder associated with a high mortality and morbidity due to a severe metabolic disturbance. There are two naturally occuring allelic mouse models for OTC deficiency, the sparse fur (Spf) and sparse fur^{ASH} (Spf^{ASH}) mice.

In this study, the focus was on the use of tissue specific promoters in driving recombinant gene expression. The main question addressed was whether expression of the OTC gene in a tissue which did not normally express this gene, namely skeletal muscle, would correct the phenotype of the deficient mouse models. The approach taken was generation of conventional transgenic mice using a creatine kinase driven OTC gene construct. In addition as a positive control, supplementation of the endogenous hepatic gene expression was also undertaken by means of hepatic-specific albumin promoter. Three indices were used as a measure of correction of OTC deficiency by transgenesis in the mouse models; a) phenotype of the mice, b) plasma ammonia levels and c) direct OTC activity.

Although transgenic mice expressing the OTC cDNA driven by the creatine kinase promoter showed high OTC activity in skeletal muscle, no metabolic or phenotypic correction of the mice was noted. Interestingly, the albumin driven OTC construct did not correct the phenotype of the sparse fur and sparse fur^{ASH} models either, although in some transgenic lines a significant amelioration of plasma ammonia levels was noted. However, this partial metabolic correction did not correlate with a significant increase in hepatic OTC activity.

In addition to these tissue-specific promoters, the potential of a novel keratin 5 minigene construct in targeting recombinant gene expression to the epidermis in a tissue and cell specific manner was assessed. The gene expression pattern of a Lac Z reporter transgenic construct driven by the regulatory sequences of the keratin 5 gene was investigated both in adult mice and during embryogenesis.

Figures and Tables

1. INTRODUCTION

1.1 GENE THERAPY FOR SINGLE GENE DISORDERS

The last decade has seen the extension of clinical genetics from a purely diagnostic and counselling service to that of a potential therapeutic speciality. This has occurred due to the advent of gene therapy, the manipulation of DNA in disease conditions. Although initially considered as a means of correcting single germline gene disorders such as cystic fibrosis (Tanne 1999), it has now been shown to have a role in the treatment of more complex genetic conditions such as cancer (Gene Therapy of Cancer 1998).

In considering gene therapy for a specific single gene disorder two distinct approaches can be envisaged. In the first approach it could be postulated that in certain conditions demonstrating a dominant inheritance pattern, gene disruption or correction could prevent the expression of an endogenous mutant gene product. In the second approach, applicable to those disorders demonstrating recessive inheritance or haploinsufficiency secondary to loss of gene function, supplementation of the deficient gene in the appropriate cells could resolve the pathological phenotype. Although this approach is of more limited potential, it has already produced results in the clinical sphere. The most advanced clinical trials for a single gene disorder have involved patients with a severe combined immunodeficiency due to the absence of adenosine deaminase (ADA). Supplementation of this enzyme by targeting gene expression and subsequent protein production to the patient's peripheral T lymphocytes has indicated a potential therapeutic role for this form of treatment, although as yet this approach has not been shown to be lasting clinical benefit (Hershfield 1998). Ideally, supplementation of an endogenous gene would result in recombinant gene expression in a highly

tissue- and cell-specific manner, which would mimic those of the endogenous gene under physiological conditions.

1.1.1 TISSUE-SPECIFIC PROMOTERS CAN BE USED TO EXPRESS RECOMBINANT GENE PRODUCTS

The most efficient method by which tight regulation of gene expression at the cellular and tissue level could be achieved is through the use of endogenous gene regulatory sequences. It is for this reason that one focus in the gene therapy field is in trying to identify genes that are expressed in a tissue-specific manner and to dissect out the cis-acting regulatory sequences governing this expression pattern. These tissue specific promoters could then be used to express recombinant genes in specific target tissues.

The advantage of tissue specific promoters over the use of their general or viral counterparts is that, by definition, they only allow gene expression in a limited number of tissues. Specific gene regulatory elements have been characterised, which can target recombinant gene expression to a variety of tissues such as the epidermis (Blessing, Nanney et al. 1993), skeletal muscle (Jaynes, Johnson et al. 1988), mammary tissue (Li, Murphy et al. 1998), liver (Pinkert, Ornitz et al. 1987) and pancreas (Ray, Fagan et al. 1998). As these gene promoter sequences are endogenous to the mammalian genome, their expression is not down-regulated by the methylation apparatus which have evolved to silence exogenous viral gene promoters (Palmer, Rosman et al. 1991). Tissue-specific promoters are therefore capable of long term expression of recombinant genes. In addition, this approach has the additional advantage that the mode of gene delivery need be less tissue-specific since gene expression pattern is tightly regulated at the cellular level.

Tissue specific expression cassettes can be integrated into viral vector for delivery to the target organ. Although viral vectors have shown good transfection efficiency, the immunogenicity of some viral vectors can have a detrimental effect on the longevity of gene expression (Ishibashi, Brown et al. 1993; Kozarsky, Grossman et al. 1993). Therefore, alternative, non-viral transfection methods have been devised. These include liposomes, particle bombardment and direct injection of 'naked' DNA. These different forms of gene delivery will described in relation to appropriate target tissues later in the thesis. However, in general these forms of gene delivery result in episomal expression of the recombinant gene which, although circumventing the risk of insertional mutagenesis, tend to decrease the time the gene is expressed.

The majority of supplementive gene therapy focuses on increasing gene expression and hence protein production in the endogenous tissue. In the case of cystic fibrosis it is vital that the gene is expressed in the lung epithelium, as it is in this tissue that the gene is normally expressed and that pathology is found when the protein is absent. At present it is often technically difficult in a clinical setting to deliver a recombinant gene to its endogenous target tissue, such as the lung. However, in enzymatic reactions or Haemophilia A or B in which the gene-product and/or substrate(s) are diffusible, it may not be as important that the gene is expressed in the endogenous tissue as long as the gene product is accessible to its substrates, and in an environment in which it can function optimally. In this scenario, it may be advantageous to express the recombinant gene in a tissue, which is clinically easily accessible, such as skin. Tissue-specific promoters can be used to target recombinant protein to tissues, which under physiological conditions do not normally expresses the gene, in order investigate if their expression in this exogenous site may be clinically beneficial.

1.1.2 ORNITHINE TRANSCARBAMYLASE DEFICIENCY IS A GOOD CANDIDATE FOR SUPPLEMENTIVE GENE THERAPY

In addition to haemophilia A and B, metabolic disorders are strong candidates for a supplementive gene therapy strategy as they are often due to single gene/enzyme deficiencies. As their substrates/products are often diffusible, it could envisaged that gene expression in an exogenous tissue may have a therapeutic benefit. Such disorders include phenylketouria, and deficiencies of α 1 antitrypsin, Haemophilia factor IX or ornithine transcarbamylase (OTC).

OTC deficiency is an excellent model system in which to investigate this form of treatment. It is an X-linked recessive, single gene disorder with high mortality and morbidity. OTC is important in the metabolism of proteins and in particular in preventing the accumulation of neurotoxic products during protein degradation. Current clinical treatments for OTC deficiency are sub-optimal, which means that the scope for devising a more effective treatment is immense. However, the major advantage that this condition has over the many other metabolic disorders in the study of novel treatment regimes, is that there are two naturally occurring mouse models in which to test possible therapeutic strategies.

This thesis concentrates on the use of tissue specific promoters to express recombinant ornithine transcarbamylase protein in OTC deficient mouse models. By this means it is possible to investigate whether the OTC gene, if expressed in ectopic tissue, can have a therapeutic effect. The two tissue-specific promoters, which have been previously well characterised, comprise the murine albumin gene promoter and enhancer targeting expression to the liver, and the murine creatine kinase gene promoter and enhancer driving gene expression to skeletal muscle. Pronuclear

technology was used to test the efficiency of the recombinant OTC expression constructs and their effect on the phenotype of the OTC deficient mouse models.

1.2 PROTEIN METABOLISM OCCURS IN THE LIVER AND PRODUCES INTERMEDIATE NEUROTOXIC METABOLITES

The liver plays a pivotal role in the metabolism of many waste and pharmacological substances prior to excretion. In particular, it is active in protein synthesis and the interconversion of amino acids. As a result of these metabolic processes the liver is involved in the formation of urea, the by-product of amino acid and protein metabolism in mammals.

As protein can only be stored to a limited extent, any imbalance in amino- acid availability versus requirement is corrected in the liver. The amino acids necessary for hepatic synthesis of protein are derived partly from the catabolism of dietary protein and partly from the metabolic turnover of endogenous proteins. During protein metabolism many neurotoxic nitrogenous compounds, such as ammonia, accumulate as by-products. In order to prevent damage due to the accumulation of these products they are incorporated into a water-soluble, non-toxic form, namely urea. Urea is safely transported to the kidneys and excreted in urine.

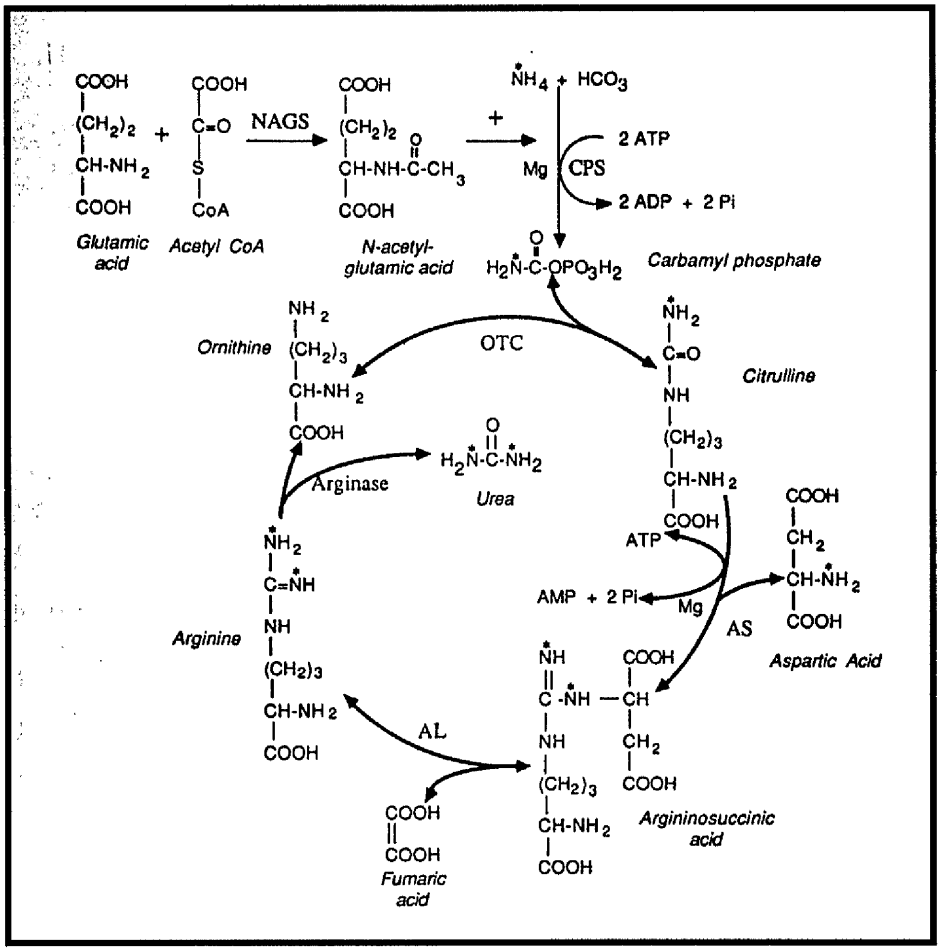
The formation of urea is controlled by a series of five biochemical reactions, called the urea cycle (Fig 1). This cycle is unidirectional, as the hydrolytic reaction catalysed by arginase is irreversible due to the excretion of the urea.

Although, the urea cycle occurs predominantly in the liver, other tissues including skeletal muscle, the epithelia of the small intestine and the kidneys also play a more

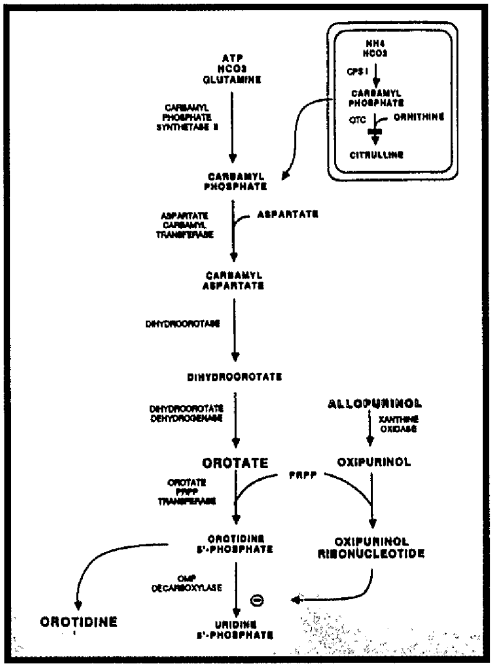
Figure 1. The urea cycle.

The urea cycle comprises five catalytic enzymes which are involved in the conversion of nitrogenous products, produced by the catabolism of amino acids, into an inert form, urea, that is excreted in the urine.

At the cellular level the urea cycle spans both the cytosolic and mitochondrial compartments and therefore involves the transportation of ornithine and citrulline across the mitochondrial membrane to complete the cycle.



A.



B.

Figure 1. The Urea cycle

peripheral role by producing or excreting many of the substrates or products involved in the cycle (Fig 2).

1.2.1 THE UREA CYCLE IS INTEGRAL TO THE CONVERSION OF NEUROTOXIC METABOLITES TO AN INERT WATER-SOLUBLE PRODUCT, UREA.

Within the liver it has been proposed that ureagenesis predominately occurs in the periportal rather than perivenular hepatocytes (Jungermann 1986). Within these periportal hepatocytes the urea cycle is further compartmentalised at the subcellular level between the mitochondria and cytoplasm. The first two enzymes in the urea cycle, ornithine transcarbamylase and carbamyl phosphate synthetase type 1 (CPS I), are mitochondrial matrix proteins, whilst later enzymes, argininosuccinate synthetase, arginosuccinate lyase and arginase, are active only in the cytoplasm (Fig 1).

In most instances, it is the non-protein amino acid, ornithine, which initiates the urea cycle, providing the molecular foundation on which urea is assembled. The initial reaction, incorporating ammonia into the urea cycle, involves the enzyme carbamyl phosphate synthetase type I. In the presence of its co-factor N-acetylglutamate (NAG), CPS I catalyses the production of carbamyl phosphate from ammonia and similar nitrogenous substrates. It has been suggested (Cooper, Nieves et al. 1988) that the extra-hepatic glutamate metabolism, predominately in skeletal muscle, provides the major source of ammonia for the carbamyl phosphate synthetase reaction, although in the liver deamination of the amino-acids histidine, tryptophan, threonine and lysine may also contribute to this ammonia pool.

In the next step in this cycle, OTC catalyses the formation of citrulline by transcarbamylation of ornithine with carbamyl phosphate. In the absence of

Figure 2. Tissues involved more peripherally in the urea cycle.

Although the urea cycle occurs predominately in the liver, it interacts with many other tissues in the body. A large proportion of the metabolic substrates for the urea cycle are formed in muscle and other tissues whilst the final product, urea, is transported to the kidneys for excretion. In addition, ornithine transcarbamylase is also expressed in the small intestine forming a partial urea cycle. As can be seen from this diagram, disruption to the urea cycle will manifest clinically as a multisystemic rather than purely hepatic disorder.

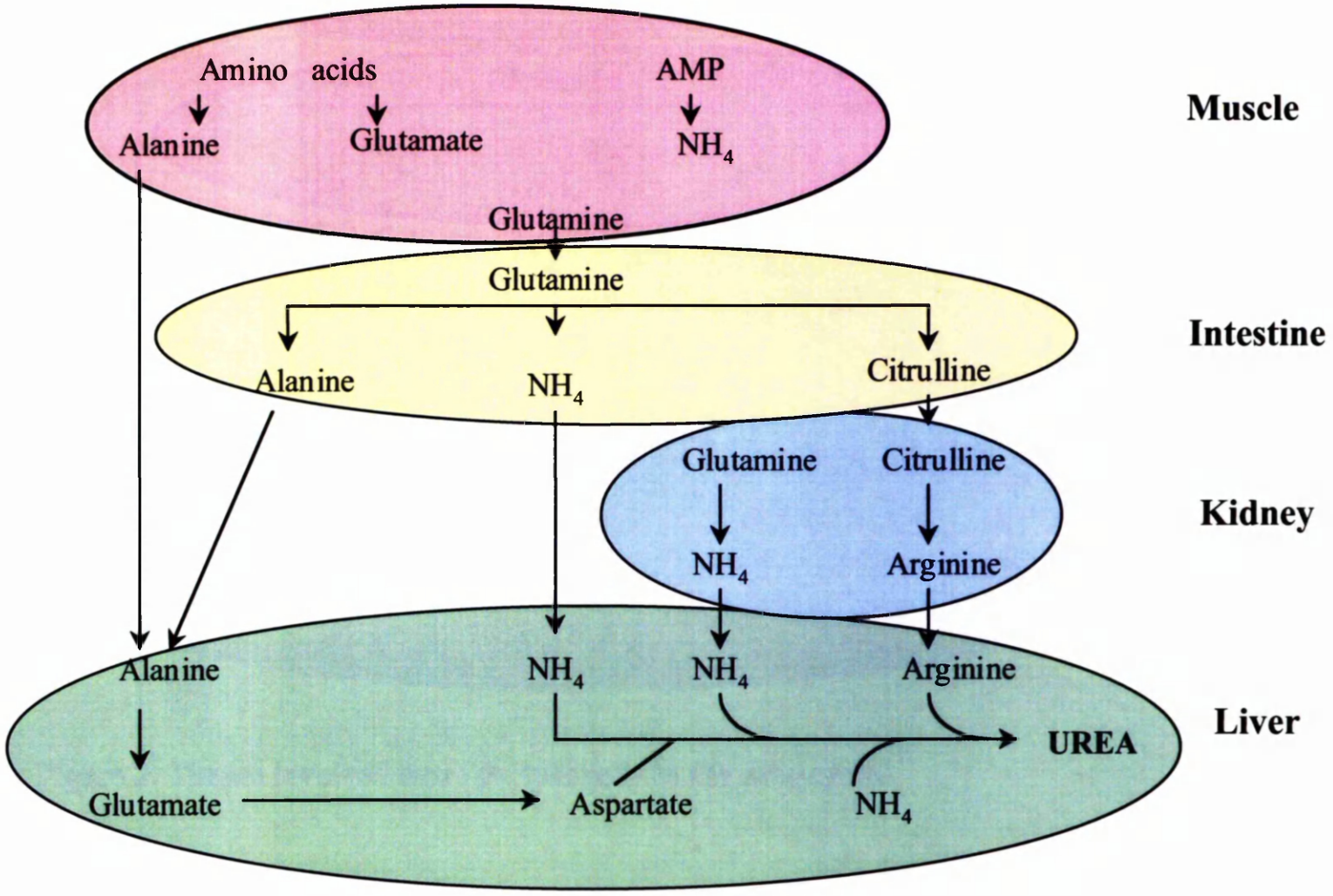


Figure 2. Tissues involved more peripherally in the urea cycle.

ornithine, CPS I activity has been shown to be impaired, due presumably to the accumulation of carbamyl phosphate, producing a negative feedback stimulus (Cohen, Cheung et al. 1980).

The citrulline formed by OTC activity is transported from the mitochondrion to the cytoplasm where it is complexed with aspartate to form argininosuccinate. This is in turn cleaved to form arginine and fumarate. The arginine is then hydrolysed by arginase to form urea, which is transported in the blood to the kidneys and excreted in urine. Ornithine is also released by the arginase reaction, and transported back into the mitochondrial matrix to complete the cycle. There is no net gain or loss of ornithine, as for each molecule of ornithine converted to citrulline in the mitochondrial matrix, a further molecule of ornithine is released by the metabolism of arginine into the cytoplasm.

In order for the urea cycle to function within two subcellular compartments, ornithine must pass from the cytosol to the mitochondrial matrix and citrulline must then leave the mitochondria in order to regenerate ornithine in the cytoplasm. This exchange between cytoplasm and mitochondrion is achieved by means of an active ornithine/citrulline carrier protein on the inner mitochondrial membrane (Indiveri, Tonazzi et al. 1992).

1.3 ORNITHINE TRANSCARBAMYLASE IS THE SECOND ENZYME IN THE UREA CYCLE.

As has been mentioned, ornithine transcarbamylase is the second enzyme of the urea cycle and is responsible for the formation of citrulline from the substrates carbamyl phosphate and ornithine. Citrulline is formed in the mitochondrial matrix of

hepatocytes through the transfer of the carbamyl moiety of carbamyl phosphate to the α-amino-nitrogen of ornithine. The major source of ornithine is from arginine in dietary proteins, and although vital for the urea cycle, it is also involved in other metabolic pathways. Ornithine metabolism is involved in the synthesis of creatine, and proline via ornithine aminotransferase (OAT) activity. Although, OAT activity is integral to the mitochondrial ornithine concentration in the liver, this enzyme is expressed in hepatocytes surrounding the pericentral vein of the hepatic lobules which do not express OTC and are not involved in the urea cycle (Kuo and Darnell 1991). Thus, there is no competition between OAT and OTC activity in their utilisation of available ornithine.

Ornithine acts as a substrate for OTC as well as a stimulator of CPS I (Stewart and Walser 1980). The concentration of ornithine in the mitochondrial matrix is dependent on the entry of ornithine from the cytoplasm and the activity of OTC. *In vitro* evidence suggests that the transported ornithine and carbamyl phosphate molecules are channelled directly to the ornithine transcarbamylase enzyme suggesting a physical organisation of the elements of the urea cycle within the mitochondrial matrix (Cohen, Cheung et al. 1987; Cohen, Cheung et al. 1992).

In addition to its presence in hepatocytes, OTC is also expressed in the small intestine in conjunction with CPS I. In this setting, it is thought to act as a nitrogen sink for glutamine, an important respiratory fuel of the bowel (Windmueller and Spaeth 1980). The citrulline produced is released into the circulation and taken up predominantly in the kidneys, where it is converted into arginine.

1.3.1 THE OTC PROTEIN COMPRISES A HOMOTRIMERIC STRUCTURE FOUND IN THE MITOCHONDRIAL MATRIX.

Mature mitochondrial human OTC was first purified in 1978 (Kalousek, Francois et al. 1978) and shown to be a homotrimer with 322 amino acids and a molecular weight of approximately 36 000 Daltons per polypeptide chain. Human OTC is translated on free cytoplasmic ribosomes as a 40 kDa precursor protein. The amino terminus of the OTC protein comprises a 32 amino acid 'leader peptide', containing eight basic but no acidic amino acids (Conboy and Rosenberg 1981; Mori, Miura et al. 1980). The basic nature of the leader peptide appears critical for the uptake of the OTC precursor polypeptide into the mitochondrion, as uptake is inhibited in the presence of histones and protamines (Mori et al 1982). When the OTC leader amino acid sequence is compared between two closely related species; namely human and rat, there is 73% sequence identity with the basic residues in the human enzyme aligning with those in the rat, again highlighting their functional importance (McIntyre, Graf et al. 1984). Site-directed mutagenesis studies indicate that the mid portion of the leader sequence with 3 highly conserved arginine residues, in particular arginine at amino acid 23, is essential for transportation of the protein subunit into the mitochondrial matrix (Horwich, Fenton et al. 1984; Horwich, Fenton et al. 1985). During transportation of the precursor protein into the mitochondrial matrix, the leader peptide is cleaved off in a two step process (Conboy and Rosenberg 1981; Kraus, Conboy et al. 1981). The resulting 36 kDa polypeptide forms a functional enzyme by homotrimerization and becomes attached to the inner mitochondrial membrane in the mitochondrial matrix. This homotrimeric structure has also been reported in rat and bovine ornithine transcarbamylase (Marshall and Cohen 1972; Clarke 1976).

Although the leader peptide only shows 73% amino acid sequence identity between rat and human, the remaining protein mRNA is 93% identical, suggesting that greater evolutionary constraint has been placed on the structure of the catalytic domains compared to the leader sequence. Two regions of particular importance correspond to the binding sites for the substrates, carbamyl phosphate and ornithine. These regions span amino acids 53-62 and 268-273, and are encoded by exons 3 and 9 respectively (Kraus, Hodges et al. 1985). These sequences are highly conserved in prokaryotic transcarbamylases.

Little is known about the tertiary structure of human ornithine transcarbamylase although models have been formed based on the 46% amino acid homology with *E.coli* aspartate transcarbamylase. These have been used to predict how mutations may alter the tertiary structure of the protein and thus affect its function (Tuchman, Morizono et al. 1995) (Fig 3).

1.3.2 OTC ACTIVITY FLUCTUATES WITH PROTEIN INTAKE

The most important factor influencing OTC activity is protein metabolism. The amount of nitrogenous waste required to be metabolised by OTC is dependent on both endogenous protein breakdown and dietary protein ingestion. A deficiency in OTC activity may only manifest when the protein load on the urea cycle exceeds that capable of being metabolised. This increase in protein degradation may be due to excessive dietary intake of protein or increased endogenous catabolism secondary to a systemic illness.

A recently described reaction, which may have a regulatory effect on the urea cycle, is the conversion of arginine to nitric oxide and citrulline (Kerwin and Heller 1994). This reaction is catalysed by nitric oxide synthetase (NOS) and although NOS plays

Figure 3. This is a representation of the speculative tertiary structure of OTC protein (Tuchman et al, 1995) based on the structure of *E. coli* aspartate transcarbamylase, which displays 50% and 75% identity at the sequence and amino acid level, respectively. The protein is a homotrimer with 322 amino acids and a molecular weight of approximately 36000 Daltons per polypeptide chain. The active protein comprises three identical polypeptides that are formed in the mitochondrion after removal of the amino terminal leader peptide. This protein model suggests that the catalytic ornithine and carbamyl phosphate binding sites form pockets in the internal core of the protein.

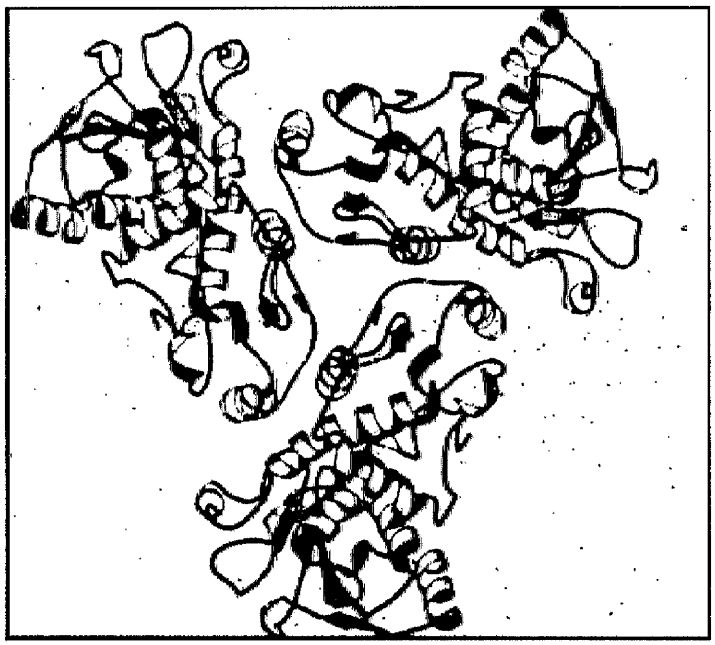


Figure 3. Speculative tertiary structure of the OTC homotrimeric protein.

a minor role in citrulline production, it is possible that alterations in its expression may be important in modifying the urea cycle disorder phenotypes.

Recent reports (Kay, Hilton-Jones et al. 1986; Honeycutt, Callahan et al. 1992; Leao 1995) have also implicated the anticonvulsant, valproate, in precipitating a hyperammonaemic crisis in OTC deficient heterozygotes and hemizygotes. It is thought this effect is due to valproate having a direct inhibitory effect on the urea cycle as well as a direct toxic effect on mitochondria.

1.3.3 OTC GENE EXPRESSION IS FOUND PRIMARILY IN THE LIVER AND SMALL INTESTINE.

Although one or more steps of protein precursor import and processing may themselves be subject to certain regulatory controls (Raymond and Shore 1981), the main elements regulating the level of OTC expression involve a range of developmental, hormonal and dietary controls over the amount of messenger RNA which accumulates in the cell (Mori et al, 1981; Cohen, 1970; Pouchelet and Shore 1981); Rozen, Noel et al. 1983; Ryall et al 1984). Thus, regulation is at the transcriptional rather than the post- transcriptional level.

Ornithine transcarbamylase is expressed in the small intestine as well as the liver, although intestinal epithelium does not possess a full urea cycle (Ryall, Nguyen et al. 1985; Raijman 1974). The citrulline produced in the small intestine due to the presence of CPS I and OTC, is primarily an end product of metabolism of ammonia. The citrulline is then transported to the peripheral tissues, predominantly the kidneys where *de novo* synthesis of arginine occurs (Windmuellar et al, 1981). Studies have shown that rodent intestinal OTC activity varies from 0.02 to 10% of hepatic values (Raijman et al. 1975, Jones et al. 1961) (Fig2), whilst intestinal mRNA is about 50%

of hepatic levels (Ryall, Nguyen et al. 1985). The ornithine transcarbamylase protein is thought to have a half-life of 6-9 days (Wallace, Knecht et al. 1986) whilst the life span of intestinal epithelium is thought to about 5 days.

1.4 FEATURES OF OTC DEFICIENCY

1.4.1 OTC DEFICIENCY IS A X-LINKED DISORDER.

Ornithine transcarbamylase deficiency is the commonest inborn error of urea synthesis with an estimated incidence of 1:40-80 000 births (Nagata, Matsuda et al. 1991). Although, mitochondria contain an intrinsic genome encoding 37 genes, the majority of mitochondrial proteins are encoded in the nuclear genome, synthesised in the cytoplasm and then transported into the mitochondria. All the enzymes in the urea cycle are encoded in the nuclear genome.

In contrast to the other enzymes in the urea cycle, which show an autosomal recessive inheritance, ornithine transcarbamylase deficiency shows an X-linked inheritance pattern. This was first demonstrated in 1972 when Scott (Scott, Teng et al. 1972) described two pedigrees which exemplified i) the severe nature of the disorder associated with almost complete absence of the protein in males, and ii) the wide variation in clinical severity and enzyme levels in heterozygous women associated with hepatic mosaicism (Ricciuti, Gelehrter et al. 1976). Further clinical studies (Short, Conn et al. 1973; Campbell, Rosenberg et al. 1973) strengthened this hypothesis as did the finding in 1976 that the OTC deficiency was X-linked in the mouse (DeMars, LeVan et al. 1976).

In 1984 the OTC gene was mapped to Xp21.1 (Lindgren, de Martinville et al. 1984) and cloned (Horwich, Fenton et al. 1984). By means of segregation analysis (Bonaiti-Pellie, Pelet et al. 1990) as well as specific mutation analysis (Tuchman, Matsuda et al. 1995) the proportion of males and females with OTC deficiency

whose mutation was inherited from one of their parents has been estimated. Of 28 total cases only 2 (7%) of OTC deficient males as opposed to 12 (80%) of affected females had new mutations. Further evidence for this high proportion of *de novo* mutation in heterozygote females has also documented more recently (Leibundgut, Liechti-Gallati et al. 1995). This information is of important clinical relevance as it suggests that the prior risk of a mother of an affected male being a carrier of the condition is about 9/10 or higher, whilst the mother of an affected daughter has only a 2/10 risk of being a carrier.

1.4.1.1 Clinical features of OTC deficiency vary in severity not only between affected males and females, but also within the hemizygous affected male group.

A deficiency of ornithine transcarbamylase was first identified in 1962 as a cause of protein intolerance and hyperammonaemia in children (Russell et al 1962). The initial cases described the clinical condition in heterozygous females which was due to the higher survival rate in females compared to males allowing time for the diagnosis to be made. As an X-linked disorder, OTC deficiency produces different clinical pictures in hemizygous males and heterozygous females.

1.4.1.1.1 The classical clinical picture of OTC deficiency occurs in neonatal males.

The most dramatic clinical presentation of OTC deficiency occurs in newborn males as a catastrophic hyperammonaemic encephalopathy associated with high mortality. This is the most commonly described clinical picture. It often occurs within a few hours or days of birth when feeding, and thus protein intake, is established. *In utero*

the maternal circulation and hepatic pathways are responsible for clearing toxic by-products of foetal protein metabolism.

Initial symptoms of lethargy, irritability and poor feeding, if not recognised and treated, can progress to convulsions, coma, apnoea, areflexia and eventually to death. The mortality rate for neonatal hyperammonaemic coma approaches 50%, with each subsequent hyperammonaemic crisis carrying a further 10% mortality rate (Batshaw, Brusilow et al. 1982). Males who do survive the neonatal period often have a poor neurological outcome, with a high incidence of mental retardation, cerebral palsy, and seizures (Msall, Batshaw et al. 1984; Nagata, Matsuda et al. 1991).

1.4.1.1.2 Late Onset presentation in males can be less severe although mortality is still high.

There have also been reports of males diagnosed with OTC deficiency without neonatal symptoms. The age of first onset of symptoms have ranged from childhood to 58 years old (Yoshino, Nishiyori et al. 1990) and the symptoms themselves may be initially quite non-specific (Oizumi, Ng et al. 1984). This group of males have been further sub-divided into those symptomatic after 28 days old but before the age of 5 (group 2) and those symptomatic at a later age (group 3) (Matsuda, Nagata et al. 1991). In a recent survey, the highest mortality rate was in those males in group 3, followed by group 1 (neonatal onset). The causes of higher mortality in this older age group may relate to a delay in diagnosis and treatment as well an impaired capacity to recover from multisystem organ failure; factors which appear to be age dependent. Group 2 had a lower mortality rate and a lower incidence of mental

abnormality compared to the other groups, which correlated with only a moderate OTC enzyme deficiency.

Interestingly, although group 3 patients had a reduction in OTC activity comparable with group 1, they had normal citrulline levels. However, it was noted that the hepatic OTC enzyme activity in group 3 was measured at *post mortem* and the lability of the OTC protein may preclude accurate OTC activity assessment after autolysis. This would explain the lack of correlation between OTC enzyme activity and plasma citrulline levels in patients in group 3.

1.4.1.1.3 Female heterozygous carriers are often asymptomatic.

Heterozygous females may be overtly symptomatic or asymptomatic depending on allelic heterogeneity (Rowe, Newman et al. 1986), the residual hepatic OTC activity due to the effect of random X-chromosome inactivation in hepatocytes, and on the endogenous and exogenous nitrogen load on the urea cycle. Obligate female carriers as determined by pedigree analysis often have an aversion to protein and therefore unwittingly often decrease the risk of an acute hyperammonaemic crisis by modifying their diet themselves.

Approximately 15% of carrier females are overtly symptomatic. A review of 13 symptomatic heterozygotes (Rowe, Newman et al. 1986) showed the age of presentation could vary greatly from birth to six years old and that, as in the case of affected males, the symptoms could be non-specific such as episodic irritability (100%), episodic lethargy and vomiting (100%), protein avoidance (92%) and ataxia (77%). However, even asymptomatic carriers are thought to be at an increased risk of hyperammonaemic coma during pregnancy (Arn, Hauser et al. 1990) or due to a prolonged catabolic period such as during a severe generalised illness.

1.4.1.2 OTC DEFCIENCY PRODUCES A MARKED BIOCHEMICAL PROFILE WHICH OFTEN ALLOWS DIAGNOSIS

1.4.1.2.1 Affected males show a marked elevation in plasma ammonia and abnormal pyrimidine metabolism.

In OTC deficiency, a pronounced plasma hyperammonaemia (~2000 mmol/L, normal <50 mmol/L) is found in association with an elevation in plasma glutamate levels. However, this is not pathognomic for the condition as it is often found in association with a more general aminoaciduria. In addition to these changes, there is the expected decrease in plasma citrulline and arginine. This metabolic disturbance leads to hyperventilation due to cerebral irritation, which exacerbates the situation producing a respiratory alkalosis rather than the metabolic acidosis usually seen in the organic acidaemias.

A more distinctive metabolic abnormality found in OTC deficiency is an increase in pyrimidine metabolites, in particular orotic acid, uridine and uracil. These are excreted in urine producing a characteristic orotic aciduria. This accumulation of pyrimidine products results from the under-utilisation and overflow of carbamyl phosphate produced in the mitochondria into the cytoplasm. These products are converted to carbamyl aspartate and thence channelled into the pyrimidine synthesis pathways (Fig 1).

In males presenting in the neonatal period, these biochemical indices, in addition to the clinical examination, allows the diagnosis to be made. The history of a previous neonatal male death within the family further compounds the diagnosis. However, in an older male, a liver biopsy may be required to differentiate OTC deficiency from Reyes syndrome, which has similar clinical picture but shows a more general

reduction in hepatic enzymes including carbamyl phosphate synthetase type 1 in addition to OTC.

1.4.1.2.2 Female Carriers only show a metabolic disturbance during a symptomatic crisis.

Symptomatic carrier females are diagnosed during an acute metabolic crisis when their biochemical indices are identical to affected males. The major diagnostic problem encountered is in an asymptomatic female with an affected sibling, the sister of a mother with an affected son or a mother with an affected daughter, as baseline metabolic indices in such cases are normal. As mentioned previously, the mothers' of affected boys have a 9 in 10 chance of being a carrier of an OTC mutation. Establishing the carrier status of women 'at risk' of ornithine transcarbamylase deficiency is important both for determining reproductive as well as medical risks for the individual.

The gold standard by which other carrier status assessments are compared is initial mutation analysis in the affected boy, followed by genomic screening of the high-risk female for this mutation. If direct DNA mutation testing is not feasible, metabolic screening procedures are employed which measure the biochemical response to hyperstimulation of the urea cycle. This hyperstimulation originally took the form of a high protein meal, but as this could trigger an acute symptomatic crisis in a heterozygote carrier, it has been replaced by the measurement of urinary orotidine or orotic acid excretion after an allopurinol challenge test (Hauser, Finkelstein et al. 1990; Burlina, Ferrari et al. 1992). Allopurinol is metabolised to oxypurinol ribonucleotide, which has an inhibitory effect on an enzyme in the pyrimidine pathway namely orotidine 5' phosphate decarboxylase. This enzyme

inhibition causes orotidinuria and orotic aciduria which is more pronounced in individuals with OTC deficiency than in normal individuals. These biochemical screening procedures are often used in association with molecular linkage analysis in suitable families in strengthening and confirming carrier status.

Interestingly, the clonal nature of epithelium found in intestinal crypts has also been used as a novel alternative to hepatic biopsy as a possible means of carrier detection (Hamano, Kodama et al. 1988). A duodenal biopsy is stained by means of immunohistochemistry for the OTC protein and if the carrier female has a null mutation two subsets of intestinal crypt cells are seen, those showing the presence of the OTC protein and those with absent protein. This technique has the advantage of having its own internal control. However, it is highly invasive and will only work if the OTC gene encodes a null mutation. Missense mutations may still produce protein capable of being recognised by the antibody.

1.5 OTC Gene Structure

1.5.1 The OTC gene is 73 kb long comprising 10 exons.

The human OTC cDNA was cloned in 1984 by Horwich *et al* (Horwich, Fenton et al. 1984). Subsequently Hata derived the full human ornithine transcarbamylase gene sequence from a human liver genomic library in 1988 (Hata, Tsuzuki et al. 1988). The genomic sequence is 73 kb long comprising 10 exons, interrupted by 9 introns of varying sizes. The exons tend to be relatively uniform in size, spanning about 140 bp, the last being the largest at 764 bp. The smallest intron, intron 7, is 80 bp whilst the largest, intron 4, spans 21.7 kb. All intron/exon boundaries comply with the well-documented GT-AG splice junction rule (Mount et al 1982). The

nucleotide sequence of an intron commences with the nucleotide pair 'GT' and finishes with the 'AG' pair. The splicesome recognises these nucleotide pairs as flanking an intron and spices out the intervening sequences to join adjacent exons together.

Four useful polymorphisms have been described, and approximately 70-80% of females are informative for one of these RFLPs (Schwartz, Christensen et al. 1986), (Spence, Maddalena et al. 1989).

Studies using a hepatoma cell line, Hep G2, revealed that in the promoter region of the rat OTC gene there are two cis-acting elements (Murakami et al, 1990), both of which are recognised by at least two members of the steroid receptor superfamily: hepatocyte nuclear factor-4 (HNF-4) (Sladek et al 1990) which activates transcription of a co-transfected OTC promoter construct, and chicken ovalbumin upstream transcription factor (COUP-TF) (Wang, Tsai et al. 1989) which represses transcription (Kimura, Nishiyori et al. 1993). The homologous OTC promoter used *in vivo* produced high OTC transcription in the small intestine but the presence of a 110bp enhancer region 11kb upstream of the promoter is capable of activating transcription in a more endogenous liver-specific manner. This enhancer contains four protein binding sites, two for hepatocyte nuclear factor-4 (HNF-4) and two for CCAAT/enhancer binding protein (C/EBP), both of which are liver-selective transcription factors and both of which have to be present for activation of the reconstituted enhancer in non-hepatic cells (Nishiyori et al 1994).

1.5.1.1 THE MAJORITY OF OTC MUTATIONS ARE NOVEL.

An up to date summary of mutations in the OTC gene currently described can be found in the human gene Mutation Database, Cardiff

(http://www.uwcm.ac.uk/uwcm/mg/ns/2/). To date there have been 142 mutations described. 74% are nucleotide substitutions (missense or nonsense), 15% nucleotide substitutions causing aberrant splicing, 7% small deletions, 2% small insertions, 1% gross deletions with as yet no descriptions of gross insertions, duplications or regulatory mutations, although this may indicate an ascertainment bias. 9 recurrent substitutions were observed, R129H, R40H, K88N, P225L, G195R, R92Q, R141Q, A209V, N161S, in order of frequency. An increase in substitutions were also noted at CpG 'hot spots' and it has hypothesised that as methylation of CpG dinucleotides is more pronounced in sperm genes than egg genes (Driscol and Migeon 1990), this may predispose mutations to arise more often in the paternal than maternal allele, as shown in previous studies into OTC inheritance.

Although the mutations are distributed throughout the OTC gene, there appears to be disproportionally higher number in exons 3 (the putative binding site for carbamyl phosphate), exon 6 and 5, in decreasing order.

1.5.1.1.1 Genotype/phenotype correlations in OTC deficiency are not always possible

Amongst the mutations found in affected males, there are almost twice as many associated with a neonatal severe phenotype than have been associated with a milder phenotype. This is probably due to a bias of ascertainment as patients with a milder phenotype may be under diagnosed and these mutations underrepresented.

Certain mutations such as R40H, R227Q and R277W have been found to be associated with a milder later onset of symptoms. It is believed that the arginine at codon 227 may interact with a conserved glutamate at position 52 and thus affect the overall tertiary structure of the enzyme.

In vitro Cos cell expression studies (Morizono, Listrom et al. 1997; Matsuura, Hoshide et al. 1994) have shown the pathogenic nature of some missense mutations, such as R227Q, R277W, T264A, D196V, E154X and G195R by demonstrating a relative decrease in mutant OTC activity compared to wild type. The level of OTC in this *in vitro* assay did correlate in general with the clinical history, the greater the residual enzymatic activity, the later the onset of clinical symptoms.

In 1995 Tuchman (Tuchman, Morizono et al. 1995)(Fig 3) generated a three dimensional model of the human OTC protein from comparisons with the crystalline structure of the *E.coli* aspartate transcarbamylase, by aligning sequences and building in gaps. In general, this model suggests that mutations associated with the more severe neonatal onset affect one of three regions, namely the interior active sites for carbamyl phosphate, the ornithine binding site or the core of the domains at the inter-chain interface. The mutations associated with a milder phenotype tend to cluster around regions on the outer surface of the protein.

Although a tentative correlation between mutation, protein structure and enzymatic function is possible, environmental and additional genetic factors also appear to play a role in determining phenotype. In family mutation tracking studies (Matsuda, Matsuura et al. 1996) the A40H mutation has been shown to be associated with a range of age of onset of symptoms of between 6 to 56 years. A 65-year old asymptomatic man was also noted, although the possibility of a mosaic genotype could not be excluded as he was at the top of the pedigree. This variation in phenotype has also been reported in the A208T mutation (van Diggelen, Zaremba et al. 1996).

This expressivity may correlate with dietary protein intake. As different mutations in the OTC protein reduce its functional capacity to different degrees, the phenotype associated with a mutation will also be dependent on the requirement of OTC

function, namely the amount of nitrogenous waste from ingested protein required to be metabolised by the urea cycle. Thus, the amount of protein ingested by a person may be a predisposing factor in genotype/phenotype correlation. This has been suggested by Matsuda (Matsuda, Matsuura et al. 1996) as an explanation to the differing phenotypes demonstrated by males with the same mutation. An alternative explanation would be the segregation of other modifier genes within the different families.

1.5.2 THE TREATMENT OF OTC DEFICIENCY IS NOT OPTIMAL

Present treatment for OTC deficiency is still sub-optimal with affected boys and symptomatic females often still developing neurological sequelae.

1.5.2.1 ACUTE PHASE TREATMENT IS AN INTENSIVE CARE SITUATION

In the acute phase the children are treated as a neonatal emergency. All protein intake is stopped to reduce the requirement for urea biosynthesis, and the plasma ammonia level monitored. Peritoneal- or haemo-dialysis is required if the ammonia continues to rise. This will also correct the profound alkalosis. It has been shown that haemodialysis is the most effective method of lowering blood ammonia levels (Donn, Swartz et al. 1979). Often the children require both ventilatory and circulatory support during the acute phase.

1.5.2.2 LONG TERM MANAGEMENT MUST BE MAINTAINED AT HOME

After the acute phase, a severely restricted protein intake is initiated, although essential amino acids are required for growth and development in young children. In the long-term management of these patients pharmacological induction of

alternative pathways through oral supplementation, in association with strict protein limitation has been shown to be effective. The former is best achieved by oral sodium phenylbutyrate or sodium benzoate supplementation. Sodium phenylbutyrate is a precursor of phenylacetate which conjugates with glutamine to form phenylacetylglutamine, a waste nitrogen compound that is excreted in the urine. By this means nitrogen is diverted from the urea cycle, which is suppressed, although if residual OTC activity is present, this can be re-activated to maintain nitrogen homeostasis (Brusilow and Finkelstien 1993).

This treatment is effective both given intra-venously, in the acute episode, or orally for more long-term control. A recent study (Maestri, Brusilow et al. 1996) indicated that sodium phenylbutyrate or -acetate was more effective in reducing the frequency of hyperammonemic episodes in symptomatic female carriers than sodium benzoate. Mortality was also reduced to 10% over 5 years. Prior to this form of treatment a high rate of mortality of 82% from hyperammmonaemic-induced coma had been reported in affected males (Batshaw et al, 1986).

1.5.2.3 SURGICAL LIVER TRANSPLANTATION IS AN OPTION

Ultimately for long term correction of OTC deficiency, orthotopic liver transplantation has been shown to be successful, both for symptomatic females and neonatal onset male patients (Hasegawa, Tzakis et al. 1995). However, this surgical procedure, rarely performed due to the paucity of suitable livers, is associated with a high mortality and morbidity.

1.6 OTC DEFICIENCY IS AMENABLE TO GENE THERAPY APPROACHES

Both surgical and pharmacological approaches to the treatment of OTC patients have inherent problems. Liver transplantation and subsequent immunosuppressive therapy is associated with a high morbidity and mortality, while a pharmacological approach is less effective in both the acute and chronic situation. As these treatments are sub-optimal, a more novel gene therapeutic approach to treatment would certainly be beneficial. Furthermore the study of OTC will advance our understanding of gene therapy in general. The advantage of OTC as a model system for investigating gene therapeutic strategies is the availability of two naturally occurring mouse models.

