A STUDY ON EXOGENOUS GENE TRANSFER IN MAMMALS

by

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SUMMARY

This thesis describes the development and utilisation of a transgenic system, a technology that was not established at Glasgow Veterinary School prior to the commencement of these studies. Satisfactory embryo culture is essential for the production of transgenic mice and Chapter 2 describes this aspect of the work. In this chapter different media were compared and protocols for culturing mouse embryos were established. Various aspects of the system were tested to ensure that conditions were optimal for embryo culture.

Chapter 3 describes the generation of transgenic mice using pronuclear microinjection and those aspects of the system that influence the efficacy of this procedure were examined. Pronuclear microinjection is associated with immediate and delayed mortality of the embryos, this chapter concentrates on the timing of the embryo loss and discusses the causes of post-injection embryo and foetal death.

The experience gained from the work described in Chapter 2 and 3 was adapted to investigate transient transgene expression in the murine preimplantation embryos using the bacterial lac Z reporter gene, the results of which are presented in Chapter 4. As preimplantation development proceeded there was an observed decline in the activity of the lac Z product - β-galactosidase. The chapter concludes with an investigation into the effects of DNA methylation on the expression of the introduced construct.

Chapter 5 describes an experiment designed to investigate the potential of retroviruses to act as vectors for exogenous gene transfer in the ovine species. Wild type Feline Leukaemia Virus (FeLV) was used to infect early ovine embryos and subsequent analysis of foetuses was used to assess the efficacy of introducing viral gene sequences into the mammalian genome. Two out of the 17 foetuses examined contained FeLV proviral sequences.

The transgenic system was utilised to investigate the role of the human *myc* proto-oncogene in the induction of murine T-cell leukaemia, this work is presented in Chapter 6. This is the first study to exclusively target murine T-cells with the c-*myc* oncogene and these transgenic mice developed thymic lymphoma at low frequency despite the apparent absence of transgene expression in healthy pre-lymphomatous mice. However the presence of the oncogene accelerates Murine Leukaemia Virus (MuLV) induced lymphoma development. The results presented in Chapter 6 indicate that the exogenous *myc* oncogene can co-operate in the tumourigenic process but that subsequent events are required for the development of the fully malignant phenotype.

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DECLARATION

I hereby declare that the work presented in this thesis was conducted by the author under supervision except were stated in the text. In particular Chapter 6 was a collaborative project involving a number of workers. The various areas of responsibility are clearly stated in the material and methods of Chapter 6.

I also certify that no part of this thesis has been submitted previously for the award of a degree to any University.

Ewan R. Cameron

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During the course of my work I was assisted by many members of the staff of Glasgow University Veterinary School but I would particularly like to thank the following:-

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ABBREVIATIONS

BSA -	Bovine Serum Albumin
d.f. -	degrees of freedom
CL -	Corpora Lutea
CMV-IE -	Cytomegalovirus - Immediate Early
CSF -	Colony Stimulating Factor
DCR -	Dominant Control Region
DIC -	Direct Interference Contrast
DLH -	DL-homocysteine
DTT -	Dithiothreitol
EDTA -	Ethylenediaminetetraacetic acid
FeLV -	Feline Leukaemia Virus
hCG -	human Chorionic Gonadotrophin
HEPES -	N-2-hydroxyethylpiperazine, N-2-ethanesulfonic acid
HSA -	Heat Stable Antigen
HSV-Tk -	Human Simplex Virus - Thymidine kinase
i.u. -	International Units
Ig -	Immunoglobin
Il -	Interleukin
LTR -	Long Terminal Repeat
MMTV -	Murine Mammary Tumour Virus
Mo-MuLV -	Moloney Murine Leukaemia Virus
MOET -	Multiple Ovulation and Embryo Transfer
MPSA -	Myeloproliferative Sarcoma Virus
NaN_3	Sodium Azide
PCR -	Polymerase Chain Reaction
PDGF -	Platelet Derived Growth Factor
PgF2α -	Prostaglandin F2α
PIPES -	Piperazine - N, N' - bis; 1,4 Piperazine diethanesulfonic acid
PMSF -	Phenylmethylsulfonyl fluoride
PMSG -	Pregnant Mare Serum Gonadotrophin
SAM -	S-adensylmethionine
S.D. -	Standard Deviation
SDS -	Sodium dodecyl sulphate
SV40	Simian Virus 40
TCR -	T-cell receptor
Tris -	Trisma Base

CHAPTER 1 - GENERAL MATERIAL AND METHODS

1.1. Mice Stocks

Different breeding groups were maintained to supply the animals used in this work. Original stocks of inbred strains, C57Bl/6J and CBA/Ca, were purchased from Harlan Olac (Blackthorn, Oxford.) as were the MF1 outbred mice. Balb/c mice were purchased from Bantin and Kingman (Grimston, Hull).