1.6.1 OTC DEFICIENT MOUSE MODELS OCCUR IN NATURE.

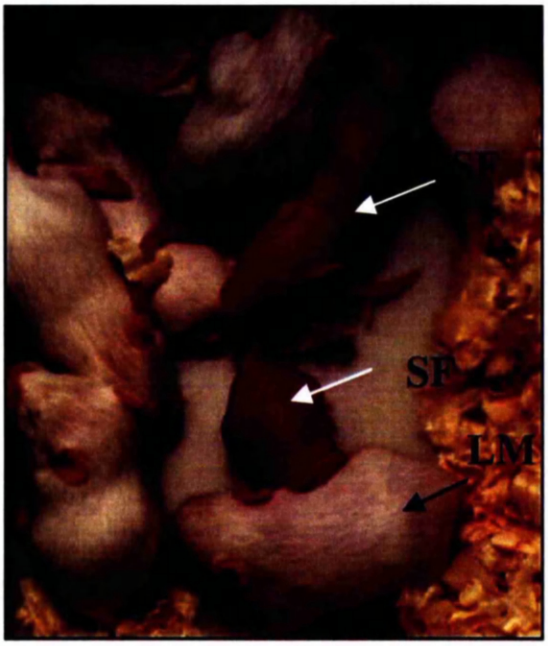
There are two laboratory mouse models for OTC deficiency; namely the sparse fur (Spf) and sparse fur and abnormal skin and hair (Spf^{ASH}). As implied, these mice have a distinctive dermatological phenotypes associated with growth retardation, reduced survival and biochemical abnormalities seen in OTC deficiency (Fig 4).
Both mouse models have low OTC activity in the liver and small intestine (Malo, Qureshi et al. 1986), (Dubois, Cavard et al. 1988).

1.6.1.1 SPARSE FUR MOUSE

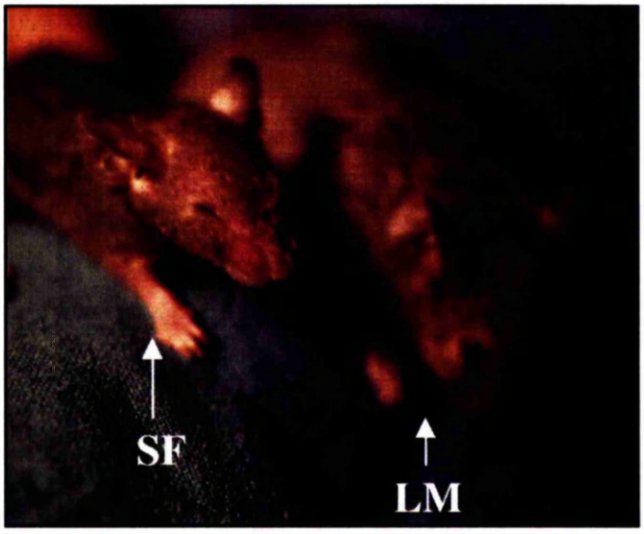
In 1976 DeMars and colleagues (DeMars, LeVan et al. 1976) demonstrated that the Spf mouse had a deficiency in OTC. The hepatic enzyme activity in these mice was subsequently demonstrated to be 10-15% of normal levels at physiological pH 7.0

Figure 4. The OTC deficient Spf mouse phenotype.

There are two naturally occurring mouse models of OTC deficiency, namely the sparse fur and sparse fur^{ASH} mice. On the mixed (FVB) x (C57Bl/6J) genetic background used in this study, there was little difference noted between the phenotypes of affected adult males in the two different mouse colonies, although there was a higher and earlier mortality rate in the Spf^{ASH} colony. The affected males are first noted due to growth retardation and delayed hair development at a week of age. There is a large variation in both these characteristics within the affected male population. The majority of the affected males survive although they often have delayed weaning due to their growth retardation. As adults, the phenotype improves although the mice often remain small and they have a thinner, waxy appearance to their coat. In figure 4 A) Spf pups (SF) are shown at 3 weeks of age with their larger litter mates (LM). Note the delayed hair growth, wrinkled skin and severe growth retardation. Figure 4 B) shows an adult Spf affected male (SF) at 6 weeks with litter mate (LM). Note the growth retardation and poor hair growth



A



B

Figure 4. The OTC deficient Sparse fur mouse phenotype

and 200% at pH 10 (Qureshi, Letarte et al. 1979). Molecular analysis characterised the mutation as a cytidine to thymidine nucleotide transition causing a histidine to asparagine substitution at amino acid 117 (Veres, Gibbs et al. 1987). Recent biochemical analysis of these mice (Batshaw, Yudkoff et al. 1995) indicated a mean hepatic OTC activity of 13% of normal, plasma glutamate levels 160% of normal and citrulline levels 25% of control values. Urinary orotic acid was elevated to a mean of 13 fold above controls. The longevity of affected males was documented as <10% compared to unaffected males on C3H/Hej and C57Bl/6J backgrounds. Behavioural and learning deficits were also noted. The level of hepatic OTC activity in the mice was found not to be related to age, contrary to results from Gushiken (Gushiken, Yoshimura et al. 1985) who demonstrated a mild improvement in OTC activity with increasing age.

1.6.1.2 Sparse Fur^{ASH} Mouse

A second sex-linked mutation affecting hair development in mice was discovered by Hulbert and Doolittle in 1971. This was subsequently found to be allelic to the Spf mouse mutation (Doolittle, Hulbert et al. 1974). The molecular cause of this OTC deficiency was described in 1989 (Hodges and Rosenberg 1989) as a guanine to adenine transition in the last nucleotide of the fourth exon of the OTC gene resulting in ~5% of wildtype OTC activity. This mutation causes a dual molecular pathology. Firstly, as a splice site mutation, it causes the transcription of two mRNAs, one correct mRNA and a second elongated mRNA incorporating 48 bases of the subsequent fourth intron. In addition, to the production of two mRNAs, transcription is inefficient and the total OTC mRNA level is reduced to about 10% normal. Secondly the missense mutation results in a change of the amino acid at position 129

from arginine to histidine. This alteration appears to have little effect on the activity of the OTC protein produced. The total effect is that the hepatic OTC activity in Spf^{ASH} mice is 3% of wildtype mice.

Interestingly, two Spanish patients have also been described with the same mutation as seen in Spf^{ASH} mice (Garcia-Perez, Sanjurjo et al. 1995). These patients have a similar residual OTC activity as the murine model when measured in the small intestine by jejunal biopsy. Despite low OTC activity, the males that survived the initial episode developed normally on treatment and the age of the initial symptomatic episode varied from an acute and fatal neonatal form to a milder form presenting only age 3 and half years.

1.6.2 PREVIOUS APPROACHES TO GENE MANIPULATION OF OTC DEFICIENCY HAVE BEEN TRIED IN THESE MURINE MODELS

The two main models for investigating *in vivo* supplementive gene therapy in the Spf and Spf^{ASH} mice have been I) the use of adeno-viral vectors, due to their natural hepatotrophic properties and II) transgenic mouse generation. Both of these systems have been used to target the OTC gene expression to its endogenous target tissues- the liver and small intestine. The transgenic model is a powerful system in which to study the *in vivo* efficacy of expression of different promoter/OTC cDNA constructs in correcting the enzymatic deficiency, whilst the use of adenoviruses provides a good model for delivery of the OTC construct to the liver, which may in the future be more directly applicable in the clinical sphere.

1.6.2.1 ADENOVIRAL APPROACHS HAVE BEEN SUCCESSFULLY USED

Recombinant adenoviruses have been evaluated as vectors for gene therapy in a variety of metabolic disorders (Ishibashi, Brown et al. 1993), (Engelhardt, Yang et al. 1993). The advantage of adenoviruses over the very commonly used gene transfection vector, the retrovirus, is that they can incorporate much larger gene expression constructs, they do not require cell proliferation for productive transfection and they can easily be produced and purified at high titres. Recombinant retroviruses (Grompe, Jones et al. 1992) and adenoviruses driven by the SRα viral promoter (Morsy 1993) have been to shown to correct *in vitro* cultures of both murine Spf and Spf^{ASH} and human OTC deficient hepatocytes.

Adenovirus is efficiently targeted to hepatocytes *in vivo* following intravenous infusion and high levels of transgene expression can be achieved in virtually 100% of hepatocytes, most of which are fully differentiated and non-dividing. However, a major drawback in adult mouse model gene transfer to the liver by means of adenoviral vectors is that the transgene expression is usually transient, often lasting less than 21 days, and it is associated with a severe hepatitis (Ishibashi, Brown et al. 1993; Kozarsky, Grossman et al. 1993; Yang et al 1994, 1994b). This is probably due to immunological rejection of infected cells.

In 1990 Stratford-Perricaudet injected newborn Spf^{ASH} with adenovirus carrying the rat OTC cDNA under the control of the viral major late promoter (Stratford-Perricaudet, Levrero et al. 1990). The mice were then sacrificed at different time points. Although the hepatic OTC activity achieved varied greatly, in those five out of the seventeen mice whose activity was ~50% of normal, phenotypic correction was noted. In addition, in the two mice who survived to 15 months, the orotic aciduria was also partially corrected. The OTC activity obtained using adenoviral expression vectors seemed to vary with different promoters and species of OTC

cDNA used. A recent report (Kiwaki, Kanegae et al. 1996) infusing the human OTC cDNA driven by a CAG promoter (a modified β-actin promoter with CMV-IE enhancer) showed good amelioration of the OTC deficiency in Spf^{ASh} mice, in contrast to a similar construct under the transcriptional control of a SRα promoter (the SV40 early promoter with the R segment and part of the U5 segment of the HTLV-1 LTR).

The use of newborn mice in both this study and the previous Stratford-Perricaudet study avoided the issue of adeno-viral induced hepatitis. Newborn mice are more tolerant to the immunological sequelae of adenovirus treatment as their immune systems are still relatively immature and therefore less likely to mount a full reaction to the viral epitopes. This would of course not be the case in the clinical setting of adenoviral exposure. In order to address this, Ye et al (Ye, Robinson et al. 1996) took a similar adenoviral approach but targeted mature mice between 6-8 weeks old. Initial studies using a human OTC cDNA driven from a CMV enhancer and β–actin promoter showed little effective correction of the metabolic abnormality in Spf male mice, but further studies using stronger CMV promoter and enhancer linked to an isogenic murine OTC cDNA produced a marked improvement in the metabolic indices, with urine orotate and plasma glutamine normalising within 2-3 weeks. This improvement persisted for 2-3 months. Obviously when targeting mature mice, the phenotypic changes associated with OTC deficiency have already been established and therefore they cannot be used as a marker of metabolic correction by the OTC construct. In addition, in adult Spf mice the levels of OTC produced after adenoviral infusion of the murine OTC cDNA was sufficient to protect against the metabolic and neurological insult produced by an intraperitoneal ammonium challenge (Ye, Robinson et al. 1997).

1.6.2.2 TRANGENIC APPROACHES HAVE ALSO BEEN SUCCESSFUL

1.6.2.2.1 Transgenic technology

The term 'transgenic' is used to denote animals that have integrated foreign DNA into their genome as a consequence of experimental introduction of DNA. The first transgenic mice were produced in 1974 by microinjecting SV40 viral DNA into the blastocoele cavity of early embryos (Jaenisch and Mintz 1974). The majority of transgenic animals are now produced by micro-injecting recombinant DNA into the pronuclei of fertilised eggs as first performed by Gordon *et al* in 1980 (Gordon, Scangos et al. 1980). The DNA integrates into the chromosomal DNA and can be transmitted through the germ-line. When more than one transgene copy integrates, which is not uncommon with micro-injected DNA, the multiple copies are typically arranged in tandem head to tail arrays. This arrangement is thought to be the result of homologous recombination between the injected molecules (Brinster, Chen et al. 1981). Although the integration of recombinant DNA occurs at random, endogenous cis-acting sequences at the site of integration can have an effect on the expression pattern of the exogenous transgene. In addition, the integration of the transgene may disrupt an endogenous gene and cause embryonic lethality when the transgenic line is bred to homozygosity (Jaenisch, Harbers et al. 1983), (Wagner, Covarrubias et al. 1983). Thus, the phenotype is not necessarily defined by the endogenous characteristics of the transgene, but by the integration site. It is therefore important to characterise more than one line to prove a transgene-phenotype correlation.

1.6.2.2.2 OTC transgenic work has relied on general viral promoter

Transgenic research in the OTC field has concentrated on the expression of the OTC gene in liver by means of the endogenous OTC promoter or a strong general viral gene promoter. The first preliminary report of transgenic correction of murine OTC deficiency was in 1988 by Cavard *et al* (Cavard, Grimber et al. 1988) .One founder mouse was produced which expressed the rat OTC cDNA from a SV40 early promoter. Phenotypic and partial metabolic correction of a Spf^{ASH} phenotype was observed in the male progeny from this founder mouse, despite Southern blot results which suggested the transgene had been rearranged at the genomic level during integration. Transgenic mRNA expression and OTC activity were found in the endogenously expressing tissues, namely the small intestine and liver. Despite no detectable mRNA, OTC activity was also detected in the spleen and lung. The transgene OTC activity in both liver and small intestine was 80% of wildtype control mice compared to a mRNA level of only 50%. This result, based on only one founder transgenic line was particularly interestingly as the mRNA tissue specificity of the transgene was very similar to the endogenous OTC gene expression despite being driven from a general viral promoter. This could suggest a cell specific mRNA stability phenomenon.

A similar approach was taken by Shimada *et al* in 1991 (Shimada, Noda et al. 1991). In this study a transgenic mouse expressing the rat OTC cDNA driven by 1.3 Kb of the 5' flanking region of the rat OTC promoter was bred onto a Spf^{ASH} background. This expression construct had previously shown a low hepatic specificity due to the absence of the 110 bp hepatic-specific enhancer element identified 11 Kb upstream of the promoter sequence (Murakami, Takiguchi et al. 1989). The transgenic Spf^{ASH} males had corrected phenotypes, with an increase in hepatic OTC activity of 1.9 fold (equivalent to 12% of control mice) and in the small intestine of 6.3 fold (27% of

control mice). In addition, there was a significant decrease in urinary orotic acid production and elevation of citrulline. These mice were also studied with regard to OTC activity during overnight-starvation and nitrogen loading (Saheki, Mori et al. 1995). After a nitrogen load the transgenic Spf^{ASH} male mice excreted much less orotic acid than their non-transgenic Spf^{ASH} littermates, suggesting the transgene had an ameliorating but not normalising effect on orotic acid production under stressed conditions. The milder stress imposed by overnight starvation was not found to have any effect on urinary orotic acid levels in transgenic mice. Further evidence suggested the orotic acid levels in the transgenic mice correlated better with intestinal rather than hepatic OTC activity, a surprising finding as orotic aciduria is also associated with arginosuccinate synthetase and arginase deficiency, which are known to be confined to liver and not the small intestine. However, these results were only preliminary and may in fact indicate an indirect effect of intestinal OTC activity on hepatic production of orotic acid.

A similar transgenic approach was taken by Jones *et al* resulting in comparable findings. Utilising 750 bp of the mouse OTC 5' flanking region linked to the human OTC cDNA, an increased small intestine compared to hepatic OTC activity was found, due again to lack of the hepatic specific gene regulatory sequences (Jones, Grompe et al. 1990). This increase in intestinal expression alone was sufficient to correct the phenotype of the Spf mice and reduce orotic aciduria.

1.7 ECTOPIC GENE EXPRESSION

Although, the previously discussed body of work has concentrated on the supplementation of OTC gene expression in its endogenous tissue, many studies

have shown that the expression of genes in target tissues which do not normally express them can also produce functional proteins. Two commonly studied organs used for this ectopic expression of genes are striated muscle and epidermis. These tissues have the advantage of being comprised of a large mass of homogenous cells with a good blood supply and relatively easily accessible.

1.7.1 The Epidermis is a versatile organ for exogenous gene expression

The epidermis is the outer layer of the skin and, as a stratified squamous epithelium arising from cells of ectoderm origin, provides a strong, protective barrier against physical, chemical and bacterial insults. It is comprised mainly of keratinocytes forming two major compartments, the basal and supra-basal (Fig 5). In the human the epidermis is thick and the suprabasal layer can be further subdivided into distinct layers, the stratum spinosum, stratum granulosum, and the outer stratum corneum. Undifferentiated proliferating keratinocytes reside in the basal layer at the junction between the epidermis and dermis. Keratinocyte terminal differentiation, concomitant with a movement towards the surface of the epidermis, involves the sequential expression of many proteins including keratins, integrins and involucrin, until the outermost, stratum corneum, layer comprises flat, anuclear squames. The tactile strength of the epidermis is gained from the presence of a strong cytoskeleton formed from keratin fibres and tight junction formation between the keratinocytes and hemidesmosome contacts to basement membrane

Figure 5 Histological section of human skin

This histological section is from a normal human skin biopsy stained with haematoxylin and eosin. The basal proliferative layer of the epidermis is at the junction of the epidermis and dermis, and is the site of Keratin 5 and 14 co-expression. This layer contains the epidermal stem cells, an important target for gene therapy. As the keratinocytes mature, they move outward towards the surface of the epidermis and form the suprabasal layers in which the keratins K1/K10 are co-expressed. In comparison to mouse skin (Fig 31), note the increased thickness of the human epidermis. This is due to the presence of multiple suprabasal layers of keratinocytes, which in the human has been classified morphologically into distinct layers, the stratum spinosum, stratum granulosum, and the outer stratum corneum.

This photograph is courtesy of Dr Fallowfield, Dept. of Dermatology, Glasgow.

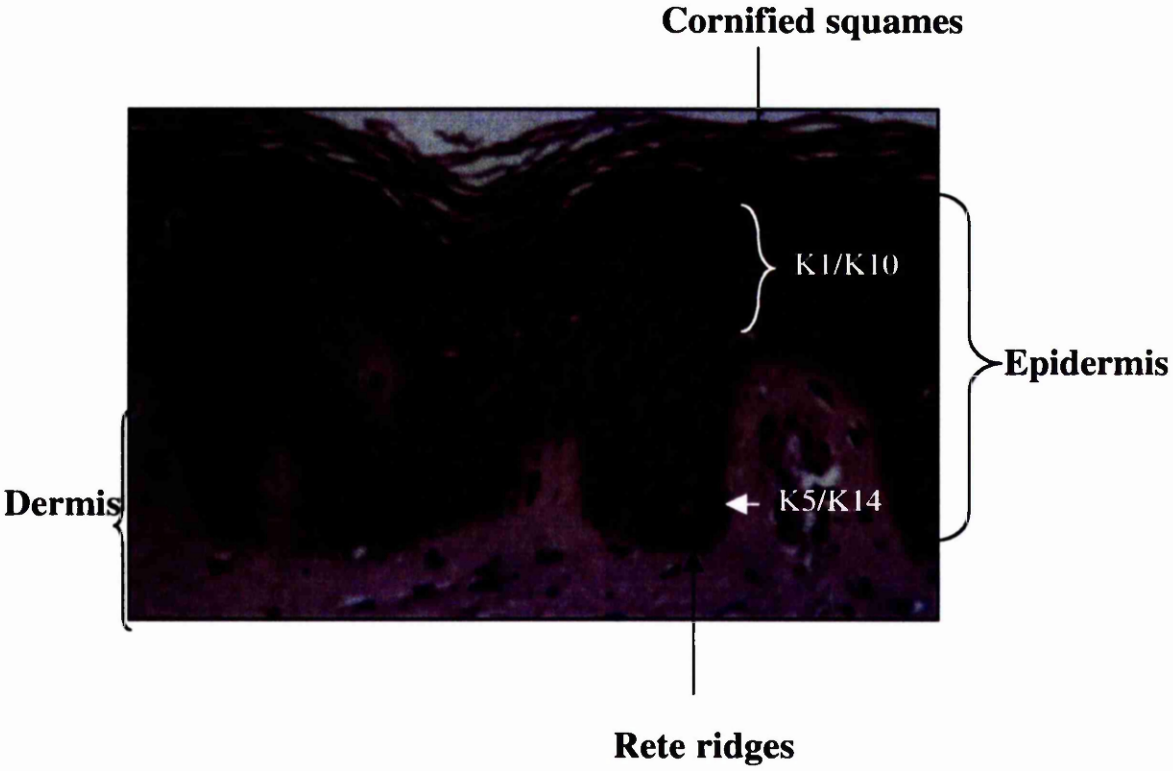


Figure 5. Histological section of human skin.

1.7.1.1 THE EXPRESSION AND FORMATION OF KERATIN FIBRES IS CELL SPECIFIC

The keratins are a family of about 20 proteins which belong to the family of intermediate filament (IF) proteins. They can be subdivided into two distinct groups by sequence homology, pH, and chromosomal location. Type I keratins (K9-20) are acidic and generally small (40-56kD), whereas the type II (K1-8) are larger (53-67kD) and more basic. Keratins form obligate heterodimers, from the co-expression of type 1 and type 2 proteins, which then assemble into a 10nm cytoskeletal or nuclear matrix protein (Albers and Fuchs 1992) with the typifying feature of a central, 310 amino acid α-helical coil, containing sequences that enable it to entwine with a second IF protein. These heterotetramers then interact to form a stable 10nm keratin fibre.

1.7.1.1.1 Keratin 5 gene expression is a marker of a stratified epithelium

The keratin co-expression pair, keratins 5 and 14 is ubiquitously expressed by mitotically active basal epidermal cells in conjunction with specific integrins (Breitkreutz, Stark et al. 1997). They are considered to be a marker of stratified epithelia. As the keratinocyte differentiates, K5 and K14 gene expression is down regulated and supra-basal keratin genes are up-regulated in a cell type specific manner, with K1 and K10 being expressed in the suprabasal layers of the skin, K3 and K12 in the cornea, and K4 and K13 in the suprabasal layers of the oesophagus (Makin, Bobrow et al. 1984). These keratins form an early marker of differentiated, committed keratinocyte. Hair follicles display a more elaborate pattern of keratin expression. K5 and K14 are expressed in the outer root sheath, K1 and K10 in the inner root sheath and the hair –specific keratins in the cortex and medulla.

Mutations in the gene products of either keratins 5 or 14 have been found to be the cause of the genetic condition epidermolysis bullosa simplex (Korge and Krieg 1996) (Fig 6). This condition is due to loss of regular stratification in the epidermis resulting from an abnormal mitotic basal layer. Mild degrees of trauma to the skin cause shearing of the epidermis at the epidermal/dermal boundary and concomitant scarring and stricture formation, which can severely affect the child's quality of life.

1.7.1.1.2 The keratin regulatory sequences have been widely used to drive exogenous gene expression

The epidermis has been used as the target organ for the expression of many exogenous genes, through the use of both viral and tissue specific promoters (Trainer and Alexander 1997). As the regulatory sequences of the keratin genes have been well documented (Byrne, Tainsky et al. 1994; LaPres et al. 1996), their use allows targeting of ectopic gene expression to the basal as well as the suprabasal compartments in the epidermis in a tissue-and differentiation-specific manner. Through the use of different keratin gene promoters, keratinocytes have been shown to be amenable to the expression of exogenous genes, such as the coagulation cascade protein Factor IX (Gerrald et al, 1996) and growth hormone (Wang, Zoppe et al. 1997) indicating that keratinocytes have the capability to modify these exogenous gene products correctly at a post-translational level. Furthermore, the epidermis has been shown to have a secretary capability, allowing recombinant gene products expressed in the epidermis to be secreted into the circulation, indicating its potential for the treatment of systemic disorders.

Tissue specific promoters have the additional advantage over viral promoters in sustaining longer gene expression (Wang, Zoppe et al. 1997). This may be due to

Figure 6. Histological skin section from patient with epidermolysis bullosa simplex.

This histological skin section was obtained from a patient with epidermolysis bullosa simplex secondary to a mutation in the keratin 5 gene. The mutant gene product disrupts the keratinocyte cytoskeleton. Note the lack of organisation within the stratified layers caused by an abnormal basal proliferative stratum. The skin loses its structure and is sensitive to shear forces, which separate the epidermis from the dermis. This causes severe blistering on minimal trauma. As keratin 5 is also expressed in other stratified epithelia, these patients also suffer severe corneal and oesophageal problems

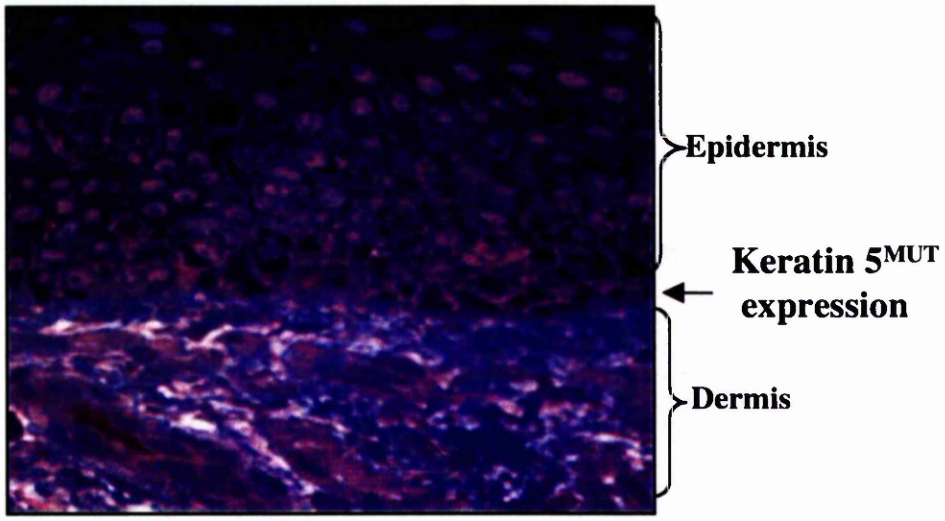


Figure 6. Histological skin section from patient with epidermolysis bullosa simplex.

sensitivity of viral promoter gene expression to down-regulation by methylation (Palmer, Rosman et al. 1991). Although, the epidermis has been used to secrete exogenous protein, in theory it also has the potential of acting as a 'metabolic sink'. The introduction of enzymes capable of acting on substrate either present in the skin or diffusible into the skin may be an alternative method of correcting metabolic insufficiencies.

1.7.2 STRIATED SKELETAL MUSCLE IS ANOTHER VERSATILE ORGAN FOR EXOGENOUS GENE EXPRESSION

Another tissue which has been widely investigated for its potential to express ectopic genes is skeletal muscle. Skeletal muscle forms a large percentage of body mass and has a variety of morphological forms, nevertheless all have the same basic structure. It is composed of extremely elongated, multinucleate contractile unicellular fibres, bound together by collagenous supporting tissue. The individual

muscle fibres are bound together into elongated bundles called fasiculi. During embryonic development mesenchymal cells differentiate into myoblasts which proliferate and fuse into myotubes. However, the mature muscle cells are highly differentiated and, if damaged, have a very limited capacity for repair and regeneration. The longevity of the muscle fibre and the ability to express any ectopic gene targeted to this tissue has attracted much interest from groups devising a means of targeting myocytes by direct DNA injection (Hauser, Amalfitano et al. 1997) with the ultimate goal of gene therapy regimes.

In addition, the use of muscle as a target tissue for ectopic expression of metabolic genes has increased over the last few years due to the availability of strong muscle

specific promoters such as creatine kinase (Sattler, Levak-Frank et al. 1996; Hoefler, Noehammer et al. 1997; Kuang, Xu et al. 1998; Chang, Benecke et al. 1994; Chang, Jensen et al. 1996).

Muscle creatine kinase is critical for energy metabolism of the skeletal muscle as it plays a pivotal role in the phosphorylcreatine shuttle between mitochondria and the myofibrils (Bessman and Geiger 1981). It occurs as three known isoforms of creatine kinase- M (muscle), -B (brain) and -mt (mitochondrial). Creatine kinase exists as a dimer and the cytoplasmic, non-mitochondrial forms M and B can form homo- or hetero-dimers, BB in the brain, MM in skeletal muscle and MB heterodimers in the heart. The mitochondrial form does not dimerise with the two cytoplasmic forms. Proliferating myoblast cultures initially show low levels of the B-isoform of creatine kinase, which is the predominant form in the brain, whilst postmitotic myocyte cultures contain high levels of the M-form of creatine kinase which is characteristic of adult muscle (Eppenberger, Eppenberger et al. 1964)

3.3 kb sequence 5' to the muscle creatine kinase gene has been shown to be sufficient to regulate transcription of a heterologous gene product in striated muscle. In addition, it has been shown that the mRNA levels and protein levels of the gene were under transcriptional rather than posttranscriptional regulation (Jaynes, Chamberlain et al. 1986). Later work delineated an enhancer, 1,000-1,250 nucleotides upstream from the transcription start site, responsible for tissue- and differentiation- specific gene transcriptional high levels (Jaynes, Johnson et al. 1988)

1.7.3 THE LIVER IS FOCUS OF MANY GENE SUPPLEMENTIVE THERAPIES AS IT IS THE SITE OF A PLETHORA OF METABOLIC PATHOLOGIES

The albumin and α-fetoprotein genes are evolutionarily related both in protein sequence, function and genome map location on chromosome 5 in the mouse. During embryonic development both genes are activated in concert in the visceral endoderm of the yolk sac, the foetal liver and the foetal gastrointestinal tract (Krumlauf, Hammer et al. 1985). However, whereas levels of hepatic albumin expression are maintained in the adult mouse, the α-fetoprotein expression level declines to undetectable levels shortly after birth. The albumin gene is hepatocyte specific in the adult mouse and is regulated primarily at the transcriptional level, with its mRNA being amongst the most abundant polymerase II transcripts in the liver and encoding for one of the most abundant serum proteins in the adult (Tilghman and Belayew 1982).

It had been shown that murine cis-acting albumin promoter sequences, containing 300 bp of the 5' albumin promoter were sufficient for tissue-specificity, (Gorski, Carneiro et al. 1986). Further studies by Pinkert et al (Pinkert, Ornitz et al. 1987) identified another element in the 5' region of the albumin gene required for high level, hepatic gene expression. It was shown that by juxtaposing a 2 Kb enhancer region, localised 10.4 Kb upstream of the promoter site, hepatocyte-specific expression could be obtained comparable to endogenous levels of albumin mRNA.

1.8 AIM OF THIS RESEARCH

The aims of this research were to investigate the use of tissue specific promoters in directing expression of recombinant proteins to different target tissues. OTC deficiency was used as the mouse model to test whether exogenous expression of recombinant OTC protein targeted to skeletal muscle cells would have a physiological effect in ameliorating the mouse OTC deficient phenotypes of Spf and Spf^{ASH}. In addition, the recombinant OTC was also targeted to the liver by means of the murine albumin promoter/enhancer as a positive control.

The previous work was possible as the regulatory sequences used to target gene expression to skeletal muscle and liver had already been investigated for their tissue specificity. However, another potentially interesting target tissue for gene therapy is the skin. A novel keratin 5 minigene expression construct was therefore investigated as to its ability to drive tissue- and cell-specific gene expression in the epidermis. The keratin 5 minigene was linked to a reporter gene cassette and the expression pattern of this gene was assayed both in the adult mouse and during embryonic development. In the future it is hoped that this expression construct may be used to target therapeutic proteins specifically to the epidermal compartment of the skin proximal to the basement membrane. This could be used for treatment of keratin disorders, or for delivery of diffusible gene products to the blood system.

2. MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

All chemicals were obtained from Sigma and molecular biology reagents from Gibco, unless otherwise stated. All solutions were made with Millipore filtered water and autoclaved, where applicable.

Solutions, tips and eppendorfs for RNA analysis were soaked overnight in Depc water and autoclaved prior to use.

Radioactive Chemicals were obtained from Amersham and were disposed of according to local COSSH guidelines

2.1.2 TRANSGENICS

All work undertaken was in strict keeping with the Scottish Home Office project licence no PPL 60/01479 and personal licence No. PIL 60/04565.

Mice were obtained from Harlan Olac and were maintained on standard rat and mouse diet (B and K Universal Ltd) in a strict 12 hrs light and dark regime.

All dissecting equipment, unless otherwise stated, was obtained from Raymond A. Lamb or R&J Wood.

2.1.3 PLASMIDS

The plasmids containing the rat OTC cDNA (pSP019), the murine albumin enhancer and promoter (p2335A-1), the murine creatine kinase promoter and enhancer (pEtCAT) and the bovine keratin 5 and 6 regulatory elements were kindly donated by G. Shore (Nguyen, Argan et al. 1986), R. Palmiter (Pinkert, Ornitz et al. 1987), J. Buskin (Jaynes, Johnson et al. 1988) and M. Blessing (Blessing, Nanney et al. 1993) respectively.

2.1.4 CELL CULTURE

Cell culture was performed in a BIOMAT-2 microbiological laminar flow hood. Sterile technique was observed throughout. Sterile plastic 10 ml pipettes were obtained from Falcon^{R} (Becton Dickinson) and 25 ml pipettes were obtained from Costar and cell culture dishes and flasks from Corning.

The SN161 cell line was kindly donated by A. Balmain, Beatson Institute, Glasgow.

2.2 MICROBIOLOGICAL TECHNIQUES

Unless otherwise specified the methods were based on the laboratory manual by Sambrook (Maniatis, Sambrook et al. 1982).

2.2.1 PREPARATION OF COMPETENT CELLS

The bacterial strain DH5α was used for all DNA transformations. 10 mls of pre-warmed L-broth (10 g Bactotryptone, 5 g yeast extract, 5g NaCl per litre, pH7.4), inoculated with 10 µl of DH5α glycerol stock was incubated in the orbital shaker at 37°C overnight. 1ml of this starter culture was expanded to 100 mls with fresh L-broth incubated for a further 2-3 hours until the culture reached an optical density of 0.3, mid-log phase.

50 mls of the culture were then centrifuged in a sterile Falcon tube (Becton Dickson) at 2 500 rpm for 5 mins at 4°C, the supernatant discarded and the bacterial pellet re-suspended in 10 mls of sterile 10 mM $MgSO_4$. The cells were cooled on ice for 20mins, centrifuged as previously, supernatant discarded and the cells re-suspended in 5 mls of sterile ice-cold 50mM $CaCl_2$. They were left on ice for a minimum of 30

mins. The transformation efficiency was approximately of 10^7 colony forming units/μg DNA.

2.2.2 TRANSFORMATION OF COMPETENT CELLS

50 ng of plasmid DNA was added to 100 μl of competent cells, left on ice for 30 mins after which the cells were heat-shocked at 42°C for 1 min. The cells were cooled briefly on ice, 800 μl of L-broth was added and the cells incubated for 45-60 mins at 37°C. After the cells were gently pelleted, all but 100μl of the supernatant was discarded. The cells were re-suspended and spread onto L-agar plates (10 g Bactotryptone, 5 g yeast extract, 5g NaCl, 10.5 g Bacto-agar (Difco) per litre, pH 7.4) containing the appropriate selection antibiotic(s), and allowed to dry before being inverted and incubated overnight at 37°C.

After the overnight incubation individual colonies were picked from the plate using a sterile loop and used for further culture inoculation.

2.2.3 GLYCEROL STOCKS OF BACTERIAL CULTURES

700 μl of an overnight bacterial culture was gently mixed with 300 μl of sterile glycerol and the resultant suspension stored at -70°C. Scrapings from these frozen stocks were used to inoculate L-broth used for further culture.

2.2.4 SMALL SCALE DNA PREPARATIONS OF PLASMID DNA FROM BACTERIAL CULTURE

5 mls of L-broth, containing the appropriate selection antibiotic, was inoculated with a single bacterial colony and incubated overnight at 37°C in an orbital shaker. 1.5 mls of this culture, in an Eppendorf microtube, was centrifuged at 1 400 rpm for 30 s before the supernatant was discarded. The cell pellet was re-suspended in 100 μl of

Solution 1 (50 mM glucose, 10mM EDTA, Tris.HCl pH 8) before 200µl of solution II (0.2 M NaOH, 1% SDS, prepared fresh) was added and the tube inverted gently 5 times. After the further addition of 150 µl of Solution III (3M Potassium acetate pH4.8) the solution was gently mixed, left on ice for 1 min and then centrifuged at 1400 rpm for 15 mins. The supernatant was then removed and added to 350 µl of ice-cold isopropanol, vortexed and then immediately centrifuged for 1 min at 1 400 rpm. The residual pellet was washed in 70% ethanol before being allowed to dry and resuspended in 30 µl of TE buffer (10 mM Tris.HCl, 1 mM EDTA pH 7.4).

2.2.5 LARGE SCALE PREPARATIONS OF PLASMID DNA FROM BACTERIAL CULTURE

Large scale preparations of plasmid from DNA was prepared using Qiagen columns (Qiagen^{TM}). This was suitable for dsDNA sequencing and eukaryotic cell transfection without the need for further purification. DH5α cells containing the plasmid of interest were streaked onto an L-agar plate containing the necessary selection antibiotic. After the plate had been incubated at 37°C overnight, a single colony was used to inoculate 5 mls of L-broth plus selection antibiotic. This seed culture was grown overnight at 37°C in a shaker at 200 rpm before 1 ml of it was used to inoculate a further 100 ml of L-broth, which was cultured under similar conditions.

The bacterial cells were pelleted by centrifugation at 5 000 rpm for 10 min at 4°C (Sorvall Rc 5B centrifuge), re-suspended in 10 mls of Solution I (50 mM Glucose, 25mM Tris-HCl. 10 mM EDTA pH8, 100 µg/ml RNAse A), incubated for 5mins at room temp before 10 mls of solution II (0.4 M NaOH, 1% SDS) was added. The tube was inverted slowly until the solution cleared and then allowed to incubate for a further 5 mins at room temp, before 10 mls of solution III (3 M Potassium acetate -

acetic acid pH 4.9) was added and the solution left on ice for 15 mins. The plasmid-containing supernatant from this solution, obtained by centrifugation at 8 000 rpm for 30 mins at 4°C, was added to a Qiagen Maxi column which had been previously equilibrated with 10mls of solution QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol pH 7.0, 15% Triton-X). The column was then washed twice with 30 mls of QC (1 mM NaCl, 50 mM MOPS, 15% ethanol pH 7.0). 15 mls of QF (1.25 M NaCl, 50 mM MOPS, 15% ethanol pH7.0) was used to elute the plasmid DNA from the column which was then precipitated by the addition of 12 mls of ice-cold isopropanol and centrifuged at 10 000 rpm for 30 mins at 4°C. The DNA pellet was washed twice with 70% ethanol and re-suspended in 100 µl of TE buffer.

The DNA concentration was measured at OD_{260} and stored at -40°C

2.2.6 RESTRICTION ENDONUCLEASE DIGESTION

In general, restriction enzymes were supplied by Gibco or Boehringer Mannheim companies. Digestions were performed at the salt concentrations and temperatures recommended and provided by the relevant enzyme buffer system. In circumstances where double digestions were necessary, if a mutually compatible buffer was not available to give 100% digestion, the enzyme digestion requiring the lower salt concentration was performed first. The salt concentration was subsequently adjusted using a low-to-medium or low-to-high salt conversion buffer prior to the addition of the second enzyme. Plasmid digests were incubated for 2-3 hours, whilst genomic were allowed to proceed overnight. Digestion products were either electrophoresed directly or reactions were terminated by a phenol/chloroform extraction step followed by ethanol precipitation of DNA.

2.2.7 FILLING IN OF 5' AND 3' DNA OVERHANGS

Some restriction enzymes cleave double stranded DNA to leave 3' or 5' single stranded DNA overhangs which are re-converted to double strand DNA by means of either the Klenow fragment of DNA polymerase or T4 polymerase treatment.

2.2.7.1 KLENOW TREATMENT

To fill in 3' recessed termini, the large fragment of the DNA polymerase I (Klenow fragment) from *E.coli* was used. It comprises 5'-3' polymerase activity and 3'-5' exonuclease activity but not 5'-3' exonuclease activity.

Blunt ending of 200 ng DNA was performed in a final volume of 20 µl comprising 10 mM dNTP, 1X TMM buffer (10X: 100 mM Tris-HCl pH7.9, 100mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 mM NaCl), and 1U large fragment DNA polymerase 1 (Klenow) (Gibco). The reaction was incubated at 37°C for one hour and the DNA polymerase and free nucleotides were removed subsequently by phenol / chloroform extraction.

2.2.7.2 T_4 DNA POLYMERASE

This enzyme was used when the restriction enzyme digestion resulted in overhanging 5' ends. It has a strong 3'-5' exonuclease activity and a weaker 5'-3' polymerase activity. Reactions were incubated in T_4 DNA polymerase buffer for 15 mins at 37°C and the products purified as described previously.

2.2.8 PHENOL/CHLOROFORM EXTRACTION

Phenol (Sigma) was added to the DNA sample (v/v) and vortexed before centrifuging at 14 000rpm for 5 mins. The supernatant was added to an equal

volume of choroform / isoamylalcohol (24:1 v/v), vortexed and again centrifuged for 5 mins at 14 000rpm. The DNA-containing supernatant was stored at -40°C.

2.2.9 LIGATION OF LINEARISED INSERT INTO PLASMID VECTOR

Ligations were performed using a modification of the method of Weiss *et al* (Weiss, Thompson et al. 1968). Ligations were performed in a final volume 20 µl comprising 100 ng vector DNA, insert DNA at 1:3 molar excess to the vector DNA, 1x T4 ligase buffer (5X: 250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol) (Gibco) and 5 U of T4 ligase (Gibco). The ligation was carried out for 16 hrs at 16°C if the DNA termini were compatible and 24 hrs at 10°C if the ends were blunt. The ligation mixture was diluted 1:1 with TE buffer prior to competent cells transformation.

2.2.10 REMOVAL OF 5' TERMINAL PHOSPHATE GROUPS FROM DNA FRAGMENTS

Calf intestinal alkaline phosphatase (CIP) was used to remove the terminal 5' phosphate of restriction endonuclease digested DNA molecules. 200 ng of linearised DNA was de-phosphorylated in a final reaction volume of 20µl comprising 1x dephosphorylation buffer (10x: 0.5 M Tris-HCl, 1 mM EDTA pH8.5) (Gibco), and 2 u CIP (Gibco, BRL). Incubation was carried out at 37°C for 30mins, followed by CIP denaturation at 65°C for 10 min. The DNA was then obtained by two phenol/chloroform extractions and ethanol precipitation.

2.2.11 ETHANOL PRECIPITATION OF DNA

DNA was precipitated by the addition of 5M sodium acetate pH5.5 to a final concentration of 3.5 M. 2 volumes of 100% ethanol were added, the sample left on

dry ice for 30 mins and then the DNA pelleted by centrifugation at 14 000 rpm for 15 min. The DNA pellet was then washed in 70% ethanol before being dissolved in TE buffer.

2.2.12 AGAROSE GEL ELECTROPHORESIS

DNA was generally electrophoretically fractionated in 1X TAE (50x: 2 M Tris, 25 ml/l glacial acetic acid, 0.05 M EDTA pH 8.0) agarose gels. Ethidium bromide was included in the running buffer at 0.5 μg/ml. The agarose concentration of each gel was determined by the fragment sizes to be resolved. DNA samples were electrophoresed after the addition of loading buffer (25 mM EDTA, 30% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanole) in a Pharmacia LKB gel apparatus system at 1-5 V/cm. Unless otherwise indicated a 1kb ladder (Gibco, BRL) was used as a size marker. The DNA fragments were then visualised by means of UV illumination.

2.2.13 DNA DILUTION FROM AGAROSE GEL

DNA was extracted from agarose gels using a commercial kit QIAquick gel extraction kit (Qiagen). In brief, the DNA was excised from the gel with a clean scalpel and placed in a sterile microcentrifuge tube. Three volumes of buffer QG were added and the tube was incubated at 50°C for 10 min until the gel had completely dissolved. One volume of isopropanol was then added prior to the sample being applied to a QIAquick column and centrifuged for 1min. The flow-through was discarded and the column washed with 0.75 ml of buffer PE and centrifuged for 1min. The flow-through was discarded and the DNA was eluted from the column by the addition of 30 μl of TE buffer, incubation for 1 min at room

temperature and centrifugation for 1 min at 13 000 rpm.

2.2.14 DNA EXTRACTION FROM TISSUE

This was performed according to Laird *et al* 1991 (Laird, Zijderveld et al. 1991). 0.5 cm of murine tail, amputated under general anaesthetic (2% halothanem (Rhone Menieux) in 2L O_2 (BOC), was added to 600 µl of lysis buffer (100mM Tris.HCl pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl). 100 µg proteinase K/ml (Boehringer Mannheim) was added prior to overnight incubation at 55°C. Following complete lysis the samples were vortexed and spun at 14,000 rpm for 5 mins. The DNA was precipitated from the supernatant by the addition of one volume of isopropanol and then re-suspended in 100 µl of TE buffer. Further purification of the DNA, if necessary, could be obtained by phenol/choloroform extraction (see 2.2.2.3.)

2.2.15 SOUTHERN BLOT

2.2.15.1 CAPILLARY BLOT TRANSFER TO NYLON

8 µg of genomic DNA was digested in a total volume of 30 µl overnight with the appropriate restriction enzyme under optimal conditions. The DNA samples were then electrophoretically fractionated. Having electrophoresed the DNA fragments to best resolve the size range of interest, gels were photographed alongside a ruler for future size correlation. The DNA was denatured by submerging the gel at room temperature for 45 min in denaturation solution (0.5 M NaOH/1.5 M NaCl). The gel was then neutralised for 30 mins in 1.5 M Tris.HCl/1.5 M NaCl.

Hybond -N (Amersham) was cut to the size of the gel, wetted in 2xSSC and the gel was blotted onto the membrane overnight using 10 x SSC (1.5 M NaCl, 0.015 M Na citrate, pH7.0) transfer buffer on a conventional Southern blot apparatus. The DNA

was then cross linked to the membrane by UV irradiation for 4 mins. The filter was then wrapped in Saran wrap and stored at 4°C until further use.

2.2.15.2 Labelling of DNA probe for Southern and Northern filters

DNA random priming was performed using Prime-It™ RmT Random primer (Stratagene) as described in their instructions.

50 ng of DNA was added to the commercial Prime-It™ tube in a final volume of 42 µl, prior to boiling at 95°C for 5 min. 6 µl of ^{32P}dCTP and 12U of magenta DNA polymerase was added and the tube incubated for 15 mins at 37°C. The reaction mix was separated through a nick column, which had been previously equilibrated with 2 mls of TE buffer, in 400 µl of TE buffer and the labelled probe was eluted in 400 µl of TE and 2 µl counted in a scintillation counter

2.2.15.3 Probing of Southern filters

This was performed using a commercial hybridisation solution DigEasy Hyb™ (Boehringer Mannheim).

The filter was prehybridised in a bottle for one hour in 25 ml of DigEasy Hyb™ at 50°C in a hybridisation oven (Hybaid). The labelled probe was then added to 100 µl of DigEasy Hyb and boiled for 5 mins before being added to 6ml of fresh DigEasy Hyb at 50°C. The 25 mls of prehybridisation fluid were replaced with the 6ml of DigEasy hyb containg the probe and the filter left to hybridise overnight at 50°C. The filter was then washed twice in 2xSSC/0.1%SDS for 5 mins, 0.2 SSC/0.1%SDS for 10 mins until the background radioactive count was <5. The filter was wrapped

in Saran wrap and exposed to X-ray film (X-Omat, Kodak 651454) overnight at -20°C, before the film was developed.

2.2.16 RNA EXTRACTION FROM TISSUES

This method is based on the commercial TRIzol™ method (Biogenesis Ltd). All equipment and solutions, excluding those containing Tris, were soaked overnight in DEPC containing dH_2O and then baked/autoclaved for 2 hours at 120°C.

100 mg mouse tissue, stored in liquid N_2, was ground in a mortar and pestle, added to 2 ml of TRIzol™ and incubated for 5 mins at room temperature. Subsequently 0.4 ml of chloroform was added, the sample shaken and incubated for a further 3 mins at room temperature. The samples were then centrifuged at 12 000 rpm for 15 mins at 4°C. The upper aqueous layer was removed to a fresh tube and the RNA precipitated from this with 0.5 ml isopropanol by incubating at room temperature for 10 mins and centrifuged at 12 000 rpm for 10 min at 4°C. The RNA pellet was then washed once with 70% ethanol and resuspended in 40 µl of DEPC treated water and stored at -70°C.

2.2.16.1 QUANTIFICATION OF RNA

In order to ascertain the quality and quantity of RNA obtained, 1 µl of RNA was added to 1.3 µl dH_2O, 1.65µl formaldehyde, 5 µl formamide and 1 µl of 10X MOPS running buffer (10X; 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0), boiled for 10 mins at 60°C, then quenched on ice. 2 µl of RNA loading buffer (50% glycerol, 1 mM EDTA, pH8.0, 0.25% bromophenol blue, 0.25% xylene cyanole FF) was added, prior to loading into a 1.5% agarose RNA gel (0.45 g agarose, 5 ml formaldehyde, 3 ml 10x MOPS, 22 ml H_2O) and run for 45 mins in 1x

MOPS buffer. In addition to the samples 4 μg of *Escherichia coli (E.coli)* ribosomal RNA (Boehringer Mannheim) was loaded as a standard. After electrophoresis the gel was soaked in dH_2O for 15min prior to staining in 0.5 μg/ml ethidium bromide for 5 mins. The gel was then de-stained in dH_2O overnight and the integrity and concentration of RNA noted by UV illumination.

2.2.17 NORTHERN BLOTTING

15 μg of RNA sample was added to Depc dH_2O to a volume of 4.7 μl prior to the addition of 3.3 μl formaldehyde, 10 μl formamide and 2 μl of 10X MOPS running buffer. The sample was loaded onto a 1.5% RNA agarose gel (4.5 g agarose, 50ml formaldehyde, 30 ml 10x MOPS, 220 ml H_2O) with a size marker and a RNA sample of known size (16 μg of *E. coli* RNA) and run at 40 V for 8 hrs. The size marker and RNA sample of known size were then separated from the rest of the gel, stained in 5 μg/ml ethidium bromide for 5 mins at room temperature and then de-stained overnight. This was then used as size comparison for RNA fragment sizes. Hybond -N (Amersham) was cut to the size of the remainder of the gel, wetted in 2x SSC and the gel was blotted onto the membrane overnight using 10x SSC (1.5 M NaCl, 0.015 M Na citrate, pH7.0) transfer buffer on a conventional Southern blot apparatus. The RNA was then cross linked to the membrane by UV irradiation for 4 mins. The filter was then wrapped in Saran wrap and stored at 4°C until further use. DNA probes were labelled and hybridised to the Northern blot as has been previously described for Southern blot analysis.

2.2.18 DOUBLE STRAND SEQUENCING

The modified rat OTC cDNA was sequenced to determine successful site-directed mutagenesis as well as to confirm the absence of other unintentionally introduced mutations.

Double stranded sequencing was based on the dideoxy chain teremination method (Sanger, Nicklen et al. 1977) and performed using Sequenase™ version 2.0 DNA sequencing kit (USB).

A final concentration of 0.2 M NaOH and 0.2 mM EDTA (pH 8.0) was added to 4 μg of purified dsDNA, incubated for 20 mins at 37°C and then neutralised by the addition of 1/10th volume of 3 M sodium acetate (pH5.5). The samples were then ethanol precipitated and washed twice with 70% ethanol. The air-dried pellets were then re-suspended in 6 μl of dH_2O. 2 μl of Sequenase™ reaction buffer was then added in addition to 2 pmol of the appropriate sequencing primer. The sample was incubated for 2 mins at 65°C and then allowed to cool to 35°C over 1-2 hours and then quenched on ice. 1 μl of 100 mM DTT was added in addition to 2 μl of labelling mix (diluted 1/5 with dH_2O), 1 μCi of ^{35}S-dATP and 2 μl of diluted Sequenase 2.0 enzyme (diluted 1/15 in TE buffer). The samples were incubated at room temperature for 5 mins. 3.5 μl of each sample was added to 2.5 μl of each dNTP, in tubes pre-warmed to 37°C, vortexed momentarily and then incubated for 5 mins at 37°C. 4 μl of STOP solution was added to each reaction. The samples were stored at 4°C until required. Prior to loading the samples, they were incubated at 75°C for 2 mins and quenched on ice immediately.

2.2.18.1 PREPARATION OF SEQUENCING GELS

Sequencing gels containing 7.2% polyacrylamide were prepared by mixing 33 g urea (Sigma), 13 mls of 40% acrylamide (Sigma), 7.7 mls of 10xTBE and 33 mls dH_2O. This was polymerised by the addition of 500 µl 10% ammonium persulphate and 75 µl TEMED (Sigma). Gels were set between glass plates 4 mm apart, run in 1 X TBE buffer and pre-heated to 55°C, prior to loading the sample. The samples were run at 34 W for a minimum of 2 hours at 55°C, and then fixed in 10% methanol, 10% acetic acid for 10 mins prior to transfer onto Whatmann 3mm paper. The gel was dried for 30 mins in a Biorad gel drier at 80°C and then exposed to X-ray film overnight at room temperature. The gels were then read manually.

2.2.19 POLYMERASE CHAIN REACTION

All primers were ordered from Oswel

2.2.19.1 MODIFICATION OF RAT OTC cDNA FOR EXPRESSION CONSTRUCT

PCR amplification of rat OTC cDNA incorporated the consensus Kozak sequence (G C C A/G C C **AUG** G), and EcoRV restriction sites either end of the cDNA for sub-cloning. Additionally a 'G' missense mutation was incorporated at position +4 in order to convert the original sequence from a cytosine to a guanine, thus achieving the consensus optimal Kozak sequence. This missense mutation had the effect of changing the second codon from 'CTG' to 'GTG' and thereby altering the second amino acid in the OTC polypeptide leader sequence from leucine to valine. This was achieved using the primer pair A1 (Oswel).

Rat OTC cDNA PCR primers-A1

A1 Forward

5'**G G C G A T A T C A A G A T G G* T G T C T A A**

EcoRV

A1 Reverse

5'**G G C G A T A T C T T C G T C C T C T T G C A**

EcoRV

As fidelity of the PCR product was important, Pfu Polymerase (Stratagene) was used in the reaction due to its 3'-5' exonuclease proofreading activity that enables nucleotide–misincorporation errors to be corrected. The reaction was performed on 100 ng of plasmid template with 2.5 u Pfu in 1X buffer (10 mM KCl, 10 mM $(NH_4)_2$ SO_4, 20 mM Tris-Cl (pH 8.75), 20 mM $MgSO_4$, 0.1% Triton X-100, 100 μg/ml BSA), 200 μM each dNTP, 0.5 μM primer. The reaction protocol was 94°C for 3 mins followed by 30 cycles of 94 °C for 1 min, 60°C for 1 min and 72 °C for 4 min followed by 72 °C for 10mins annealing time. The 1.1 kb PCR product was then visualised on a 1% agarose gel, extracted, purified and stored at –20 °C prior to use.

2.2.19.2 SCREENING PCR FOR TRANSGENIC BREEDING

The primers were designed using the GCG software programme. Primers were located in different exons so that only the transgene incorporating the OTC cDNA and not the endogenous OTC gene was amplified.

OTC Forward

5' **T C C T T C T T T T C T T A C C A C A C**

OTC `Reverse

5' **T G A C T A T A T T A G G A T C T G G C T C**

The PCR was performed on 0.5 μg of genomic DNA using 0.5 U of Taq DNA polymerase (Boehringer-Mannheim) in 1X buffer (10mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.2 μM of each dNTP and 0.5μM of each primer in a final volume of 100 μl. The reaction protocol was 94°C for 3 mins followed by 30 cycles of 94 °C for 1 min, 55°C for 1 min and 72 °C for 1 min followed by 72 °C for 10 mins annealing time. The PCR product was then visualised on a 1.5% agarose gel.

2.2.19.3 **2.2.11** *AMPLIFICATION REFRACTORY MUTATION DETECTION SYSTEM*

In order to confirm a direct correlation between the scoring of the sparse fur mouse phenotype and the sparse fur genomic mutation, an amplification refractory mutation system was designed for the sparse fur mutation based on the protocol by Ferrie *et al* (Ferrie, Schwarz et al. 1992).

Common primer

5' GGC ATT ATC TAA GGA GAA GCATCA TCT TCT C

Wild type Primer (sparse fur)

5' ACA CCC TTC CTT TCT TCA CAC ACA AGA CAT GC

Sparse fur primer

5' ACA CCC TTC CTT TCT TCA CAC ACA AGA CAT GA

Sparse fur^{ASH} primer

GTG AAT GAA AGT CTC ACA GAC ACC GCT CA

Wild type primer (sparse fur^{ASH})

GTG AAT GAA AGT CTC ACA GAC ACC GCT CG

The penultimate nucleotide in the wildtype primer was altered to destabilise the PCR reaction on a Spf and Spf^{ASH} genomic template.

The PCR was performed on 0.5 μg of genomic DNA using 0.5 U of Taq DNA polymerase (Boehringer-Mannheim) in 1X buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.2 μM of each dNTP and 0.5 μM of each primer in a final volume of 100 μl. The reaction protocol was 94°C for 3 mins followed by 30 cycles of 94 °C for 1 min, 60°C for 1 min and 72 °C for 1 min followed by 72 °C for 10 mins annealing time. The 250 bp PCR product was then visualised on a 2% agarose gel.

2.2.20 FLUORESCENT IN SITU HYBRIDISATION (FISH)

Metaphase fluorescent *in situ* hybridisation was performed to establish the presence of dual transgene integration sites in one founder mouse.

2.2.20.1 PRODUCTION OF MURINE CHROMOSOMAL METAPHASES

Spleens were obtained from 2 mice from the same founder line. Each spleen was collected into PBS. The splenic capsule was pierced with a sterile 22 gauge needle and the splenocytes were washed out with a RPMI culture medium. The resultant cells were centrifuged at 1 200 rpm for 6 mins at room temperature. The cell pellet was re-suspended in 5 ml of RPMI, 20% foetal calf serum, and divided into 2 round bottomed plastic universals. 50 mg of lipopolysaccharide (LPS, Sigma) was added prior to incubation at 37°C for 44-46hrs. 50 μl colemid (10 mg/ml) was added to the culture 30 min prior to harvesting. This was performed by centrifugation of the cells at 1 200 rpm for 6 mins. 10 mls of 0.075 M KCl was added to the re-suspended cells and incubated for 10 mins at room temperature The cells were centrifuged as described previously and the cells re-suspended in fresh fixative (3:1 methanol; acetic acid by volume) and left overnight at 4°C. The cells were pelleted again prior

to being re-suspended in 1 ml fresh fixative and metaphase spreads were obtained by dropping one drop of the suspension onto an alcohol-cleaned slide from 10 cm height. The metaphase slides were air-dried at room temperature for 5 hrs and stored at -20°C prior to use.

2.2.20.2 LABELLING AND PROBING OF CHROMOSOMAL METAPHASES

The transgenic construct probe was labelled using the Gibco BRL kit, using Biotin 11 dUTP. 1 μg of linearised construct DNA in a total of 45 μl including 5μl reaction mix (A4), 1 μl enzyme (solution C), 3.4 μl biotin-11-dUTP (Sigma) Tris-HCl pH7.5. was mixed, and incubated at 15°C for 90 mins and the reaction stopped. The DNA was ethanol- precipitated and resuspended in TE buffer to a concentration of 100 ng/μl. The metaphase slides were dehydrated through a series of ethanols, 50%, 70%, 90% and 100% for 2 mins in each and denatured for 2mins in 70% formamide/30% 2X SCC at 70°C. 2 ng of labelled DNA in 10 μl of Hybridisation mix (50% formamide, 10% dextran sulphate, 2xSSC, salmon sperm 10 μl/ml)) was layered over the slides, covered, and incubated overnight at 37°C. The slides were washed twice in 2xSSC at 42°C for 5 mins, twice in 50% formamide in SCC at 42°C for 5 mins, the SSC washes repeated and finally in 4xT. The blocking reagent (15% human AB serum in 4XSSC) was then added and the slides incubated for 10-20 mins at 37°C. The signal was then detected by the addition of avidin-FITC, followed by two 4x SSC(T) washes, biotinylated anti-avidin, two 4x SSC(T) washes and finally avidin-FITC. The slides were then washed twice in blocking solution, twice in 4x SSC(T) and dehydrated 50, 70, 90 and finally 100% ethanol for 1 min each. The chromosomes were counter-stained with 0.4 ng/ml DAPI. The slides were

mounted in Citifluor prior to visualisation using a Zeiss Axioskop epifluoresence microscope.

2.3 TRANSGENIC TECHNOLOGY

All animal procedures were carried out under project and personal licence numbers PPL 60/01479 and PIL 60/04565 respectively. The techniques described are based 'Manipulating the mouse embryo' by Hogan et al (Hogan, Costantini et al. 1986) An overall view is found in Figure 7.

2.3.1 TOXICITY TESTING

All solutions were tested for embryo toxicity prior to use. 10-15 healthy fertilised eggs were cultured to blastocyst stage (~4 days). If less than 70-80% of eggs achieved blastocyst stage or if there was delay in reaching this stage, the solution was discarded and a different batch number of the solution ordered and tested

2.3.2 PREPARATION OF DNA FOR MICROINJECTION

The expression construct was excised from the vector plasmid by restriction enzyme digestion, and the digest run out on a 1% TAE agarose gel. The construct was purified from the gel as described previously (2.2.13). The DNA was eluted with 30 µl of sterile injection buffer (10 mM Tris.HCl, 0.1 mM EDTA pH 7.4 in sterile H_2O (Antigen Ltd) at 55°C by a 20 s centrifugation at 14 000 rpm, then dialysed in

Figure 7. Summary of transgenic mouse production.

This figure is summary of pronuclear transgenic mouse production. Six week old females are supra-ovulated to increase and synchronise egg production. Twelve hours after fertilisation the embryos are collected and incubated. The DNA expression cassettes are injected into the male pronucleus and the embryos are further incubated until the two-cell stage. At this stage, the embryo is transferred into the oviduct of a pseudo-pregnant female and the pregnancy is allowed to continue until birth of the pups. The pups are screened at weaning for genomic integration of the transgene.

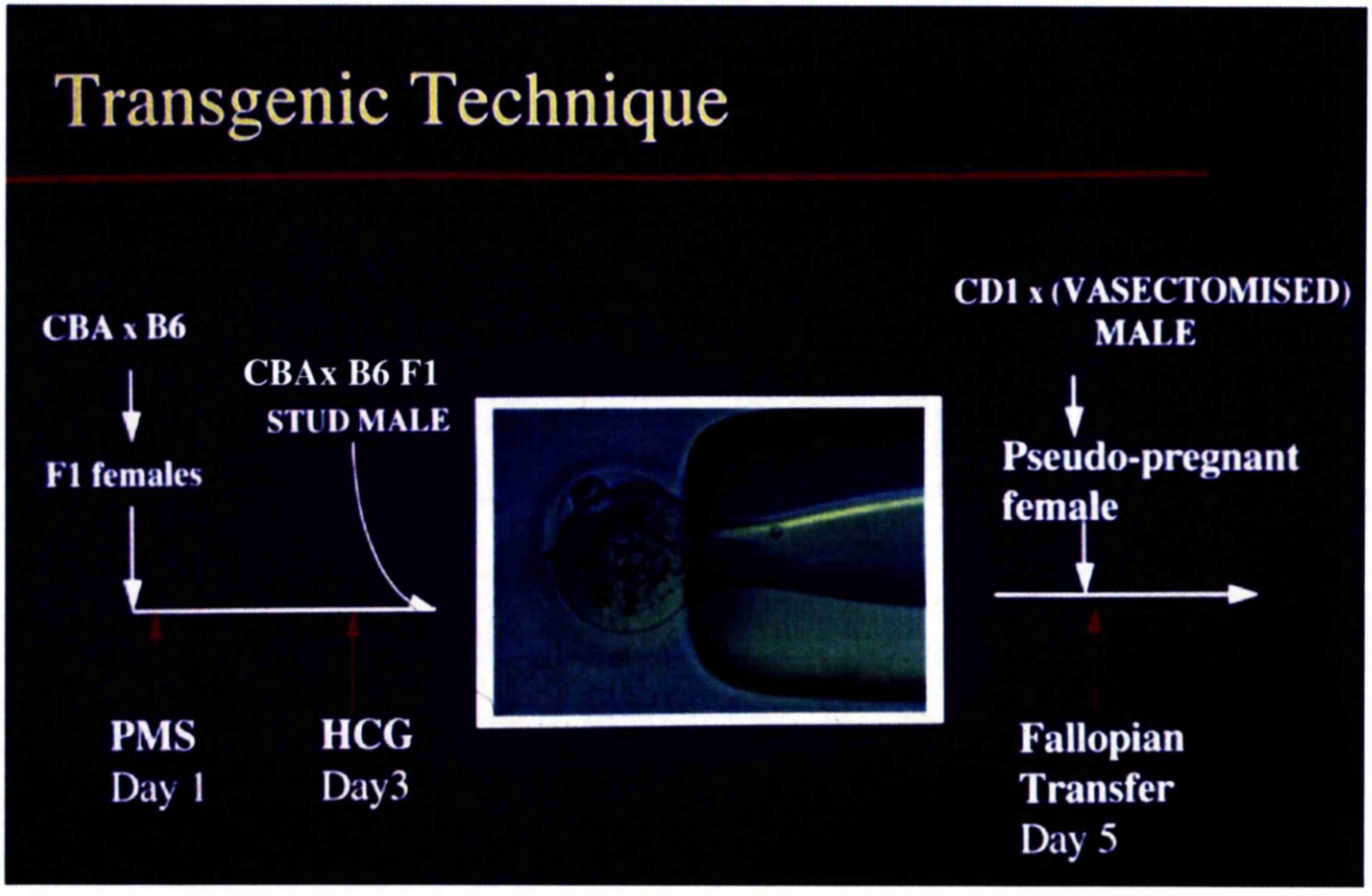


Figure 7. Summary of transgenic mouse production

dialysis tubing (Sartorius) at 4°C for 48 hrs against 800 ml of injection buffer (10 mM Tris.HCl, 0.1 mM EDTA pH 7.4 in sterile H_2O (Antigen Ltd) with 3 changes of dialysis fluid.

Serial dilutions of the DNA were run against a standard DNA titration set to estimate the DNA concentration. The DNA was stored in the concentrated form at -40°C and micro-injected at 5 ng/ml.

2.3.3 PRODUCTION OF NEEDLES FOR MICROINJECTION

Transfer needles and holding needles: (Clark electromedical Instruments GC100-15) were pulled by hand over a naked flame. Transfer needles were pulled to 1.5X diameter of the embryos, broken with a diamond pen and the ends smoothed and tapered on a microforge until 0.5-0.8X the diameter of the embryos (Research Instruments Ltd).

Injection Needles (Clark electromedical Instruments with inner filamant GC100TF-15) were pulled daily on a micropipette puller (Falming Brown Mod. P-87) using a cycle which was optimised for the heating filament used.

2.3.4 MOUSE VASECTOMY

C57Bl/6J male mice, aged 6 weeks were anaesthetised with general anaesthetic (2:2:8, Hypnorm (Janssen):Hypovel(Roche):dH_2O) i.p. An incision was made on the ventral surface in the midline at the level of the upper thigh. Both testes were identified and externalised, before the vas deferens were then identified and cauterised bilaterally. The testes were then replaced in the abdominal cavity and the incision sutured using a dissolvable suture (Ethicon, Vicryl coated 3/0, W91140). The mouse was then placed in an incubator at 37°C until fully recovered. In order to test the effectiveness of the vasectomy, the male was set up with three fertile female

mice and coital plugs noted. After three proven matings that did not result in a successful pregnancy, the vasectomised male was deemed as infertile.

2.3.5 SUPEROVULATION OF F1 MICE FOR EMBRYO HARVEST

Female mice, the progeny of a CBA male X Bl6 female cross, were used at either 3 or over 6 weeks of age. 5iu of pregnant mare serum in sterile 0.9% saline (Folligon, Intervet) was injected intra-peritoneally at time 0 (1600hrs). 46hrs later 5iu of human chorionic gonadotrophin (Chorulon, Intervet) in sterile 0.9% saline (Baxter) was injected intra-peritoneally and each mouse set up with a C57Bl/6J stud male.

2.3.6 HARVESTING OF EMBRYOS

Super-ovulated females were sacrificed at 10 hrs post conception by cervical dislocation. The abdomen was swabbed with 70% alcohol and the Fallopian tubes removed into 0.8% saline pre-warmed to 37°C. Under a dissecting microscope (Wild Heering M8) the embryos within the Fallopian tubes were visualised and dissected out into a Petri dish (Falcon 3004 easy grip tissue culture dish, Becton Dickson) containing 2 mls H6 medium (Appendix 1), pre-warmed to 37°C. Embryos transferred into 50 µl H6 medium at 37°C, overlaid with liquid paraffin (colourless, light paraffin BDH 294365H). 6µl of hylauronidase (Sigma, 1.5 mg/l in H6) was added to aid dispersion of the cumulus cells, before the embryos were washed 3 times in 100 µl of fresh H6 medium over-laid with liquid paraffin. The embryos were then added to 100 µl T6 (Appendix 1) overlaid with liquid paraffin, which had been equilibrated for 3 hrs at 37°C in 5% CO_2 (Scotlab-VSL incubator), and further incubated at 37°C in 5% CO_2 for approximately 2-3 hrs until the pronuclei were clearly formed.

2.3.7 MICROINJECTION OF EMBRYOS

Microscope slides (Select micro slides Chance propper Ltd, R.J.Wood), washed in absolute alcohol, were dipped in silican (Repelcote^{TM}, BDS) for 10s, allowed to dry then washed in dH_2O. A bilaterally-open chamber was formed on these slides, comprising anterior and posterior walls formed from glass rods, held in place with vaseline. In the centre of the chamber 300 µl of H6, prewarmed to 30°C, was placed and overlaid with a coverslip. Sterile paraffin oil sealed both the open lateral sides of the chamber. 20 embryos were transferred into the centre of the H6 medium and the slide transferred to the platform of the microscope (Olympus) for microinjection. The air-filled holding pipettes by means of mouth control picked up and held an embryo, allowing adjustment so that the pronuclei were in the same plane of vision as the central circumference of the embryo. The injection needle was then inserted into the embryo until it penetrated a pronucleus, and sufficient DNA was injected to temporarily inflate the pronucleus. The needle was then quickly withdrawn after injection and the embryos were transferred into T6 medium and cultured overnight at 37°C in 5% CO_2. Those embryos which had cleaved into 2-cell stage were then transferred back into a recipient female.

2.3.8 TRANSFER OF EGGS

CD1 females, due to their natural capacity to rear large litters, were mated with vasectomised males. Those females with 'post coital plugs' were then anaesthetised with 0.6 mls of general anaesthetic (2:2:8V/V Hypnorm(Janssen) : Hypnovel(Roche) : dH2O) i.p.. The flank was swabbed with 70% alcohol and an incision made on the left flank. The peritoneum was visualised, incised and immobilised with a suture (Ethicon, Vicryl coated 3/0, W9114). The fat pad overlying the ovary was identified, externalised and immobilised with a blallock clip

(Downs surgical). The mouse was then moved under the dissecting microscope (Olympus S2H10) and the membrane surrounding the ovary and Fallopian tube opened. The presence of multiple corpi lutei in the ovary confirmed ovulation and the mouse as a good recipient for egg transfer. The open end of the Fallopian tube was identified. 25 eggs were taken up into the transfer needle, followed by an air bubble and transferred into the Fallopian tube by mouth pipetting. Successful transfer could be monitored by the visualisation of the air bubble in the Fallopian tube. The ovary was then returned to the peritoneal cavity and the peritoneum sutured. The overlying skin was also sutured and the mouse placed in 37°C incubator overnight until recovery was complete.

2.3.9 BREEDING OF TRANSGENIC AND OTC DEFICIENT MOUSE LINES

Matings were set up between 6 week old or older mice and the litters were weaned at 3 weeks, screened for presence of the transgene from tail DNA. At weaning all mice were given a unique identification number, which was noted in both personal and project licence records. At weaning the mice were designated phenotypically affected or unaffected and this was also entered into the records. All matings and subsequent dealing with the mice were given a procedure number, and multiple procedures, other than transgene screening and mating, were not carried out on a single mouse.

2.4 BIOCHEMISTRY

2.4.1 TOTAL PROTEIN ASSAY

This was undertaken by a modified version of Lowry *et al* 1951 using Bio-Rad DC protein assay (Bio-Rad).

0.4 mg of murine tissue was homogenised in 1ml mitochondrial lysis buffer (0.5% Triton X-100, 10 mM Hepes, 2 mM DTT), sonicated for 2 min at maximal intensity and then incubated on ice for 30 mins. An aliquot was then further diluted 1:50 in dH_2O and used for protein and OTC assay. The initial concentrate protein solution was stored at -70^{0}C.

100 µl of protein sample/ bovine serum albumin standard was added to 500 µl of solution A (2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4$ in water, 2% Na K tartate in water, combined just prior to use in a ratio of 98:1:1 respectively) after 20 µl/ml Solution C had been added, and vortexed. 4mls of solution B was added, vortexed and left to incubate for 15 min at room temperature in the dark. The samples were read at OD_{750} and compared to a standard graph of serial dilutions from 0-1500 µg/ml of bovine serum albumin (Sigma) dissolved in mitochondrial lysis buffer, to estimate protein concentration

2.4.2 OTC ASSAY

This assay was performed according to Lee *et al* 1989 (Lee and Nussbaum 1989). In brief, 2-10 µg total cellular protein, in mitochondrial lysis buffer as described previously were added, to 700 µl of OTC reaction mix (5 mM ornithine, 15 mM carbamyl phosphate and 270 mM triethanolamine, pH7.7), which was incubated at 37^{o}C for 30 mins. The reaction was stopped with the addition of 250 µl of 3:1 absolute phosphoric acid/sulfuric acid (by volume).

Citrulline production was measured by adding 50 µl of 3% 2,3-butanedione monoxime, incubating at 95-100 ^{o}C in the dark for 15 mins, and measuring OD absorbance at 490 mM. The concentration of citrulline produced/hr/mg protein could then be calculated by means of comparison with a graph of standard citrulline

concentrations undertaken at the same time. However, as this assay was used to comparing relative levels of OTC activity between different mouse populations, an arbitrary unit of OTC activity was used OD_{490} / mg protein. All samples were assayed in triplicate and the average was then calculated. Samples from all the different mouse populations were used every time the assay was performed.

2.4.3 PLASMA AMMONIA LEVEL ANALYSIS

The mice were anaesthetised in 30% halothane and bled by cardiac puncture (This technique was performed by Frances Coussins). The mice were then sacrificed by cervical dislocation prior to tissue removal. The blood was added to a lithium heparin-containing tube and taken to the Department of Paediatric Biochemistry, within 30 mins, where the ammonia level was measured in the diagnostic laboratory, which is the regional centre for metabolic disorders The commercial assay used was Vitros AMON slides (Vitros Chemistry products). The Vitros AMON slide is a dry, multilayered, analytical element coated onto a polyester support. The ammonium ions are converted to gaseous ammonia, which passes through a semi-permeable membrane to reach the indicator layer. This is then detected in a colorimetric assay at a wave length of 600nm.

2.4.4 GALACTOSIDASE STAINING OF EMBRYOS AND ADULT TISSUES

Galactosidase staining of tissues and embryos was performed according to the protocol published by Cheng *et al* (Cheng, Wallace et al. 1993).

Freshly dissected whole embryos from d9.5post-coitum up to d13pc., or tissues cut into 3mm sections, were added immediately to and incubated in fixative (2% paraformaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$ in PBS) for 90 mins at 4°C on a rolator (Jencons), washed three times for 3 mins in PBS, prior to being

incubated in the dark overnight in LacZ stain (5 mM $Fe_3(CN)_6$, 5 mM $Fe_4(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-Gal) at room temperature on the rolator. The tissues were the de-stained for 24 hrs in PBS at room temperature before being re-fixed in 10% formalin for a minimum of 4 hrs. Whole mount embryos were photographed at this stage prior to being sectioned.

2.4.5 WHOLEMOUNT GALACTOSIDASE STAINING OF THE INTESTINE.

The intestine was removed post morteum and flushed through once with cold PBS. Cotton thread was used to close one end of the intestine and cold 4% formalin was then infused into the open end of the intestine and the tissue was left to fix for 90 min. The base of a Petri dish was coated with RalwaxII (BDH) to a depth of 1cm. The wax had been softened by the addition of 10% heavy grade liquid paraffin (BDH) prior to pouring the dish. Post fixation the intestine was opened up longitudinally and pinned onto the wax covered Petri dish. The 4% formalin was replaced with a demucifying agent (20% ethanol, 10% glycerol (Fisons), 0.01 M Tris pH8.2, 0.65%w/v NaCl, 3 mg/ml dithiothreitol (Sigma) added immediately prior to use) and left to incubate for 30 mins at room temperature. This removes the bacteria-containing mucin and thus, any false positive bacterially encoded galactosidase activity. The intestine was then stained with lacZ stain as described previously in 1.2.4.4, de-stained in PBS and photographed.

2.4.6 TESPA COATING OF MICROSCOPE SLIDES

Microscope slides (Select micro slides Chance Propper Ltd, R.J.Wood) were dipped sequentially in 10% HCl in ethanol, distilled H_2O, and 95% ethanol, then dried at 150°C for 30 mins and cooled. The slides were then dipped for 10 s in 2% 3-aminopropyltriethoxy-silane (TESPA) (Sigma) in acetone, washed twice in acetone,

twice in dH_2O and dried at 42°C overnight. The slides were stored in a dust proof container at 4°C until use.

2.4.7 SECTIONING OF TISSUE SECTIONS

Embryos and tissues, which were previously fixed in 10% formalin for β-galactosidase staining, were placed in histosettes (Simport) and processed further by a Shandon Citadel Automatic Processor, courtesy of the Department of Pathology. This processing comprised 3 washes in methylated spirit for 90 min, and 150 min x 2 respectively, followed by a 2 hr dehydration in absolute alcohol, three 30min washes in xylene and lastly 2 paraffin washes for 2 hrs and 4 hrs respectively, before the tissue was mounted by hand in paraffin wax and stored at 4 °C prior to sectioning.

7 μM tissue sections were cut, floated on water at 45°C and mounted on TESPA coated slides. The slides were then left to dry at 52°C for 5-6 hrs and stored at 4^0C.

2.4.8 COUNTER-STAINING OF TISSUE SECTIONS WITH EOSIN

Tissue section slides had the paraffin removed by two 10 min washes in Histoclear (National Diagnostics) prior to being dehydrated through a series of alcohols, 2 mins in 100% x2, 90%, 70%, 50%, 30% alcohol in dH_2O respectively. The sections were then incubated in freshly filtered eosin (BDH) for 2 mins, with intermittent checks for optimal staining, before the sections were re-hydrated through the alcohols and washed for 10 min X2 in Histoclear. The sections were then mounted in DeePeeX (Sigma) and left to dry overnight before being visualised and photographed.

2.5 IN VITRO CELL CULTURE

2.5.1 CULTURE OF SN161 CELL LINE

Transient transfection of the SN161 cell line was undertaken with this keratin 5/LacZ expression construct prior to the formation of transgenic founder murine lines.

The SN161 cell line, a squamous carcinoma cell line known to express the keratin 5 gene, was kindly obtained from Prof. Balmain, Beatson CRC Institute, Glasgow.

The SN161 cell line was cultured in 25 cm^2 flasks (Nunc) at 37°C, 5% CO_2 in a Sanyo incubator in 14 mls of Dulbecco's modified Eagle medium (DMEM)(Gibco) with 10% bovine fetal serum (Gibco), 2 mM glutamine, 1% penicillin and streptomycin (Gibco) The culture was never allowed to become fully confluent. Fresh medium was added every third day after the culture was washed twice in PBS (Gibco). The culture was passaged at 80% confluency level by the addition of 2.5 mls of 1X Trypsin-EDTA (Gibco) and incubation until the cells lose their adherence. 9 mls of culture medium was added and pipetted to form a single cell suspension. 1 ml of cell suspension was added to 13 mls of fresh culture medium in a 50cm^2 flask (Nunclon^{TM}) and incubated at 37°C, 5% CO2 as normal.

2.5.2 TRANSFECTION OF SN161 CELLS

2.5.2.1 REMOVAL OF LIPOPOLYSACCHARIDES (LPS) FROM DNA PREPARATION.

As contamination of plasmid DNA with bacterial lipopolysaccharides(LPS) can be toxic to mammalian cells during transfection, LPS was removed by means of Triton-X114 to improve transfection efficiency. 0.01 volumes of Triton X-114 was added to DNA (1 mg/ml), mixed gently and incubated on ice for 5 mins, followed by a

further incubation at 37°C for 5 min. Centifugation at 14 000 rpm for 5 mins at room temperature was performed and the upper phase removed and extracted twice more with Triton X-114. The DNA was then ethanol-precipitated and re-suspended in TE buffer.

2.5.2.2 TRANSIENT TRANSFECTION OF SN161 CELL LINE

This technique is based on O'Mahoney and Adams 1994. 20 μg of LPS-free plasmid DNA, was added to 0.5 ml of 0.25 M $CaCl_2$ and gently mixed. The DNA solution was added dropwise to 0.2 ml of 2 x Hepes buffered saline pH7.04, gently shaken to form a fine precipitate and then added immediately, in fresh culture medium, to a 25-50% confluent cell culture. The cells were left to incubate for 15-20 hours at 37°C, 5% CO_2, then washed in PBS, prior to having fresh culture medium added and incubated for a further 24 hrs before any assay being performed.

2.5.2.3 X-GAL STAINING OF SN161 CELLS

Cell cultures at 50-80% confluency, were fixed in cell fixative (2% formaldehyde, 0.1% glutaraldehyde in PBS) for 10 mins at room temperature, washed three times with PBS and then incubated with 14 ml of X-gal stain (5mM $Fe_3(CN)_6$, 5mM $Fe_4(CN)_6$, 2mM $MgCl_2$, 1mg/ml X-Gal) for 2 hours at room temperature The cell cultures were then de-stained by washing three times in PBS prior to examination for X-Gal staining.

2.5.3 2.5. CRYOPRESERVATION OF CELL CULTURES

Cells cultured until 80% confluency were washed twice in PBS and incubation at 37°C in 2.5 mls of trypsin for 3 minutes. The cell suspension was then centrifuged at

1 200 rpm for 5 mins and the supernatant discarded. The cell pellet was then gently re-suspended in 1ml cryoprotectant medium (10% dimethyl sulphoxide (DMSO) in foetal calf serum), placed in a 2 ml Biofreeze vial (Costar) and placed at -70°C overnight before transferring to liquid nitrogen for long term storage.

2.5.4 Statistical Analysis of OTC activity and plasma Ammonia results

All statistical analysis of plasma ammonia levels and OTC activity was performed using the computer statistics programme package SPSS for windows 8.0 (SPSS Inc. 444n Michigan Ave, Chicago, Illinois 60611). As it was not possible to confirm the parametric basis of the spread of OTC activity or plasma ammonia levels within a population, non-parametric index which analyses the results obtained within two independent groups was used, namely the Mann Whitney test.

3. RESULTS

3.1 DESIGN OF TRANSGENIC EXPRESSION CONSTRUCT

3.1.1 GENERATION OF MODIFIED RAT O TC CDNA (OTC^{MOD}) WAS ACHIEVED BY SITE-DIRECTED MUTAGENESIS.

The rat OTC cDNA (Accession No. M11266) comprises the entire 1.1 kb open reading frame from nucleotides –40 from the AUG to 1170, including 40 bp and 360 bp of the 5' and 3' untranslated region respectively. This OTC cDNA had been cloned previously into the pSP64 plasmid and designated pSP019 (Nguyen, Argan et al. 1986).

In order to maximise protein production and OTC activity, the rat OTC cDNA was modified to potentially enhance translation. Site-directed mutagenesis was achieved by incorporating a single nucleotide mismatch into the 5' end of the OTC cDNA forward PCR primer sequence, The nucleotide at position +4 of the Kozak translation initiation motif of the rat OTC cDNA was converted from a cytosine to a guanine, thus achieving the consensus optimal Kozak sequence. This missense mutation had the effect of changing the second codon from 'CTG' to 'GTG' and thereby altering the second amino acid in the OTC polypeptide leader sequence from leucine to valine. This is a very conservative amino acid change involving the loss of one methyl group. There was no overall change in amino acid polar charge.

In addition to this modification, the PCR primer sequences used to amplify the OTC cDNA were designed to truncate the 5' and 3' non-coding sequences The resultant PCR encoded the entire open reading frame of the rat OTC cDNA and only 3 bp of the 5' and 6 bp of the 3' untranslated sequence. This modified rat OTC cDNA was termed OTC^{MOD} and used to form the transgenic expression constructs after cloning into pIC20H plasmids by means of EcoRV restriction enzyme recognition sites, placed into the 5' ends of both the forward and reverse A1 primer sequences.

The incorporation of the nucleotide substitution at position +4, as well as the absence of any other PCR-introduced mutations was confirmed by manual double-stranded direct sequencing of the rat OTC cDNA in the pIC20H plasmid clones (Fig 8). A total of 10 clones were sequenced before a high fidelity clone (pP20MOD) was identified. This OTC cDNA clone was then used in the formation of the tissue-specific expression vectors.

3.1.2 GENERATION OF GENE EXPRESSION CONSTRUCTS

The tissue-specificity of the OTC gene expression constructs resides in promoter/enhancer regulatory sequences used to drive expression of the cDNA. In addition to being tissue-specific, the promoters required to be associated with a high level of gene transcription. As the rationale for the expression of OTC activity in exogenous tissues required the diffusion of the substrates to the target tissues, an excellent blood supply to the target tissue was a prerequisite. In addition, tissues were chosen that had previously been shown to integrate, transcribe and post-translationally modify other exogenous genes. The two target tissues identified in which to express recombinant OTC protein were skeletal muscle and, as a positive control, the liver. The rationale for using the murine creatine kinase promoter/enhancer was to target the OTC gene expression to skeletal muscle. This tissue is large and homogenous with a good blood supply and low cellular turnover rate. This has the advantage that ectopic gene expression should be highly persistent even if terminally differentiated cells are targeted. The good blood supply should enable the appropriate enzyme substrates to diffuse into the targeted cells.

Figure 8. Sequence modification of rat OTC cDNA

The OTC^{MOD} cDNA was produced by incorporating a mutation in the Kozak sequence into the PCR primers used to amplify the sequence. Incorporation of the mutation was confirmed by manual double strand sequencing of Kozak translation consensus sequence of reverse strand of OTC cDNA. Figure 8A shows the endogenous sequence of the reverse strand of rat OTC cDNA at the translation initiation codon. Figure 8B shows the rat OTC cDNA with incorporation of 'C' at +4 and 'G' at –4 (OTC^{MOD}). Site-directed mutagenesis was used to convert the normal OTC Kozak sequence to a form thought to be associated with a higher translational efficiency. This sequence change caused a leucine to valine missense change in the second amino acid of the OTC leader sequence in the expression constructs. The 'G' at -4 was formed from the EcoRV site incorporated into the 5' end of the primer for subcloning purposes.

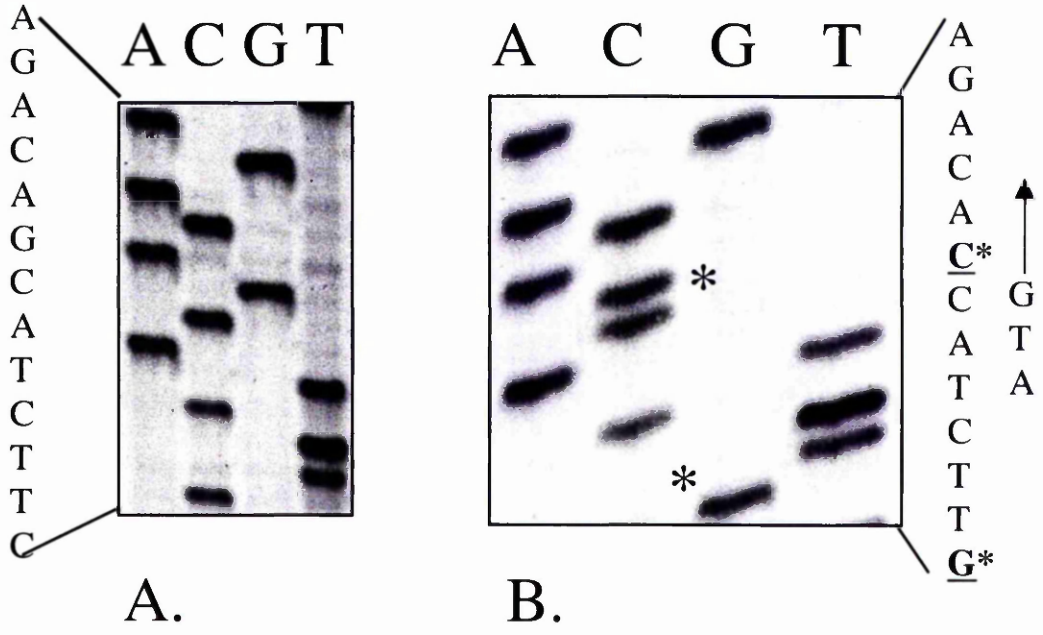


Figure 8. Sequence modification of rat OTC cDNA.

3.1.2.1 THE LIVER- TARGETED OTC^{MOD} EXPRESSION CONSTRUCT

The plasmid (p2335A-1), containing 2.3 Kb of the murine albumin promoter and enhancer sequences was obtained from R.D. Palmiter (Pinkert, Ornitz et al. 1987). This plasmid included 22 bp of the albumin gene 5' untranslated region, which had previously been used to express the human growth hormone as a reporter gene, producing high gene and protein expression levels, and consequent overgrowth of the transgenic mice.

The strategy used to clone the albumin driven OTC cDNA expression vector is shown in figure 9. In brief, the albumin promoter/enhancer sequence was removed from its plasmid vector by double restriction enzyme digestion with SacI and EcoRV. This fragment was ligated into a pIC 20H/SV40 plasmid which contains the SV40 polyadenylation/intron sequence cloned into pIC20H and kindly provided by another member of the group, Dr D. Fowlis. The OTC^{Mod} cDNA was then ligated between the albumin enhancer/promoter sequences and the SV40 polyadenylation sequence at a unique EcoRV restriction site, and the correct orientation and mapping of the final expression cassette (pAOP) was confirmed by restriction enzyme digestion (Fig 10).

The inclusion of a 3' SV40 polyadenylation consensus sequence and small intron in the expression constructs was due to previous work suggesting a splice event and polyadenylation of primary mRNA transcripts to be important in stabilisation of the mRNA, transport of the mRNA to the cytoplasm and transcription of micro-injected genes (Brinster, Allen et al. 1988). In addition, polyadenylation may facilitate translation (Jackson and Standart 1990; Bachvarova 1992).

The complete transgene was released from the parental vector by a NruI / NotI double digestion prior to purification for micro-injection (Fig 10).

Figure 9. Formation of albumin-OTC^{MOD} expression cassette

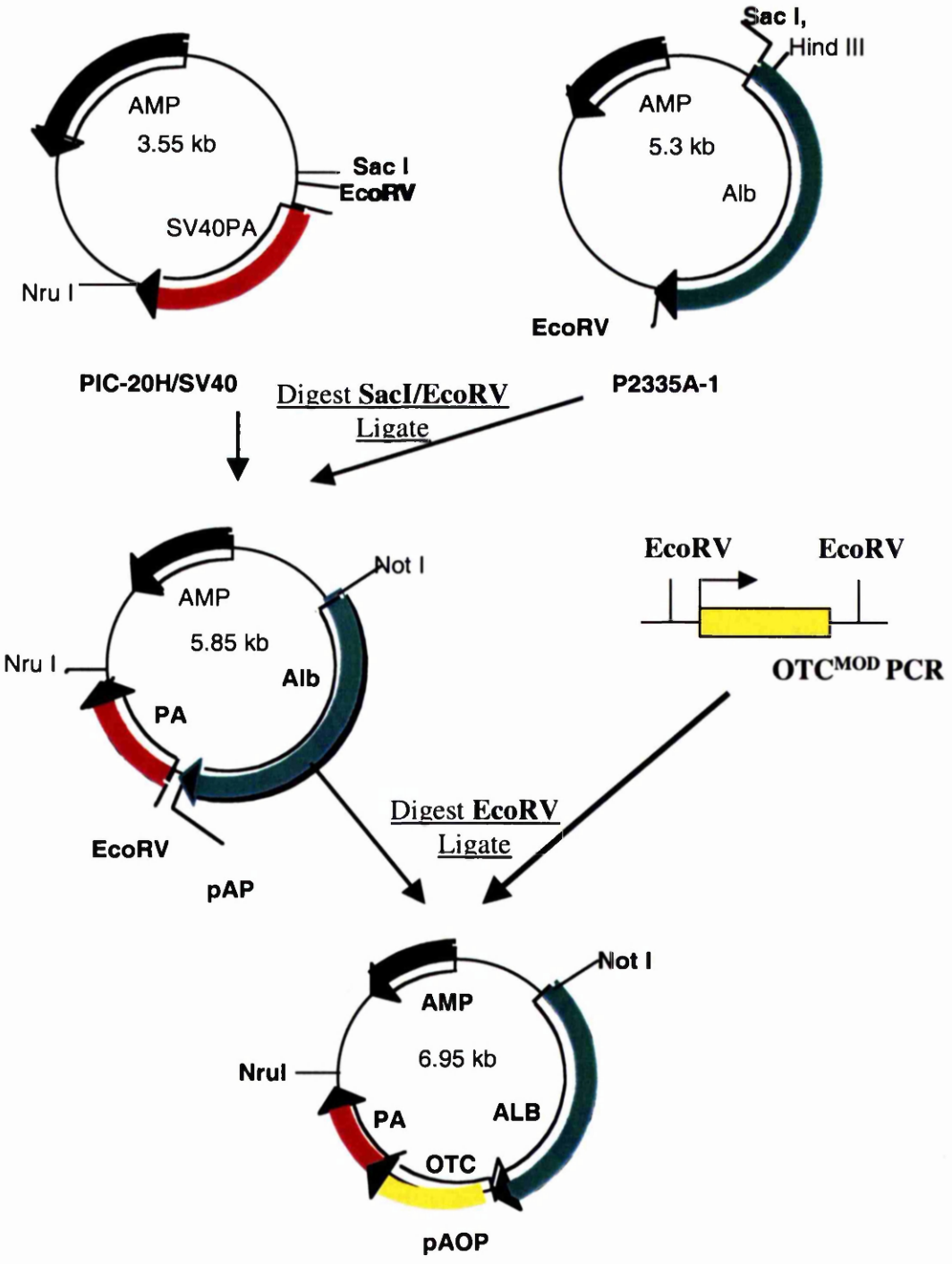
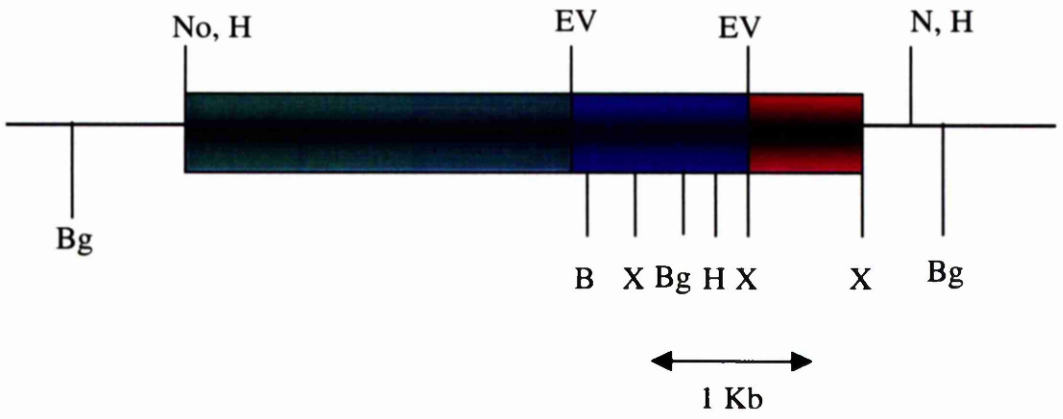


Figure 9. Formation of albumin-OTC^{MOD} expression cassette.

Figure 10 Formation of albumin-OTC^{MOD} expression cassette

A. Restriction enzyme map of the albumin-OTC^{MOD} expression construct in plasmid pIC20H.

B. **B)** Diagnostic restriction enzyme digestions of the resultant albumin-OTC^{MOD} expression construct in pIC20H H:HindIII, B:BamH I, X:Xho I, EV:EcoR V, II,Hc:Hinc II, N:NruI, No: NotI, BglII; BgII. The Xho I and Hind III digests indicate the correction orientation of the OTC cDNA.