Inbred colonies of C57Bl/6J and CBA/Ca mice were kept to produce female C57Bl/6J stock and male CBA/Ca stock. Small groups of C57BL/6J females (4-5) were housed with single CBA/Ca males, the resultant F1 hybrid (B6/CBA) offspring were weaned at between three and four weeks of age. Weaned females were pooled and used as embryo donors, some of the male offspring were retained and used for mating with the superovulated embryo donors, the remainder were culled.

In addition, a breeding colony of outbred MF1 mice was maintained. The female offspring from this group were weaned at three to six weeks of age, pooled and used as recipient females in the embryo transfer programme. A small proportion of male offspring from this breeding group was retained and later vasectomised, the remainder being culled.

1.2. Preparation Of Superovulated Females

Freeze-dried aliquots of Pregnant Mare Serum Gonadotrophin (PMSG, Folligon - Intervet) and Human Choronic Gonadotrophin (hCG, Chorulon - Intervet) were each made up to a final concentration of 50 international units (i.u.) in each millilitre of sterile water. These were then divided into 1 ml aliquots and frozen until required.

B6/CBA F1 females aged between three to six weeks of age were superovulated and used as embryo donors. The animals were maintained on a light/dark cycle with lights on from 6 am to 8 pm daily. Superovulation was accomplished by an intraperitoneal injection (i/p) of 5 i.u. of PMSG between the hours of 2 and 4 pm. Five i.u. of hCG was injected i/p 46-48 hours later. Immediately after injection each female mouse was placed with a single male mouse (B6/CBA F1) of proven fertility. The following morning (designated day 0.5 of gestation) the female mice were inspected for the presence of a copulation plug and used for embryo collection.

1.3. Preparation Of Murine Embryo Culture Media

a) Materials

Water - millipore Q filter sterilised		
Media Ingredients	NaCl	(Sigma Chemical Co.)
	KH_2PO_4	(Sigma Chemical Co.)
	KCl	(BDH - Analar)
	$CaCl.2H_2O$	(BDH - Analar)
	$MgCl.6H_2O$	(BDH - Analar)
	$NaH_2PO_4.2H_2O$	(BDH - Analar)
	$Na_2HPO_4.12H_2O$	(BDH - Analar)
	$NaHCO_3$	(Formachem)
	$MgSO_4.7H_2O$	(Sigma Chemical Co.)
	Sodium Pyruvate	(Sigma Chemical Co.)
	Sodium Lactate	(Sigma Chemical Co.)
	(DL lactic acid, 60% solution)	
	Glucose	(BDH - analar)
	Penicillin G	(Sigma Chemical Co.)
	Streptomycin Sulphate	(Sigma Chemical Co.)
	Phenol Red	(Sigma Chemical Co.)
	HEPES	(Sigma Chemical Co.)
	Bovine Serum Albumin	(Sigma Chemical Co.)
	(Fraction V)	
Liquid Paraffin	Liquid Paraffin	(Boots Plc)
	Light Paraffin Oil	(BDH)
Glassware	250ml glass beakers.	
	250ml volumetric flasks.	
Tissue Culture	35mm petri dishes	(Gibco)
	50ml tissue culture centrifuge tubes.	(Flow)
	50ml sterile syringes	(Plastipak)
	0.22um dish filters.	(Millipore)

b) Method

Millipore Q water was used for all batches of embryo culture or embryo handling media used in these experiments. Details of the embryo culture media and embryo handling media are given in Table 1.1. Embryos were either cultured in T6 - a modified Tyrodes solution (Howlett *et al.*, 1987), or M16 - a modified Krebs-Ringer solution (Whittingham, 1971). Embryos were handled or stored outside the CO2 incubator either in PB1 - a phosphate buffered medium (Whittingham and Wales, 1969) or M2 - a HEPES buffered medium (Fulton and Whittingham, 1978).

The medium was prepared by weighing out the relevant salts separately and dissolving each in a small volume of water, which was transferred to a 250ml volumetric flask and made up to 250ml with water. The medium was filter sterilised using 0.22μm millipore filters and stored at 0-4°C in 50ml tissue culture centrifuge tubes until required.

M16 and M2 were prepared following the protocol outlined by Hogan *et al.* (1986). The various salts except CaCl were weighed out into a volumetric flask and dissolved in millipore Q water. CaCl was weighed out and dissolved in water separately before being added to the main salt solution as was the penicillin/streptomycin mixture. Finally, lactate syrup was weighed out and mixed with a small volume of water and then added to the salt solution. Additional water was then added to the volumetric flask to achieve the correct concentration. The preparation of M2 was similar to that of M16 except HEPES was weighed out and dissolved separately before being added to the salt solution. The authors recommend that the final 1X concentration should be used within a week of preparation.