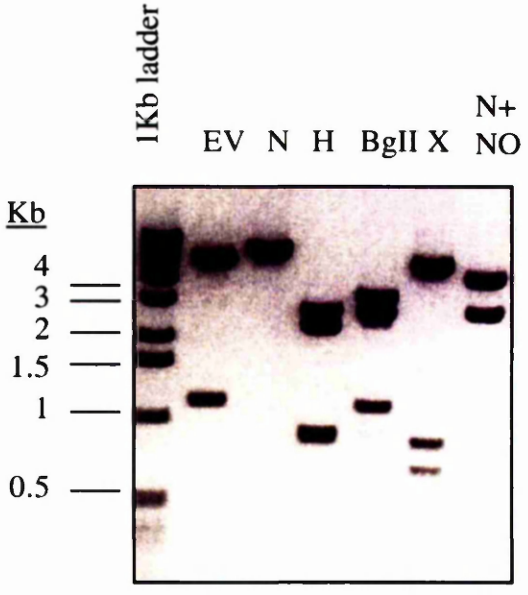


Albumin promoter /enhancer

OTC cDNA

SV40 Polyadenylation/intron

A.



B.

Figure 10. Restriction enzyme digestion map of albumin-OTC^{MOD} expression cassette.

3.1.3 SKELETAL MUSCLE DIRECTED OTC^{MOD} EXPRESSION

The pEtCAT plasmid containing 3.3 Kb of the murine creatine kinase promoter (mCK) and enhancer was obtained from J. Buskin (Jaynes, Johnson et al. 1988). The strategy for the formation of the creatine kinase driven OTC cDNA expression construct is summarised in figure 11. In brief, the mCK promoter and enhancer were excised from the pEtCAT vector with a HindIII/BSYTEII digestion. The 5' ssDNA overhangs were filled with Klenow enzyme and ligated into pIC20H/SV40. The OTC^{Mod} cDNA was then subcloned into the unique EcoRV site and the correct orientation selected (pMOP). The expression construct could then be excised at unique EcoRI and NruI prior to use. Correct orientation and mapping of the construct was confirmed by restriction enzyme analysis (Fig 12).

Figure 11. Formation of creatine kinase-OTC^{MOD} expression cassette.

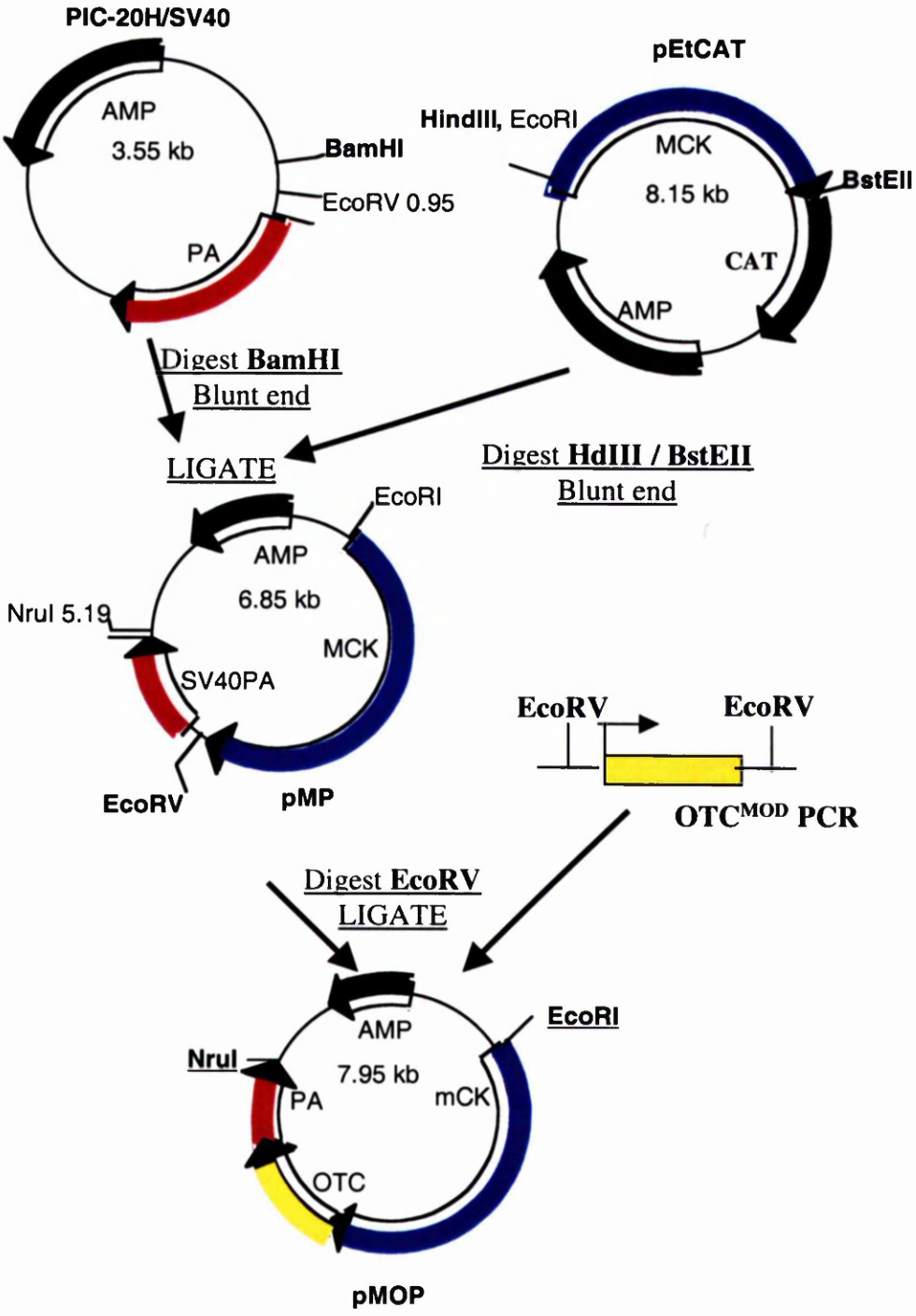
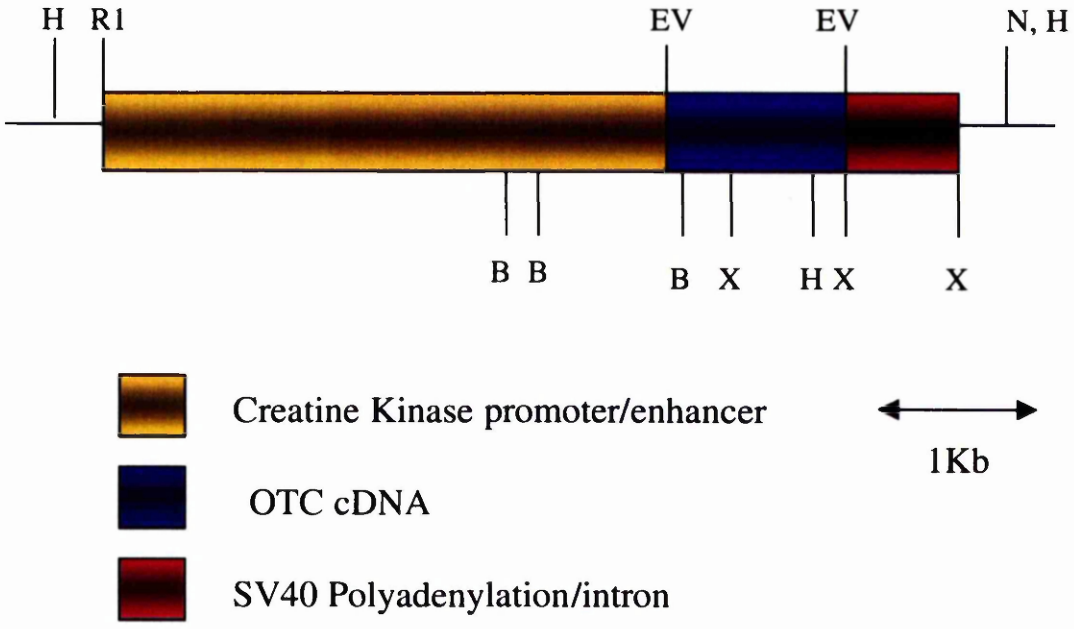


Figure 11. Formation of creatine kinase-OTC^{MOD} expression cassette.

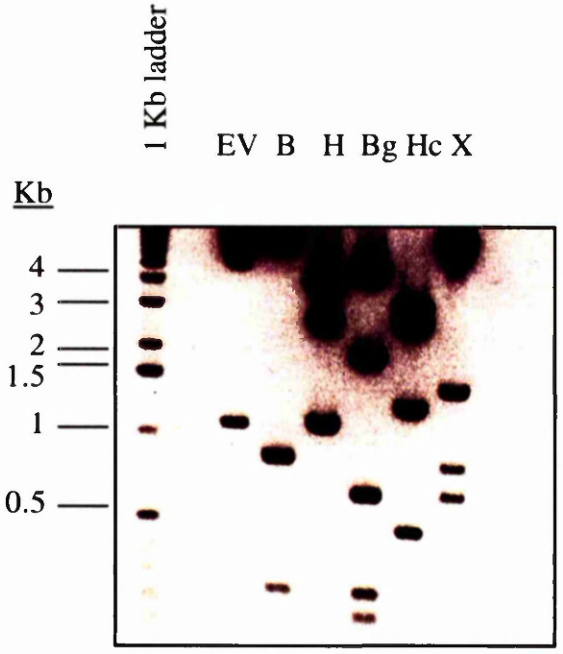
Figure 12 Restriction enzyme digestion map of creatine kinase-OTC^{MOD} expression cassette.

A) This is a restriction enzyme map of the creatine kinase-OTC^{MOD} expression construct in plasmid pIC20H.

B) Diagnostic restriction enzyme digestions of the resultant creatine kinase-OTC^{MOD} expression construct in pIC20H H:Hind III, B:BamH I, X:Xho I, EV:EcoR V, Bg:Bgl II, Hc:Hinc II, N:NruI,.



A.



B.

Figure 12. Restriction enzyme digestion map of creatine kinase-OTC^{MOD} expression construct.

3.2 4.GENERATION OF TRANSGENIC MOUSE LINES BY PRONUCLEAR INJECTION

3.2.1 MICRO-INJECTION OF EXPRESSION CONSTRUCTS

Standard micro-injection technique was employed as described in 'Manipulating the mouse embryo' by Hogan et al (Hogan, Costantini et al. 1986). The expression constructs were excised from the parental plasmid vector and purified by dialysis against injection buffer. The purified, linearised expression constructs were originally micro-injected at a concentration of 2 ng/μl. No transgenic pups were obtained after screening 25 resultant pups. Subsequently a concentration of 5 ng/μl of DNA was used in the micro-injection.

This table indicates the efficiency of the technique

Table 1. Efficiency of pronuclear injection after the technique was optimised.

Expression Construct	Concentration Of DNA	Number of Pups born	Number of transgenic mice	Efficiency
Albumin - driven OTC	5 μg/ml	40 (+5 dead)	3	7.5%
Creatine Kinase driven OTC	5 μg/ml	22	3	13.6%
Keratin V driven LacZ (see later)	5 μg/ml	13	3	23%

3.2.2 5. ANALYSIS OF INTEGRATION SUGGESTS MOST LINES HAVE INSERTED MULTPLE COPIES OF THE TRANSGENE.

3.2.2.1 SOUTHERN BLOT ANALYSIS

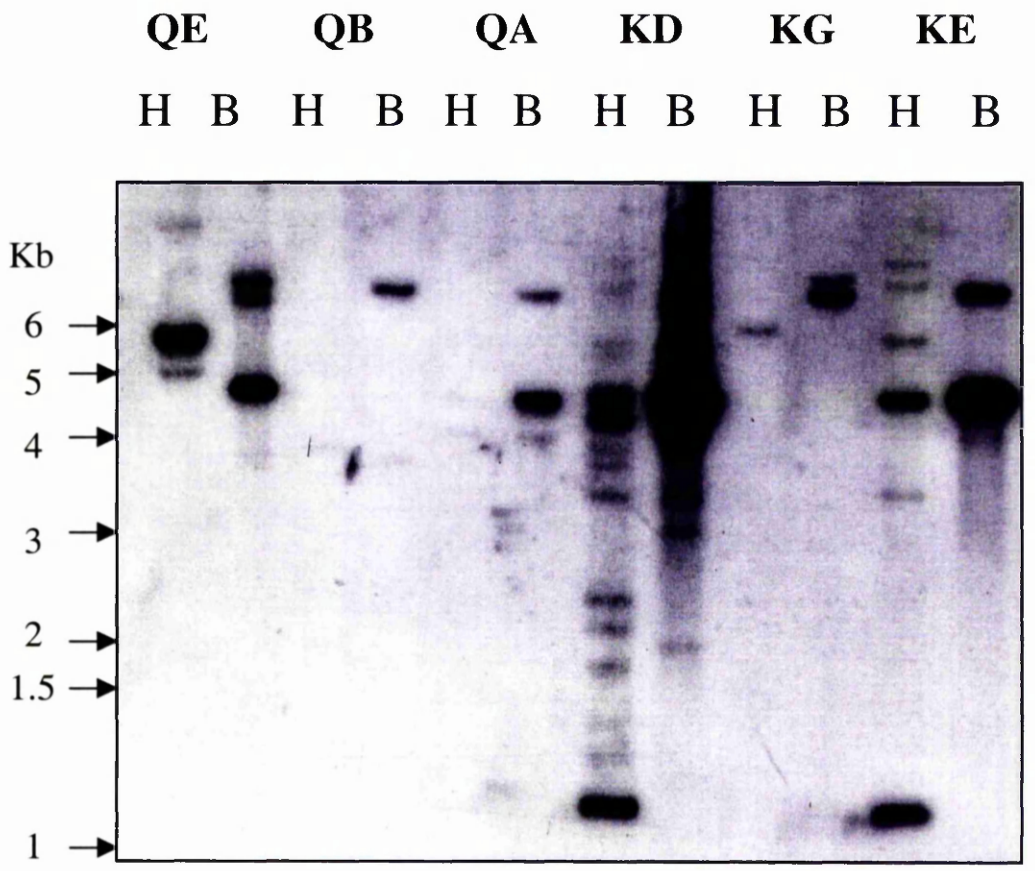
All founder mice resulting from pronuclear injection were screened for the presence of the transgene by Southern blot analysis performed on 5 μg of genomic DNA extracted from a tail tip biopsy (Fig 13). The DNA was digested overnight with HindIII or BamHI, blotted overnight and the resultant blot probed using radioactively labelled 850 bp SV40 intron and polyadenylation sequence. This sequence is of viral origin and has no homology to any murine genomic sequence.

Using this method three founder mice were identified from each expression construct. The albumin founder lines were termed the 'K' lines and comprised KE, KD and KG, whilst the creatine kinase founder lines were deemed the 'Q' lines and comprised QA, QB and QE.

Southern blot analysis (Fig13) shows proof of transgene integration. Unfortunately, no single restriction enzyme cut twice within either transgene to produce a constant size internal band. The HindIII site 3' to both expression constructs in the pIC 20H/SV40 plasmid was lost when the expression cassettes were excised from the parental vector prior to pronuclear injection. However, as more than one copy of a transgene usually integrates in a 'head to tail'array, the presence of a HindIII site at the 5'end of the albumin-OTC^{MOD} expression construct should produce a constant 1.2 Kb band when the SV40 polyadenylation sequence is used as a probe, this band being diagnostic for tandem transgene integration. This constant band is seen in both the KD and KE line but not the KG line suggesting multiple transgene copies in KD and KE but not in KG lines.

Figure 13. Southern blot analysis of albumin and creatine kinase-OTC^{MOD} founder lines.

Southern blot analysis of all creatine kinase and albumin-OTC^{MOD} founder transgenic lines digested with BamHI or HindIII. The SV40 polyadenylation sequence was radioactively labelled as the probe. KD and KE have an internal 1.2 Kb band, which is absent in KG. This internal band is formed by 'head to tail' insertion of the transgenes at the point of integration and is therefore indicative of the integration of multiple transgenes. In addition, KD shows multiple bands of varying size suggestive of multiple chromosomal integration sites. Unfortunately in the creatine kinase–OTC^{MOD} transgenic lines, neither BamHI or HindIII digestions give an internal constant band.



B: BamH I H: Hind III

Figure 13. Southern blot analysis of albumin and creatine kinase-OTC^{MOD} founder lines.

Figure 13 also shows the presence of multiple bands in addition to the constant 1.2 Kb band in the KD founder line, therefore additional Southern blot analysis was performed with a lesser amount of genomic DNA and a higher enzyme concentration in order to rule out the possibility that the additional bands were due to incomplete digestion of the genomic DNA (Fig 14).

This second Southern blot result gave identical results to the first. Thus, the additional bands could be due to gross rearrangements of the integrated transgene. Alternatively, it might suggest that the transgene had integrated into more than one chromosomal location. In order to address the latter question, transgene integration sites were visualised in the KD founder mouse using fluorescent *in situ* hybridisation (FISH).

3.2.2.2 Fluorescence In Situ Hybridisation (FISH) was used to show chromosomal localisation of the transgenes

FISH provides a direct and powerful way to study the cytogenetic localisation of DNA sequences. In order to confirm the hypothesis that the KD founder mouse has multiple integration sites of the transgene, FISH was optimised using the entire albumin-OTC^{MOD} transgene construct as a probe (Fig 15). Murine metaphase chromosome spreads were obtained by a mixed splenic lymphocytic culture from two F_1 progeny from both the KD and KE transgenic lines. The whole albumin-OTC^{MOD} transgene was then labelled with a fluorescent epitope and used as a probe for the detection of the site of transgene integration. This technique confirmed the presence of one transgene integration site in the progeny of both these lines (Fig 15). Due to the fact that the majority of murine chromosomes are acrocentric and

Figure 14. Southern blot analysis of albumin-OTC^{MOD} founder lines KD and KE.

As previously shown (Fig 13), multiple bands were obtained in the Southern blot of KD after restriction enzyme digestion. These bands could have been an artefact due to incomplete digestion of the genomic DNA, and therefore a confirmatory Southern blot of KD and KE was performed using 5 μg genomic DNA from albumin-OTC^{MOD} lines. Genomic DNA was digested with HindIII and probed with 0.85Kb SV40 polyadenylation sequence. The KD line shows multiple bands suggestive of multiple chromosomal integration sites.

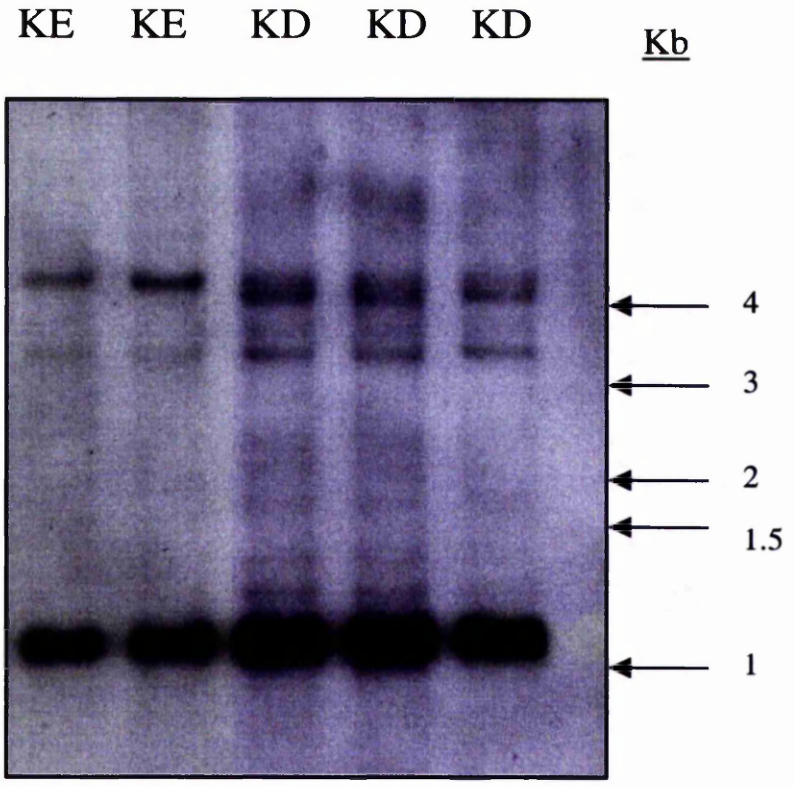
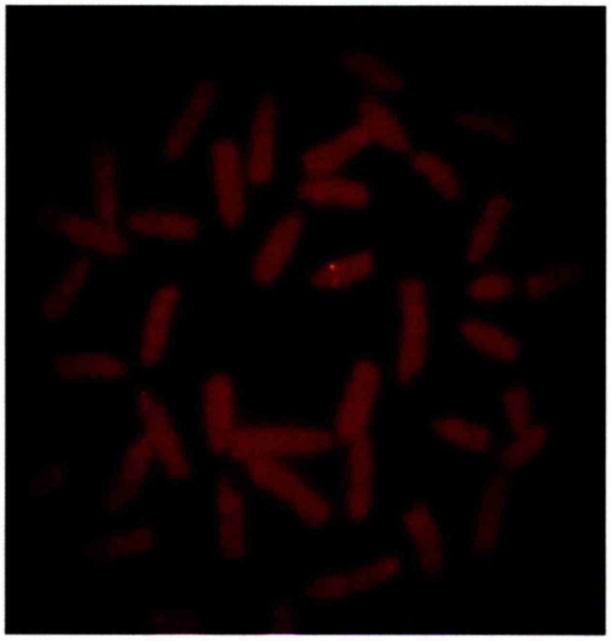


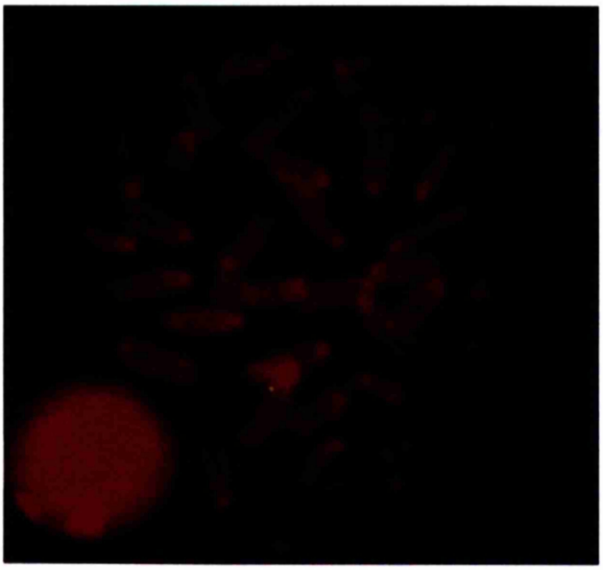
Figure 14. Southern blot analysis of albumin-OTC^{MOD} founder lines KD and KE.

Figure 15. Fluorescent *in situ* hybridisation of KE and KD line chromosome metaphase spreads.

Metaphase chromosome spreads were obtained from KE and KD transgenic lines. Fluorescent *in situ* hybridisation was performed using the entire albumin-OTC^{MOD} transgene as probe. Both lines show a single chromosomal insertion site. Female mice were used in this assay to exclude integration into the 'X' chromosome not being detected.



KD line



KE line

Figure 15. Fluorescent *in situ* hybridisation of KE and KD line chromosome metaphase spreads.

because unique centromeric probes were not available, it was not possible to determine which chromosome the transgene had integrated into in both F_1 lines.

3.2.3 DURING BREEDING THE PRESENCE OF THE TRANSGENE WAS DETECTED BY A PCR SCREENING REGIME

After confirmation of integration of the gene expression constructs by Southern Blot analysis of founder transgenic mice, a PCR based screening procedure was used to screen the progeny from subsequent breedings (Fig 16). The PCR used to detect transgene integration was designed to amplify only exogenous OTC^{MOD} gene construct DNA, by selecting primers within exons that spanned a large intronic sequence. The endogenous genomic sequence OTC gene was thus unlikely to be amplified by PCR.

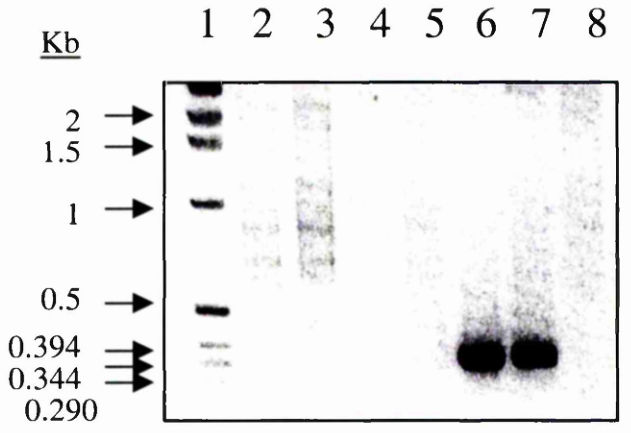
Total genomic DNA was extracted from tail tip biopsy and used as a template for the PCR reaction. The PCR product was 340 bp in positive transgenic mice (Fig 16). The same PCR was used to screen all the albumin-OTC^{MOD} and creatine kinase-OTC^{MOD} transgenic lines. During optimisation of the OTC^{MOD} cDNA PCR, a qualitative correlation was performed between PCR results and those obtained by Southern Blot analysis of the F_1 mice from the 'K' founder lines of mice. The results were identical in all cases.

3.2.4 NORTHEN BLOT ANALYSIS SHOWS THAT THE INTEGRATED TRANSGENES ARE EXPRESSED

Although integration of the transgene had been shown by Southern and FISH technology, approximately 5% of integrated transgenes are not expressed. In order to confirm expression of the transgene, total mRNA was extracted from the relevant target tissue i.e. liver or skeletal muscle The 0.85 Kb SV40 polyadenylation/intron

Figure 16. Detection of the recombinant OTC^{MOD} transgene by PCR.

A PCR was optimised to screen for the presence of the transgene during breeding of the different transgenic and OTC deficient lines. Primers were designed in two adjacent exons of the endogenous OTC gene, thereby amplifying across an intron. The presence of the intron in the endogenous OTC gene would prevent a PCR product being formed, whilst the OTC^{MOD} cDNA, which contained no intron, produced a 340 bp PCR product which could be visualised on a 1.5% agarose gel. The template for the PCR reaction was genomic DNA extracted from a tail tip biopsy.



Lanes
1. 1 Kb ladder
2. Negative for transgene
3. Negative for transgene
4. Negative for transgene
5. Negative for transgene
6. Positive for transgene
7. Positive for transgene
8. Negative for transgene

Figure 16. Detection of the recombinant OTC^{MOD} transgene by PCR.

viral sequence was used as a radioactive probe for Northern blot analysis. Figure 17 shows a Northern blot result using 15 μg of total RNA for lines KE, KD, KG, QA, QB and QE. Northern blot analysis confirmed expression of the transgenes, with an expected mRNA of 1.8 Kb in all six of the albumin-OTC^{MOD} and creatine kinase-OTC^{MOD} transgenic lines.

KE shows a doublet band at the expected 1.8 Kb mRNA size, whilst KD has only a faint band at the expected size with the majority of the transcript being much larger. This abnormal KD transcript size on Northern blot analysis taken together with the Southern blot and FISH data (Figs 13, 14 &15) strengthen the hypothesis that this line has rearranged the transgene on integration. KG shows the highest level of specific OTC^{MOD} gene expression in the albumin promoter driven group, despite Southern analysis results indicating that only one copy of the gene expression construct has integrated (Fig 13).

The Northern blot results of both hepatic and skeletal muscle recombinant OTC expression in the albumin and creatine kinase-OTC^{MOD} transgenic lines indicates that in addition to the correct sized transcript of 1.8 Kb, there were multiple other transcripts from the OTC expression constructs (Fig 17). These multiple transcripts are unlikely to be due to expression from the endogenous gene as they are absent from control lanes and the SV_{40} probe has no homology to endogenous murine genomic sequences. Although these alternative transcripts may suggest rearrangement of the transgene at the genomic level, this phenomenon of multiple transcripts had been seen in other transgenic mouse lines produced in the research group which have also incorporated the same SV40 polyadenylation / intron sequence. It has been shown that the SV40 polyadenylation sequence can cause aberrant splicing events (Huang and Gorman 1990).

Figure 17. Northern blot analysis of albumin and creatine kinase-OTC^{MOD} transgenic lines.

Northern blot analysis using total RNA from hepatic tissue in the albumin-OTC^{MOD} lines (KE, KD, KE) and total RNA from skeletal muscle from the creatine kinase-OTC^{MOD} lines (QA, QB, QE). The SV40 polyadenylation sequence was radioactively labelled as a probe. KG and KE lines show reasonable expression of the correct 1.8 Kb transcript, although KE does appear to have a doublet band at this size. Although KD does have a 1.8 kb transcript, the majority of the KD transcript appears larger than this, which may suggest that the transgene may have rearranged during integration. QE shows the highest expression of OTC^{MOD} transcription although in fact all the 'Q' lines show higher expression levels than the 'K' lines. The larger transcript sizes do appear constant in all the transgenic lines.

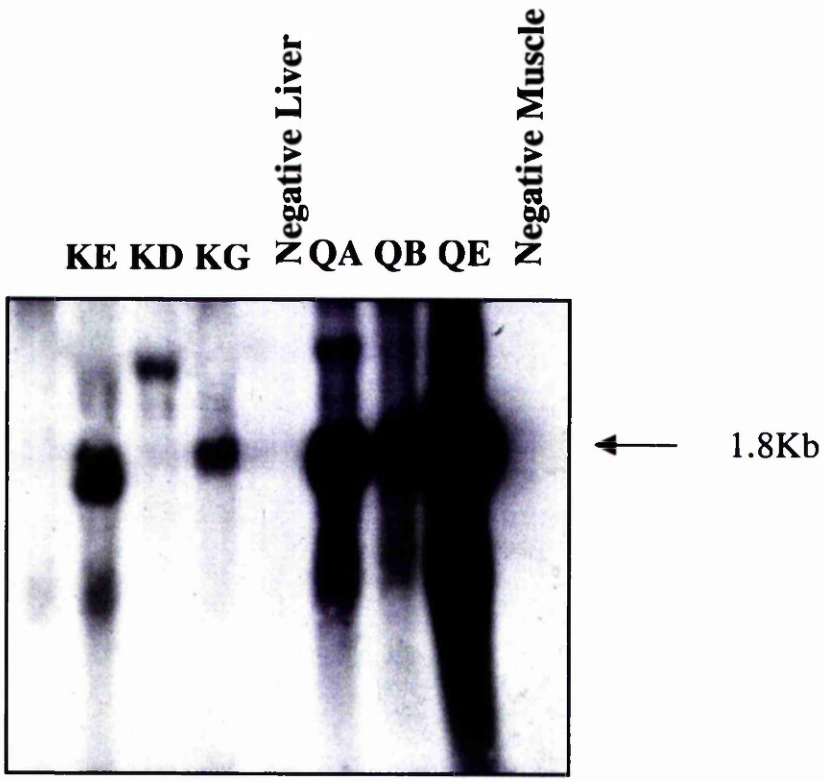


Figure 17. Northern blot analysis of albumin and creatine kinase-OTC^{MOD} transgenic lines.

3.2.5 THE EXPRESSION PATTERN OF THE TRANSGENE CORRELATED WELL WITH THE REGULATORY SEQUENCES USED IN THE CONSTRUCT

The tissue-specific expression of the albumin and creatine kinase promoters was investigated. In one representative transgenic line for each gene construct, total RNA was extracted form multiple tissues from a KE (albumin-OTC^{MOD}) and a QE (creatine kinase-OTC^{MOD}) mouse, and Northern blot analysis was performed using the unique 0.85 Kb SV40/intron polyadenylation sequence as the hybridisation probe (Figs 18 and 19).

3.2.5.1 CREATINE KINASE PROMOTER

Northern blot analysis of the QE creatine kinase-OTC transgenic founder is shown in figure 18. Total RNA was extracted from liver, small intestine, brain, lung spleen, kidney, skeletal and cardiac muscle. A 1.8 Kb transcript can be seen, as expected, in skeletal muscle RNA as well as, to a lesser degree, cardiac muscle RNA. There is also a faint band of similar size present in the renal RNA sample. This result shows that the murine regulatory sequences from the creatine kinase gene used in this construct are capable of directing high levels of gene expression in skeletal, and to a lesser extent, in cardiac muscle. This pattern of creatine kinase promoter/enhancer regulated gene expression has been previously reported (Hoefler, Noehammer et al. 1997). In the current study, the Northern result suggested there is additionally a very low level of recombinant OTC mRNA expression in renal tissue, not previously described.

3.2.5.2 ALBUMIN PROMOTER DRIVEN GENE EXPRESSION CONSTRUCT

Total RNA was extracted from multiple tissues of the KE transgenic line and Northern analysis was performed using the 0.85 Kb SV40/intron polyadenylation sequence as the probe. This Northern blot result (Fig 19) indicates regulatory sequences from the murine albumin promoter and enhancer appear to drive tight hepatic-tissue specific gene expression. No illegitimate transcripts were seen in any of the tissue samples.

3.3 THE ABILITY OF ECTOPIC RECOMBINANT OTC EXPRESSION TO CORRECT THE PHENOTYPES OF OTC DEFICIENT MOUSE MODELS

Having established successful transgene integration and tissue specific expression of the albumin-OTC^{MOD} and creatine kinase–OTC ^{MOD} constructs in transgenic founders, the aim of this study was to investigate whether exogenous expression of the recombinant OTC in skeletal muscle would correct the phenotype of the OTC deficient mouse models. This was achieved by breeding the creatine kinase-OTC^{MOD} transgenic mice onto a Spf and Spf^{ASH} mutant background. As the OTC mutation is X-linked only male mice with both the transgene and OTC endogenous mutation were analysed. In addition, as a positive control, the albumin-OTC^{MOD} mice were also bred onto the OTC deficient backgrounds.

The effect of the transgenic tissue-specific expression of recombinant OTC on the OTC deficient mouse models was assessed by three criteria namely; 1) correction of the phenotype associated with endogenous OTC deficiency, 2) reduction in plasma ammonia levels, which are elevated in OTC deficiency and finally 3) direct *in vitro* assayed tissue- specific OTC activity

Figure 18. Northern blot analysis indicating the tissue specificity of the creatine kinase-OTC^{MOD} expression cassette.

Northern blot analysis showing tissue-specific expression of creatine kinase-OTC^{MOD} transgene (QE line) using the SV40 polyadenylation sequence as the probe. Total RNA was extracted from multiple tissues from a QE transgenic mouse. High OTC^{MOD} expression levels are seen in skeletal muscle, moderate in cardiac tissue and low in renal tissue. In the skeletal muscle there is also a smaller transcript seen which was also present in Figure 17. The negative control is total RNA extracted from skeletal muscle from a non-transgenic mouse, indicating the transgene-specific nature of the SV40 probe.

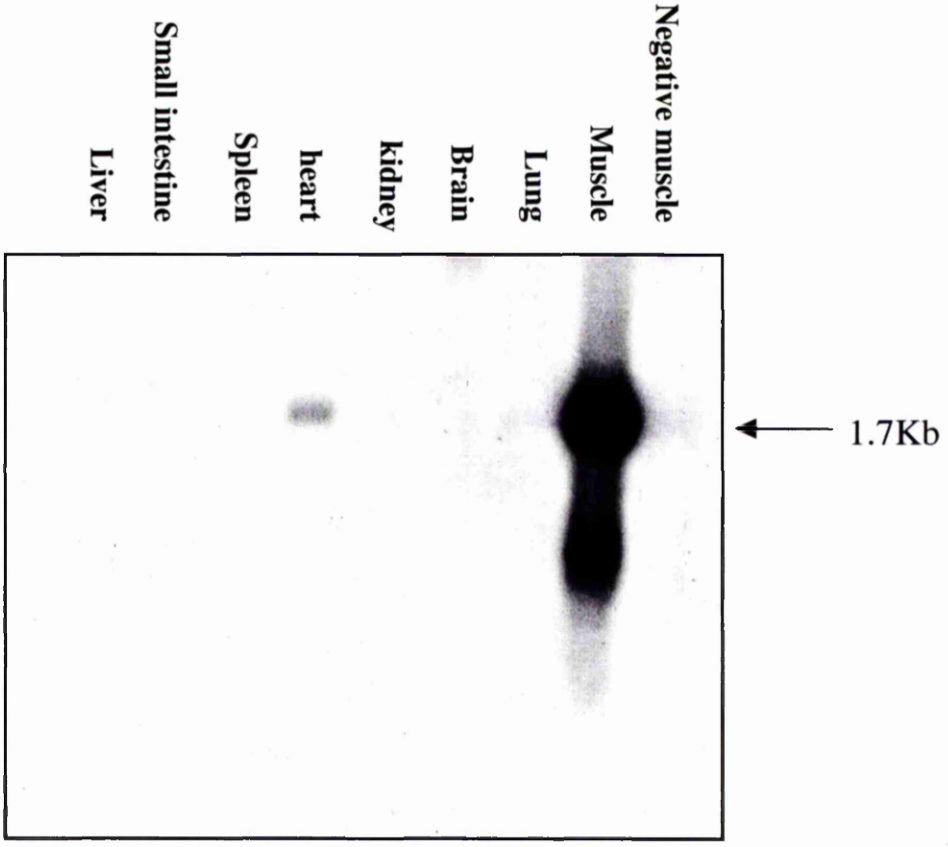


Figure 18. Northern blot analysis indicating the tissue specificity of the creatine kinase-OTC^{MOD} expression cassette.

Figure 19. Northern blot analysis showing tissue specific expression of the albumin-OTC^{MOD} transgene.

Northern blot analysis showing tissue-specific expression of albumin-OTC^{MOD} transgene (KE line). The SV40 polyadenylation sequence was radioactively labelled as the probe. Total RNA was extracted from multiple tissues from a KE transgenic mouse. High expression of the transgene is seen in hepatic extract only.

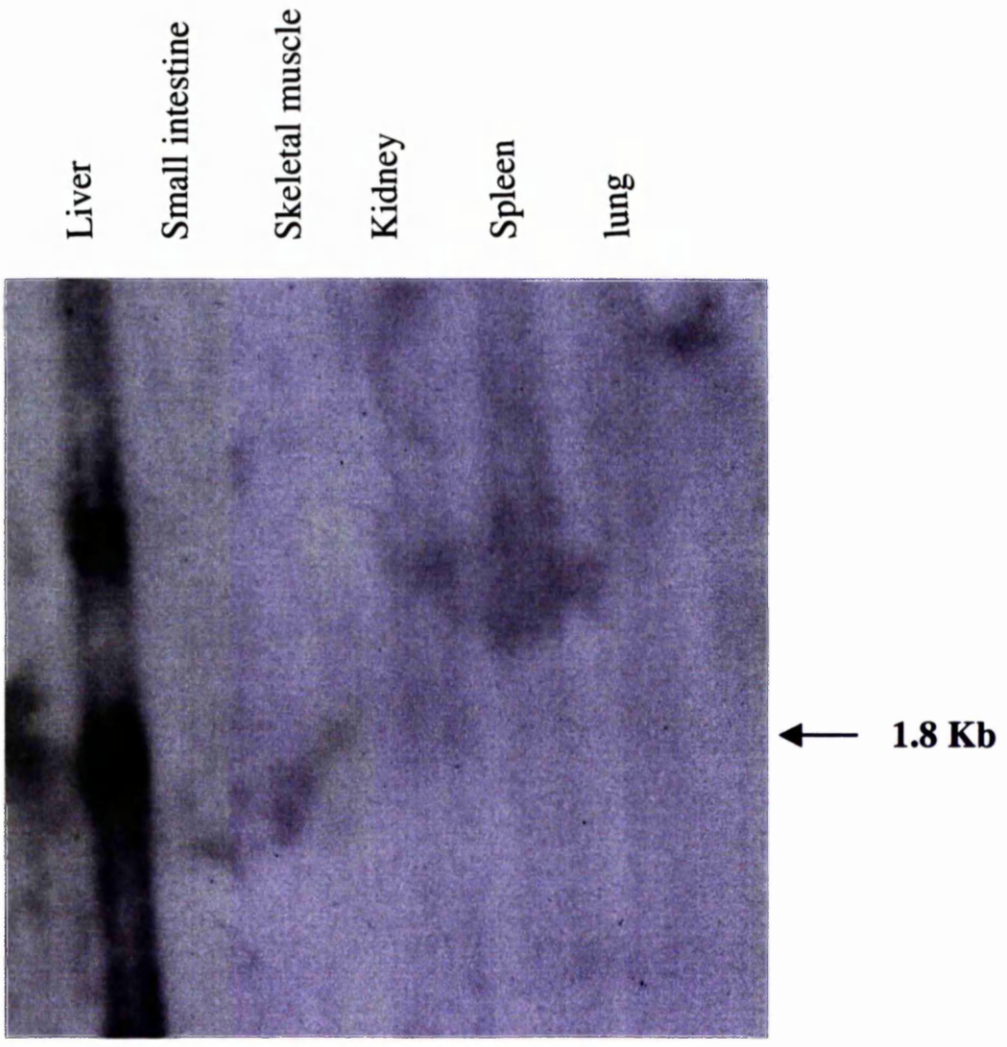


Figure 19. Northern blot analysis showing tissue specific expression of the albumin-OTC^{MOD} transgene.

3.3.1 BREEDING OF TRANSGENIC LINES

3.3.1.1 PHENOTYPE OF AFFECTED OTC DEFICENT MICE

The phenotypes of the affected Spf and Spf^{ASH} male mice were scored at weaning. At this stage affected males showed considerable variation in phenotypes. The majority of the affected males were small and failing to thrive, with sparse to absent hair growth, whilst in about 5% of cases severe runting prevented weaning and necessitated culling (Fig 4). The affected males did grow but remained noticably smaller than their unaffected male littermates. The appearance of body fur was delayed by about 2 weeks and appeared in sparse irregular clumps, which slowly outgrew to cover the entire body. By 8 weeks the only phenotype detectable was low body weight and a 'waxy' appearance to the coat texture which appeared to be due to thinning of the fine down undercoat of fur. On the mixed (FVB) x (C57Bl/6J) background, no phenotypic difference could be observed between the sparse fur and sparse fur^{ASH} mouse colonies. However, there was a difference in mortality rate between the sparse fur and the sparse fur^{ASH} mouse colonies with a higher mortality rate in the latter.

3.3.1.2 BREEDING OF THE RECOMBINANT OTC TRANSGENIC MICE ONTO AN OTC DEFICIENT BACKGROUND

The C57B/6J (B6) transgenic founder mouse from each line was bred with both Spf and the Spf^{ASH} animals, which were both outbred with a mixture of (FVB) x (C57Bl/6J) genetic background. The F_1 females from an affected male X wildtype cross were obligate carriers of the mutant OTC allele and were further crossed with wildtype males to produce 50% affected F_2 males. Unfortunately due to the higher mortality in the Spf^{ASH} colony, the number of surviving transgenic mice on this

mutant background, was very low which compromised statistical analysis of this data. The data from the three albumin-OTC^{MOD}/Spf^{ASH} lines was therefore pooled for statistical analysis. Similarly, the data from the creatine kinase–OTC^{MOD}/Spf^{ASH} lines was pooled for statistical purposes.

As mentioned previously, a complicating factor was the large variation in expressivity within the 95% of affected Spf male mice that survived past weaning. This variability could be due to interactions between the OTC gene locus and other modifier genes, as has been suggested in the human clinical setting. Regardless of cause, this large variation in expressivity precluded a meaningful score of affected phenotypes when the Spf and Spf^{ASH} were bred onto transgenic backgrounds. To minimize subjectivity, mice that showed any feature of the Spf phenotype were classified as 'affected' and no intermediate phenotypic classification was attempted.

At this juncture, transmission of the OTC transgene into F_1 and F_2 progeny was determined by PCR of the rat OTC cDNA. Due to the higher residual hepatic OTC activity present in the Spf than the Spf^{ASH} mice, it was expected that these mice would be more likely to show phenotypic correction by the transgene.

Unfortunately, despite the documented presence and expression of the transgene, no correction of either the sparse fur or sparse fur^{ASH} murine phenotype was noted after breeding with any of the transgenic founder lines, including, unexpectedly, the hepatocyte-specific transgenic K lines, KD, KE and KG.

Although the presence of Spf mutation could be assessed by phenotypic examination, in those mice with a mild phenotype the presence of the Spf mutation was confirmed at the genomic level by an amplification refractory mutation detection system (ARMS) reaction (Fig 20). This technique was also performed in those phenotypically affected transgenic mice from the K (hepato-specific) lines in order to confirm the presence of the sparse fur mutation and, thereby ruling out the

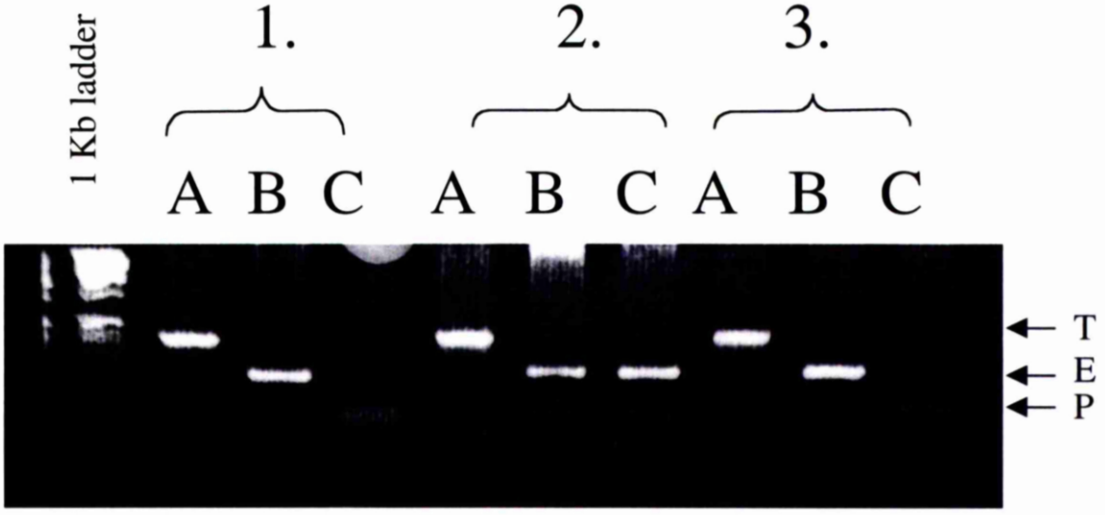
Figure 20. Amplification refractory PCR (ARMS) assay for sparse fur mutation. This allows the molecular distinction of Spf and wildtype mice based on the single Spf point mutation in the endogenous OTC gene. PCR forward primers were designed in which the 3' nucleotide of the oligonucleotide has high fidelity for either the Spf genomic mutation or the wildtype genomic sequence. The downstream reverse primer in both reactions was a common primer.

Common primer-5' GGC ATT ATC TAA GGA GAA GCATCA TCT TCT C

Wild type Primer 5' ACA CCC TTC CTT TCT TCA CAC ACA AGA CAT **GC***

Spf primer 5' ACA CCC TTC CTT TCT TCA CAC ACA AGA CAT GA*

The PCR conditions are optimised so that the Spf mutation primer will not prime off a wildtype sequence and the wildtype primer will not prime of a Spf mutation sequence. The presence of the OTC^{MOD} transgene was screened for using the PCR design-specific for the exogenous transgene (Figure 16.) PCR products amplified from genomic DNA using primers specific for A) Transgenic OTC^{MOD} cDNA, B) Wildtype endogenous OTC sequence (5' end of exon 4 ~ amino acid 117), C) Endogenous Spf mutation (5' end of exon 4 ~amino acid 117.)