1.4. Collection Of Mouse Embryos

Embryos were collected on the day of plugging (day 0.5). The superovulated mice were killed by cervical dislocation, their oviducts dissected out and placed in 35mm petri dishes (Nunc) containing 2ml of holding medium. The oviducts were then transferred to petri dishes containing 100 μl drops of holding medium and under the dissecting microscope the ampulla of each oviduct was torn to release the cumulus mass containing the one-cell embryos. After removal of the oviducts, hyaluronidase (Sigma - type II from sheep testes at a concentration of 300 μg/ml in holding medium) was added to the drop containing the embryos in order to remove the attached cumulus cells. When the embryos were free of cumulus cells they were removed and transferred through two washes (each containing approximately 2mls of holding medium) before being held in another petri dish also containing holding medium.

Table 1.1. Murine Embryo Culture Media.

	(mg/100 ml)			
	T6	PB1	M16	M2
NaCl	472	597	553	553
KCl	11	20	35.6	35.6
$NaH_2PO_4.2H_2O$	6	-	-	-
$Na_2HPO_4.12H_2O$	-	288[a]	-	-
$NaHCO_3$	210[b]	-	210	35
KH_2PO_4	-	19	16.2	16.2
$MgCl_2.6H_2O$	10	10	-	-
$CaCl_2.2H_2O$	26	14	25.2	25.2
$MgSO_4.7H_2O$	-	-	29.3	29.3
HEPES	-	-	-	497
Sodium Pyruvate	3	4	3.6	3.6
Glucose	100	100	100	100
Sodium Lactate	0.305ml (60% soln.)	-	261	261
Penicillin	6	6	6	6
Streptomycin	5	5	5	5
Phenol red	0.1	0.1	1.0	1.0
Bovine Serum Albumin (BSA)	400	400	400	400

a. $Na_2HPO_4.12H_2O$ was added to the final solution last when PB1 was being prepared.
b. $NaHCO_3$ was added to the final solution last when T6 was being prepared.

1.5. Murine Embryo Culture

Two main types of media - T6 and M16 - were used to culture murine embryos. Embryos were cultured in 5% CO_2, the pH of the medium being buffered by the CO_2. When embryos were handled outside the incubator, the medium used was either buffered by HEPES in the case of M2 or phosphate buffered in the case of PB1. Microdrops of culture medium were placed on 35mm tissue culture petri dishes (Gibco) and overlaid with liquid paraffin (Boots) or light paraffin oil (BDH) and

allowed to equilibrate in the CO2 incubator for at least two hours before the embryos were added. This method was first described by Brinster in 1963.

Embryos were washed twice before being cultured in 30 μl microdrops in groups of between 20 to 30 per drop and were examined daily using an invert microscope (Willovert - Leitz), the proportion of embryos proceeding to the next stage of development being noted. In order to replicate the results described by Lavitrano *et al.* (1989), *in vitro* fertilisation was carried out as described by this group.

1.6. Preparation Of Foster Mothers

MF1 female mice aged between two to six months of age were used as pseudopregnant recipients. A state of pseudopregnancy was induced by mating a female mouse in natural oestrus with a vasectomised male mouse. Three females were placed with each vasectomised MF1 male, those females which were plugged usually entered a state of pseudopregnancy which lasted between ten to fourteen days.

1.7. Preparation Of Vasectomised Mice

Young adult (older than 2 months of age) male mice were selected for vasectomy and were anaesthetised using a combination of Hypnorm/Hypnovel and sterile water in a 1:1:4 ratio, the dosage used in the final solution being 0.5-1.0 ml/100g body weight (Hypnorm is supplied by Janssen Animal Health, each millilitre contains 0.315 mg fentanyl citrate and 10 mg of flunisone. Hypnovel is supplied by Roche Pharmaceuticals Ltd and each millilitre contains 5mg of midazolam hydrochloride). This anaesthetic was administered by the intraperitoneal route. The operating site was prepared by clipping the hair and swabbing the area with 70% ethanol. A horizontal incision was made through the skin and body wall on the ventral abdomen at the level of the tuber coxae. The testes were exteriorised and a section of the vas deferens was removed, each end being ligated with 5/0 silk (Mersilk - Ethicon). The procedure was repeated on the other side, the body wall and skin incisions were each closed with a single suture of 5/0 silk.

1.8. Microinjection

One-cell embryos were placed under a diavert microscope (Leitz) fitted with differential interference contrast optics (DIC) and flanked with Leitz micromanipulators. A coverslip holding a 15 μl drop of handling medium covered with liquid paraffin (Boots) was placed on a 60mm petri dish which in turn was placed on the microscope stage. A square hole was cut in the base of the petri dish which the coverslip spanned - this allowed the embryos to be viewed directly through the coverslip and gave good visualisation of the pronuclei.

A flame polished glass pipette, attached to the left hand manipulator, was used to hold the embryos. These holding pipettes were fashioned by flame pulling a length of glass capillary tubing (GC 100-10 Clarks electromedical instruments). Using a microforge (Research Instruments Ltd.) two 90^{o} angles were introduced into the glass pipette and the pipette was broken just beyond the second angle. The broken end of the pipette was heated over the microforge's filament resulting in a smooth polished end with a very small opening (5-10 micrometers). This pipette was then inserted in the pipette holder and Fluorinert (FC 77 Sigma) was used to fill the air spaces of the pipette, holder and tubing so that fine adjustments of pressure could be applied.

Injection pipettes were also fashioned from the glass capillary tubing (GC100Tf-15, Clarks Electromedical instruments). Injection pipettes were pulled using a moving-coil microelectrode puller (model 753, Camden Instruments) and were filled with approximately 1 μl of DNA solution, the remainder of the pipette being filled with Fluorinert. The right hand manipulator held the injection pipette. Pressure on the injection needle was exerted using a 100ml air filled syringe with tubing connecting the syringe to the right hand pipette holder.

The embryos were injected in groups of 15-25. An embryo was picked up, held by the holding pipette and the fine focus adjusted until the outline of the pronuclear membrane was in focus. The injection pipette was brought into the same plane as the pronuclei which was then injected - swelling of the pronucleus usually indicated a successful injection.

After injection the embryos were washed once in 2 ml of the culturing medium and those embryos which had not been lysed were transferred to a 30 μl culture drop under oil and placed in the CO_2 incubator.

1.9. Embryo Transfer

Embryos were normally transferred at the two-cell stage to a foster mother which had been plugged on the morning of transfer. For experimental reasons, other embryonic stages were sometimes transferred - four-cells and early morulae were also transferred into the oviduct of foster mothers at day 0.5 of pseudopregnancy but late morulae and blastocysts were transferred into the uteri of foster mothers at day 2.5 of pseudopregnancy.

The recipient mouse was anaesthetised and the operating site prepared as described in section 1.7. Embryos were loaded into a glass transfer pipette which had an internal diameter of approximately 120 μm and a flame polished end. Plastic tubing (Portex Ltd., Kent), filled with light paraffin oil (BDH Ltd.), is attached to the other end of the glass capillary tubing and fine movements in the pipette are controlled by mouth pipetting.

Embryo transfers were performed using a dissecting microscope, the operating site being illuminated with a fibre optic light source. A small skin and body wall incision was made over the dorsal-lateral aspect of the abdominal wall and the ovary, oviduct and proximal part of the uterus were exteriorised.

The oviduct opening was located, the transfer pipette inserted into the infundibulum and the embryos delivered by gently blowing down the attached tubing. The body wall and skin were closed separately with a single horizontal mattress suture using 5/0 silk. The procedure was then repeated on the other side. Each recipient received between 15-30 embryos.

The mice were allowed to recover in an incubator to minimise hypothermia and recovery was usually complete within 1-2 hours.

1.10. Fragment And Plasmid Construction

With the exception of the CD2-*myc* construct, which was made by Dr. M. Stewart, all DNA constructs used in this work were prepared by Dr. S. Hettle who kindly provided the following information. The various gene constructs are shown in Figures 1.1. - 1.5. Preparation of the CD2-*myc* construct is described in Chapter 6. The DNA was isolated and purified for microinjection following the protocol described by Hogan *et al.*, 1986.

Tk-ENV

The entire coding sequence of the *env* gene of Moloney Murine Leukaemia Virus (MoMuLV) was cloned into an expression vector. The site chosen resulted in the *env* gene being flanked on its 5 prime side with the Herpes Simplex Virus Type 1 Thymidine Kinase (HSV1-Tk) promoter (Wagner *et al.*, 1981b) and on its 3 prime side by the HSV1-Tk polyadenylation signal. A 2.78 kb Hind III restriction fragment containing the HSV1-Tk promoter, the *env* coding region and the HSV1-Tk polyadenylation signal was isolated from this plasmid and used for microinjection.

Tk-lac Z

The Tk-lac Z gene construct was made by fusing the E coli gpt-trpS-lac Z fusion gene derived from plasmid CH110 (Hall et al, 1983) to the Human Simplex Virus Type 1 Thymidine Kinase promoter (HSV1-Tk). This fragment was excised from its vector sequences by restriction with BamH1 and consists of 240 base pairs of the HSV-Tk promoter region, ligated to the lac Z coding region at the Hind III site. The polyadenylation signal is derived from simian virus 40 (SV40).

CMV-lac Z

The CMV-lac Z gene construct was made by fusing the E coli gpt-trpS-lac Z fusion gene derived from plasmid CH110 (Hall et al, 1983) to the Human Cytomegalovirus Immediate Early promoter (HCMV-IEP) sequences (-302 to +72 relative to the start of transcription). A 4.38 kb fragment containing the HCMV-IEP promoter, the lac Z coding region, the SV40 polyadenylation signal and the HCMV-IEP terminator (+2757-3052) region was isolated by partial digestion of the plasmid with EcoR1. The resulting linear fragment was termed CMV-lac Z and used for microinjection experiments.