T: transgene, **E**: endogenous OTC gene, **P**: unincorporated primers

Mouse 1. Female, Transgene on a wildtype OTC background
(No PCR product specific to the sparse fur mutation)

Mouse 2. Female,Transgene on a heterozygous background
(PCR products specific to both the sparse fur and
wild type OTC genomic alleles)

Mouse 3. Male , Transgene on a wildtype background
(No PCR product specific to the sparse fur mutation)

Figure 20. Amplification refractory PCR assay for the Spf mutation.

possibility that the transgene may have a detrimental effect on normal OTC expression and function, and thus produce an affected phenotype. The possibility of this latter effect was also ruled out by the birth of phenotypically normal male mice carrying the transgene. ARMS was also performed in unaffected transgenic males to assess the absence of the mutant OTC allele, thus excluding the possiblity that in these unaffected male mice the normal phenotype was due to correction of a mutant allele by the transgene. Unfortunately, the ARMS reaction for the Spf^{ASH} mutation could not be reliably optimised as it gave false positives with primers for the sparse fur^{ASH} mutation in wildtype DNA. This may have been due to the high GC content at the 3' end of the Spf^{ASH} mutation specific primer. The last 5 3' bases comprised 4 G:C bonds in comparison to the Spf mutation specific primer which only contained 2 G:C bond in the last 5 3' nucleotides.

Due to the wide phenotypic variation seen within the breeding colonies of both sparse fur and sparse fur^{ASH}, it was felt that any attempt to correlate modulation of phenotypic severity with expression of transgenes bred into this colony was not possible, therefore biochemical analysis of the mice was undertaken.

3.3.2 Plasma Ammonia levels were evaluated to determine efficacy of recombinant OTC expression

The plasma ammonia level is a metabolic marker of a hypomorphic OTC allele used in clinical practice (normal range 10-35 μmol/l). Although by no means pathognomic, plasma ammonia is elevated when the urea cycle decompensates, and is therefore raised in cases of OTC deficiency. Plasma ammonia levels were investigated as a possible indicator of partial correction of the metabolic OTC defect in the Spf mice.

Ammonia levels (μmol/l) were assayed in plasma samples obtained by cardiac puncture. Only male mice were used in this assay and they were always sacrificed at the same time of day to allow for variation in NH_4 levels due to dietary intake or physical activity. The plasma was isolated immediately.

As the data set collected was small (18, 12 and 29 mice in the wild type, Spf^{ASH} and Spf control groups respectively, Table 2) a non parametric statistical analysis was performed, namely the Mann-Whitney test, which compares two independent groups. This test was performed using the SPCC statistic programme.

The wildtype, sparse fur and sparse fur^{ASH} backgrounds had median plasma ammonia levels of 33.0, 58.0 and 236.5 respectively. Figure 21 demonstrates the wide variation in NH_4 levels in both the Spf and Spf^{ASH} mice compared to the wild types, as reflected earlier in the variable expressivity of the post–natal phenotypes. Nevertheless, non-parametric testing of the plasma ammonia (μmol/l) showed a statistical difference between the levels found in both the Spf and Spf^{ASH} compared to the wildtype mice (Table 2, Fig 21). This indicated that the plasma ammonia level was a reasonable metabolic index, which could differentiate between a group of mice with mutant OTC alleles from those with wildtype alleles.

3.3.2.1 THE PRESENCE OF THE ALBUMIN-OTC^{MOD} TRANSGENE SIGNIFICANTLY REDUCED THE PLASMA AMMONIA LEVELS IN THE OTC DEFICIENT MOUSE MODELS

Plasma ammonia levels were measured in the albumin-OTC^{MOD} transgenic mice bred with the sparse fur mutant mice. Due to the X-linked inheritance of the endogenous OTC gene, only male mice were assayed. Each of the albumin-OTC^{MOD} lines was analysed separately. The results are depicted in figure 22 and tabulated in

Table 3. The KG and KE lines bred onto a Spf background had median plasma ammonia levels of 41 and 34 respectively and showed a statistically significant improvement in ammonia levels compared to non-transgenic Spf background (p=0.009 and p=0.01 respectively) (Table 3). In addition, they showed no significant difference in plasma ammonia values from the wildtype OTC background (p=0.258 and p=0.759) (Table 3). However, the number of animals analysed for the KD transgenic line was insufficient to show a statistically significant difference from Spf (p=0.071). Nevertheless, it is clear that the data from the KD transgenic line was following the same trend as in KG and KE lines (Fig 22), despite the low level of OTC^{MOD} transcript seen in the Northern Blot analysis (Fig 17). By pooling the data from the three albumin-OTC^{MOD} lines, there was a highly significant improvement in plasma ammonia levels compared to Spf background (p=0.001) and no significant difference compared to the wildtype background (p=0.380) (Table 3). It should also be noted that the large variation in plasma ammonia seen in the Spf mice is virtually eliminated in the three transgenic lines, with the exception of one KG mouse (Fig 22).

Unfortunately due to the higher mortality found in the Spf^{ASH} mice, it was not possible to analyse the albumin transgenic lines separately on this background and therefore results from all the albumin transgenic lines were amalgamated (Fig23, table 4). The results show a significant improvement in the plasma ammonia levels in the albumin-OTC^{MOD} transgenic mice on the Spf^{ASH} background compared to the Spf^{ASH} background alone (68 compared to 236.5μmol/l, p=0.028) but the ammonia level was still significantly different from those found in the wildtype mice (p=0.003) (Table 4). This is most likely because of the small sample size analysed for the K lines (N=7). The 'K' line plasma ammonia data show a tight distribution

with little variation in ammonia levels measured (Fig 23), which is similar to the spread of values obtained in the wildtype control group and distinct from the larger variation in values seen in the Spf^{ASH} group (Fig 21).

3.3.2.2 THE PRESENCE OF THE CREATINE KINASE-OTC^{MOD} TRANSGENE HAD NO EFFECT ON PLASMA AMMONIA LEVELS IN THE OTC DEFICIENT MOUSE MODELS

Due to low numbers of mice available, data from the creatine kinase-OTC^{MOD} lines was pooled together on both the Spf and Spf^{ASH} backgrounds (Figs. 24 and 25, table 5A and B) In neither case was the plasma ammonia in the 'Q' lines significantly lower than in the mutant OTC allele mice alone (p=0.152 on the Spf background and p=0.057 on the Spf^{ASH} background) (table 5). Indeed on the Spf background the median plasma ammonia level was higher than on the Spf^{ASH} background (Fig. 24 and 25, table 5). Closer examination of the original data (Table 5A) suggests that on the Spf background the ammonia levels are widely spread with the majority (5/9) of the levels being outwith the normal values. However, on the Spf^{ASH} background, the majority of the values are within normal values. If the QA line is analysed alone all the ammonia values are within then normal range and have a much smaller spread than that seen in the QE line suggesting that there may have been a lowering of the ammonia concentration in the QA line, however inadequate mouse numbers precluded statistical analysis.

In addition, creatine kinase-OTC^{MOD} lines show a wide variation in plasma ammonia level on both a Spf and Spf^{ASH} background similar to the results obtained from the mutant OTC allele background alone (Figs 24 ,25 & 21).

Figure 21. Plasma ammonia levels in wildtype, and affected male Spf and Spf^{ASH} mice.

Plasma ammonia levels were measured in blood obtained by cardiac puncture from male wild type, and affected Spf and Spf^{ASH} mice. Each data point represent the result obtained from an individual mouse. There is a statistical difference values between the three groups. In addition, there appears to be a wider spread of values in the two OTC deficient groups. This variation in plasma ammonia levels seen in the Spf and Spf^{ASH} mice is mirrored by the wide variation in severity of affected phenotypes seen in the males.

Fig 21. Plasma Ammonia levels on normal, sparse fur and sparse fur^{ASH} backgrounds

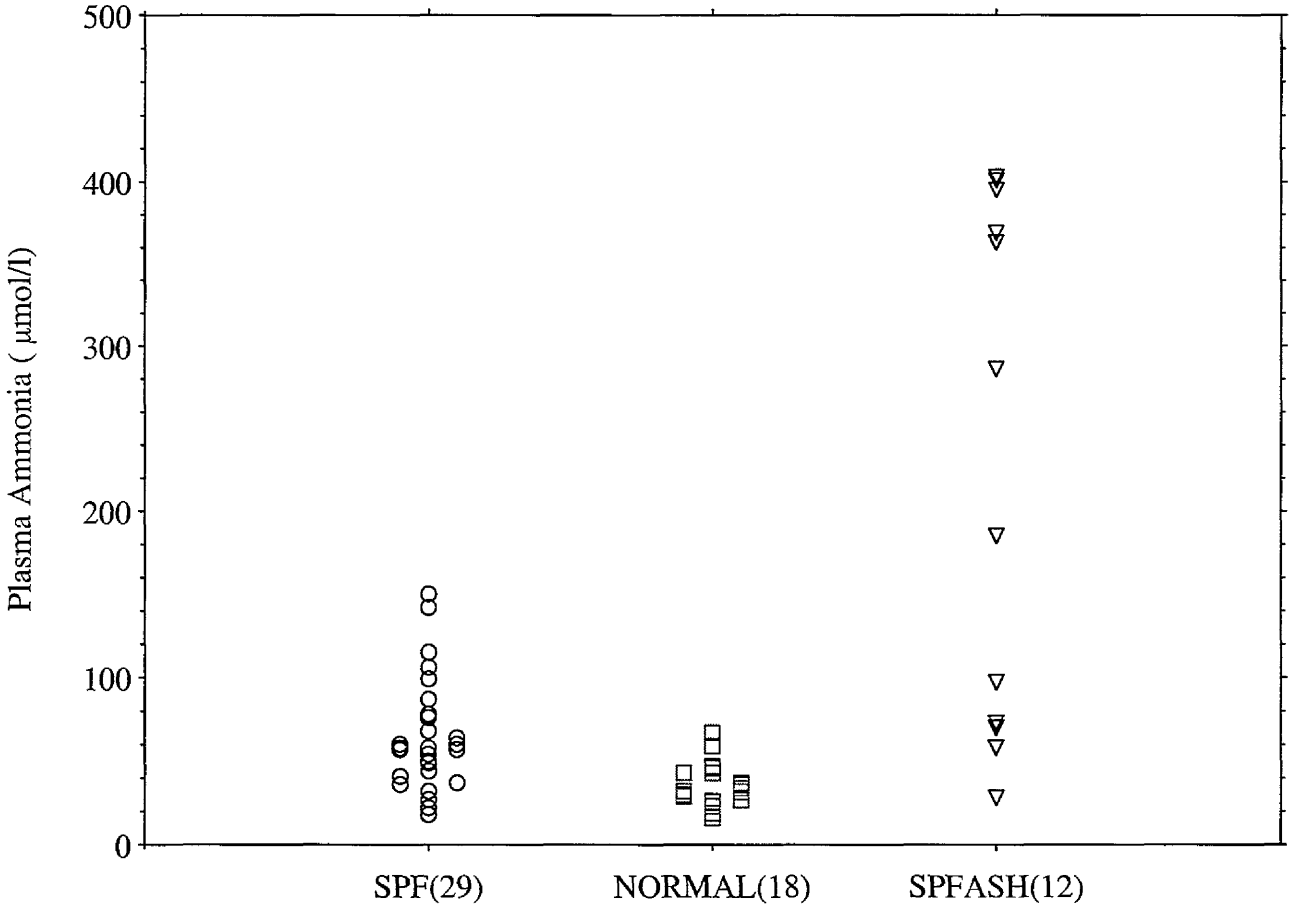


Figure 22. Plasma ammonia levels in albumin-OTC^{MOD} transgenic mice bred onto a Spf background.

Plasma ammonia values (μMol/L) were assayed in male mice from the indicated albumin-OTC^{MOD} transgenic lines were bred onto a Spf background. KG and KE show a statistically significant improvement in plasma ammonia levels compared to the non-transgenic Spf background alone. In addition, the spread in plasma ammonia levels is less than that seen in the Spf group, more in keeping with the wildtype ammonia values. The KD group, containing the smallest number of animals, does not show a statistically significant amelioration of ammonia values, although there is not the spread of values seen in the Spf group.

Fig 22. Plasma Ammonia Levels In Albumin (K) Lines on a sparse fur background

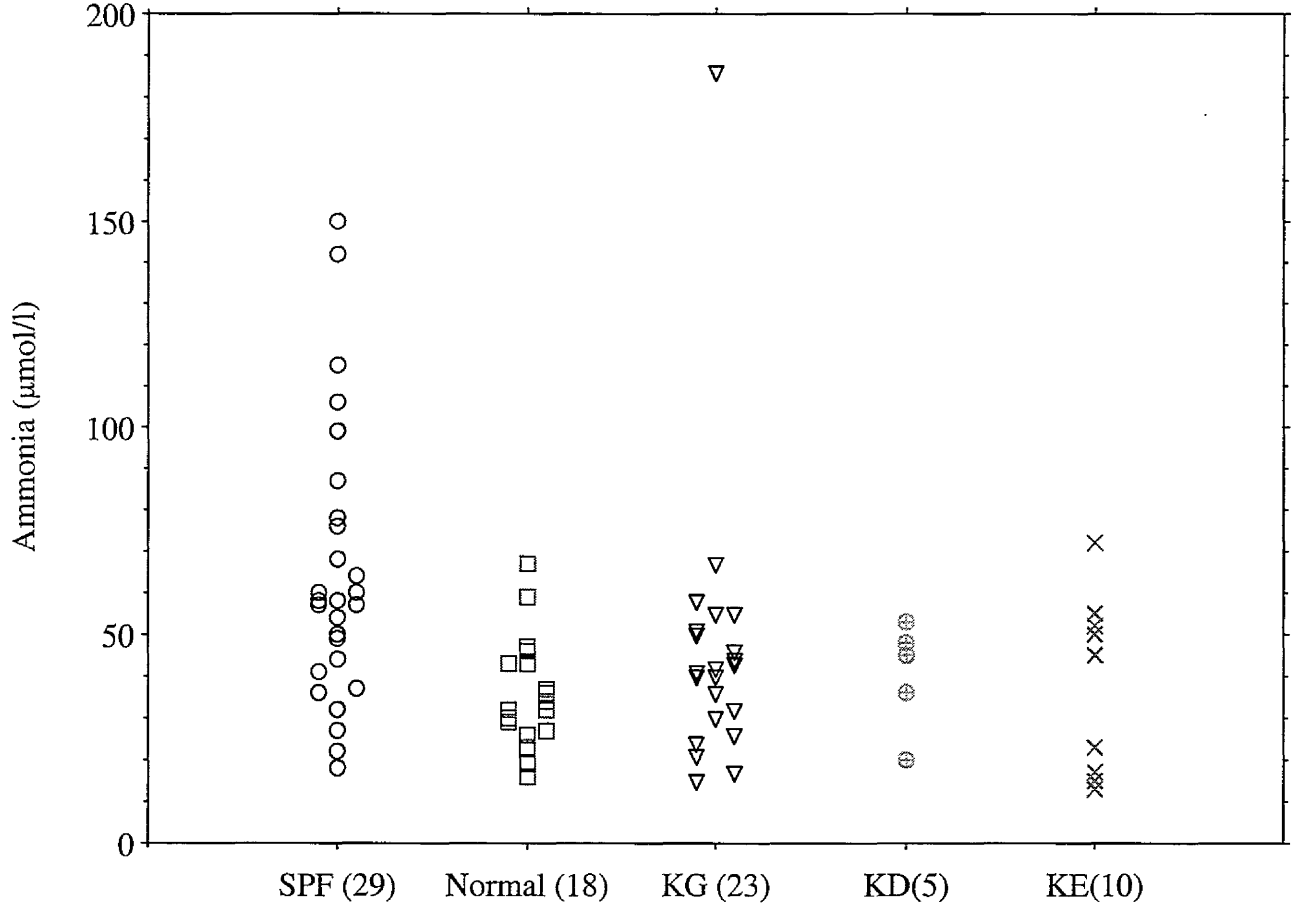


Figure 23. Plasma ammonia levels assayed in albumin-OTC^{MOD} transgenic lines bred onto a sparse fur^{ASH} OTC deficient background.

Plasma ammonia levels were assayed in male albumin-OTC^{MOD} transgenic lines bred onto a SPF^{ASH} background. Each point represents the data from an individual mouse.

Due to low numbers of animals examined for each construct, the data from all the different albumin-OTC^{MOD} transgenic lines were pooled and analysed together. There is a statistically significant improvement in plasma ammonia levels in the presence of the albumin-OTC^{MOD} transgene compared to the non-transgenic OTC deficient group alone.

Fig23. Plasma Ammonia levels in albumin-OTC^{MOD} (K) lines on a sparse furASH background

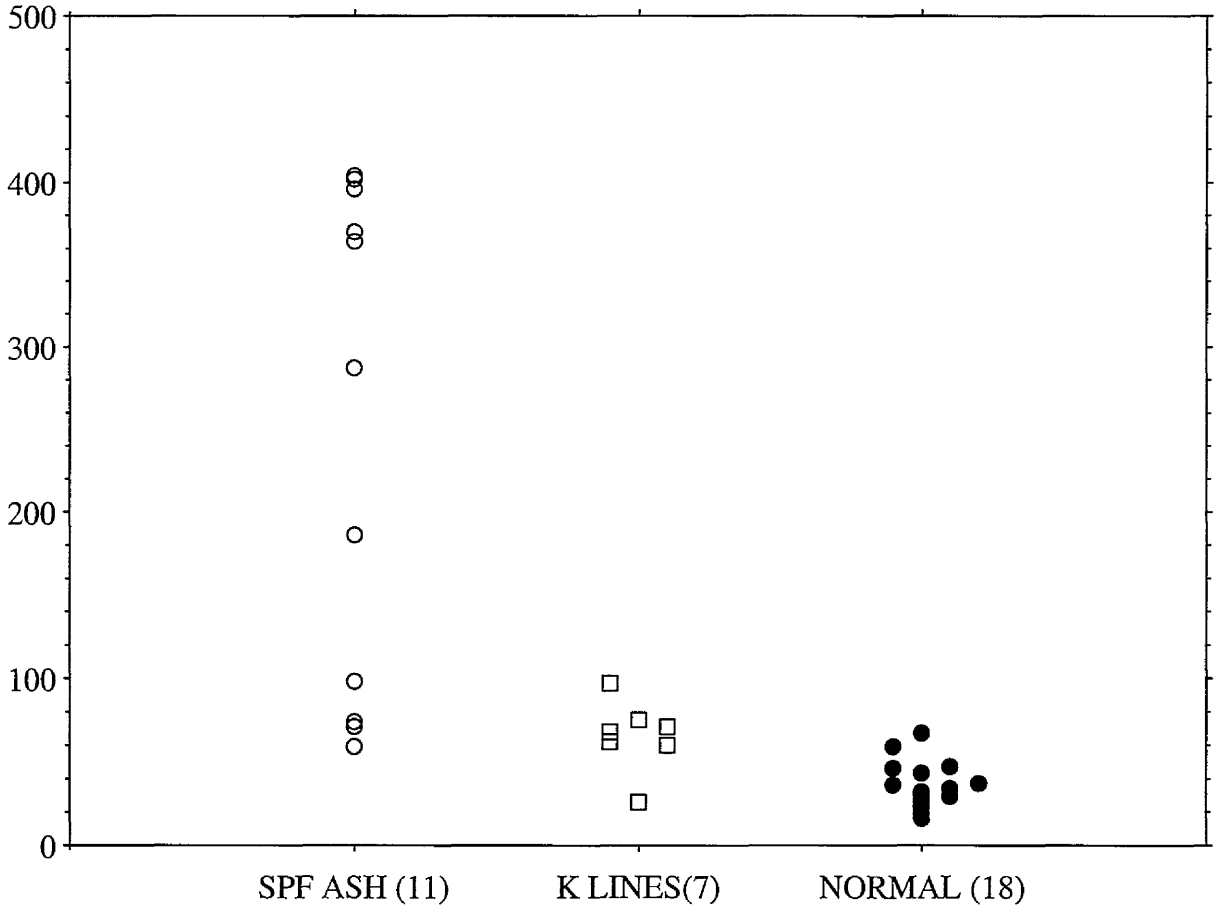


Figure 24. Plasma ammonia levels assayed in creatine kinase-OTC^{MOD} transgenic lines bred onto a Spf OTC deficient background.

Plasma ammonia values were assayed in male mice from the indicated creatine-OTC^{MOD} transgenic lines were bred onto a Spf background. Each point represents an individual mouse. There is no statistically significant improvement in ammonia values in the transgenic group compared to the non-transgenic OTC deficient group. In addition, the transgenic group shows the same wide spread distribution in values noted in the non-transgenic Spf group.

Fig 24. Plasma ammonia level in creatine kinase (Q) lines on a Sparse fur background

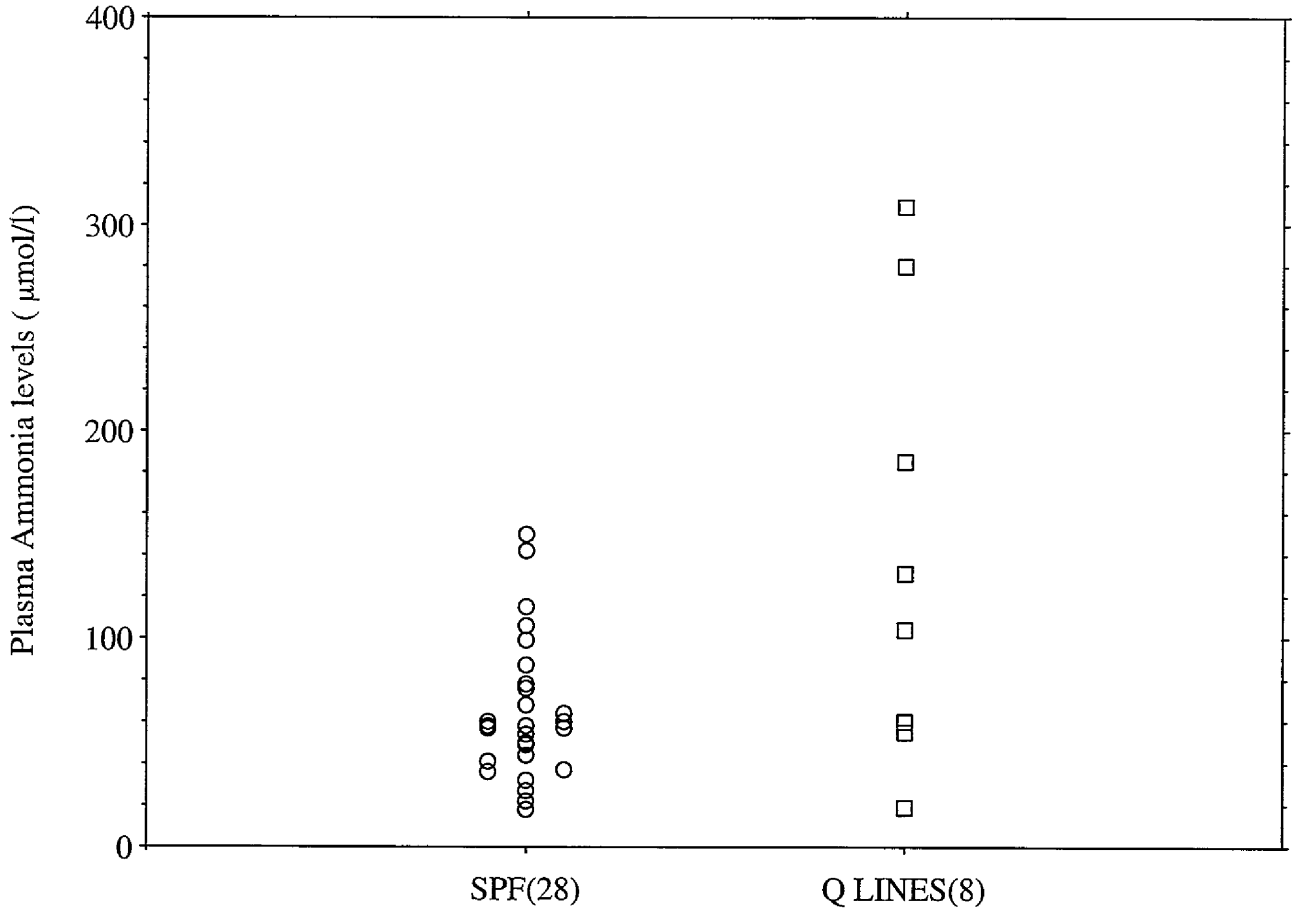


Figure 25. Plasma ammonia levels assayed in creatine kinase-OTC^{MOD} transgenic lines bred onto a Spf^{ASH} OTC deficient background.

Plasma ammonia levels were assayed in male creatine kinase-OTC^{MOD} transgenic lines bred onto a SPF^{ASH} background. Each point represents the data from an individual mouse. Due to low numbers of animals examined for each construct, the data from all the different creatine kinase-OTC^{MOD} transgenic lines were pooled and analysed together. There is no statistically significant improvement in plasma ammonia levels in the presence of the albumin-OTC^{MOD} transgene compared to the non-transgenic OTC deficient group alone.

Fig.25 Plasma Ammonia level in creatine kinase (Q) line on sparse fur^{ASH} background

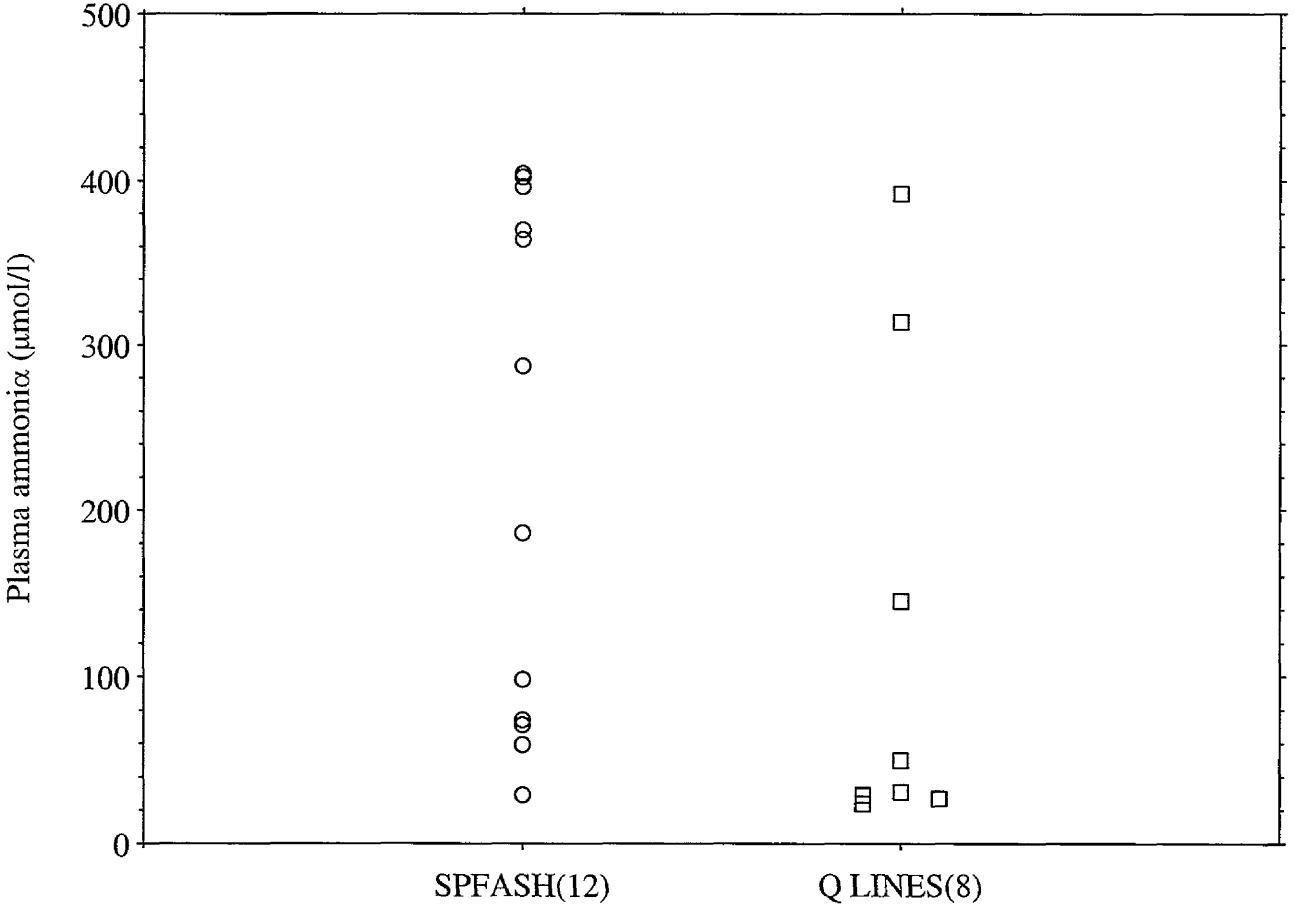


Table 6 Hepatic OTC activity (OD_{490}/mg protein) on sparse fur, sparse fur^{ASH} and wildtype background

All assays were performed in triplicate and the average of the results was taken.

	Wildtype (No 9)	Sparse fur (No 18)	Sparse furASH (No 8)
Median OTC	7.6	1.2	0.44
Wildtype		P*<0.0001	P*<0.0001

Table 7 Hepatic OTC activity (OD_{490}/mgprotein) levels in the albumin -OTC^{MOD} lines on a sparse fur background.

	Wildtype (No 9)	Sparse fur (No 18)	KD (No 9)	KE (No 10)	KG (No 15)	All 'K' Line
Median OTC	7.6	1.2	1.44	1.37	1.65	
Wildtype			P*=0.0001	P*<0.0001	P*<0.0001	P*=0<0001
Sparse fur			P=0.145	P=0.089	P*=0.006	P*=0.007

Table 8 Hepatic OTC activity (OD_{490}/mgprotein) in the albumin -OTC^{MOD} lines on a sparse fur^{ASH} background.

	Wildtype (No.9)	Sparse FurASH (No 8)	All 'K'lines (No 8)
Median OTC	7.6	0.44	0.61
Wildtype			P*<0.0001
Sparse fur^{ASH}			P=0.161

p*= statistical significance in comparison to wildtype data by Mann Whitney test

Table 9 Skeletal Muscle OTC activity (OD_{490}/mgprotein) in normal mice and in the creatine kinase -OTC^{MOD} lines.

	Baseline (No 13)	QA (No 11)	QB (No 5)	QE (No 8)	All "Q"lines
Median OTC	0.73	5.4	1.7	17.2	
Baseline		P*<0.0001	P*=0.014	P*<0.0001	P*<0.0001

Hepatic OTC activity (OD_{490}/mgprotein) levels in the albumin -OTC^{MOD} lines on a sparse fur background.

Table 5 Plasma ammonia levels (μmol/l) in creatine–kinase OTC^{MOD} lines.

Due to low numbers of mice examined, the QA, QB and QE data was pooled and analysed together.
Table 5A indicates the raw data in each founder creatine–kinase OTC^{MOD} founder line, whilst table 5B is the pooled data analysed.

Table 5A

creatine-kinase OTC^{MOD} line	NH_3 levels on sparse fur background	NH_3 levels on sparse fur^{ASH} background
QA	19	27, 50, 29, 24,
QB	185, 55, 104	
QE	27, 60, 131, 280, 309	145, 314, 31, 392

Table 5B

	Wildtype	Sparse fur (No 28)	Sparse furASH (No12)	All "Q' lines on sparse fur B/G (No 9)	All 'Q' lines on sparse fur^{ASH} (No 8)
Median Ammonia	33.0	58	236.0	104.0	40.5
Wildtype				P*=0.012	P=0.338
Sparse fur				P=0.152	
Sparse fur ASH					P=0.057

p*= statistical significance in comparison to wild type data by Mann Whitney test

3.3.3 TOTAL ORNITHINE TRANSCARBAMYLASE ACTIVITY WAS ASSESSED IN THE TRANSGENIC LINES

Direct measurement of OTC activity was undertaken in the different transgenic groups on both the Spf and Spfr^{ASH} backgrounds. An *in vitro* assay was optimised to measure OTC activity in total tissue from liver and skeletal muscle. The estimate of OTC activity was then standardised against the protein content of the individual tissue. An arbitrary unit of OTC activity was used to compare the different sample populations, namely OD_{490}/mg protein. Due to the X-linked inheritance pattern of OTC deficiency, this assay was only performed on male mice.

Baseline hepatic OTC activity was measured in the normal, Spf and Spf^{ASH} mouse populations and gave results of 7.6, 1.2 and 0.44 OD_{490}/mg protein respectively (Fig 26, Table 6). The results confirm a significant difference in hepatic OTC activity between the different OTC mutant alleles and the wildtype hepatic activity. Compared to the wildtype mice, the Spf and Spf^{ASH} mice had a hepatic enzyme activity of 16% and 5%, which is in keeping with relative OTC activities reported originally in the mutant OTC mice (Qureshi, Letarte et al. 1979; Hodges and Rosenberg1989).

In comparison with the plasma ammonia levels observed in these mice (Fig 21), hepatic OTC activites showed a smaller variation in values within each group, with wild type mice having the largest spread (Fig 26). However, as it was not possible to prove normal distribution to the values obtained within a group, the results were analysed using non–parametric statistics, namely Mann-Whitney.

3.3.3.1 HEPATIC OTC ACTIVITY IN ALBUMIN TRANSGENIC LINES ON A SPARSE FUR OR SPARSE FUR^{ASH} BACKGROUND

Each albumin transgenic line was analysed independently (Fig 27, Table 7). The KD and KE lines showed no significant increase in hepatic OTC activity compared to the Spf background alone (1.44 and 1.37 OD_{490}/mg protein, p=0.145 and p=0.089 respectively) (Table 7). However, the KG line showed a significant increase in OTC activity compared to the Spf (1.65 compared to 1.2 OD_{490}/mg protein, p=0.006), but it was still significantly lower than wildtype hepatic OTC levels (p<0.0001) (Table 7). If data from the 'K' line mice were pooled, there was a significant elevation in hepatic OTC activity in this group compared to the Spf group (p=0.007) but OTC levels were still significantly lower than in the wildtype group (p<0.0001)(Table 7). On a Spf^{ASH} background there were not enough mice to analyse each albumin transgenic line separately and therefore, they were grouped together (Fig 28 Table 8). The albumin transgenic mice on a Spf^{ASH} mice background showed no improvement in OTC activity compared to the Spf^{ASH} mice alone (0.61 compared to 0.442 OD_{490}/mg, p=0.161) (Table 8). The transgenic group had a significantly lower hepatic OTC activity than the wildtype mice did (p<0.0001)(Table 8).

3.3.3.2 SKELETAL MUSCLE OTC ACTIVITY IN THE CREATINE KINASE TRANSGENIC LINES

As neither the Spf nor the Spf^{ASH} mice have skeletal muscle OTC activity, the creatine kinase transgenic OTC activity was not measured on either of these backgrounds. Each transgenic line was analysed separately (Fig. 29, Table 9). All the creatine kinase transgenic lines had significantly higher levels of OTC activity than was found in baseline skeletal muscle with QA, QB and QE skeletal muscle

activity being 5.4, 1.7 and 17.22 OD_{490}/mg: $p<0.0001$, $p=0.014$ and $p<0.0001$ respectively) (Table 9). Line QE had an OTC activity that was three times higher than was found in normal hepatic tissue (Tables 6 and 9). This result correlated with the Northern Blot analysis of the mRNA levels which also indicated a higher transcript level in skeletal muscle in QE, than in the hepatic tissue of any of the K lines (Fig. 17)

Interestingly, when comparing with the plasma ammonia levels, the QE line has the higher values compared to those seen in the QA line despite a markedly higher level of skeletal OTC activity (Table 5A).

Figure 26. OTC activity assayed in wild type, Spf and Spf^{ASH} hepatic extracts.

OTC levels were assayed in male liver extracts wild type, Spf and Spf^{ASH} liver extracts. Each data point represents the mean of triplicate measurements from one animal. There is a statistically significant difference in OTC activity between the three groups.

Fig 26. Hepatic OTC activity in normal, sparse fur and sparse fur^{ASH} backgrounds

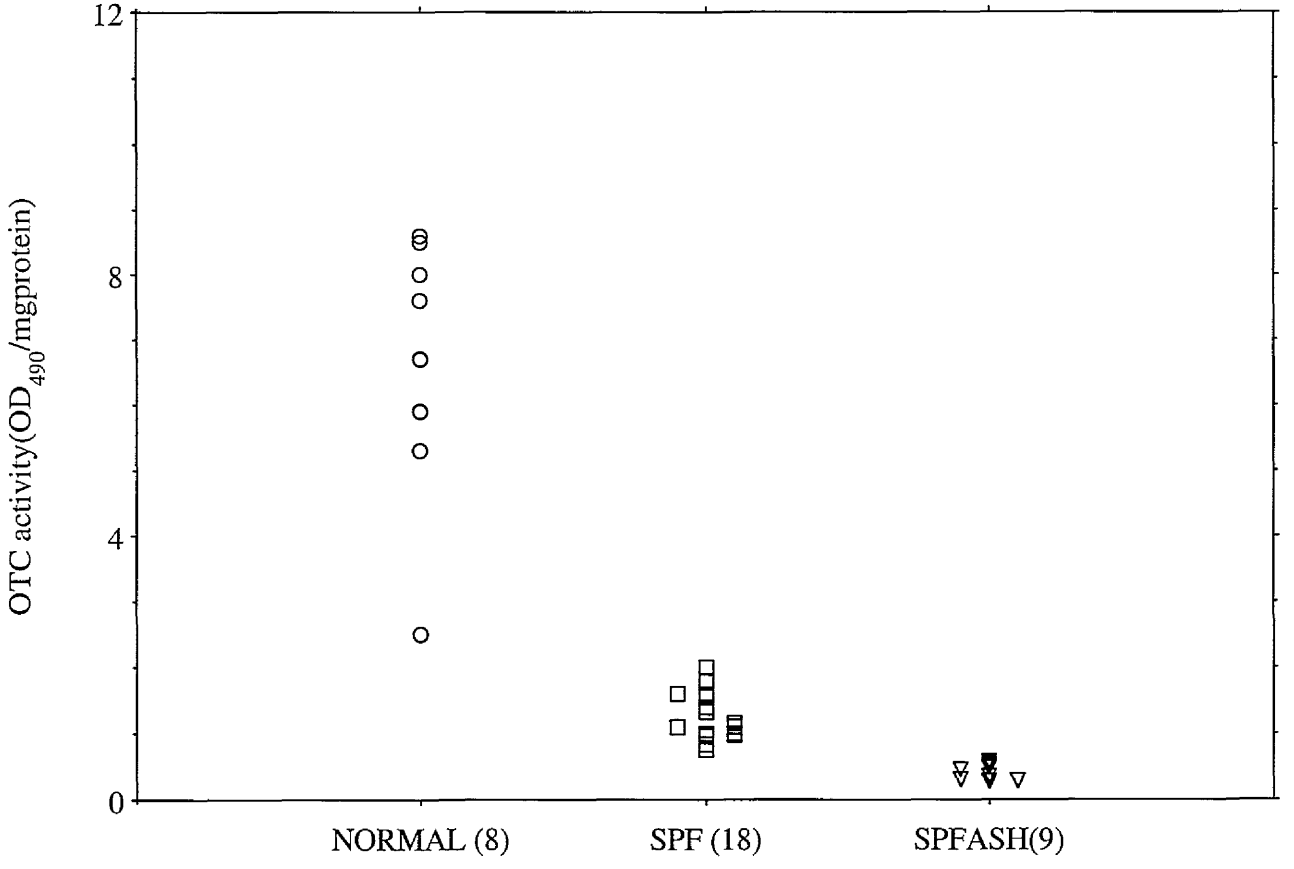


Figure 27. Hepatic OTC levels in albumin-OTC^{MOD} transgenic mice bred onto Spf background.

OTC levels were assayed in male liver extracts from the albumin-OTC^{MOD} transgenic lines bred onto mice carrying the SPF mutation. Each data point represents the mean of triplicate measurements from one animal. KG showed a statistically significant increase in OTC activity above that seen in the non-transgenic Spf liver.

Fig 27. Hepatic OTC activity in albumin (K) lines on a sparse fur background

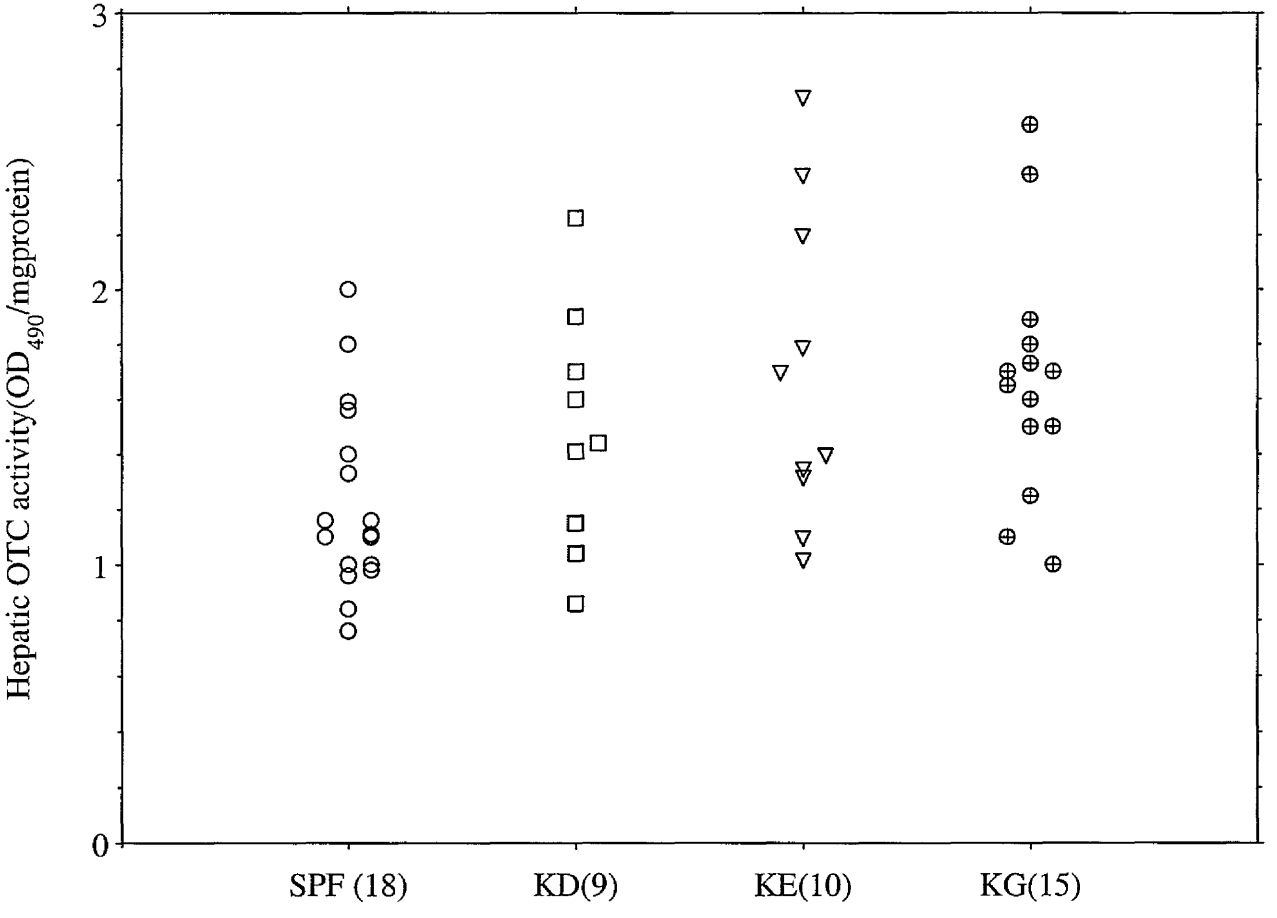


Figure 28. Hepatic OTC levels in albumin-OTC^{MOD} transgenic mice bred onto Spf^{ASH} background.

OTC levels were assayed in male liver extracts from the albumin-OTC^{MOD} transgenic lines bred onto mice carrying the SPF^{ASH} mutation. Each data point represents the mean of triplicate measurements from one animal. The transgene gave no statistically significant alteration in OTC level compared to non-transgenic SPF^{ASH} mice.

Fig 28. Hepatic OTC levels in albumin (K) line on a sparse fur^{ASH} background

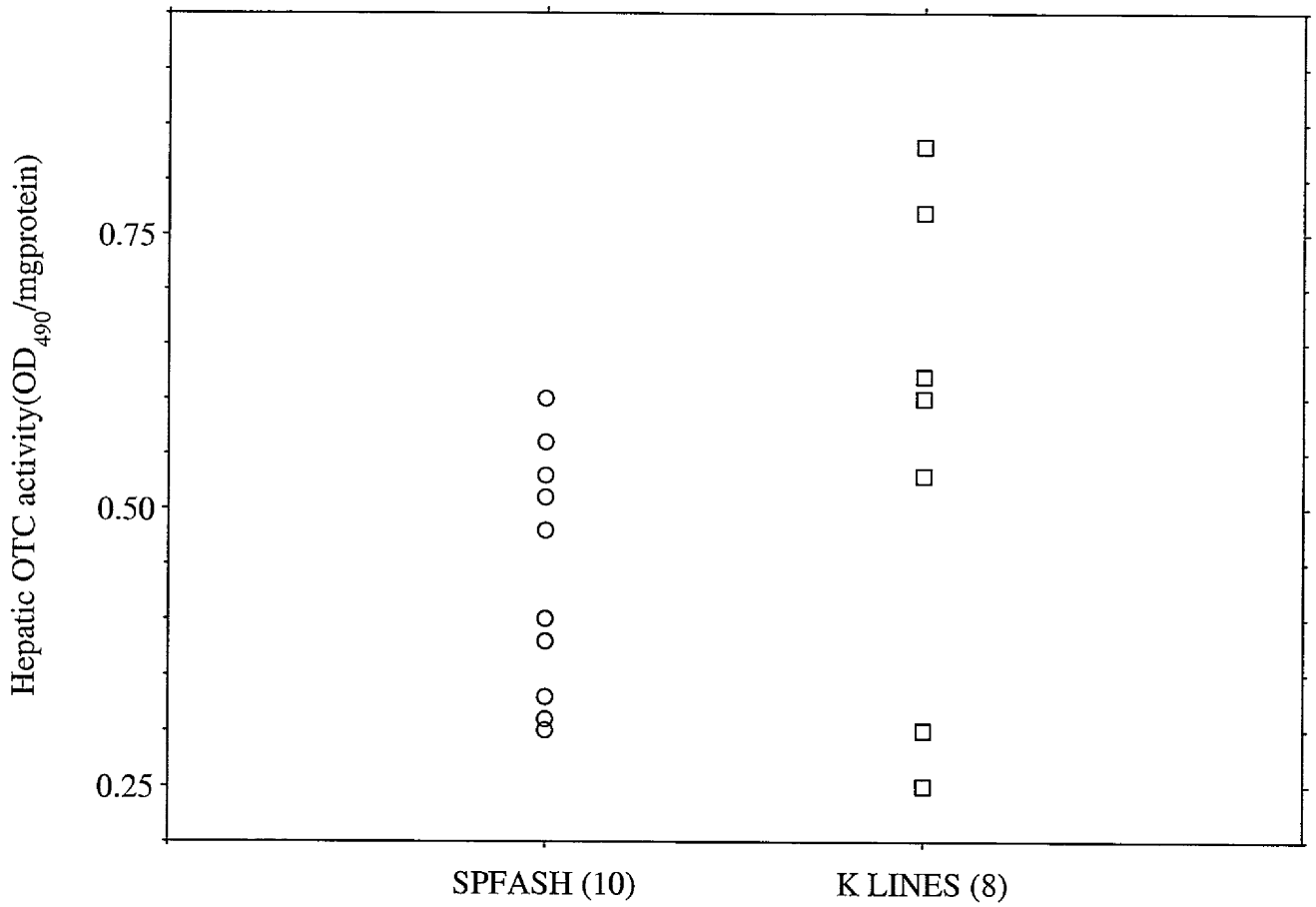


Figure 29. Skeletal muscle OTC levels in creatine kinase-OTC^{MOD} transgenic mice.

OTC levels were assayed in male skeletal muscle extracts from the creatine kinase-OTC^{MOD} transgenic lines. Each data point represents the mean of triplicate measurements from one animal. The transgene gave a statistically significant increase in skeletal OTC level compared to non-transgenic mice.