Tk-BGB and CMV-BGB

The 3.95 kb Tk-lac Z BamH1 restriction fragment consisting of the coding region of the E coli lac Z gene fused to the Herpes Simplex Virus Type 1 Thymidine Kinase promoter was cloned into the BamH1 site of pARS65 to produce Tk-BGB. The 4.38 EcoR1 partial restriction fragment CMV-lac Z comprised of the E coli lac Z coding region fused to the immediate early promoter of the Human Cytomegalovirus was also cloned into the BamH1 site of the ARS65 plasmid to produce CMV-BGB.

Plasmid pARS65 contained putative mouse autonomous replicating sequences (ARS) and has been described previously (Ariga et al, 1987). This plasmid was supplied by H. Ariga, to whom I am most grateful.

1.11. Transgenic Screening

Those animals born to foster mothers which had received manipulated embryos were screened as follows. After weaning, the offspring were anaesthetised as described in section 1.7. and approximately 2 cm of the tail tip was removed, the wound was cauterised and the animals were assigned an identification number using an ear punch. Tail biopsies were then analysed for the presence of the transgene by Southern blotting after extracting DNA from the tissue.

DNA Extraction

This work was carried out by Dr. Hettle who kindly provided the following information, the protocol used for DNA isolation was outlined by Hogan et al (1986).

Tail tissue was placed in 1.5 ml microforge tubes to which was added O.7 ml of 50 mM EDTA, 0.5% SDS and 35 μl of a 10 mg/ml solution of proteinase K. The tissue was incubated overnight at 55°C using a waterbath with a moving arm (Gallenkamp Ltd.). The following day 0.7 ml of phenol was added and the tubes shaken. After shaking the tubes were centrifuged for 3 minutes before the aqueous phase was removed and transferred to a fresh microfuge tube. To the aqueous phase was added 0.7 ml phenol:chloroform (1:1) and again the samples were shaken, centrifuged and the aqueous phase transferred. The DNA was precipitated by adding 70 μl of 3M sodium acetate and 0.7 ml of 100% ethanol to the aqueous phase, the precipitate was pelleted by centrifugation, the ethanol poured off and the DNA dried by leaving the tubes in a warmed room with the lids open. The DNA was dissolved by adding 0.1 ml of 10 mM Tris (pH8) 1 mM EDTA to the pellet. DNA was digested by adding the appropriate restriction enzyme and incubating for the recommended period of time, to each sample was added 5 μg of DNase free RNase A to remove contaminating RNA.

A similar procedure was used for both foetal sheep and mouse tissues. The procedures used for Southern blotting, nick translation, hybridisation and autoradiography were those described by Sambrook et al (1989). DNA extraction and Southern blotting for the c-*myc* studies was carried out by Dr. M. Stewart and Dr. M. Campbell.