Fig 29. OTC in skeletal muscle of creatine kinase(Q) line

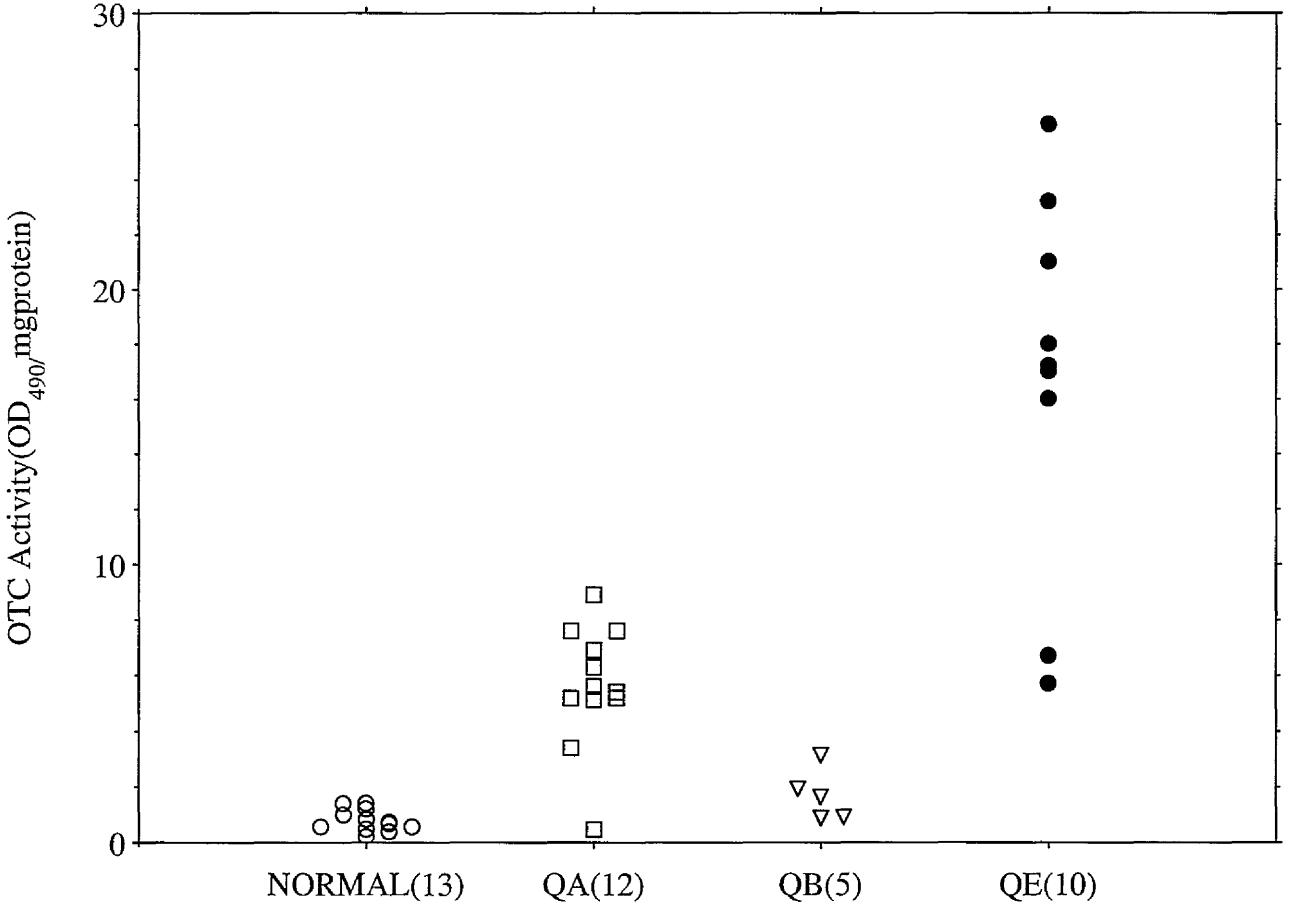


Table 6 Hepatic OTC activity (OD_{490}/mg protein) on sparse fur, sparse fur^{ASH} and wildtype background

All assays were performed in triplicate and the average of the results was taken.

	Wildtype (No 9)	Sparse fur (No 18)	Sparse furASH (No 8)
Median OTC	7.6	1.2	0.44
Wildtype		P*<0.0001	P*<0.0001

Table 7 Hepatic OTC activity (OD_{490}/mgprotein) levels in the albumin -OTC^{MOD} lines on a sparse fur background.

	Wildtype (No 9)	Sparse fur (No 18)	KD (No 9)	KE (No 10)	KG (No 15)	All 'K' Line
Median OTC	7.6	1.2	1.44	1.37	1.65	
Wildtype			P*=0.0001	P*<0.0001	P*<0.0001	P*=0<0001
Sparse fur			P=0.145	P=0.089	P*=0.006	P*=0.007

Table 8 Hepatic OTC activity (OD_{490}/mgprotein) in the albumin -OTC^{MOD} lines on a sparse fur^{ASH} background.

	Wildtype (No.9)	Sparse FurASH (No 8)	All 'K'lines (No 8)
Median OTC	7.6	0.44	0.61
Wildtype			P*<0.0001
Sparse fur^{ASH}			P=0.161

p*= statistical significance in comparison to wildtype data by Mann Whitney test

Table 9 Skeletal Muscle OTC activity (OD_{490}/mgprotein) in normal mice and in the creatine kinase -OTC^{MOD} lines.

	Baseline (No 13)	QA (No 11)	QB (No 5)	QE (No 8)	All "Q"lines
Median OTC	0.73	5.4	1.7	17.2	
Baseline		P*<0.0001	P*=0.014	P*<0.0001	P*<0.0001

Hepatic OTC activity (OD_{490}/mgprotein) levels in the albumin -OTC^{MOD} lines on a sparse fur background.

3.4 INVESTIGATION OF THE EPIDERMIS AS A POSSIBLE TARGET ORGAN FOR RECOMBINANT GENE EXPRESSION

The first section of this project, to investigate the effect of exogenous OTC expression on OTC deficient mouse models, was only possible because the tissue-specific promoters employed, namely the albumin and creatine kinase regulatory sequences had already been well characterised and shown to be capable of directing gene expression to the relevant target tissues.

With the long term goal of developing epidermal specific expression constructs work was also undertaken to assess the ability of a novel bovine keratin 5 minigene construct to drive expression of recombinant genes to the epidermis.

The epidermis is a highly attractive tissue in which to express many exogenous genes as it is easily accessible allowing the optimisation of non-invasive techniques such as liposomes for gene delivery. In addition, *in vitro* culture and transplantation of skin is well established in clinical practice. The skin has the additional benefit of being easily monitored after gene delivery (review Trainer and Alexander 1997). As the epidermis forms the outer layer of the skin, it provides the body's first line of defence and as such has attracted much attention as a target for tissue-specific promoters. However, regulation of gene expression in the epidermis is very complex, as there are both temporal and cell type-specific controls of different gene promoters. Thus, in order to investigate the bovine keratin 5 gene expression profile both in adult mouse and during embryogenesis, a reporter gene construct comprising a Keratin 5 expression cassette and the *E.coli* β galactosidase (Lac Z) reporter gene cDNA was formed.

3.4.1 FORMATION OF A KERATIN 5 DRIVEN LACZ REPORTER CONSTRUCT

A plasmid containing a bovine keratin 5 and 6 'minigene' expression cassette was obtained from Manfred Blessing (Blessing, Nanney et al. 1993). This plasmid had been generated by cloning the bovine genomic sequence containing the contigous keratin 5 and 6 genes and then deleting from this genomic sequene, the keratin 5 and keratin 6 translated sequences. In the case of the keratin 5 gene all that remains is 5.5 Kb of the 5' regulatory sequence including the first 30 bp of the 5' untranslated sequence, and the last intron, non-translated exon and endogenous polyadenylation sequence, at the 3' end. Between these two K5 gene elements was a unique SalI site for insertion of cDNAs.

The strategy for formation of the Keratin 5 driven lacZ reporter construct is summarised in figure 30. In brief, the keratin 5 minigene regulatory sequence were separated from the keratin 6 minigene using a KpnI digest. This was then ligated into the plasmid pIC20R, in which the endogenous SalI site had been destroyed by restriction enzyme digestion, end filling of over hanging termini and then ligation of the 'flush' ends. The entire *E.coli* β- lactamase coding sequence (Lac Z) cDNA including an 'ATG' translation initiation sequence to a 'STOP' codon was then amplified by means of PCR, incorporating SalI restriction enzyme sites into the 5' ends of the amplicon primers. This was ligated into the keratin 5 minigene construct downstream from the promoter sequences and upstream from the endogenous polyadenylation sequences using its unique SalI site (Fig 31A).

Figure 30. Formation of Keratin 5-LacZ expression cassette.

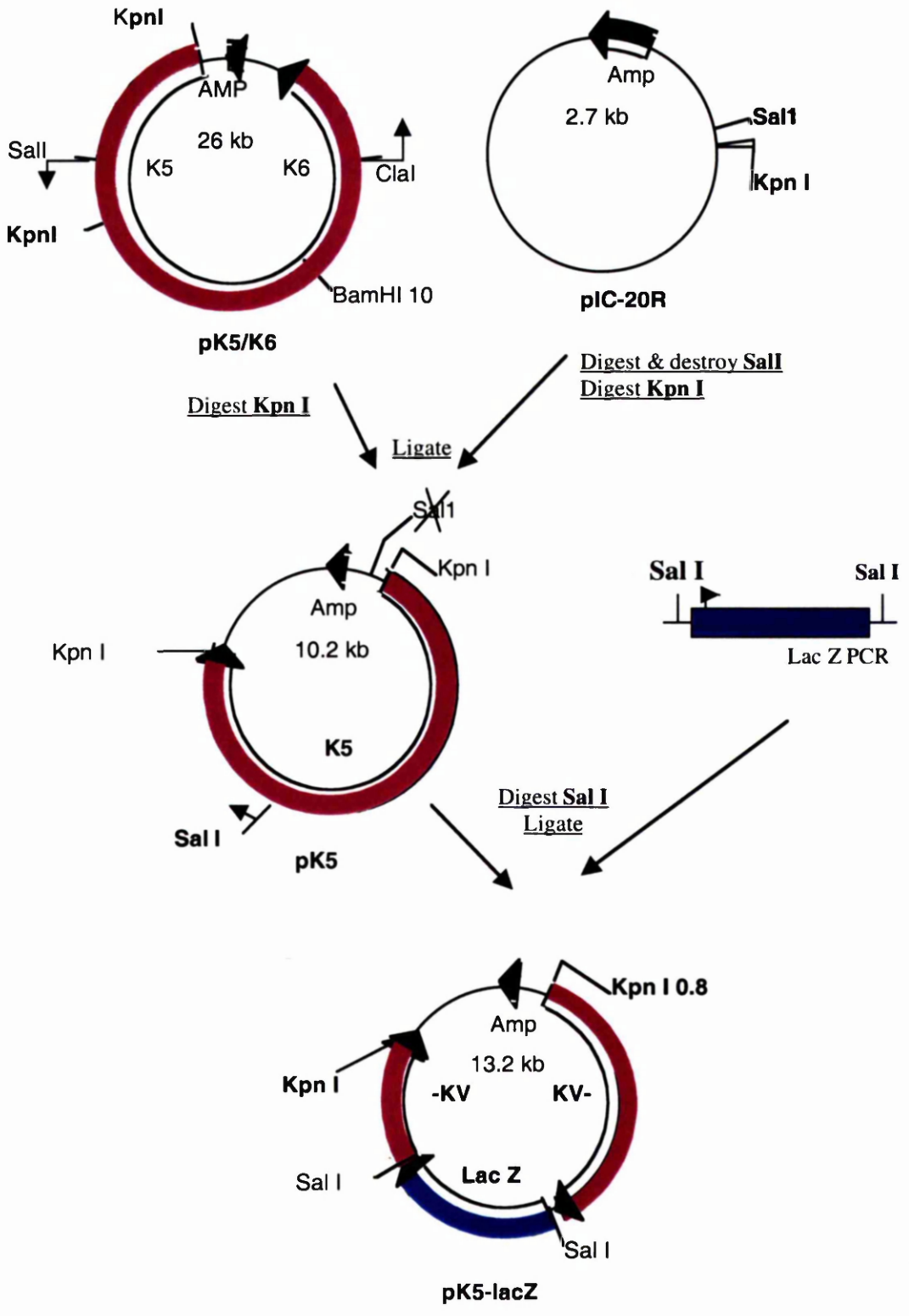


Figure 30. Formation of Keratin 5-LacZ expression cassette.

Figure 31. Southern blot analysis of Keratin 5-Lac Z founder

C. A restriction enzyme digestion map of the Keratin 5-LacZ expression construct in pIC20H

D. Southern blot analysis of 10 μg genomic DNA extracted from the Keratin V-Lac Z transgenic founders lines (WA, WB, WD). The DNA was digested with BamHI and the 3Kb Lac Z cDNA was radioactively labelled as the probe. The Southern blot shows the expected 3 Kb transcript in all three founder lines.

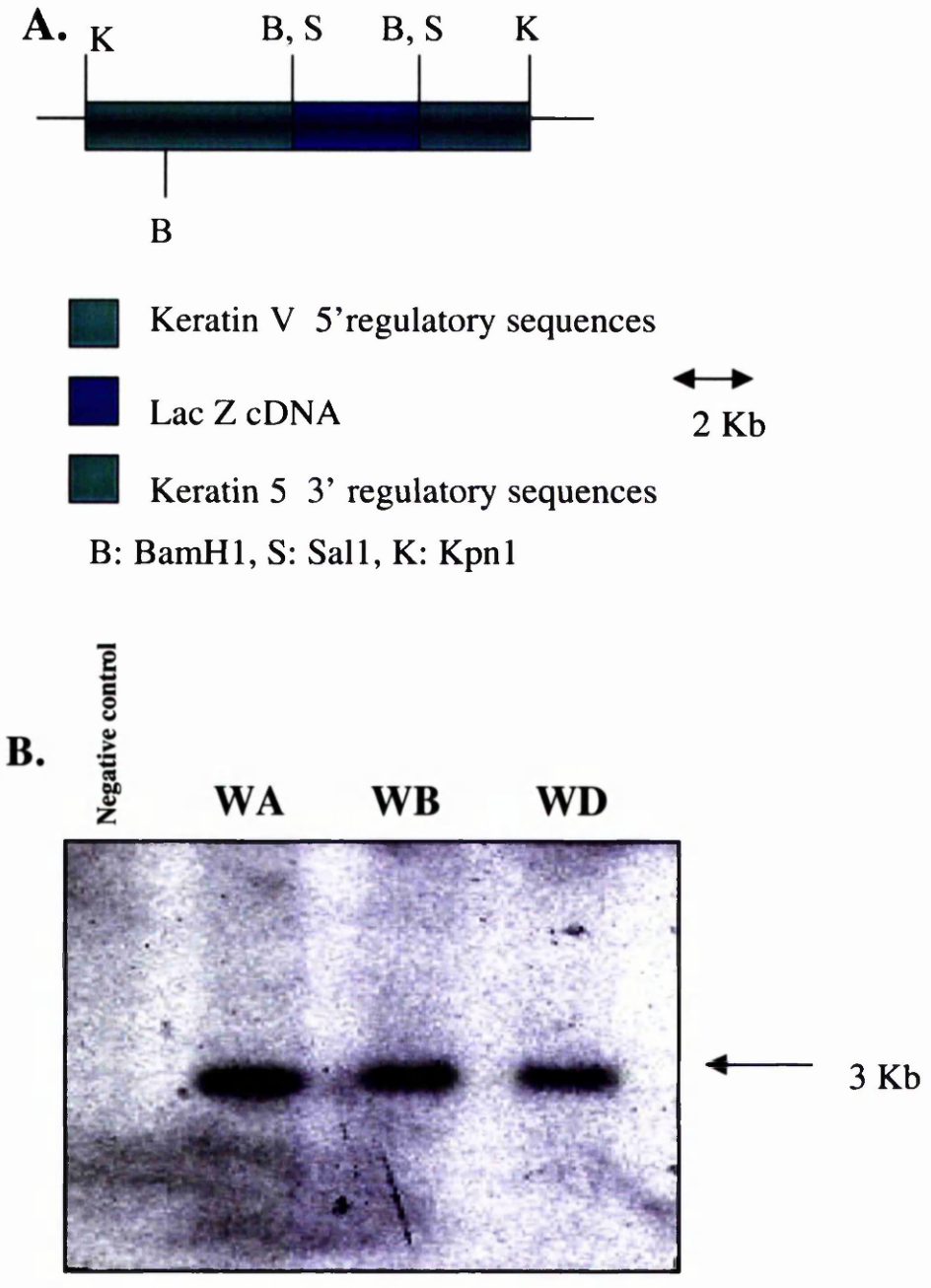


Figure 31.Southern blot analysis of Keratin 5-Lac Z founder lines.

3.4.2 CONFIRMATION OF THE REPORTER ACTIVITY OF KERATIN 5 CONSTRUCT BY TRANSIENT IN VITRO TRANSFECTION.

In order to confirm the functional capacity of the expression construct, the resultant Keratin 5 driven lac Z and empty pK5 vector plasmids were separately transiently transfected into a squamous

carcinoma cell line SN161 which is known to express the keratin 5 gene. 48 hours post transfection the *in vitro* cell culture was stained with X-Gal, the substrate for β galactosidase activity. The resultant blue-staining cells confirmed β-Galactosidase expression and protein activity (Fig 32) in the pK5-acZ activity. The control trasnfection with the empty pK5 vector remained negative (data not shown). The low number of stained cells was mostly likely due to a low level of transfection efficiency.

3.4.3 FORMATION OF TRANSGENIC LINES

The K5-LacZ transgene was excised from the parental vector by a KpnI restriction enzyme digestion and used for pronuclear injection as described in material and methods previously. Three transgenic founder mice (WA, WB, WD) were obtained (see section 3.2.1.).

Integration of the transgene was confirmed by Southern Blot analysis. Genomic DNA was digested with BamHI and then the filter was probed with the entire 3 Kb lacZ cDNA. This should produce a 3 Kb internal band on Southern Blot analysis as shown in figure 31B. The founder transgenic mice were each bred onto a NIH background. Screening for the transgene in the founder mice progeny was performed by direct staining of the tail tip biopsies with X-Gal.

Figure 32. Expression of keratin 5-Lac Z expression construct in SN161 cell line.

In order to assess the integrity of the K5-Lac Z expression cassette. The construct was transfected using a liposome-mediated method into SN161 carcinoma cell line, which is known to express the keratin 5 gene. The cells were then fixed and stained for β galactosidase expression. Blue staining cells indicate Lac Z expression. The low number of cells expressing the K5-Lac Z construct is due to the transient nature of the transfection and poor transfection efficiency.

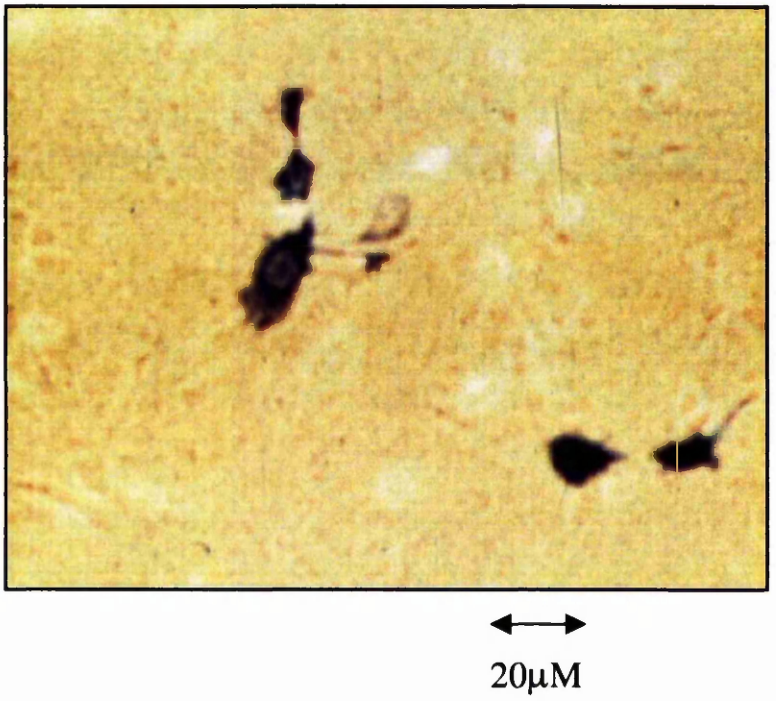


Figure 32. Expression of keratin 5-Lac Z expression construct in SN161 cell line.

3.4.4 THE EXPRESSION PATTERN OF KERATIN 5-LAC Z CONSTRUCT IN ADULT MOUSE TISSUE CORRELATES WELL WITH THE EXPRESSION OF THE ENDOGENOUS KERATIN 5 GENE.

Transgenic mice were sacrificed by cervical dislocation and fresh tissues were fixed and stained for β–Galactosidase activity. The tissues were then embedded in paraffin and 7 μM sections cut. The slides were counter-stained with eosin. In the case of the orbital eye sections, after the tissue was stained they were sectioned by Dr B Clarke in the Dept. of Ophthalmopathology, Western Infirmary.

The results discussed here are from two out of the three mouse lines studied namely, WB and WD.

The epidermis (Fig 33) shows high levels of Lac Z expression. In the section shown, the staining appears to be mainly confined to the basal epidermal layer as expected (Lersch and Fuchs 1988), although there is some staining in the suprabasal layers which could be due to leeching out of the cells of the Lac Z gene product or the X-Gal cleavage product. The skin section also shows high levels of expression in the bulbs of the hair follicles found deep in the dermis, but which are also epithelial in nature and known to express K5. The Lac Z staining pattern seen in the tongue (Fig 33) correlates with endogenous keratin 5 gene expression (Dhouailly, Xu et al. 1989). B galactosidase activity is seen in both the basal and parabasal layers of the two distinct epithelia making up the filliform papillae found on the upper surface of the tongue. From the oral cavity the keratin 5 expression is then seen to continue in the basal layers of the epithelium covering the oesophagus continuing down into the basal layers of the forestomach (fig 34). This is the expected pattern of the keratin gene associated with the basal layer of stratified and pseudostratified epithelium (Moll, Dhouailly et al. 1989). Both the control and the keratin 5 transgenic mice

Figure 33. Expression of keratin 5-Lac Z transgene in skin and tongue.

A) Section of transgenic skin stained with X-Gal and eosin. The X-gal staining of the skin from a keratin 5–Lac Z transgenic mouse shows expression in the basal layer of the epidermis and hair follicle e) epidermis f) hair follicle. There appears to be a quantitative difference in staining within the epidermal section as shown with the more intense Lac Z staining in the basal layers being associated with staining in the suprabasal compartment. This suprabasal staining pattern could be due to diffusion of the gene product or the cleavage product of X-Gal. Alternatively it could be produced by residual Lac Z protein activity in differentiated keratinocytes. **B)** X-gal, haematoxylin and eosin stained epidermis from a non-transgenic mouse shows no Lac Z staining.

C) Lac-Z staining of the tongue showing expression in the anterior surface papillae (p) and basal layer of the epithelium papilla compared to control tissue (**D**). This Lac Z staining pattern obtained from the transgene expression mirrors the expression pattern of the endogenous keratin 5 gene in these tissues.

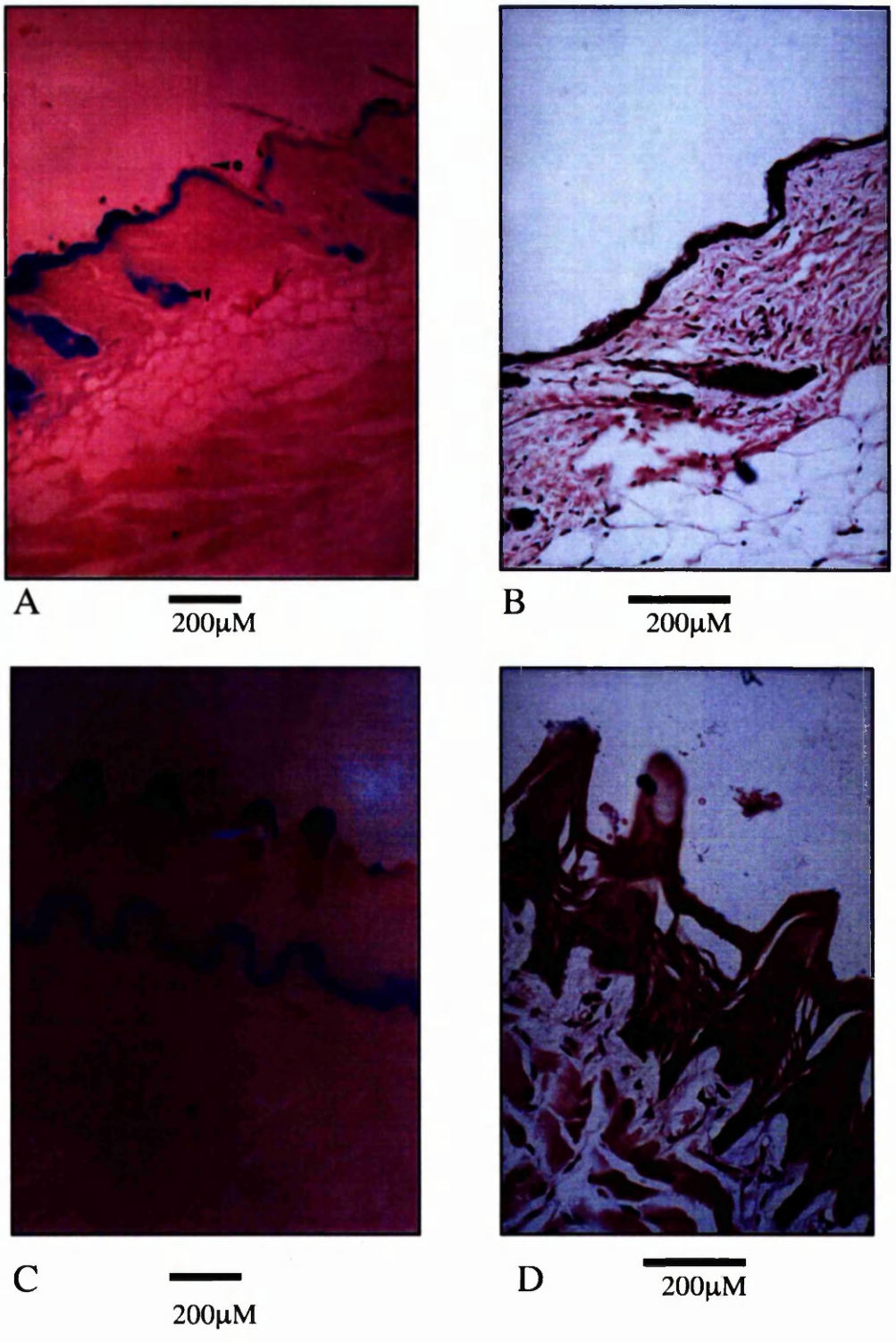


Figure 33. Expression of keratin 5-Lac Z transgene in skin and tongue.

Figure 34. Expression of Keratin 5-Lac Z transgene in the eye and gastrointestinal tract.

A) Histological section of oesophagus and stomach from a keratin 5-Lac Z transgenic mouse stained with Lac Z and eosin. Lac Z staining shows expression in the basal layer of the epithelium in the oesophagus (o) and forestomach (s) compared to a non-transgenic control mouse stained with haematoxylin and eosin (**B**).

B) Histological section of the orbit of the eye (This section was kindly prepared by Dr B. Clarke, Dept of Ophthalmopathology, Western Infirmary, Glasgow.) Lac Z staining is clearly seen in the proliferative limbic area of the eye. The limbic area (La) is found at the junction of the iris (i) and cornea (c): and is thought to contain the corneal stem cells. The corneal cells downregulate K5 and K14 as they mature and form the avascular cornea, which covers the iris. The mouse was brown agouti in colouring, which accounts for the normal dark pigment granules seen in the iris. Retina; (r), lens; (l).

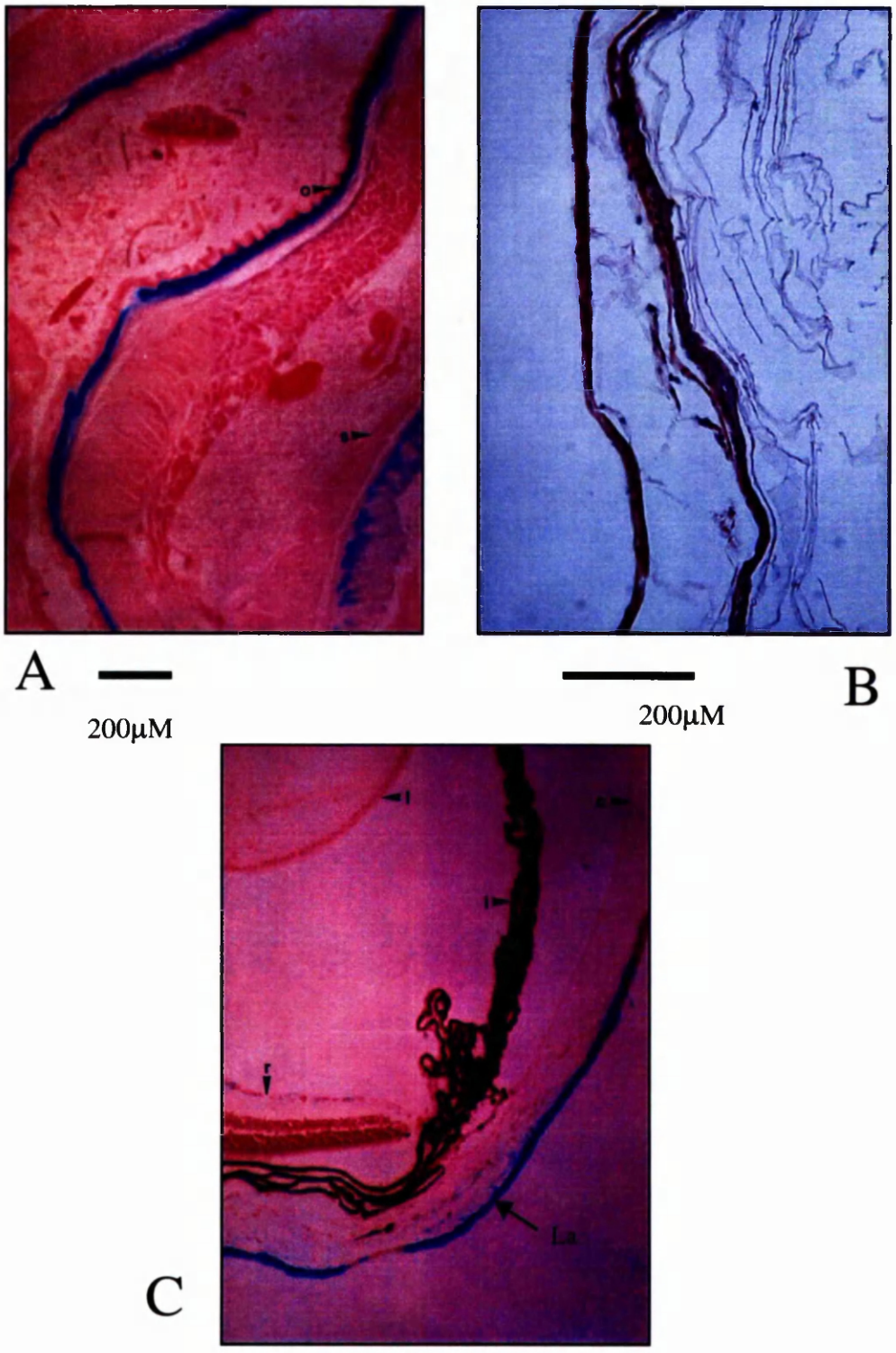


Figure 34. Expression of Keratin 5-Lac Z transgene in the eye and gastrointestinal tract.

Figure 35. Expression of Keratin 5 -Lac Z transgene in epithelium of small intestine.

A) Paraffin-embedded histological section of intestinal tissue from K5-Lac Z mouse stained with Lac Z. This section shows generalised staining of epithelium in all villi (v) including the Peyers Patches. This result may be a staining artefact due to the presence of Lac Z-expressing bacteria in the intestine. Therefore, the staining was repeated on a whole-mount intestine preparation after removal of intestinal bacteria (**B**). This staining results still indicates Lac Z expression in the epithelial lining of the villi. This staining pattern was not seen in the control, non-transgenic mouse (**C**). There is no endogenous keratin 5 expression in this tissue as the intestine is lined with simple columnar epithelium.

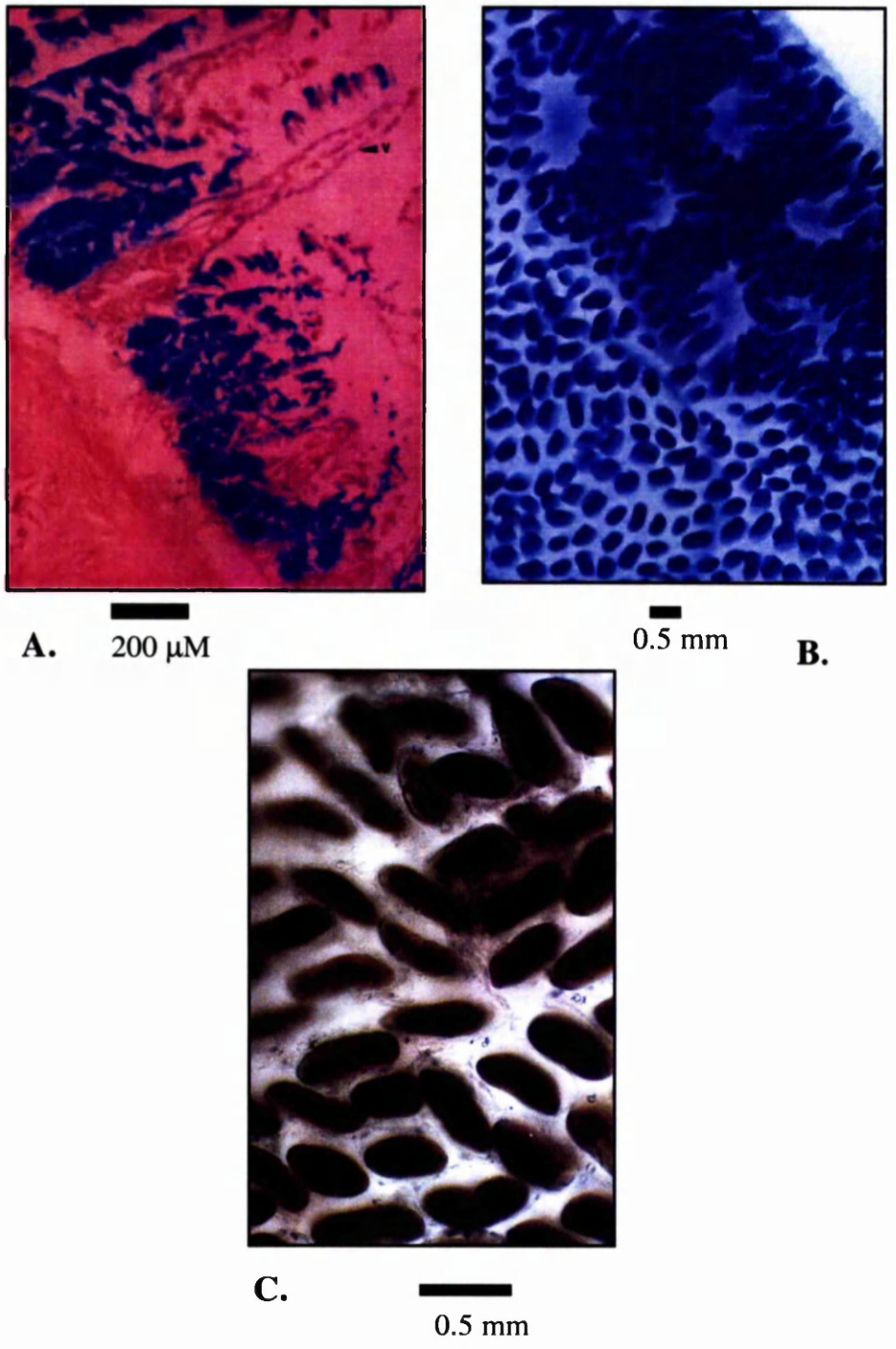


Figure 35. Expression of Keratin 5 -Lac Z transgene in epithelium of small intestine.

showed Lac Z staining in the hindstomach (data not shown) so no comment could be made as to keratin 5 expression pattern in that particular tissue.

In the small and large intestine, there appears to be expression of the keratin 5-Lac Z construct in the basal layers of epithelium in these tissue. Although the original results were obtained by staining of whole intestine preparations and therefore may have included false positive staining from bacterial Lac Z expression in the lining of the intestine (Fig 35), the same results were obtained when the intestine was fixed as a wholemount and treated with mucin and bacteria removing agent prior to staining (Fig 35). The intestinal Lac Z staining pattern was was seen in both transgenic lines investigated and are therefore not due to a chromosomal positional effect secondary to the site of integration of the transgene but must be due to the absence of the regulatory sequences governing cell specific control of gene expression in this tissue. The expression pattern appears to be in the simple columnar epithelium lining the intestine. The control murine gastrointestinal tract did not show this lac Z expression on wholemount staining (Fig 35).

The keratin 5 driven transgene also showed very specific cellular expression in the proliferative region of the cornea as expected from the expression pattern of the endogenous keratin 5 gene (Fig 34). Other tissue, such as liver, lung, spleen and brain were also stained for Lac Z but none was observed.

In conclusion, with the exception of the intestinal staining, the expression of the transgene correlated well with the documented endogenous keratin 5 expression pattern.

Figure 36. Expression pattern of keratin 5 -Lac Z transgene at d9.5 and d10.5 during embryonic development.

A.) d9.5 embryo with first noticeable gene expression as shown by low level β galactosidase staining on the lateral aspect, indicated by the arrow

B.) d10.5 transgenic embryo showing K5 expression in first branchial arch (BA), nasal pit (NP) and over the developing somites. There is goes expression the forelimb (VL). There is a cranial to caudal diminution of gene expression, in keeping with the normal embryo developmental gradient **C.)** K5 expression is higher in the ectoderm directly overlying an individual somite compared to intervening tissue.

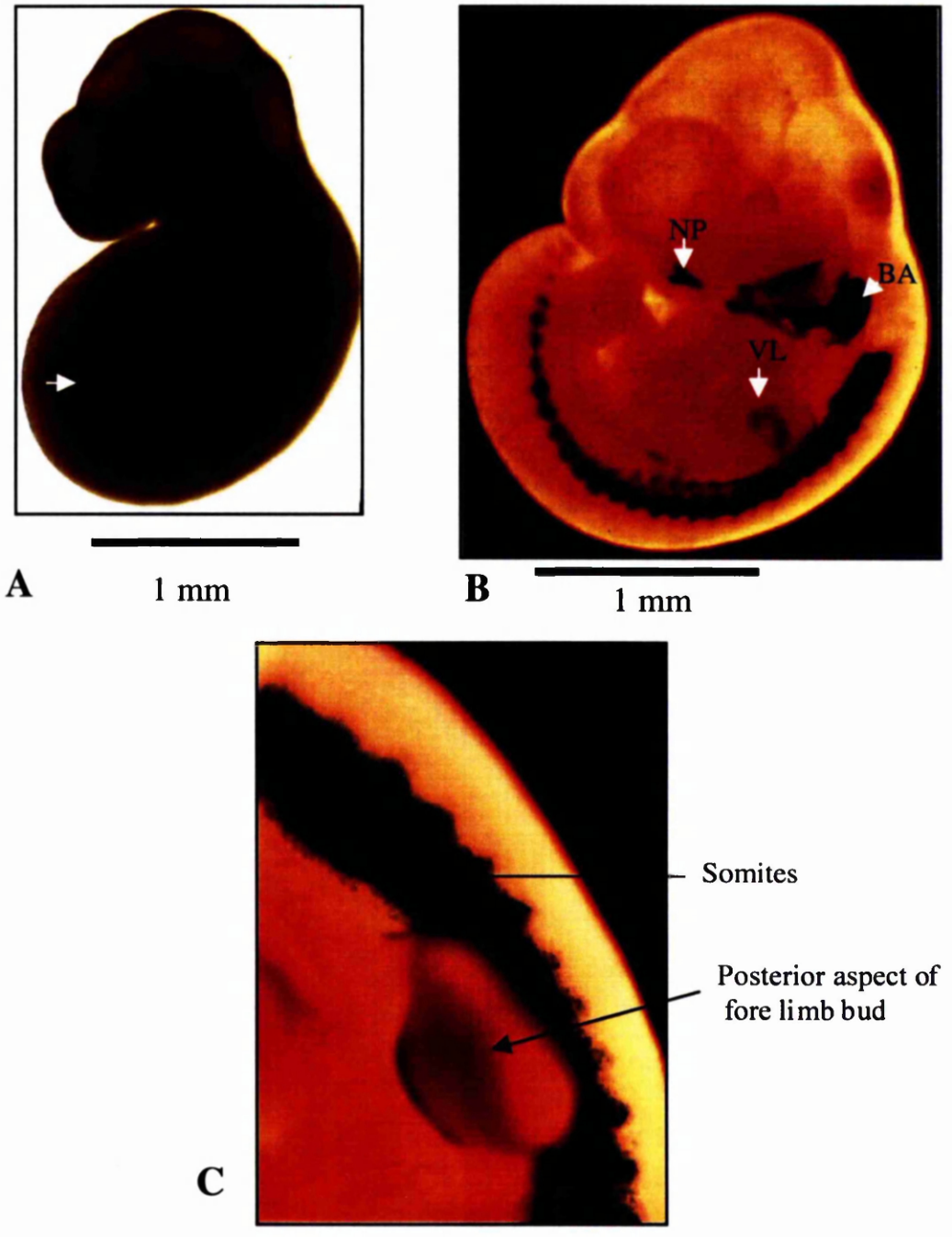


Figure 36. Expression pattern of keratin 5 -Lac Z transgene at d9.5 and d10.5 during embryonic development.

3.4.5 EMBRYONIC EXPRESSION OF KERATIN 5-LAC Z REPORTER CONSTRUCT

In addition to examining the keratin 5–Lac Z expression pattern in adult tissue, the expression pattern during embryonic development was also documented. Embryos from d8 to d14 postcoitum matings on a mixed (FVB) x (C57Bl/6J) genetic background were harvested, fixed and stained for Lac Z activity as described previously. Initial expression could be detected over the caudal aspect of the lateral ridge from d9.5 (Fig 36). The staining was very weak and difficult to photograph but was reproducible. No staining was observed prior to this. By d10.5 the expression pattern increased in the ectoderm covering the lateral aspect of the embryo with relative sparing of the cranial vault, dorsal and ventral aspects. Marked staining was observed over the somites with strong cranial somite staining with dissipation of staining occurring in the more caudal somites. This staining is very specific to the individual somites and not the intervening cells. Gene expression was easily visible in the first branchial arch. In particular, there is strong expression in the mandibular branch with weaker staining detectable in the maxillary branch of the first branchial arch. There was also a strong focus of expression in the nasal pit. At d10.5 there was also initiation of gene expression at a focus in the proximal central position on the fore limb bud (Fig 36).

LacZ expression increased in intensity over the next 24 hours and at d11.5 the expression pattern increased over the lateral aspect of the embryo in a more ventral direction, although there was still marked sparing of the dorsal and cranial vault regions. The individual somites could still be clearly visualised but there was less distinction between somatic and intersomatic regions. The eye and branchial arch show increased staining. In addition, the staining in the proximal limb extended more distally whilst expression in the proximal aspect of the hind limb bud was

Figure 37. Expression pattern of Keratin 5-Lac Z transgene at d11.5 during embryonic development.

Two d11.5 embryos stained for β galactosidase activity (**A**) Negative control from wildtype litter. (**B**) K5-Lac Z transgenic embryo showing expression in the first branchial arch, somites and in the periphery of the limb buds. The staining was more intense in the proximal fore- rather than hind limb. This is not unexpected as the fore limb develops earlier and therefore is more advanced developmentally than the hind limb. There is also a diminution of Lac Z expression over the anterior and posterior aspects of the embryos. The β galactosidase staining in the brain was not reproducible within and between the two transgenic lines and may be due to retention of the Lac Z gene product or the staining product. Wildtype embryos showed no β-galactosidase staining at any point studied during embryogenesis.

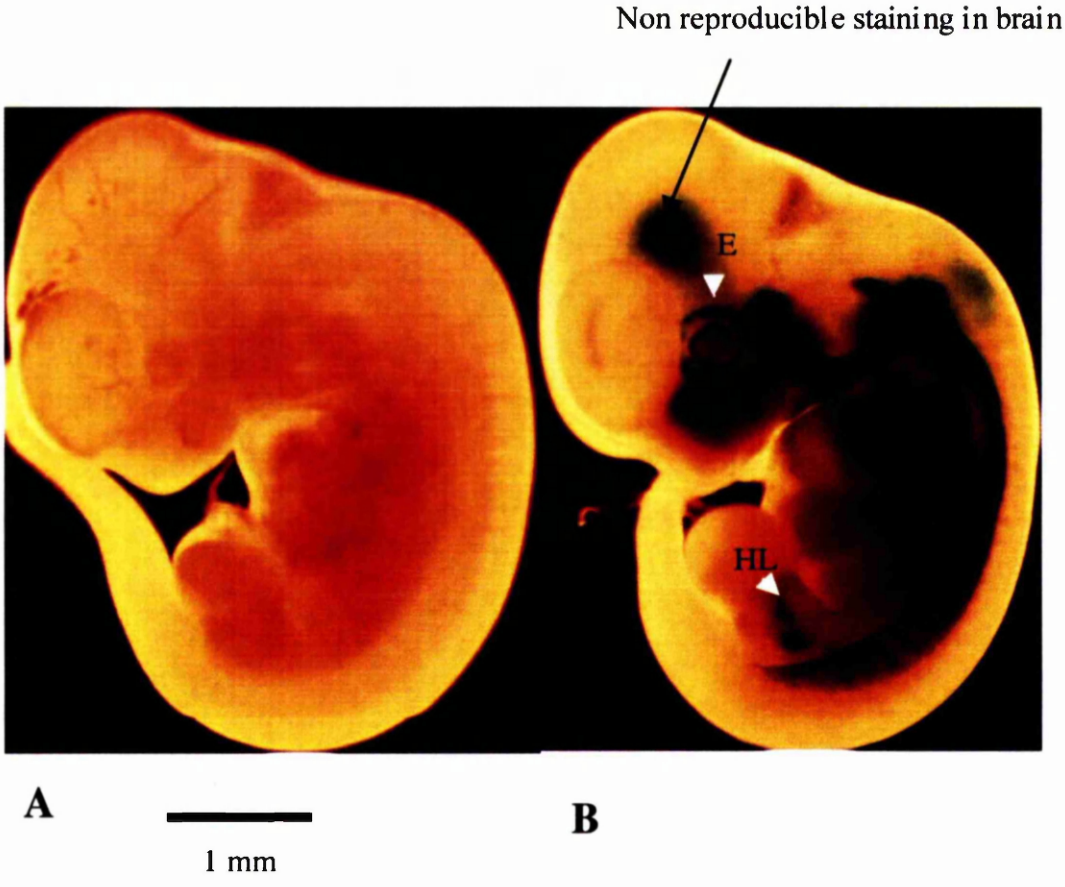


Figure 37. Expression pattern of Keratin 5-Lac Z transgene at d11.5 during embryonic development.

Figure 38. Expression pattern of keratin 5-Lac Z transgene at d12.5 during embryonic development.

Transgenic d12.5 embryo showing increased gene expression in the maxillary area as well as over the fronto-lateral aspect of the embryo. The staining pattern over the limbs has increased and extends more distally, although the hind-limb still shows delayed expression pattern compared to the fore limbs (**A**). There is noticeable sparing of staining overlying the dorsal and cranial tissue (**B**) forming a sharp demarcation line (**D**). Staining is marked in the developing maxillary hair follicles, supra orbital fibrillae and limbic area of the eye (**C**).