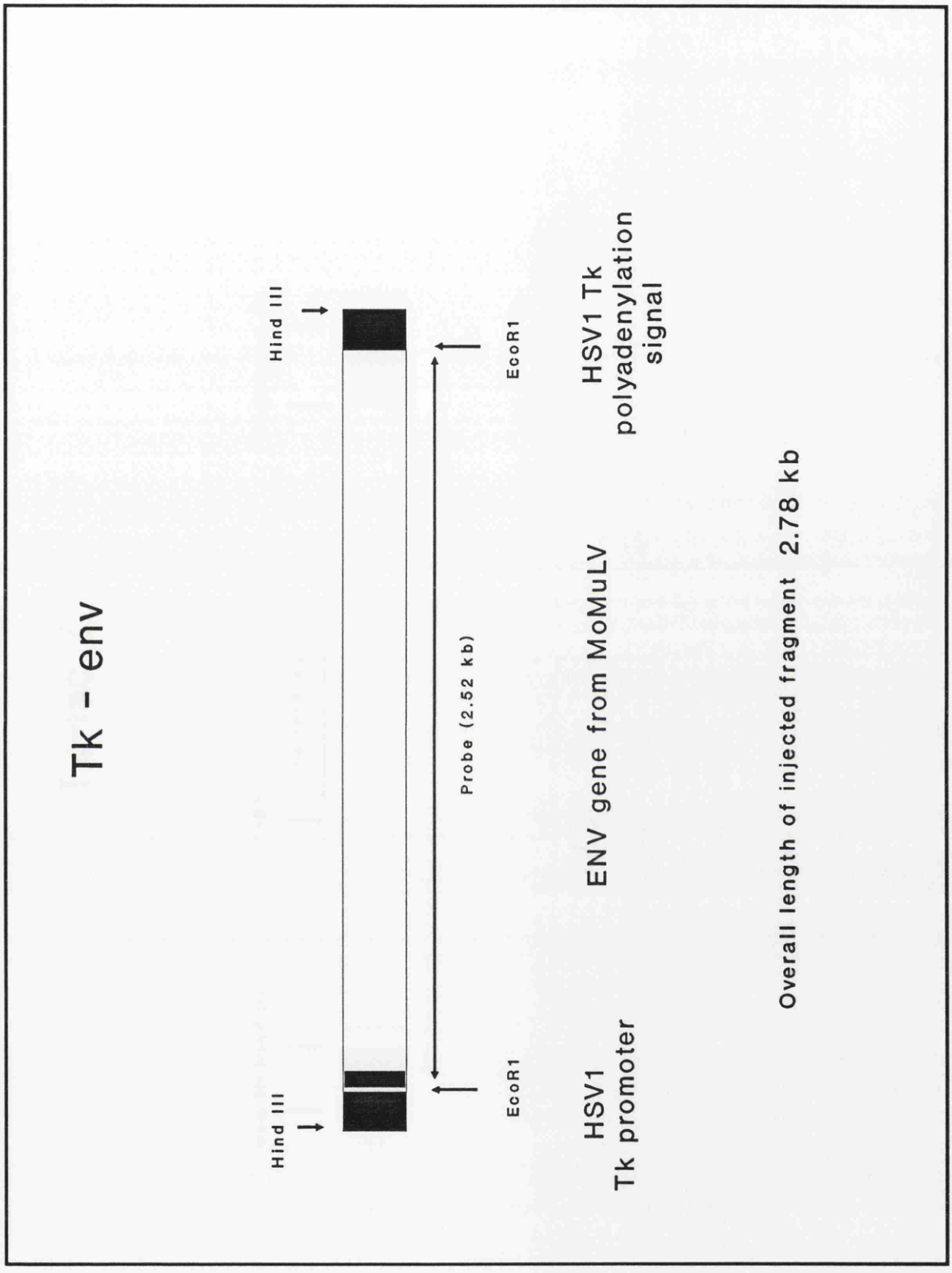


Figure 1.1.

Diagram of the Linear Construct Tk-env Showing Relevant Restriction Enzyme Sites.

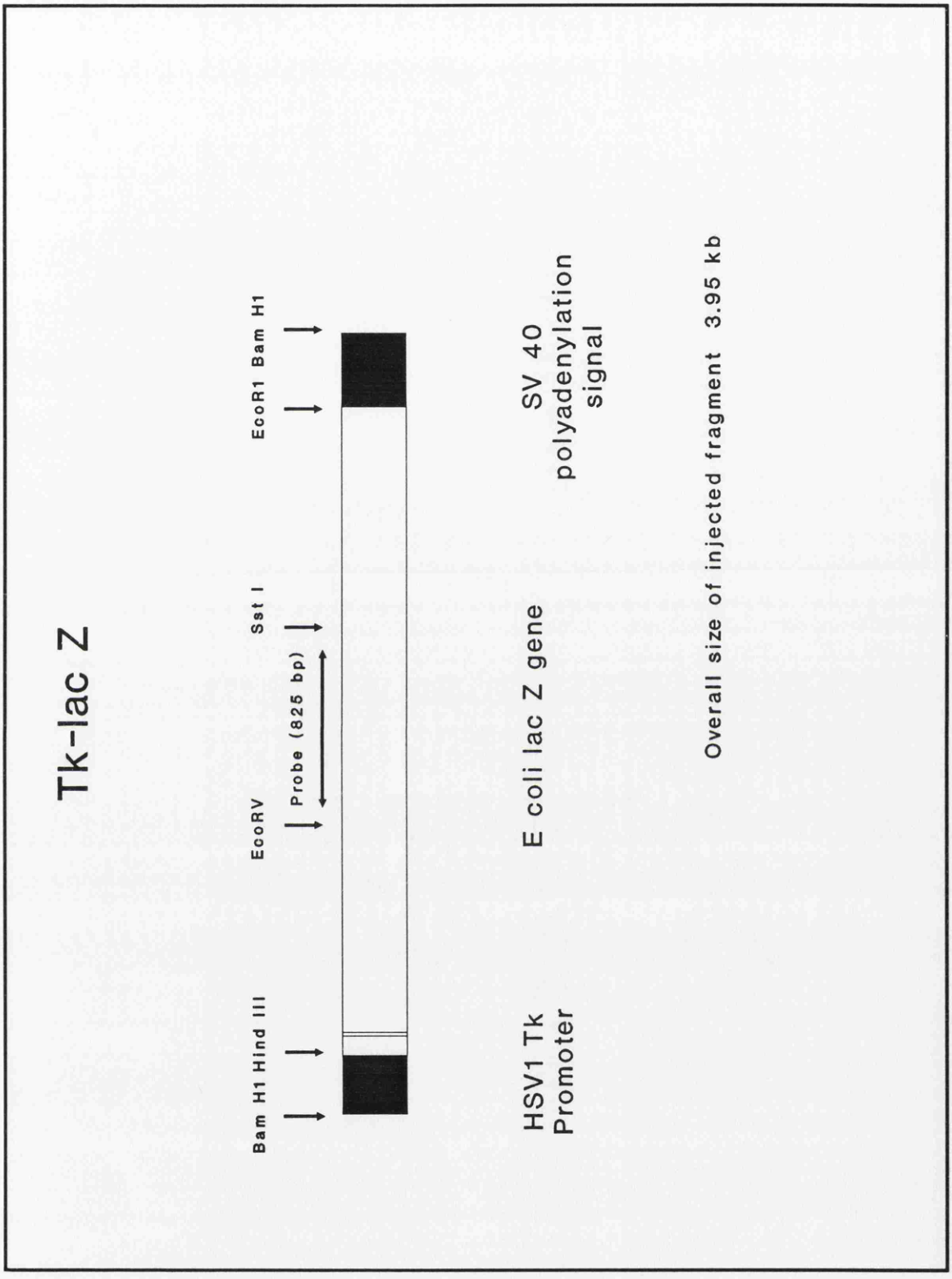


Figure 1.2.

Diagram of the Linear Construct Tk-lac Z Showing Relevant Restriction Enzyme Sites.

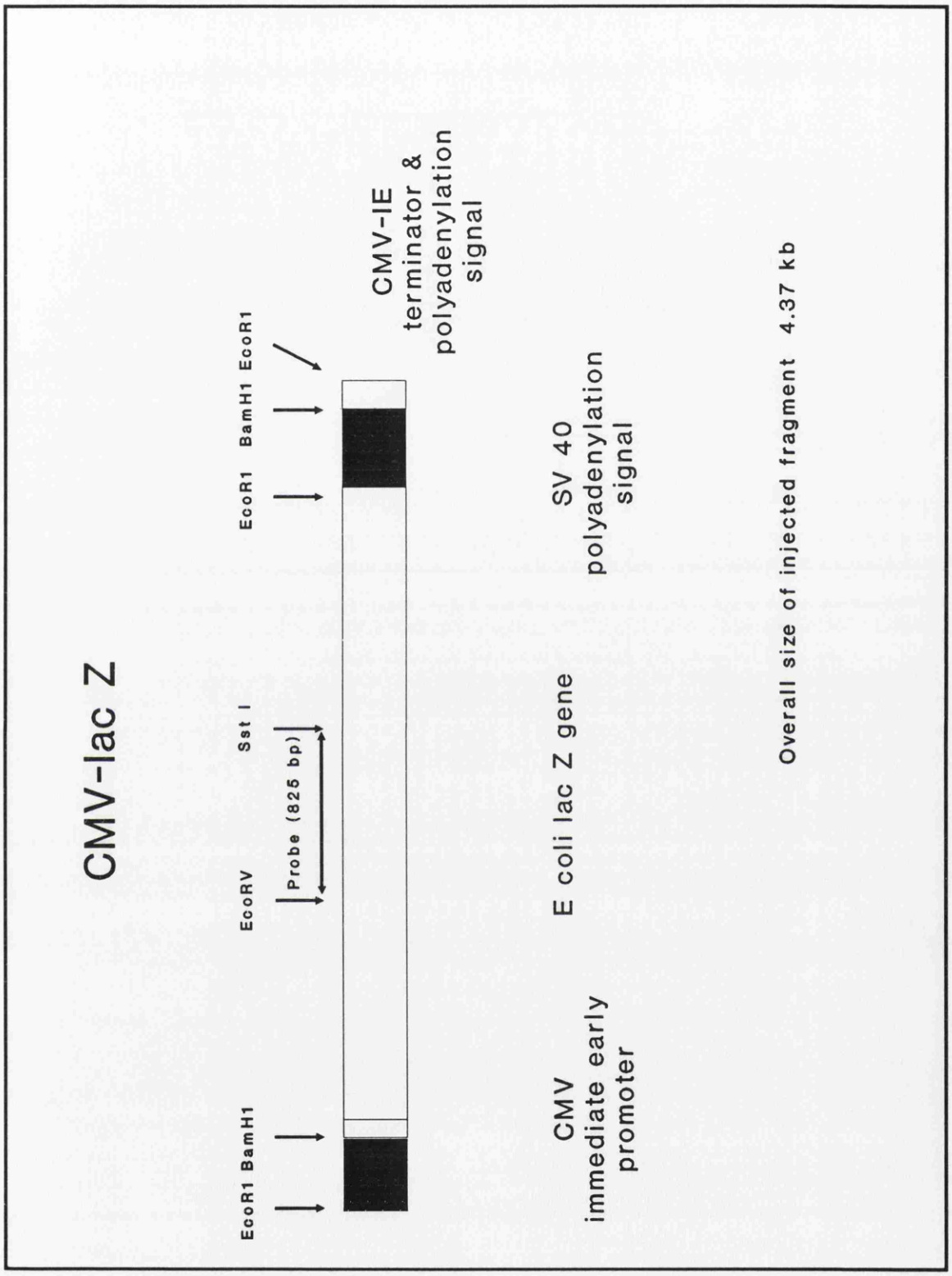


Figure 1.3.