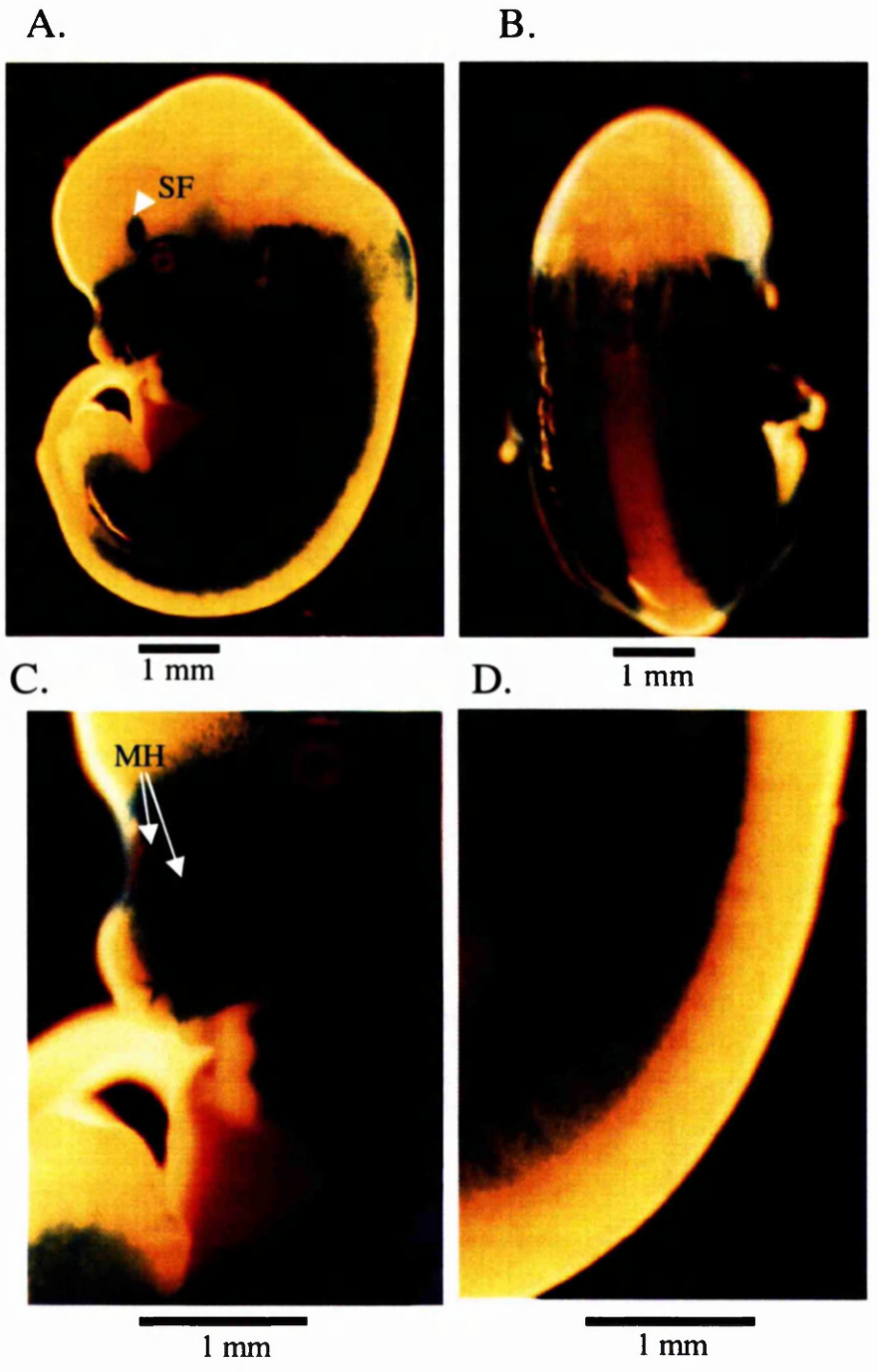


Figure 38. Expression pattern of keratin 5-Lac Z transgene at d12.5 during embryonic development.

clearly visible. In the day 11.5 embryo there was also marked staining in the brain. This staining pattern was not reproducible and was found intermittently in differing embryonic stages in the different founder transgenic lines. Therefore, it is likely that this is a staining artefact. As can be seen in the d11.5 negative control, staining in the brain was not seen when lac Z expression was totally absent and it may therefore result from trapping of Lac Z in the brain vesicles (Fig 37). The staining in the brain may represent trapping of the b-galactosidase gene product or the X-Gal cleavage product.

By day 12.5 (Fig 38) there is strong expression over the lateral and anterior aspects of the embryo with continued sparing of the areas over the developing central nervous system; namely the cranial vault and spinal column.

The maxillary and mandibular regions are highly stained with the whisker follicles showing increased K5 expression before the rest of the pelage hair follicles. In particular the primordia of the two prominent tactile hair follicles in the supraorbital region are clearly defined as well as their two counterparts in the infraorbital region. The K5 expression pattern has moved distally along the both limb buds with the proximal limb remaining more advanced. Histological sections of the embryo at this stage indicate that the Lac Z staining is not limited to the ectodermal tissue but in some instances appears to extend into the underlying mesoderm (Fig 39 A, B, C, D). In figure 39 marked Lac Z staining can be seen in A) the ectodermal tissue in the maxillary area, and the developing whisker hair follicles. Weaker staining due to diffusion can be seen in the mesoderm underlying the ectoderm. This does not appear to be cellular and may represent a staining artefact ; B) in all the layers of the ectodermal tissues in the nasal pit; (C) There is also expression in a distinct region of mesoderm near the optic nerve underlying the area adjoining the developing eye, in addition to the epithelium coating the posterior aspect of the eye. In particular,

Figure 39. Histological sections through a stained keratin 5 -Lac Z transgenic d12.5 embryo.

Histological sections through a stained transgenic d12.5 embryo. There is ectodermal expression of the transgene in the nasal epithelium in keeping with the adult Keratin 5 endogenous expression pattern (**A**). In addition, there is expression in the maxillary hair follicles and interfollicular ectoderm (**D**). However, expression is not restricted to the ectoderm in the developing skin and Lac Z staining is also noted in the underlying mesoderm (**B**). It is difficult to determine whether this mesodermally staining is specific or due to diffusion of the very high level of staining seen in the overlying ectoderm. In the developing eye, there also appears to be mesoderm expression which appears to be site specific rather than due to a staining artefact. (**C**).

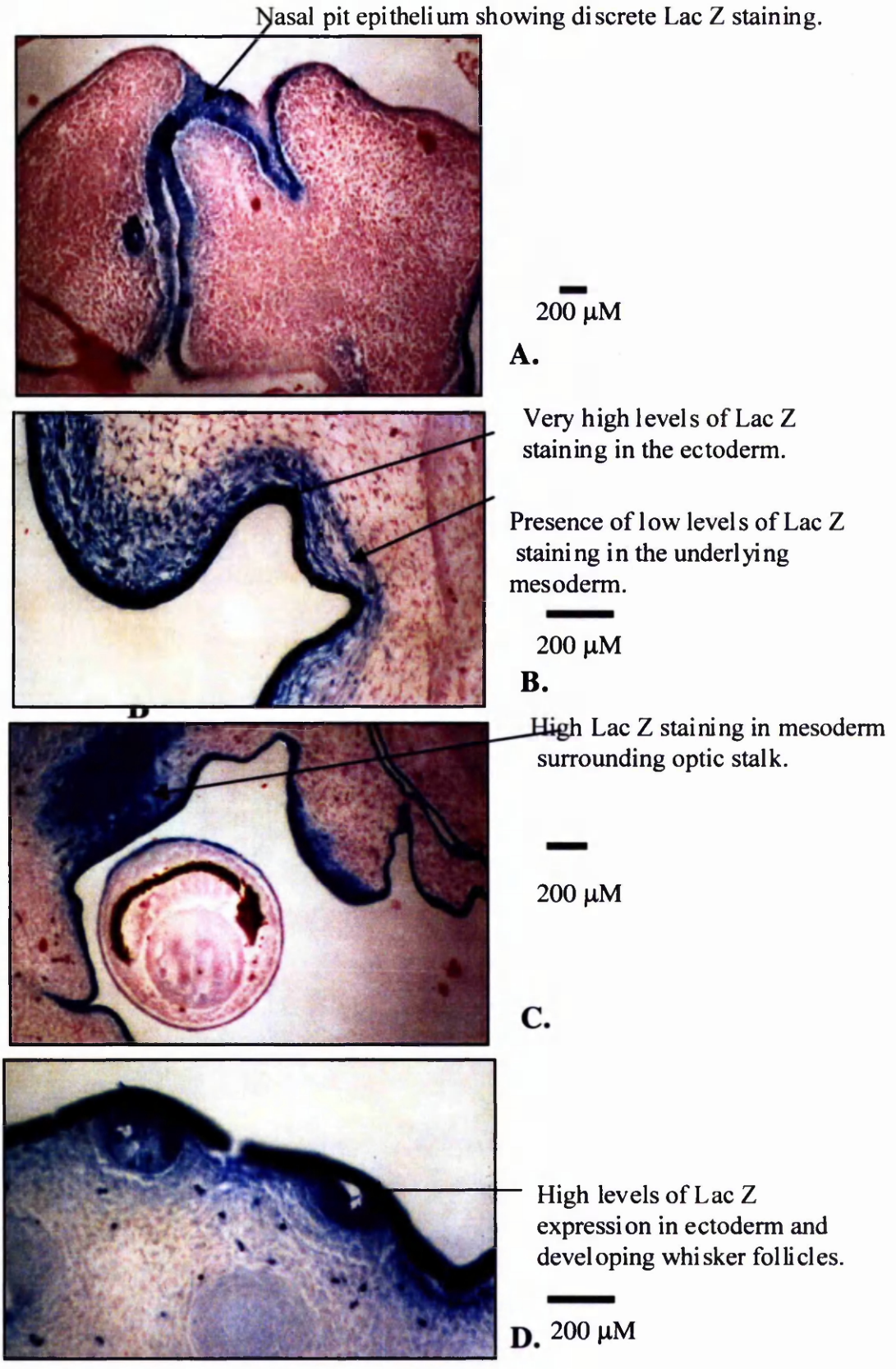


Figure 39. Histological sections through a stained keratin 5 -Lac Z transgenic d12.5 embryo.

Figure 40. Expression pattern of Keratin 5-Lac Z transgene at d13.5 in embryonic development.

d13.5 transgenic embryo showing marked K5 expression over almost entire embryonic epidermis with noted sparing of the distal aspects of the developing hind limbs and tail There is a high level of gene expression in the pelage hair follicles. The epidermis at this juncture is friable and if damaged there is no expression in the underlying mesoderm. This suggests that previously described mesodermal Lac-Z staining could be due to diffusion of either β-galactosidase gene product or X-Gal cleavage product.

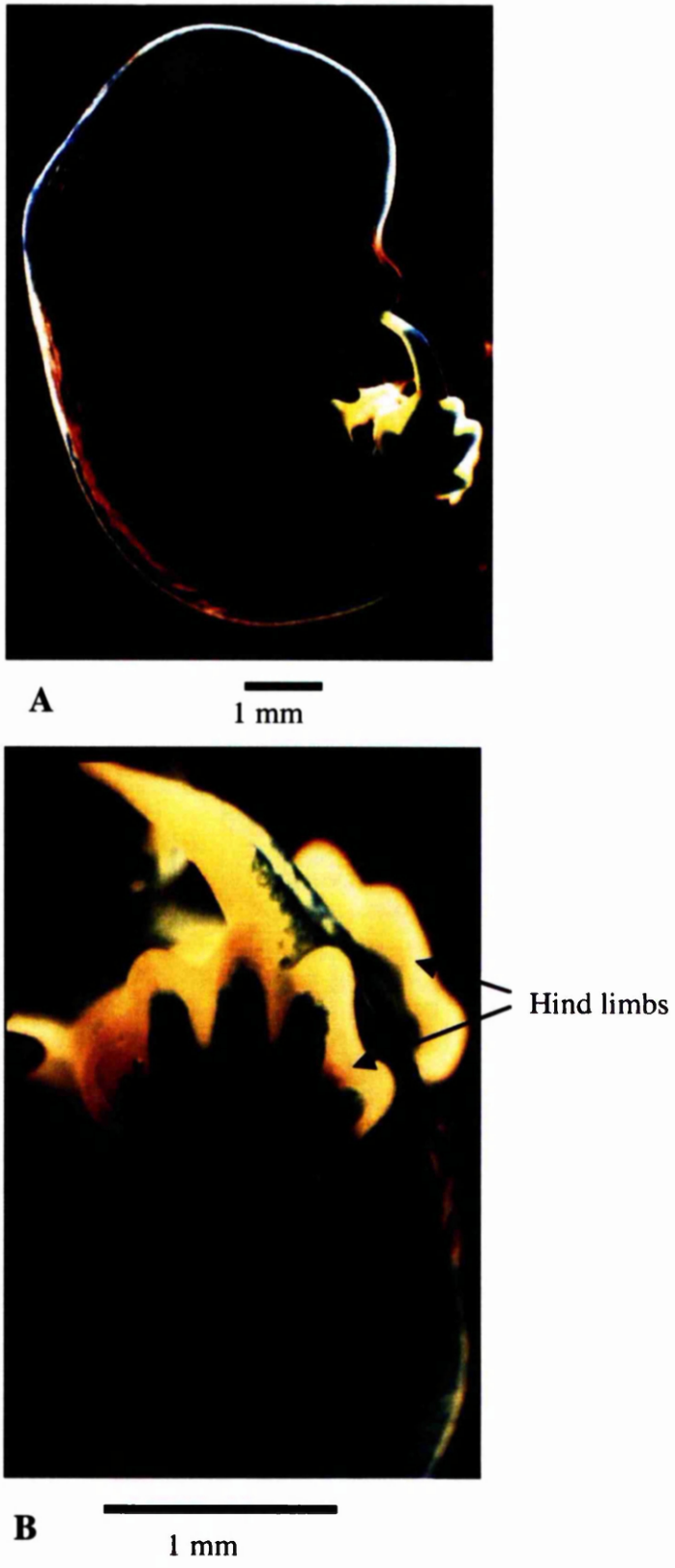


Figure 40. Expression pattern of Keratin 5-Lac Z transgene at d13.5 in embryonic development.

this section would suggest that the K5 expression pattern visualised is specific as the staining pattern seems too localised for it to be attributable to leeching out of the lac Z gene products causing a staining artifact; (D) Lastly, marked lac Z staining can be seen in the mesoderm underlying the main body ectodermal covering at d12.5. It should be noted that although the Lac Z staining is of a much higher intensity in the ectoderm, there does appear to be cell specific Lac Z expression in the underlying meosderm cells. By day 13.5 (Fig 40) the body is almost completely covered with an epithelium expressing keratin 5. However, there is still a noticable diminution of Lac Z staining in the caudal region. The pelage hair follicles now express high levels of keratin 5 and the skin has become more friable during handling.

By day 14.5 there is generalised expression of K5 over the entire embryonic surface. The epithelium is as this juncture very friable and in areas where it has become detached there is no underlying Lac Z expression seen. This suggests that the underlying mesodermally staining described previously may indeed be due to diffusion of transgene product or stain into the lower tissues.

4. DISCUSSION

4.1 DESIGN OF EXPRESSION CONSTRUCTS

4.1.1 MODIFICATION OF RAT OTC cDNA

The ornithine transcarbamylase protein has been highly conserved in evolution, especially within its catalytic sites. The human OTC cDNA has 95% amino acid and 90% DNA sequence identity to the rat OTC. In addition, the rat OTC cDNA (Genbank accession No. M11266) used in the current study (Nguyen, Argan et al. 1986), has 98% homology with the mouse OTC gene. Previous work (Cavard, Grimber et al. 1988; Shimada, Noda et al. 1991) has shown that hepatic expression of the rat OTC cDNA produces a protein capable of substituting for and correcting the enzyme defect in the Spf and Spf^{ASH} OTC deficient mouse models.

Initiation of translation in multicellular eukaryotes has been shown to be influenced by five elements in mRNA structure namely 1) the 5' m7G cap; 2) the primary sequences surrounding the AUG codon; 3) the position of the AUG codon; 4) the leader sequence, and finally 5) the secondary RNA structure surrounding the AUG codon (Kozak 1996). In the rat OTC cDNA the largest open reading frame is commensurate with a translation initiation codon at nucleotide position 101 of the cDNA present in the surrounding context of G A A G **AUG** C T G T C T.

Sequence data compilation from 699 vertebral mRNAs (Kozak 1987), and experimentally-derived sequences (Kozak 1986; Kozak 1989) suggests that the optimum translation consensus sequence surrounding the AUG codon which ensures fidelity and efficiency of translation initiation is G C C A/G C C **AUG** G. In this motif the two base pair positions most critical for optimum high level translation are the 'A' at position -3 and 'G' at position +4. Their importance is emphasised by their highly conserved nature within different genes (Kozak 1986). As shown previously, although the OTC cDNA conformed to the consensus optimum Kozak sequence at -

3 base position, the nucleotide at +4 position was a cytidine. Therefore, site-directed mutagenesis was used to convert the nucleotide at position +4 to a 'G', in an attempt to increase the translational efficiency of the rat OTC cDNA from an ectopic promoter. However, a more recent study (Kozak 1997) called into question the effect of including this transversion event, suggesting that the enhancement in translation conferred by a 'G' at position +4 is lost when the subsequent base is a 'U'. Unfortunately this is indeed the case in the rat OTC cDNA and thus this modification may indeed be neutral if not detrimental to translational efficiency.

This alteration in nucleotide sequence at position +4 has the effect of changing the second amino acid in the leader sequence of the OTC protein from a leucine to a valine. The evidence that this amino acid substitution is not detrimental to enzymic activity is seen most clearly in the creatine kinase transgenic mouse lines, where high levels of muscular OTC activity were assayed compared to background levels found in non-transgenic skeletal muscle. Since the enzyme produced in the transgenic skeletal muscle cells, which can only have originated from the transgene, was capable of OTC, it can be assumed that the recombinant polypeptide is able to form the normal active OTC homotrimeric configuration required.

It is difficult to access whether truncation of the 5' and 3' untranslated sequences in the OTC^{MOD} gene construct had any beneficial or detrimental effect on resultant OTC production in the transgenic animals since expression constructs containing the full-length sequences were not made. The rationale for shortening the 5' untranslated region from the OTC cDNA was based on the observation of an inhibitory effect on translation due to secondary structure formation in the long 5'UTRs of other genes (Kozak 1986; Manzella, Rychlik et al. 1991; Yukht, Davis et al. 1995). Although the 3' UTR of certain genes has been associated with a translational enhancer effect (Lorenzini and Scheffler 1997; Wang, Browning et al.

1997), in many genes this region may contain tissue-specific translational inhibitory motifs for mRNA degradation (Lai and Posakony 1997). The 3' UTR of the OTC gene construct was therefore limited to 8 bp following the OTC translation termination codon plus either the SV40 3' non-coding region or, in the case of the keratinocyte –specific construct, the endogenous K5 3'UTR. Once again both the results obtained from the Northern blot as well as in the direct OTC activity seen in the creatine kinase and albumin-OTC^{MOD} transgenic mice would suggest that neither of these alterations had an adverse effect either on transcription or translation of the rat OTC cDNA in hepatic or skeletal muscle, although this was not directly investigated.

4.1.1.1 CREATINE KINASE DRIVEN OTC EXPRESSION CASSETTE

The targeting of somatic gene therapy to muscle cells has been under investigation for almost a decade, since it was first noted that direct intramuscular injection of naked DNA resulted in plasmid uptake and gene expression (Wolff, Malone et al. 1990). Due to the long survival and low replicative index of muscle cells, this ectopic gene expression could persist with no requirement for vector replication for up to 19 months despite being targeted to a post-mitotic cell (Wolff, Ludtke et al. 1992). The classical example of muscle cell targeted gene expression has been the use of human dystrophin to supplement a mouse dystrophin deficient model (Acsadi, Dickson et al. 1991), or the expression of utrophin to counter the effect of dystrophin deficiency in a similar murine model (Rafael, Tinsley et al. 1998; Tinsley, Deconinck et al. 1998). Both the utrophin and dystrophin gene products are normally expressed in skeletal muscle and thus it can assumed *a priori* that the machinery required for expression and translation of the recombinant forms of these

proteins is present in muscle fibres. This is especially pertinent as this study involved the expression of recombinant protein not normally expressed in muscle cells. A recent study combined both fluorescence *in situ* hybridisation and immunohistochemistry in monitoring the presence of introduced genes and the expression of their gene products (Gussoni, Wang et al. 1996). Their results suggested that the efficiency of gene transfer is affected not only by gene delivery but also by cellular controls on gene expression.

Proof of principle that dystrophin deficient muscular phenotypes can be ameliorated by expression of utrophin was established using transgenic technology. More recently, targeting of expression vectors has utilised adenoviruses injected directly into muscle as a means of increasing transfection efficencies. This approach has shown good results in expressing genes whose products supplement and correct an abnormal muscular phenotype (Gilbert, Nalbanoglu et al. 1998; Greelish, Su et al. 1999). In addition, an intra-muscular adenovirus approach has successfully been used in the treatment of canine Haemophilia B (Herzog, Yang et al. 1999). A series of intramuscular injections caused partial correction of the whole blood clotting time in a canine model for Christmas disease.

An alternative method for the investigation of muscle-directed gene therapy is the culture and transfection of myoblasts *in vitro* followed by their injection into adult muscle. Following myoblast transplantation the donor nuclei appear to be either surrounded by host nuclei or they fuse into the preexisting multinucleated myofibers that are vascularised and innervated (Gussoni, Wang et al. 1996). This method of myoblast transplantation has been shown to be amenable for the expression of both endogenous mucle proteins such as dystrophin (Gussoni, Pavlath et al. 1992) as well as exogenous recombinant protein such as growth hormone (Dhawan, Pan et al. 1991), and may well form a reliable vehicle for the systemic delivery of

recombinant proteins. One major drawback to this method of gene transfer is that myogenic cells have a limited life span *in vitro* and, in comparison to skin fibroblasts, this limits their clinical application in cell replacement. Interestingly one group recently immortalised primary myogenic cells using SV40 large –T antigen, which could be excised by means of the Cre-lox recombination system (Berghella, De Angelis et al. 1999). Although using a xenotransplantation model, no tumour formation was seen after transplantion of the floxed myogenic cells, they did only report >90% efficiency of excision of the large T antigen after application of Cre protein which would make transformation of the cells a plausible and worrying proposition.

In the current study the muscle-directed gene product was not normally expressed at this site and therefore it was of interest that muscle cells contain the post translational machinary necessary for formation of a catalytically active ornithine transcarbamylase homotrimer. In fact, the QE founder line produced a higher OTC activity in muscle than was normally found in hepatic tissue and it is worthy of note that this over-expression had no overt pathological sequelae for the mouse.

Despite the high level of recombinant OTC activity detected *in vitro* from skeletal tissue, no correction of the Spf or Spf^{ASH} phenotype was observed when the transgenic mice were bred onto these OTC deficient backgrounds. In addition, there was no statisitical amelioration of the plasma ammonia level noted in these OTC deficient/transgenic mouse crosses. Taken together, these results suggest that the OTC enzyme activity present in the skeletal muscle was not accessible to the urea cycle.

It is difficult to explain the finding that the 'Q' lines on a Spf background should have a higher plasma ammonia level than the same transgene on a sparse fur^{ASH} background. In addition, there appears to be a reduction of plasma ammonia levels

in the QA line compared to the QE line despite a lower level of OTC activity in QA skeletal muscle. This could indicate that the plasma ammonia level is not a reliable metabolic indicator of OTC activity, although in all other respects the plasma ammonia levels correlated well with hepatic OTC activity.

The results from the ammonia levels in the individual mice in the Spf^{ASH} group indicate that only three out of the eight mice had an elevation in plasma ammonia levels outwith the range found in the wildtype mice. The number of mice analysed is each group was small especially as the plasma ammonia levels showed an overlap in values between the control wildtype and affected groups. If more creatine kinase-OTC^{MOD} mice had been analysed, it would have been easier to clarify whether there was no real improvement in ammonia levels or whether there was an occult improvement masked in statistical terms by the overlap in ammonia values.

It should be noted that the OTC enzyme assay used was a whole tissue *in vitro* assay, and therefore it does not rule out the possibility that the OTC peptide in the myocyte was not being correctly targeted to the mitochondrial membrane *in vivo*. Thus, there is the possibility that although the OTC enzyme is catalytically active it is sequestered in the wrong sub-cellular compartment due to the intentional leucine to valine missense mutation engineered into the recombinant OTC^{MOD} protein. This argument could account for the lack of phenotypic or metabolic correction of the Spf and Spf^{ASH} mice seen despite the presence of high OTC activity in skeletal muscle.

Evidence refuting defective peptide targeting as a possible explanation of the inability of OTC activity in the muscle cells to correct the OTC deficient phenotype, comes from two distinct sources. Firstly from sequence data which indicates that the first amino acid in the leader peptide is not highly conserved between species and indeed valine, the amino acid formed through the site–directed mutagenesis, is

actually a normal polymorphic amino acid variant at this site in the leader peptide (Tuchman, Morizono et al. 1995). Secondly, evidence from deletion studies highlight the mid portion of the leader sequence and in particular the presence of 3 highly conserved arginine residues as being the essential region for the targeting and transportation of polypeptide into the mitochondrial matrix rather than the amino terminal amino acids (Horwich, Fenton et al. 1984; Horwich, Fenton et al. 1985; Horwich, Kalousek et al. 1986)].

A more likely explanation for the inability of high levels of skeletal muscle targeted OTC activity to correct the metabolic and phenotypic abnormalities in Spf and Spf^{ASH} mice is the absence of one of the substrates for the catalytic reaction to occur. There is little evidence that CPS I is expressed in skeletal muscle cells. If this is indeed the case, then despite ammonia being a highly diffusible substrate, no carbamyl phosphate would be produced in skeletal muscle. Carbamyl phosphate, as mentioned previously, is a substrate for OTC which is normally produced within the mitochondrion and it is highly likely that it will not diffuse over large distances. In the liver any excess carbamyl phosphate which leaves the mitochondrion is quickly metabolised in the pyrimidine pathway. If carbamyl phosphate was absent in myocytes even in the presence of high levels of OTC there would be little effect in lowering plasma ammonia levels in the mice as the substrate for ornithine transcarbamylase activity would be absent. This is certainly appears to be the case in all the creatine kinase driven transgenic lines; despite high levels of gene expression and OTC activity in skeletal muscle of transgenic animals, there is no difference in the plasma ammonia levels from the control, non- transgenic sparse fur and sparse fur^{ASH} animals

Although at first glance, muscle cells are an unlikely target for the expression of hepatic metabolic enzymes, and despite the failure of OTC^{MOD} recombinant protein

expressed in skeletal muscle to correct the OTC deficiency in Spf mice, the underlying hypothesis has been proven recently. Harding *et al* (Harding, Wild et al. 1998) produced transgenic mice in which the phenylalanine hydroxylase gene (PAH) was targeted to skeletal muscle under the transcriptional control of the creatine kinase promoter. The transgenic mice were then bred to PAH hepatic-deficient mice. The resulting mice, which had no endogenous hepatic PAH activity but did have exogenous skeletal muscle-specific PAH activity, showed an underlying basal hyperphenylalaninaemia. The serum phenylalaninaemia in these mice showed a marked decrease when further supplemented in the diet with tetrahydrobiopterin (BH4) a required cofactor for PAH. In this system, compared to the OTC metabolic pathway, the substrate for PAH namely phenylalanine is highly diffusible in plasma and is indeed the direct cause of the clinical pathology found in PAH deficient patients. It is through monitoring plasma levels of phenylalanine that the clinical diagnosis is made.

It could well be envisaged that in order for creatine kinase–OTC^{MOD} mice to utilize the skeletal OTC activity effectively a double transgenic with the CPS I gene also under the control of the creatine kinase promoter would be required. It is certainly within the realms of possiblity to foresee the use of plasmid or viral vectors with both transcriptional units as a method of delivery for both recombinant proteins. In addition, it would also be possible to express both recombinant proteins in myoblasts *in vitro* to check for their catalytic activity, and to subsequently transplant the myoblasts in adult muscle. These are certainly avenues worthy of future consideration.

4.1.1.2 THE LIVER IS A WELL RESEARCHED TARGET ORGAN FOR SUPPLEMENTIVE ENDOGENOUS GENE EXPRESSION

The fact that the liver is a major target organ for supplementive gene therapy lies both in the number of clinical disorders attributable to hepatic pathology, and in the hepatotrophic affinity of the viral gene expression vectors used in gene therapy studies. For these reasons, models involving gene expression studies in the liver have concentrated on supplementation of endogenous hepatic genes rather than the expression of exogenous genes as in the case of muscle or skin targeted gene expression. Due to the natural hepatic affinity of adenoviruses, recombinant forms of these viruses, when injected intravenously, can cause high levels of hepatocyte transfection and gene expression, allowing the potential treatment of a large number of gene disorders (Patijn and Kay 1999). The recent advances in modifying recombinant adenoviral vectors (Morral, Parks et al. 1998), so that they are devoid of all viral encoding sequences, are hoped to decrease the inflammatory responses induced by classical adenoviruses which limit the duration of their gene expresion and thus their clinical potential. However, there may also be an immune response dependent on the recombinant protein expressed (Morral, Parks et al. 1998). The power of the intravenous injection route is that it lends itself directly to clinical application. Evidence that even the application of a single intravenous injection can have a benefical effect on a murine model for glycogen storage disease type II is potentially very exciting (Amalfitano, McVie-Wylie et al. 1999). Similarly, although surgically invasive, high levels of hepatic gene expression can also be achieved by portal vein infusion of either retroviral or adenoviral recombinant vectors (Patijn, Terpstra et al. 1998). This method of intra-biliary administration (Peeters, Patijn et al. 1996) has the advantage of reducing the transfection of non-

hepatic cells, which also occur with recombinant viral administration by the intra venous route. Even when administered by intramuscular injection in an attempt to target muscle cells, recombinant adeno-associated virus was found to be expressed and have a therapeutic effect in the liver due to dissemination of the virus in the blood (Daly, Okuyama et al. 1999). This study also highlights a danger in that it indicates that although the route of administration was designed to be muscle–specific, the hepatic affinity of the expression vector changed the target organ, which could have potentially hazardous implications.

An alternative means of targeting recombinant protein to liver is by culture and transfection of the hepatocytes *ex vivo* and then seeding of the genetically-modified hepatocytes back into the patient. This has been successfully achieved in the clinical setting for hypercholesterolaemia (Grossman, Raper et al. 1994; Grossman, Rader et al. 1995) and well as murine model systems (Overturf, Al-Dhalimy et al. 1996; Overturf, Al-Dhalimy et al. 1998). Although the use of autologous hepatocytes in these studies circumvents problems with immune rejection, the culture of any cell type *in vitro* can induce deleterious genetic cellular changes, which have detrimental consequences after the hepatocytes have re-populated the liver.

In the current study the albumin driven-OTC^{MOD} transgene was initially devised as a positive control for expression of the rat OTC^{MOD} cDNA in the endogenous hepatic tissue. Therefore, it was counterintuitive to find that expression of the albumin driven expression construct did not correct the phenotype of either the Spf or the Spf^{ASH} OTC deficient mice.

4.1.2 PARTIAL METABOLIC CORRECTION OF THE SPARSE FUR PHENOTYPE BY THE ALBUMIN DRIVEN RAT OTC CDNA TRANSGENE.

Although the albumin transgenic lines did not correct the phenotype of the Spf and Spf^{ASH} mice, there does appear to be a slight improvement in OTC metabolism as measured by plasma ammonia levels. Using plasma ammonia as an intermediate metabolic index for OTC supplementation, transgenic lines KG and KE showed a significant decrease in plasma ammonia levels on a Spf background. Furthermore, the ammonia levels in these lines could not be differentiated from those in the wildtype mice. Although, the KD line was not found to have a statistically significantly lower plasma ammonia level, the plasma ammonia values appeared to be following the trend of the other two transgenic lines in having a lower and smaller spread of values. This trend was further highlighted if all the 'K' line mice were grouped together, as a significant difference in ammonia levels was found not only on the Spf but also the Spf^{ASH} background. The KD group contained the fewest mice and it is probable that if this group was expanded the difference in plasma ammonia levels may have reached a higher statistical significance. The small number of animals in this group particularly weakens the statistical significance, as there is a marked overlap in plasma ammonia levels between the control wildtype and OTC deficient groups of mice. This overlap necessitates a higher statistical power to separate these two sets of data into two statistically significant groups, which would in turn translate into large numbers of animals in each group.

Alternatively, as opposed to plasma ammonia levels, it could be argued that a better index of metabolic correction would have been urinary orotic acid levels as this has been found both in the clinical setting and in other murine studies to correlate well with hepatic OTC activity. However, due to technical difficulties in collecting and analysing 24 hour murine urine samples it was not possible to undertake this analysis. Although, it would have been interesting to compare the results obtained from both the orotic acid urine and plasma ammonia levels.

Having obtained results suggesting a partial metabolic correction of both OTC deficient mouse models, it was hoped that an increase in direct OTC hepatic activity would correlate with the improvement in plasma ammonia levels. Unfortunately when the OTC activity was measured in hepatic tissue only the KG transgenic line showed a significantly increased OTC activity when bred onto a Spf background, compared to the endogenous hepatic Spf OTC activity. This increase in the KG line was equivalent of a 6% increase in total hepatic OTC activity, bringing the total activity to 21% of wildtype. However, when all the 'K' line mice on a Spf background were pooled they showed a significantly increased OTC activity compared to endogenous Spf OTC activity. Studies (Cavard, Grimber et al. 1988; Stratford-Perricaudet, Levrero et al. 1990) have suggested that the hepatic OTC activity must be in the region of 50-80% of normal to correct the phenotype of the Spf mice. As the hepatic OTC activity in the albumin-OTC^{MOD} mice was still well below this value, it is not surprising that the transgene failed to correct the mouse OTC deficient phenotype.

On the Spf^{ASH} background the 'K' lines did not show a significant increase in OTC activity. The median activity rose from 5 to 8% of the wildtype hepatic value. Interestingly, although one group found an increase of hepatic OTC activity to only 12% of normal was sufficient to correct the Spf^{ASH} phenotype, this transgenic line was also found to increase in intestinal OTC activity to 27% normal (Murakami, Takiguchi et al. 1989). It is difficult therefore to determine if it was the increase in OTC activity in the liver or the small intestine that had most influence on the correction of the Spf^{ASH} phenotype. It may well be that the small intestine OTC activity would counter the low hepatic activity in correcting the Spf^{ASH} phenotype. The Northern blot results from one of the current albumin-OTC^{MOD} transgenic lines showed a reasonable level of expression of the transgenic rat OTC transcript which

was as expected hepatocyte-specific and therefore this transgene would have no effect on intestinal OTC activity.

Although the recombinant OTC^{MOD} protein produced high levels of OTC activity in skeletal muscle cells, the increase in hepatic OTC activity was marginal. The use of the identical OTC^{MOD} cDNA in the formation of both the creatine kinase and albumin-OTC^{MOD} constructs eliminates the possibility of a PCR-induced mutation in the OTC^{MOD} cDNA accounting for this low enzyme activity in the liver. However, from the data given, it is not possible to exclude the possibility that the recombinant OTC^{MOD} protein has an intrinsically lower catalytic activity compared to the endogenous hepatic protein, as the levels of mRNA were also higher in the creatine kinase OTC^{MOD} transgenic lines. In addition, the information from the creatine kinase transgenic mice also precludes the alteration of the first amino acid of the leader peptide sequence affecting OTC activity.

The first possible hypothesis to account for this difference in OTC activity between the hepatic and muscle transgenic mice could be that the albumin promoter/enhancer is a relatively weaker promoter compared to the creatine kinase promoter. From the Northern data there does appear to be a lower quantity of transcript in the liver of the hepatic driven transgenic mice than in the skeletal muscle of the creatine kinase driven transgenic mice. However, albumin is one of the most predominant proteins in plasma and the gene shows a high level of transcriptional control in gene expression. It has been used to drive high expression of growth hormone in another transgenic mouse model (Pinkert, Ornitz et al. 1987) and therefore it would be surprising, but not implausible, for the albumin to be the weaker promoter. However, it is more plausible that all the endogenous regulatory elements, which allow high transcription level *in vivo* may be absent from the 2.3 Kb promoter/ enhancer elements present in the albumin-OTC^{MOD} expression construct.

A more technically based explanation is that the creatine kinase driven OTC transgenic mice may have integrated a higher number of copies of the OTC transgenes. There is no data comparing the transgene copy number in the different transgenic lines. The number of transgenes integrating into the genome during pronuclear injection can vary greatly from one to thousands of copies of the transgene and an increase in transcription levels in the creatine kinase-OTC^{MOD} transgenic mice may be due to the presence of a high number of the transgene integrations. Future work must include a Southern blot to estimate the copy numbers in all the transgenic lines produced.

Another plausible explanation could be that the albumin promoter is directing recombinant OTC^{MOD} gene expression to the subset of hepatocytes that are not intricately involved in the urea cycle pathway. The urea cycle predominately occurs in the periportal and not in the perivenule areas of the hepatic lobule. Again although a plausible explanation to account for the lack of murine phenotypic correction *in vivo*, the OTC assay employed measured total OTC activity from whole hepatic tissue and therefore would have detected OTC activity in both the hepatic compartments, periportal and perivenule. The lack of an increase in total hepatic OTC activity would suggest that *in vivo* there is no pool of sequestered recombinant OTC^{MOD} that is not accessible to the urea cycle. The use of tissue *in situ* hybridisation studies using the SV40 polyadenylation sequence as the probe could determine the expression pattern of the albumin-OTC^{MOD} transgene at a cellular level.

One further theory, which may have important implications despite many published examples of correction of the OTC deficient mouse models, is the possibility that endogenous mutant polypeptides produced in the heptocytes of Spf or Spf^{ASH} animals may have a dominant negative effect via a protein-protein interaction with

the recombinant polypeptide. As discussed in the introduction, ornithine transcarbamylase functions as a homotrimer. The translated OTC polypeptides are transported into the mitochondrion where the leader peptide is cleaved off and the peptides are joined to form the enzymatically active OTC homotrimer. In both OTC deficient mouse models, endogenous OTC mRNA is produced, although in the Spf^{ASH} hepatocytes the level is 10% that of wildtype OTC mRNA. Thus, in both the Spf and Spf^{ASH} mice OTC polypeptides with a missense mutation are being produced. When the albumin-OTC^{MOD} transgene is bred onto these mutant OTC lines, two populations of OTC polypeptides will be present within the same hepatocyte, namely the recombinant OTC^{MOD} polypeptide and a mutant form from the endogenous gene. Therefore, it is possible to postulate that the endogenous mutant OTC polypeptides may have a dominant negative effect by forming a homotrimer with the transgenic wild type OTC polypeptides and thereby decrease the formation of functional OTC protein. This is never a normal physiological possibility as in female heterozygotes, due to X chromosome inactivation, each hepatocyte expresses either the normal or the mutant but not both forms of the OTC polypeptide. This possible dominant negative effect could account for the small increase in OTC activity despite the presence of high levels of transgenic mRNA. Evidence that this is a real possibility and not just supposition, was provided in a recent study (Morsy, Zhao et al. 1996) in which higher levels of adenoviral driven OTC expression was required for a lower OTC activity in cell lines with endogenous aberrant OTC production than cell lines with no endogenous aberrant OTC activity. Western blotting confirmed the presence of equal amounts of OTC protein formation but lower OTC activity in a hepatocyte cell lines derived from Spf mice compared to a OTC non-expressing Cos cell line. In addition, this group studied hepatocytes from two patients with OTC deficiency, one had a missense

mutation in exon 3, which did not affect protein expression but totally abolished protein activity, whilst the other patient had no protein on Western blot which correlated with no enzymatic activity (mutation unknown). Morsey et al (Morsy, Zhao et al. 1996) found that despite equal levels of adenoviral mediated recombinant OTC expression in both these cell lines, the OTC activity was substantially inhibited in the hepatocyte cell expressing a mutant form of OTC.

Although a valid possible explanation, the results from the Spf^{ASH} mice in this study would suggest that this is not the total answer. The Spf^{ASH} hepatocytes have been shown to have only 10% of normal OTC mRNA levels in keeping with 5% of normal OTC activity. Therefore, assuming this level of OTC mRNA is translated at the same level as wildtype, there should be less aberrant OTC polypeptide available in the Spf^{ASH} hepatocyte compared to the sparse fur hepatocyte to form a dominant negative effect. The result of this would be that the albumin-OTC^{MOD} transgene should increase the level of hepatic OTC activity in the Spf^{ASH} hepatocyte proportionately higher than the Spf hepatocyte. This was not seen in this study although the numbers of mice for the Spf^{ASH} / transgenic crosses were low.

If, however, this dominant negative effect of diminution of exogenous OTC activity in the presence of endogenous aberrant protein formation holds true, it could be clinically important. It would suggest that hepatocyte-targeted OTC gene therapy might be most relevant only for a subset of patients with mutations causing an effective null allele and therefore producing no endogenous protein. In the human disease about 74% of all mutations found to date are nucleotide substitutions causing missense or nonsense alterations, causing a diminution in OTC protein activity but most patients do conserve some residual OTC activity suggesting the presence of mutant protein. Therefore, it is conceivable that the majority of patients with OTC may not be suitable for a direct hepatic OTC replacement approach unless

high levels of the normal protein could be achieved. In addition, it would also highlight the importance of pursuing means of expressing ectopic OTC in tissues, other than liver, in which endogenous aberrant OTC protein is absent.

4.1.3 Pathophysiology of the Sparse fur and sparse fur^{ASH} mouse models

It is of interest that the phenotype of the naturally occurring OTC deficient mice predominantly involves ectodermal tissue, as is highlighted by the terms used to describe them namely, sparse fur (Spf) and sparse fur and abnormal skin and hair (Spf^{ASH}). Although these mice have been shown to have allelic mutations in the X-linked OTC gene identical to that describe in the equivalent human condition, the ectodermal phenotype found in the OTC deficient mice has not been described as a manifestation of the human presentation of OTC deficiency. The skin and hair in either OTC deficient males or obligate female carriers is normal, although the biochemical abnormalities are indeed identical to those described in the mouse models.

Argininosuccinicaciduria is caused by a defect in a urea cycle enzyme, arginosuccinase. Clinical manifestations include sparse hair and skin lesions, in association with other phenotypic changes such as mental and physical retardation and liver enlargement. This condition is due to the deficiency in argininosuccinase (Omin *207900), the cytosolic enzyme preceding OTC in the urea cycle and responsible for the production of arginine. The degree of ectodermal involvement appears to depend on the total protein intake of the patient (Coryell, Hall et al. 1964). 'Normalisation' of the hair shafts has been shown with the commencement of a low protein, arginine supplemented diet (Batshaw, Roan et al. 1980). This evidence associating low arginine levels with pathological ectodermal features, and

the correction of these features with supplementation of arginine, suggest that sparse hair and skin lesions can result directly from low levels of arginine.

In the Spf and Spf^{ASH} OTC deficient mouse models, the characteristic phenotype may result indirectly from an arginine deficiency rather than directly as a result of OTC deficiency. Although this possibility certainly does not preclude the use of phenotypic scoring of the OTC deficient mouse as a correlate of OTC deficiency, it would however temper caution on relying on this solely as a direct correlate of hepatic OTC values as it may be more highly influenced by small intestine OTC activity. Evidence for this can also be seen in the study by Jones et al (Jones, Grompe et al. 1990) in which an increase in OTC activity in the small intestine alone was sufficient to correct the phenotype of Spf mice. The OTC activity in the small intestine is thought to metabolise excess nitrogen and the citrulline produced, when transported to the kidneys, is important in the *de novo* production of arginine. Previous transgenic studies (Cavard, Grimber et al. 1988; Jones, Grompe et al. 1990) using the enodgenous OTC promoter would have increased the small intestinal as well as the hepatic OTC activity although small intestinal OTC activity is less commonly commented on. In the current study the albumin-OTC^{MOD} construct is hepato-specific and would have no effect on intestinal OTC activity, which may also have contributed to the lack phenotypic correction if this theory is true.

4.2 THE SKIN IS AN EXCELLENT TARGET ORGAN FOR EXOGENOUS RECOMBINANT PROTEIN EXPRESSION

The skin is an excellent tissue as a target for gene therapy and as an organ for basic research studies on gene transfer and expression. It is easily accessible, monitored, has a good blood supply and has been shown to have the post translational machinery required to express many exogenous genes (Trainer and Alexander 1997). Unlike the two other important tissues already discussed namely muscle and liver, the skin has been used primarily as an organ in which to express recombinant protein which has a more widespread function rather than the supplementation of an protein defective in the skin itself. The major limitation in treatment of intrinsic skin pathologies is their generalised nature, necessitating treatment of the entire skin. In addition, the keratin mutations causing epidermolysis bullose simplex (Korge and Krieg 1996) and epidermolytic hyperkeratosis (Bickenbach, Longley et al. 1996) tend to be dominant and therefore difficult to rectify by supplementive gene therapy due to the possibility of a dominant negative effect. However, the epidermis has been used to express gene products important in many differing fields. Applications have included the expression of insulin-like growth factors (Bickenbach, Longley et al. 1996) and epidermal growth factors (Bickenbach, Longley et al. 1996) to promote wound healing; the expression of coagulation cascade factor IX (Bickenbach, Longley et al. 1996) and growth hormone (Wang, Zinkel et al. 1997) in systemic disorders; antigenic epitopes (Raz, Carson et al. 1994) or active immunological gene products as genetic vaccines or to ellict a immunomodulatory effect towards neoplasm (O'Malley, Chen et al. 1995; Chong, Hutchinson et al. 1996).

Keratinocytes can be classified into three types with respect to their clonal proliferative capacity: (i) holoclones or stem cells with extensive growth capacity: (ii) differentiated paraclones with a limited growth capacity; and (iii) intermediate meroclones which are thought to constitute long lived progenitor cells *in vivo*

(Barrandon and Green 1987). Ideally, epidermal gene targeting would involve integration of an exogenous gene into the genome of holoclone keratinocytes, whose progeny form both a self-renewing population of stem cells allowing long-term expression, and a more differentiated suprabasal population of keratinocytes. It is for this reason that it is the regulatory sequences from the basally expressed keratins, namely keratin 5 and 14 have been focused on to drive expression of exogenous gene products. In particular, the regulatory elements of the keratin 14 gene has been used extensively for delivery of many growth control molecules (Wysolmerski, Broadus et al. 1994; Wang, Zinkel et al. 1997).

It was due to this potential for both basic and preclinical studies in the skin that the bovine keratin 5 regulatory sequences (obtained from Manfred Blessing (Blessing, Nanney et al. 1993) were chosen for study in the current work. The keratin 5 gene was examined for its ability to regulate exogenous gene expression in both a tissue and cell specific manner. This was done means of linking the regulatory sequences to a reporter β galactosidase gene.

4.2.1 KERATIN 5 MINIGENE EXPRESSION CASSETTE DOES MIMIC ENDOGENOUS K5 EXPRESSION IN THE ADULT MOUSE

The expression data obtained using the bovine keratin 5 minigene driven β galactosidase transgenic lines mimics the expected expression pattern of the endogenous keratin 5 gene. This pattern of expression was identical in the two founder transgenic lines investigated, suggesting that the expression pattern was dictated by the K5 regulatory gene sequences rather than being influenced by transgene integration site.

In adult transgenic mice X-Gal staining showed high levels of βgalactosidase activity in the basal, proliferative layers of the epidermis and well as in the root bulb

of the hair follicles which lies deep in the dermis (Lersch and Fuchs 1988). There does appear to be some staining in the epidermal suprabasal layers which is unexpected as keratinocytes down-regulate K5 and K14 in this area and up-regulate the keratin co-expression pair K1 and K10 (Fuchs and Green 1980; Moll, Franke et al. 1982). This suprabasal Lac Z staining could be an artefact caused by leeching of the X Gal into the neighbouring cells due to the high levels of Lac Z activity or simply that residual Lac Z protein remains after differentiation and suprabasal migration. The β-galactosidase cDNA used in the formation of the expression cassette did not include a nuclear localisation sequence (NLS) which would have reduced the non–specific staining of the lac Z gene product out of the cell. Another group (Ramirez, Bravo et al. 1994) using a similar bovine keratin 5-lac Z expression also found suprabasal expression of the Lac Z which did not correlate with endogenous keratin 5 gene expression. This group were also using *E. Coli* Lac Z with no NLS.

Interestingly though, there is the suggestion of a quantitative difference in the staining pattern within different areas of the basal layer of the epidermis. This may correlate with differing degrees of ongoing proliferation/differentiation in the epidermis. Hair growth, for example, which is also associated with interfollicular growth, occurs as waves of proliferation in a cranial to caudal direction. Staining of other stratified epithelia in the tongue (Dhouailly, Xu et al. 1989) and oesophagus indicated a lower degree of lac Z expresion and a more distinctly basal pattern with no suprabasal staining. In the transgenic mice the epithelium of the forestomach (Schweizer, Rentrop et al. 1988) also stained in the basal layer indicating keratin 5 expression. In contrast the epithelium of the hindstomach stained with X Gal in both

the transgenic and the control mouse. This background staining precluded any comment being made regarding keratin 5 expression at this site.

Cellular proliferation in the cornea of the eye occurs at the junction of the iris and the cornea in a region called the limbic area. This area is ideal for proliferation as its is highly vascular. The cells in the cornea then leave the proliferative area and differentiate over the region of the cornea overlying the pupil. Any vascular elements, vital for proliferating cells, in this region would severely affect vision. The transgenic mice show a very discrete expression pattern limited to the limbic region in keeping with the normal endogenous expression of keratin 5 and its down-regulation in the more differentiated corneal cells (Fig 30), which then co-express the suprabasal keratins K3 and K12 (Schermer, Galvin et al. 1986).

Interestingly, it is known that patients with the severe form of Epidermolysis bullosa simplex due to mutations in the keratin 5 gene can have oesophageal and corneal problems in addition to the severe epidermal blistering problems that it normally associated with the condition.

The only tissue in which expression of the transgene deviated from that of the endogenous gene was in the basal layer of the intestinal epithelium, where the endogenous gene is not expressed, but a low level expression pattern of the transgene was detected. The original staining was noted in paraffin embedded sections of the small intestine. However, the small intestinal lining is coated with mucin and colonised with bacteria that may themselves have β-galactosidase activity. As mentioned previously, the cDNA sequence of the β galactosidase used in the keratin 5 expression construct was originally cloned from *E. Coli*. Therefore, it was necessary to confirm this staining result using small intestine that had been treated to remove the mucin and bacterial coating. A whole mount of the small

intestine was treated with an anti-mucogen and stained for β-galactosidase activity. The lacZ-staining pattern in the intestinal epithelium was also observed in whole mount preparation suggesting that it was intrinsic to the intestinal epithelium and not the concomitant bacterial flora. The Lac Z staining was not seen in the control mouse, which had been similarly treated. As this expression pattern was seen in both transgenic founder lines, it suggests the expression pattern is due to the specific regulatory sequences found in the bovine keratin 5 minigene rather a secondary effect due to the influence of cis-acting modifying sequences at the site of the transgene integration. This is interesting, as the epithelium lining the intestinal villi is a simple columnar rather than a stratified epithelium.

Allowing for this intestinal tissue expression deviation, the results from this transgene indicates that the 8.5 kb fragment containing both 5' and 3' regulatory sequences of the bovine keratin 5 gene are sufficient for high levels of ectopic gene expression in a keratin 5 tissue and cell specific manner. The presence of this exogenous gene transcribed from the keratin 5 regulatory sequences does not appear to have a detrimental impact on the cell.

A similar study (Byrne and Fuchs 1993) using 5 kb of the 5' keratin 5 regulatory sequences found comparable results to those described here with the exception that Byrne *et al* found no staining in either the epithelium of the forestomach or oesphagus of the transgenic mice. The internal control in this study, the use of immunohistochemistry for endogenous keratin 5 protein did detect the endogenous protein at these sites, suggesting that the regulatory sequences used in the transgenic construct lacked the ability to target full tissue specificity. In view of this, it was surprising that deleting the promoter to only 90 bp was sufficient to target gene expression, albeit to a lesser extent, to those same tissue (Byrne and Fuchs 1993;

Ramirez, Bravo et al. 1994). However, using this severely truncated form of the promoter it was noted that although the tissue specificity of the expression pattern was retained, the cellular differentiation pattern was lost, as staining was seen in the suprabasal cells within the epithelium. It is difficult to reconcile this result in light of results by Ramirez et al (Ramirez, Bravo et al. 1994) in which they failed to obtain any tissue-specific keratin5 Lac Z expression using 1.5 Kb of the keratin 5 5'regulatory sequences. They examined 10 transgenic lines and found no expression, with the exception of a few epithelia cells in the nasal cavity. It can only be assumed that there was a secondary reason for the intrinsic failure of expression from this expression cassette, although it is very difficult to draw any conclusions from such a negative result. It is worth noting that in none of the transgenic mice produced by Byrne *et al* was expression seen in the intestine.

From the results shown here, the keratin 5 minigene expression cassette appears to bean excellent vector for the expression of exogenous genes in an epidermal population of keratinocytes which are capable of proliferation and therefore may, in theory, harbour long term gene expression. In addition, the keratin5-lac Z transgenic mice would form an ideal positive control in studying modes of gene transfer to the epidermis as the epidermis of these mice would represent the 100% transfection scenario. Possible means of non-viral epidermal gene transfer have included application of 'naked' DNA- intradermal 'tatooing' (Hengge, Chan et al. 1995), particle bombardment (Ciernik, Krayenbuhl et al. 1996) and liposome-mediated (Li and Hoffman 1995). These methods would allow tissue-specific targeting of recombinant proteins and the additional use of a keratin–specific promoter would prevent promiscuous gene expression in other tissues, which can occur with viral vectors as mentioned previously (Daly, Okuyama et al. 1999).

It was unfortunate that time prevented the formation of a keratin 5-OTC^{MOD} especially in view of the unexpected expression pattern in the intestinal epithelium. Although there is no evidence for the expression of CPS I in the epidermis, the intestinal expression driven by the keratin 5 minigene in the presence of a partial OTC cycle may have had an interesting phenotype.

In the clinical setting, it is the adult expression pattern of the keratin 5 minigene that is of interest. However, in future murine studies utilising this expression cassette to express less inert proteins, it is important to know the expression pattern during development, as this will have repercussions on the phenotype seen in the mouse

4.2.2 KERATIN 5/L ACZ EXPRESSION PATTERN DURING EMBRYOGENESIS.

During embryogenesis expression of K5 and K14 have been detected as early as the bi-layered epithelial phase (Dale, Holbrook et al. 1985; Moll, Moll et al. 1982). K14 expression has been noted in the inner embryonic layer and on later stratification is restricted to the innermost layer (Kopan and Fuchs 1989). In conjunction with the later temporal expression of keratin 1 and 10, this body of work suggests that there are strong parallels between embryonic and adult epidermal keratin expression patterns.

Studies by Byrne *et al* (Byrne, Tainsky et al. 1994) using a 5 kb keratin 5 promoter driven LacZ reporter sequence showed initial K5 expression over the dorsal lateral region of the embryo overlying the somites at day 9.5. In addition, there appeared to be some expression over the head region. In the results presented here, there was faint staining over the lateral somites but no staining was observed over the head area. At this gestational stage even the staining pattern over the somites was very

faint and difficult to visualise. However, the later Lac Z expression pattern appeared to mirror those found in the earlier study. The staining at d10.5 also showed a very similar staining pattern except that in addition, the results obtained in this study showed moderate expression in the mandibular branch and weaker expression in the maxillary branch of the first branchial arch as well as the nasal pit. In general the embryo at d10.5 in this study appeared to be slightly more advanced that the equivalent d10.5 embryo demonstrated in the Byrne *et al* study which could account for this variation in expression seen (Byrne, Tainsky et al. 1994). In both transgenic systems the expression included the first branchial arch and then slowly extended rostrally and caudally over the dorsal lateral ectoderm overlying the somites. Evidence that this expression pattern represented the true endogenous gene expression came from *in situ* hybridisation data performed by Byrne *et al* which was indistinguishable from the lac Z staining pattern from the transgenic embryo although it was significantly less sensitive.

The K5 induction in the first branchial arch ectoderm followed soon after the proposed migration of the underlying mesenchyme, of neural crest origin (Nichols 1981). This is particularly interesting as the formation of many later epidermal appendage such as hair follicles etc are dependent on the underlying dermal cues (Hardy 1992).

K5 expression was found throughout the epidermis at day 12.5 but with marked sparing of the head and dorsal aspects of the embryo relating perhaps to the rapidly expanding neural tissue underlying these areas of epithelium. This is particularly interesting as other cranial features such as the maxillary hair follicles showed markedly increased staining compared with the more caudal pelage hair follicles.

Histological sections taken at d12.5 interestingly showed that the lac Z staining was not limited to ectodermal tissue alone. Althought the nasal cavity and developing

fibrissae in the maxillary area show distinct ectodermal staining, the sections through the developing eye and skin appear to show mesodermal cell-specific Lac Z staining, which was not commented upon in the study by Bryne et al. It should be noted in the current study that the ectodermal tissue shows very high levels of Lac Z staining compared to Byrne et al.

It is difficult to ascribe the staining seen in these tissues to a staining artefact, although this could be easily investigated by means of *in situ* hybridisation. This technique would separate staining due to cell expression of the keratin 5 gene from a staining artefact due to cellular overspill of the lac Z gene product. However, in the future more specific staining could be achieved by utilising a Lac Z cDNA with a nuclear localisation sequence. By day 13.5 almost the entire embryo surface is expressing keratin 5 as indicated by the Lac Z staining, although the most distal areas of the fore and hind limbs still stained negative with marked interdigital sparing.

Interestingly, the results obtained here did correlate well temporally and spatially with those obtained by Byrne et al using 6 kb of the regulatory sequences. However, another group using 5 kb of 5' regulatory sequences of the bovine keratin 5 showed very delayed expression pattern. The transgene was not expressed until d13.5 and their control comprising immunohistochemistry for the endogenous keratin 5 did not show endogenous expression before d11.5. It should be noted that this group did not study the d10.5 embryo but concentrated on 48 hr increments in developmental patterning. The difference in control endogenous keratin 5 gene expression pattern seen between these two groups (Byrne, Tainsky et al. 1994; Ramirez, Bravo et al. 1994), is most likely attributable to the sensitivites of the two techniques used with *in situ* hybridisation being the more sensitive. With regard to the Lac Z staining

pattern, Bryne et al found this technique to be more sensitive than *in situ* hybridisation whilst Ramirez found it to be less sensitive than immunohistochemistry. The results presented here correlate well with those found by Byrne *et al* showing expression 3 days prior to that seen by Ramirez et al. This body of work emphases the need to carefully characterise specific regulatory elements used in the formation of tissue-specific expression constructs rather than extrapolate expression patterns from previously published mouse models.

4.2.3 CONCLUSION

The major conclusion from this study is that the skeletal muscle may not be an effective target organ for the exogenous expression of recombinant OTC to correct OTC deficiency. This is most likely due to lack of other enzymatic components of the urea cycle. One other study has demonstrated proof of principle for the use of genetically modified muscle to act as a 'metabolic sink' and it is conceivable that viral vectors which drive expression of two or more urea cycle enzymes could be used for correction of OTC deficiency in muscle tissue. Nevertheless, much work remains to be performed to explore this avenue effectively.

In the meantime the liver remains the obvious choice as the clinically relevant tissue for OTC gene supplemantation therapy. However, the current study and other published works suggest that fundemental questions concerning the spectrum of molecular pathologies of OTC need to be addressed before gene supplementation therapy for OTC deficiency in humans can be regarded as appropriate to all patients. In particular it should be ascertained whether any mutation can positively interfere with oligomerisation of wild type proteins, which would preclude the use of gene supplementation therapy in the liver.

(1998). "7th International Conference on Gene Therapy of Cancer. San Diego, California, USA. November 19-21, 1998. Abstracts [In Process Citation]." Cancer Gene Ther **5**(6 Suppl): S1-35.

Acsadi, G., G. Dickson, et al. (1991). "Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs [see comments]." Nature **352**(6338): 815-8.

Albers, K. and E. Fuchs (1992). "The molecular biology of intermediate filament proteins." Int Rev Cytol **134**: 243-79.

Amalfitano, A., A. J. McVie-Wylie, et al. (1999). "Systemic correction of the muscle disorder glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid-alpha-glucosidase." Proc Natl Acad Sci U S A **96**(16): 8861-8866.

Arn, P. H., E. R. Hauser, et al. (1990). "Hyperammonemia in women with a mutation at the ornithine carbamoyltransferase locus. A cause of postpartum coma [see comments]." N Engl J Med **322**(23): 1652-5.

Bachvarova, R. F. (1992). "A maternal tail of poly(A): the long and the short of it." Cell **69**(6): 895-7.

Barrandon, Y. and H. Green (1987). "Three clonal types of keratinocyte with different capacities for multiplication." Proc Natl Acad Sci U S A **84**(8): 2302-6.

Batshaw, M. L., S. Brusilow, et al. (1982). "Treatment of inborn errors of urea synthesis: activation of alternative pathways of waste nitrogen synthesis and excretion." N Engl J Med **306**(23): 1387-92.

Batshaw, M. L., Y. Roan, et al. (1980). "Cerebral dysfunction in asymptomatic carriers of ornithine transcarbamylase deficiency." N Engl J Med **302**(9): 482-5.

Batshaw, M. L., M. Yudkoff, et al. (1995). "The sparse fur mouse as a model for gene therapy in ornithine carbamoyltransferase deficiency." Gene Ther **2**(10): 743-9.

Berghella, L., L. De Angelis, et al. (1999). "Reversible immortalization of human myogenic cells by site-specific excision of a retrovirally transferred oncogene [In Process Citation]." Hum Gene Ther **10**(10): 1607-17.

Bessman, S. P. and P. J. Geiger (1981). "Transport of energy in muscle: the phosphorylcreatine shuttle." Science **211**(4481): 448-52.

Bickenbach, J. R., M. A. Longley, et al. (1996). "A transgenic mouse model that recapitulates the clinical features of both neonatal and adult forms of the skin disease epidermolytic hyperkeratosis." Differentiation **61**(2): 129-39.

Blessing, M., L. B. Nanney, et al. (1993). "Transgenic mice as a model to study the role of TGF-beta-related molecules in hair follicles." Genes Dev **7**(2): 204-15.

Bonaiti-Pellie, C., A. Pelet, et al. (1990). "A probable sex difference in mutation rates in ornithine transcarbamylase deficiency." Hum Genet **84**(2): 163-6.

Breitkreutz, D., H. J. Stark, et al. (1997). "Integrin and basement membrane normalization in mouse grafts of human keratinocytes--implications for epidermal homeostasis." Differentiation **61**(3): 195-209.

Brinster, R. L., J. M. Allen, et al. (1988). "Introns increase transcriptional efficiency in transgenic mice." Proc Natl Acad Sci U S A **85**(3): 836-40.

Brinster, R. L., H. Y. Chen, et al. (1981). "Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs." Cell **27**(1 Pt 2): 223-31.

Brusilow, S. W. and J. Finkelstien (1993). "Restoration of nitrogen homeostasis in a man with ornithine transcarbamylase deficiency [published erratum appears in Metabolism 1994 Jul;43(7):929]." Metabolism **42**(10): 1336-9.

Burlina, A. B., V. Ferrari, et al. (1992). "Allopurinol challenge test in children." J Inherit Metab Dis **15**(5): 707-12.

Byrne, C. and E. Fuchs (1993). "Probing keratinocyte and differentiation specificity of the human K5 promoter in vitro and in transgenic mice." Mol Cell Biol **13**(6): 3176-90.

Byrne, C., M. Tainsky, et al. (1994). "Programming gene expression in developing epidermis." Development **120**(9): 2369-83.

Campbell, A. G., L. E. Rosenberg, et al. (1973). "Ornithine transcarbamylase deficiency: a cause of lethal neonatal hyperammonemia in males." N Engl J Med **288**(1): 1-6.

Cavard, C., G. Grimber, et al. (1988). "Correction of mouse ornithine transcarbamylase deficiency by gene transfer into the germ line." Nucleic Acids Res **16**(5): 2099-110.

Chang, P. Y., H. Benecke, et al. (1994). "Expression of a dominant-negative mutant human insulin receptor in the muscle of transgenic mice." J Biol Chem **269**(23): 16034-40.

Chang, P. Y., J. Jensen, et al. (1996). "Overexpression of hexokinase II in transgenic mice. Evidence that increased phosphorylation augments muscle glucose uptake." J Biol Chem **271**(25): 14834-9.

Cheng, T. C., M. C. Wallace, et al. (1993). "Separable regulatory elements governing myogenin transcription in mouse embryogenesis." Science **261**(5118): 215-8.

Chong, H., G. Hutchinson, et al. (1996). "Expression of co-stimulatory molecules by tumor cells decreases tumorigenicity but may also reduce systemic antitumor immunity." Hum Gene Ther **7**(14): 1771-9.

Ciernik, I. F., B. H. Krayenbuhl, et al. (1996). "Puncture-mediated gene transfer to the skin." Hum Gene Ther **7**(8): 893-9.

Clarke, S. (1976). "The polypeptides of rat liver mitochondria: identification of a 36,000 dalton polypeptide as the subunit of ornithine transcarbamylase." Biochem Biophys Res Commun **71**(4): 1118-24.

Cohen, N. S., C. W. Cheung, et al. (1980). "The effects of ornithine on mitochondrial carbamyl phosphate synthesis." J Biol Chem **255**(21): 10248-55.

Cohen, N. S., C. W. Cheung, et al. (1987). "Channeling of extramitochondrial ornithine to matrix ornithine transcarbamylase." J Biol Chem **262**(1): 203-8.

Cohen, N. S., C. W. Cheung, et al. (1992). "Kinetic properties of carbamoyl-phosphate synthase (ammonia) and ornithine carbamoyltransferase in permeabilized mitochondria." Biochem J **282**(Pt 1): 173-80.

Conboy, J. G. and L. E. Rosenberg (1981). "Posttranslational uptake and processing of in vitro synthesized ornithine transcarbamoylase precursor by isolated rat liver mitochondria." Proc Natl Acad Sci U S A **78**(5): 3073-7.

Cooper, A. J., E. Nieves, et al. (1988). "Short-term metabolic fate of 13N-labeled glutamate, alanine, and glutamine(amide) in rat liver." J Biol Chem **263**(25): 12268-73.

Coryell, M. E., W. K. Hall, et al. (1964). "A familial study of a human enzyme defect, argininosuccinic aciduria." Biochem Biophys Res Commun **14**: 307-12.

Dale, B. A., K. A. Holbrook, et al. (1985). "Expression of epidermal keratins and filaggrin during human fetal skin development." J Cell Biol **101**(4): 1257-69.

Daly, T. M., T. Okuyama, et al. (1999). "Neonatal intramuscular injection with recombinant adeno-associated virus results in prolonged beta-glucuronidase

expression in situ and correction of liver pathology in mucopolysaccharidosis type VII mice." Hum Gene Ther **10**(1): 85-94.

DeMars, R., S. L. LeVan, et al. (1976). "Abnormal ornithine carbamoyltransferase in mice having the sparse-fur mutation." Proc Natl Acad Sci U S A **73**(5): 1693-7.

Dhawan, J., L. C. Pan, et al. (1991). "Systemic delivery of human growth hormone by injection of genetically engineered myoblasts." Science **254**(5037): 1509-12.

Dhouailly, D., C. Xu, et al. (1989). "Expression of hair-related keratins in a soft epithelium: subpopulations of human and mouse dorsal tongue keratinocytes express keratin markers for hair-, skin- and esophageal-types of differentiation." Exp Cell Res **181**(1): 141-58.

Donn, S. M., R. D. Swartz, et al. (1979). "Comparison of exchange transfusion, peritoneal dialysis, and hemodialysis for the treatment of hyperammonemia in an anuric newborn infant." J Pediatr **95**(1): 67-70.

Doolittle, D. P., L. L. Hulbert, et al. (1974). "A new allele of the sparse fur gene in the mouse." J Hered **65**(3): 194-5.

Driscol, D. J. and B. R. Migeon (1990). "Sex difference in methylation of single-copy genes in human meiotic germ cells: implications for X chromosome inactivation, parental imprinting, and origin of CpG mutations." Somat Cell Mol Genet **16**(3): 267-82.

Dubois, N., C. Cavard, et al. (1988). "Compared expression levels of ornithine transcarbamylase and carbamylphosphate synthetase in liver and small intestine of normal and mutant mice." Biochim Biophys Acta **950**(3): 321-8.

Engelhardt, J. F., Y. Yang, et al. (1993). "Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses." Nat Genet **4**(1): 27-34.

Eppenberger, H. M., M. Eppenberger, et al. (1964). "The ontogeny of creatine kinase isoenzymes." Developmental Biology **10**: 10-16.

Ferrie, R. M., M. J. Schwarz, et al. (1992). "Development, multiplexing, and application of ARMS tests for common mutations in the CFTR gene." Am J Hum Genet **51**(2): 251-62.

Fuchs, E. and H. Green (1980). "Changes in keratin gene expression during terminal differentiation of the keratinocyte." Cell **19**(4): 1033-42.

Garcia-Perez, M. A., P. Sanjurjo, et al. (1995). "Demonstration of the spf-ash mutation in Spanish patients with ornithine transcarbamylase deficiency of moderate severity." Hum Genet **95**(2): 183-6.

Gilbert, R., J. Nalbanoglu, et al. (1998). "Efficient utrophin expression following adenovirus gene transfer in dystrophic muscle." Biochem Biophys Res Commun **242**(1): 244-7.

Gordon, J. W., G. A. Scangos, et al. (1980). "Genetic transformation of mouse embryos by microinjection of purified DNA." Proc Natl Acad Sci U S A **77**(12): 7380-4.

Gorski, K., M. Carneiro, et al. (1986). "Tissue-specific in vitro transcription from the mouse albumin promoter." Cell **47**(5): 767-76.

Greelish, J. P., L. T. Su, et al. (1999). "Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector." Nat Med **5**(4): 439-43.

Grompe, M., S. N. Jones, et al. (1992). "Retroviral-mediated gene transfer of human ornithine transcarbamylase into primary hepatocytes of spf and spf-ash mice." Hum Gene Ther **3**(1): 35-44.

Grossman, M., D. J. Rader, et al. (1995). "A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia." Nat Med **1**(11): 1148-54.

Grossman, M., S. E. Raper, et al. (1994). "Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia [see comments]." Nat Genet **6**(4): 335-41.

Gushiken, T., N. Yoshimura, et al. (1985). "Transient hyperammonemia during aging in ornithine transcarbamylase- deficient, sparse-fur mice." Biochem Int **11**(5): 637-43.

Gussoni, E., G. K. Pavlath, et al. (1992). "Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation." Nature **356**(6368): 435-8.

Gussoni, E., Y. Wang, et al. (1996). "A method to codetect introduced genes and their products in gene therapy protocols." Nat Biotechnol **14**(8): 1012-6.

Hamano, Y., H. Kodama, et al. (1988). "Use of immunocytochemical analysis of a duodenal biopsy specimen to identify a carrier of ornithine transcarbamylase deficiency." N Engl J Med **318**(23): 1521-3.

Harding, C. O., K. Wild, et al. (1998). "Metabolic engineering as therapy for inborn errors of metabolism-- development of mice with phenylalanine hydroxylase expression in muscle [In Process Citation]." Gene Ther **5**(5): 677-83.

Hardy, M. H. (1992). "The secret life of the hair follicle." Trends Genet **8**(2): 55-61.

Hasegawa, T., A. G. Tzakis, et al. (1995). "Orthotopic liver transplantation for ornithine transcarbamylase deficiency with hyperammonemic encephalopathy." J Pediatr Surg **30**(6): 863-5.

Hata, A., T. Tsuzuki, et al. (1988). "Structure of the human ornithine transcarbamylase gene." J Biochem (Tokyo) **103**(2): 302-8.

Hauser, E. R., J. E. Finkelstein, et al. (1990). "Allopurinol-induced orotidinuria. A test for mutations at the ornithine carbamoyltransferase locus in women" N Engl J Med **322**(23): 1641-5.

Hauser, M. A., A. Amalfitano, et al. (1997). "Improved adenoviral vectors for gene therapy of Duchenne muscular dystrophy." Neuromuscul Disord **7**(5): 277-83.

Hengge, U. R., E. F. Chan, et al. (1995). "Cytokine gene expression in epidermis with biological effects following injection of naked DNA." Nat Genet **10**(2): 161-6.

Hershfield, M. S. (1998). "Adenosine deaminase deficiency: clinical expression, molecular basis, and therapy." Semin Hematol **35**(4): 291-8.

Herzog, R. W., E. Y. Yang, et al. (1999). "Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector [see comments]." Nat Med **5**(1): 56-63.

Hodges, P. E. and L. E. Rosenberg (1989). "The spfash mouse: a missense mutation in the ornithine transcarbamylase gene also causes aberrant mRNA splicing." Proc Natl Acad Sci U S A **86**(11): 4142-6.

Hoefler, G., C. Noehammer, et al. (1997). "Muscle-specific overexpression of human lipoprotein lipase in mice causes increased intracellular free fatty acids and induction of peroxisomal enzymes." Biochimie **79**(2-3): 163-8.

Hogan, B., F. Costantini, et al. (1986). Manipulating the mouse embryo, Cold spring Harbour Laboratory.

Honeycutt, D., K. Callahan, et al. (1992). "Heterozygote ornithine transcarbamylase deficiency presenting as symptomatic hyperammonemia during initiation of valproate therapy." Neurology **42**(3 Pt 1): 666-8.

Horwich, A. L., W. A. Fenton, et al. (1985). "Expression of amplified DNA sequences for ornithine transcarbamylase in HeLa cells: arginine residues may be required for mitochondrial import of enzyme precursor." J Cell Biol **100**(5): 1515-21.

Horwich, A. L., W. A. Fenton, et al. (1984). "Structure and expression of a complementary DNA for the nuclear coded precursor of human mitochondrial ornithine transcarbamylase." Science **224**(4653): 1068-74.

Horwich, A. L., F. Kalousek, et al. (1986). "Targeting of pre-ornithine transcarbamylase to mitochondria: definition of critical regions and residues in the leader peptide." Cell **44**(3): 451-9.

Huang, M. T. and C. M. Gorman (1990). "The simian virus 40 small-t intron, present in many common expression vectors, leads to aberrant splicing." Mol Cell Biol **10**(4): 1805-10.

Indiveri, C., A. Tonazzi, et al. (1992). "Identification and purification of the ornithine/citrulline carrier from rat liver mitochondria." Eur J Biochem **207**(2): 449-54.

Ishibashi, S., M. S. Brown, et al. (1993). "Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery [see comments]." J Clin Invest **92**(2): 883-93.

Jackson, R. J. and N. Standart (1990). "Do the poly(A) tail and 3' untranslated region control mRNA translation?" Cell **62**(1): 15-24.

Jaenisch, R., K. Harbers, et al. (1983). "Germline integration of moloney murine leukemia virus at the Mov13 locus leads to recessive lethal mutation and early embryonic death." Cell **32**(1): 209-16.

Jaenisch, R. and B. Mintz (1974). "Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA." Proc Natl Acad Sci U S A **71**(4): 1250-4.

Jaynes, J. B., J. S. Chamberlain, et al. (1986). "Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts." Mol Cell Biol **6**(8): 2855-64.

Jaynes, J. B., J. E. Johnson, et al. (1988). "The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer." Mol Cell Biol **8**(1): 62-70.

Jones, S. N., M. Grompe, et al. (1990). "Ectopic correction of ornithine transcarbamylase deficiency in sparse fur mice." J Biol Chem **265**(24): 14684-90.

Jungermann, K. (1986). "Functional heterogeneity of periportal and perivenous hepatocytes." Enzyme **35**(3): 161-80.

Kalousek, F., B. Francois, et al. (1978). "Isolation and characterization of ornithine transcarbamylase from normal human liver." J Biol Chem **253**(11): 3939-44.

Kay, J. D., D. Hilton-Jones, et al. (1986). "Valproate toxicity and ornithine carbamoyltransferase deficiency [letter]." Lancet **2**(8518): 1283-4.

Kerwin, J. F., Jr. and M. Heller (1994). "The arginine-nitric oxide pathway: a target for new drugs." Med Res Rev **14**(1): 23-74.

Kimura, A., A. Nishiyori, et al. (1993). "Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor- 4 (HNF-4)." J Biol Chem **268**(15): 11125-33.

Kiwaki, K., Y. Kanegae, et al. (1996). "Correction of ornithine transcarbamylase deficiency in adult spf(ash) mice and in OTC-deficient human hepatocytes with

recombinant adenoviruses bearing the CAG promoter." Hum Gene Ther **7**(7): 821-30.

Kopan, R. and E. Fuchs (1989). "A new look into an old problem: keratins as tools to investigate determination, morphogenesis, and differentiation in skin." Genes Dev **3**(1): 1-15.

Korge, B. P. and T. Krieg (1996). "The molecular basis for inherited bullous diseases." J Mol Med **74**(2): 59-70.

Kozak, M. (1986). "Influences of mRNA secondary structure on initiation by eukaryotic ribosomes." Proc Natl Acad Sci U S A **83**(9): 2850-4.

Kozak, M. (1986). "Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes." Cell **44**(2): 283-92.

Kozak, M. (1989). "Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems." Mol Cell Biol **9**(11): 5073-80.

Kozak, M. (1996). "Interpreting cDNA sequences: some insights from studies on translation." Mamm Genome **7**(8): 563-74.

Kozak, M. (1997). "Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6." Embo J **16**(9): 2482-92.

Kozarsky, K., M. Grossman, et al. (1993). "Adenovirus-mediated correction of the genetic defect in hepatocytes from patients with familial hypercholesterolemia." Somat Cell Mol Genet **19**(5): 449-58.

Kraus, J. P., J. G. Conboy, et al. (1981). "Pre-ornithine transcarbamylase. Properties of the cytoplasmic precursor of a mitochondrial matrix enzyme." J Biol Chem **256**(21): 10739-42.

Kraus, J. P., P. E. Hodges, et al. (1985). "A cDNA clone for the precursor of rat mitochondrial ornithine transcarbamylase: comparison of rat and human leader sequences and conservation of catalytic sites." Nucleic Acids Res **13**(3): 943-52.

Krumlauf, R., R. E. Hammer, et al. (1985). "Regulated expression of alpha-fetoprotein genes in transgenic mice." Cold Spring Harb Symp Quant Biol **50**: 371-8.

Kuang, W., H. Xu, et al. (1998). "Merosin-deficient congenital muscular dystrophy. Partial genetic correction in two mouse models J Clin Invest **102**(4): 844-52.

Kuo, F. C. and J. E. Darnell, Jr. (1991). "Evidence that interaction of hepatocytes with the collecting (hepatic) veins triggers position-specific transcription of the glutamine synthetase and ornithine aminotransferase genes in the mouse liver." Mol Cell Biol **11**(12): 6050-8.

Lai, E. C. and J. W. Posakony (1997). "The Bearded box, a novel 3' UTR sequence motif, mediates negative post- transcriptional regulation of Bearded and Enhancer of split Complex gene expression." Development **124**(23): 4847-56.

Laird, P. W., A. Zijderveld, et al. (1991). "Simplified mammalian DNA isolation procedure." Nucleic Acids Res **19**(15): 4293.

Leao, M. (1995). "Valproate as a cause of hyperammonemia in heterozygotes with ornithine- transcarbamylase deficiency." Neurology **45**(3 Pt 1): 593-4.

Lee, J. T. and R. L. Nussbaum (1989). "An arginine to glutamine mutation in residue 109 of human ornithine transcarbamylase completely abolishes enzymatic activity in Cos1 cells." J Clin Invest **84**(6): 1762-6.

Leibundgut, E. O., S. Liechti-Gallati, et al. (1995). "Ornithine transcarbamylase deficiency: new sites with increased probability of mutation." Hum Genet **95**(2): 191-6.

Lersch, R. and E. Fuchs (1988). "Sequence and expression of a type II keratin, K5, in human epidermal cells." Mol Cell Biol **8**(1): 486-93.

Li, B., K. L. Murphy, et al. (1998). "A transgenic mouse model for mammary carcinogenesis." Oncogene **16**(8): 997-1007.

Li, L. and R. M. Hoffman (1995). "The feasibility of targeted selective gene therapy of the hair follicle." Nat Med **1**(7): 705-6.

Lindgren, V., B. de Martinville, et al. (1984). "Human ornithine transcarbamylase locus mapped to band Xp21.1 near the Duchenne muscular dystrophy locus." Science **226**(4675): 698-700.

Lorenzini, E. C. and I. E. Scheffler (1997). "Co-operation of the 5' and 3' untranslated regions of ornithine decarboxylase mRNA and inhibitory role of its 3' untranslated region in regulating the translational efficiency of hybrid RNA species via cellular factor." Biochem J **326**(Pt 2): 361-7.

Maestri, N. E., S. W. Brusilow, et al. (1996). "Long-term treatment of girls with ornithine transcarbamylase deficiency." N Engl J Med **335**(12): 855-9.

Makin, C. A., L. G. Bobrow, et al. (1984). "Monoclonal antibody to cytokeratin for use in routine histopathology." J Clin Pathol **37**(9): 975-83.

Malo, C., I. A. Qureshi, et al. (1986). "Postnatal maturation of enterocytes in sparse-fur mutant mice." Am J Physiol **250**(2 Pt 1): G177-84.

Maniatis, T., J. Sambrook, et al. (1982). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Manzella, J. M., W. Rychlik, et al. (1991). "Insulin induction of ornithine decarboxylase. Importance of mRNA secondary structure and phosphorylation of eucaryotic initiation factors eIF-4B and eIF-4E." J Biol Chem **266**(4): 2383-9.

Marshall, M. and P. P. Cohen (1972). "Ornithine transcarbamylase from Streptococcus faecalis and bovine liver. II. Multiple binding sites for carbamyl-P

and L-norvaline, correlation with steady state kinetics." J Biol Chem **247**(6): 1654-68.

Matsuda, I., T. Matsuura, et al. (1996). "Phenotypic variability in male patients carrying the mutant ornithine transcarbamylase (OTC) allele, Arg40His, ranging from a child with an unfavourable prognosis to an asymptomatic older adult." J Med Genet **33**(8): 645-8.

Matsuda, I., N. Nagata, et al. (1991). "Retrospective survey of urea cycle disorders: Part 1. Clinical and laboratory observations of thirty-two Japanese male patients with ornithine transcarbamylase deficiency." Am J Med Genet **38**(1): 85-9.

Matsuura, T., R. Hoshide, et al. (1994). "Expression of four mutant human ornithine transcarbamylase genes in cultured Cos 1 cells relates to clinical phenotypes." Hum Genet **93**(2): 129-34.

McIntyre, P., L. Graf, et al. (1984). "A highly basic N-terminal extension of the mitochondrial matrix enzyme ornithine transcarbamylase from rat liver." FEBS Lett **177**(1): 41-6.

Moll, R., D. Dhouailly, et al. (1989). "Expression of keratin 5 as a distinctive feature of epithelial and biphasic mesotheliomas. An immunohistochemical study using monoclonal antibody AE14." Virchows Arch B Cell Pathol Incl Mol Pathol **58**(2): 129-45.

Moll, R., W. W. Franke, et al. (1982). "The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells." Cell **31**(1): 11-24.

Moll, R., I. Moll, et al. (1982). "Changes in the pattern of cytokeratin polypeptides in epidermis and hair follicles during skin development in human fetuses." Differentiation **23**(2): 170-8.

Mori, M., S. Miura, et al. (1980). "Processing of a putative precursor of rat liver ornithine transcarbamylase, a mitochondrial matrix enzyme." J Biochem (Tokyo) **88**(6): 1829-36.

Morizono, H., C. D. Listrom, et al. (1997). "'Late onset' ornithine transcarbamylase deficiency: function of three purified recombinant mutant enzymes." Hum Mol Genet **6**(6): 963-8.

Morral, N., R. J. Parks, et al. (1998). "High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity [In Process Citation]." Hum Gene Ther **9**(18): 2709-16.

Morsy, M. A., J. Z. Zhao, et al. (1996). "Patient selection may affect gene therapy success. Dominant negative effects observed for ornithine transcarbamylase in mouse and human hepatocytes." J Clin Invest **97**(3): 826-32.

Msall, M., M. L. Batshaw, et al. (1984). "Neurologic outcome in children with inborn errors of urea synthesis. Outcome of urea-cycle enzymopathies." N Engl J Med **310**(23): 1500-5.

Murakami, T., M. Takiguchi, et al. (1989). "Tissue- and developmental stage-specific expression of the rat ornithine carbamoyltransferase gene in transgenic mice." Dev Genet **10**(5): 393-401.

Nagata, N., I. Matsuda, et al. (1991). "Retrospective survey of urea cycle disorders: Part 2. Neurological outcome in forty-nine Japanese patients with urea cycle enzymopathies." Am J Med Genet **40**(4): 477-81.

Nagata, N., I. Matsuda, et al. (1991). "Estimated frequency of urea cycle enzymopathies in Japan [letter]." Am J Med Genet **39**(2): 228-9.

Nguyen, M., C. Argan, et al. (1986). "Import and processing of hybrid proteins by mammalian mitochondria in vitro." J Biol Chem **261**(2): 800-5.

Nichols, D. H. (1981). "Neural crest formation in the head of the mouse embryo as observed using a new histological technique." J Embryol Exp Morphol **64**: 105-20.

O'Malley, B. W., Jr., S. H. Chen, et al. (1995). "Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model." Cancer Res **55**(5): 1080-5.

Oizumi, J., W. G. Ng, et al. (1984). "Partial ornithine transcarbamylase deficiency associated with recurrent hyperammonemia, lethargy and depressed sensorium." Clin Genet **25**(6): 538-42.

Overturf, K., M. Al-Dhalimy, et al. (1998). "Ex vivo hepatic gene therapy of a mouse model of Hereditary Tyrosinemia Type I." Hum Gene Ther **9**(3): 295-304.

Overturf, K., M. Al-Dhalimy, et al. (1996). "Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I Nat Genet **12**(3): 266-73.

Palmer, T. D., G. J. Rosman, et al. (1991). "Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes." Proc Natl Acad Sci U S A **88**(4): 1330-4.

Patijn, G. A. and M. A. Kay (1999). "Hepatic gene therapy using adeno-associated virus vectors." Semin Liver Dis **19**(1): 61-9.

Patijn, G. A., O. T. Terpstra, et al. (1998). "Method for continuous infusion into the portal vein of mice." Lab Anim Sci **48**(4): 379-83.

Peeters, M. J., G. A. Patijn, et al. (1996). "Adenovirus-mediated hepatic gene transfer in mice: comparison of intravascular and biliary administration." Hum Gene Ther **7**(14): 1693-9.

Pinkert, C. A., D. M. Ornitz, et al. (1987). "An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice." Genes Dev **1**(3): 268-76.

Pouchelet, M. and G. C. Shore (1981). "Chloramphenicol inhibits hormone-dependent induction of cytoplasmic mRNA coding for the mitochondrial enzyme, carbamyl phosphate synthetase, in Rana catesbeiana tadpoles." Biochim Biophys Acta **654**(1): 67-76.

Qureshi, I. A., J. Letarte, et al. (1979). "Ornithine transcarbamylase deficiency in mutant mice I. Studies on the characterization of enzyme defect and suitability as animal model of human disease." Pediatr Res **13**(7): 807-11.

Rafael, J. A., J. M. Tinsley, et al. (1998). "Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice." Nat Genet **19**(1): 79-82.

Raijman, L. (1974). "Citrulline synthesis in rat tissues and liver content of carbamoyl phosphate and ornithine." Biochem J **138**(2): 225-32.

Ramirez, A., A. Bravo, et al. (1994). "Sequences 5' of the bovine keratin 5 gene direct tissue- and cell-type- specific expression of a lacZ gene in the adult and during development." Differentiation **58**(1): 53-64.

Ray, M. K., S. P. Fagan, et al. (1998). "A mouse model for beta cell-specific ablation of target gene(s) using the Cre-loxP system." Biochem Biophys Res Commun **253**(1): 65-9.

Raymond, Y. and G. C. Shore (1981). "Processing of the precursor for the mitochondrial enzyme, carbamyl phosphate synthetase. Inhibition by rho-aminobenzamidine leads to very rapid degradation (clearing) of the precursor." J Biol Chem **256**(5): 2087-90.

Raz, E., D. A. Carson, et al. (1994). "Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses." Proc Natl Acad Sci U S A **91**(20): 9519-23.

Ricciuti, F. C., T. D. Gelehrter, et al. (1976). "X-chromosome inactivation in human liver: confirmation of X-linkage of ornithine transcarbamylase." Am J Hum Genet **28**(4): 332-8.

Rowe, P. C., S. L. Newman, et al. (1986). "Natural history of symptomatic partial ornithine transcarbamylase deficiency." N Engl J Med **314**(9): 541-7.

Rozen, R., C. Noel, et al. (1983). "Effects of glucagon on biosynthesis of the mitochondrial enzyme, carbamoyl-phosphate synthase I, in primary hepatocytes and Morris hepatoma 5123D." Biochim Biophys Acta **741**(1): 47-54.

Ryall, J., M. Nguyen, et al. (1985). "Expression of nuclear genes encoding the urea cycle enzymes, carbamoyl- phosphate synthetase I and ornithine carbamoyl transferase, in rat liver and intestinal mucosa." Eur J Biochem **152**(2): 287-92.

Saheki, T., K. Mori, et al. (1995). "Importance of ornithine transcarbamylase (OTC) deficiency in small intestine for urinary orotic acid excretion: analysis of OTC-deficient spf-ash mice with OTC transgene." Biochim Biophys Acta **1270**(1): 87-93.

Sanger, F., S. Nicklen, et al. (1977). "DNA sequencing with chain-terminating inhibitors." Proc Natl Acad Sci U S A **74**(12): 5463-7.

Sattler, W., S. Levak-Frank, et al. (1996). "Muscle-specific overexpression of lipoprotein lipase in transgenic mice results in increased alpha-tocopherol levels in skeletal muscle." Biochem J **318**(Pt 1): 15-9.

Schermer, A., S. Galvin, et al. (1986). "Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells." J Cell Biol **103**(1): 49-62.

Schwartz, M., E. Christensen, et al. (1986). "Detection and exclusion of carriers of ornithine transcarbamylase deficiency by RFLP analysis." Clin Genet **29**(5): 449-52.

Schweizer, J., M. Rentrop, et al. (1988). "The intermediate filament system of the keratinizing mouse forestomach epithelium: coexpression of keratins of internal squamous epithelia and of epidermal keratins in differentiating cells." Cell Tissue Res **253**(1): 221-9.

Scott, C. R., C. C. Teng, et al. (1972). "X-linked transmission of ornithine-transcarbamylase deficiency." Lancet **2**(7787): 1148.

Shimada, T., T. Noda, et al. (1991). "Correction of ornithine transcarbamylase (OTC) deficiency in spf-ash mice by introduction of rat OTC gene." FEBS Lett **279**(2): 198-200.

Short, E. M., H. O. Conn, et al. (1973). "Evidence for x-linked dominant inheritance of ornithine transcarbamylase deficiency." N Engl J Med **288**(1): 7-12.

Spence, J. E., A. Maddalena, et al. (1989). "Prenatal diagnosis and heterozygote detection by DNA analysis in ornithine transcarbamylase deficiency." J Pediatr **114**(4 Pt 1): 582-8.

Stewart, P. M. and M. Walser (1980). "Short term regulation of ureagenesis." J Biol Chem **255**(11): 5270-80.

Stratford-Perricaudet, L. D., M. Levrero, et al. (1990). "Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector." Hum Gene Ther **1**(3): 241-56.

Tanne, J. H. (1999). "US trial of gene therapy for cystic fibrosis." Bmj **318**(7191): 1096.

Tilghman, S. M. and A. Belayew (1982). "Transcriptional control of the murine albumin/alpha-fetoprotein locus during development." Proc Natl Acad Sci U S A **79**(17): 5254-7.

Tinsley, J., N. Deconinck, et al. (1998). "Expression of full-length utrophin prevents muscular dystrophy in mdx mice." Nat Med **4**(12): 1441-4.

Trainer, A. H. and M. Y. Alexander (1997). "Gene delivery to the epidermis." Hum Mol Genet **6**(10): 1761-7.

Tuchman, M., I. Matsuda, et al. (1995). "Proportions of spontaneous mutations in males and females with ornithine transcarbamylase deficiency." Am J Med Genet **55**(1): 67-70.

Tuchman, M., H. Morizono, et al. (1995). "The molecular basis of ornithine transcarbamylase deficiency: modelling the human enzyme and the effects of mutations." J Med Genet **32**(9): 680-8.

van Diggelen, O. P., J. Zaremba, et al. (1996). "Asymptomatic and late-onset ornithine transcarbamylase (OTC) deficiency in males of a five-generation family, caused by an A208T mutation." Clin Genet **50**(5): 310-6.

Veres, G., R. A. Gibbs, et al. (1987). "The molecular basis of the sparse fur mouse mutation." Science **237**(4813): 415-7.

Wagner, E. F., L. Covarrubias, et al. (1983). "Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line." Cell **35**(3 Pt 2): 647-55.

Wallace, R., E. Knecht, et al. (1986). "Turnover of rat liver ornithine transcarbamylase." FEBS Lett **208**(2): 427-30.

Wang, L., M. Zoppe, et al. (1997). "A factor IX-deficient mouse model for hemophilia B gene therapy." Proc Natl Acad Sci U S A **94**(21): 11563-6.

Wang, L. H., S. Y. Tsai, et al. (1989). "COUP transcription factor is a member of the steroid receptor superfamily." Nature **340**(6229): 163-6.

Wang, S., K. S. Browning, et al. (1997). "A viral sequence in the 3'-untranslated region mimics a 5' cap in facilitating translation of uncapped mRNA." Embo J **16**(13): 4107-16.

Wang, X., S. Zinkel, et al. (1997). "Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy." Proc Natl Acad Sci U S A **94**(1): 219-26.

Weiss, B., A. Thompson, et al. (1968). "Ezymatic breakage and joining of deoxyribonucleic acid. VII. Properties of the enzyme-adenylate intermediate in the polynucleotide ligase reaction." J Biol Chem **243**(17): 4556-63.

Windmueller, H. G. and A. E. Spaeth (1980). "Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate." J Biol Chem **255**(1): 107-12.

Wolff, J. A., J. J. Ludtke, et al. (1992). "Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle." Hum Mol Genet **1**(6): 363-9.

Wolff, J. A., R. W. Malone, et al. (1990). "Direct gene transfer into mouse muscle in vivo." Science **247**(4949 Pt 1): 1465-8.

Wysolmerski, J. J., A. E. Broadus, et al. (1994). "Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development." Proc Natl Acad Sci U S A **91**(3): 1133-7.

Ye, X., M. B. Robinson, et al. (1996). "Prolonged metabolic correction in adult ornithine transcarbamylase- deficient mice with adenoviral vectors." J Biol Chem **271**(7): 3639-46.

Ye, X., M. B. Robinson, et al. (1997). "Adenovirus-mediated in vivo gene transfer rapidly protects ornithine transcarbamylase-deficient mice from an ammonium challenge." Pediatr Res **41**(4 Pt 1): 527-34.

Yoshino, M., J. Nishiyori, et al. (1990). "Ornithine transcarbamylase deficiency in male adolescence and adulthood." Enzyme **43**(3): 160-8.

Yukht, A., R. C. Davis, et al. (1995). "Regulation of lipoprotein lipase translation by epinephrine in 3T3-L1 cells. Importance of the 3' untranslated region." J Clin Invest **96**(5): 2438-44.

Stock Solutions for Pronuclear Injection

	Component	g/100ml
F (10x)	NaCl	5.534
	KCl	0.110
	$NaH2PO_4$	0.047
	$MgCl_26H_2O$	0.100
	Na lactate	3.4mls of 60% syrup
	Glucose	1.00
B (10x)	$NaHCO_3$	2.16
G(100x)	Na pyruvate	0.3
	Penicillin	0.06 $x10^5$i.u.
	Streptomycin	0.050
H(100x)	$CaCl_22H_2O$	2.60
E(10x)	Hepes	5.97
Adjust to pH 7.4 with NaOH		
BSA		0.400

Stock solution	T6 pH 7.6	H6 pH7.4
F	1.0	1.0
B	1.0	1.0
G	0.1	0.1
H	0.1	0.1
E	0	0.84
Phenol red	50µl	0
Water(Analar)	7.8	7.8
BSA(Sigma)	40mg/10ml	40mg/10ml

Check pH and store at –4°C

Appendix I :Solutions used in transgenic technology