Diagram of the Linear Construct CMV-lac Z Showing Relevant Restriction Enzyme Sites.

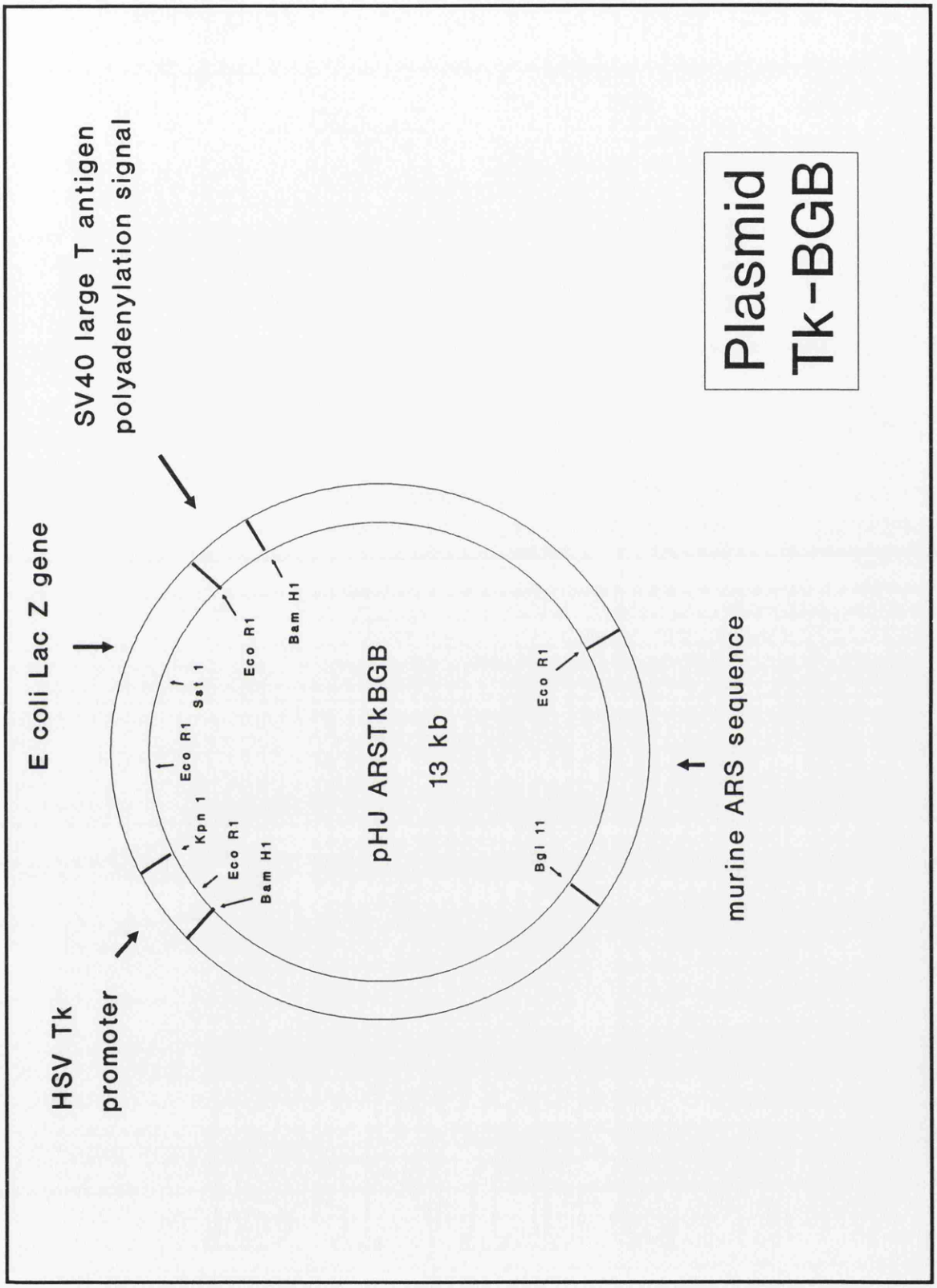


Figure 1.4.

Diagram of Plasmid Tk-BGB Showing Relevant Restriction Enzyme Sites.

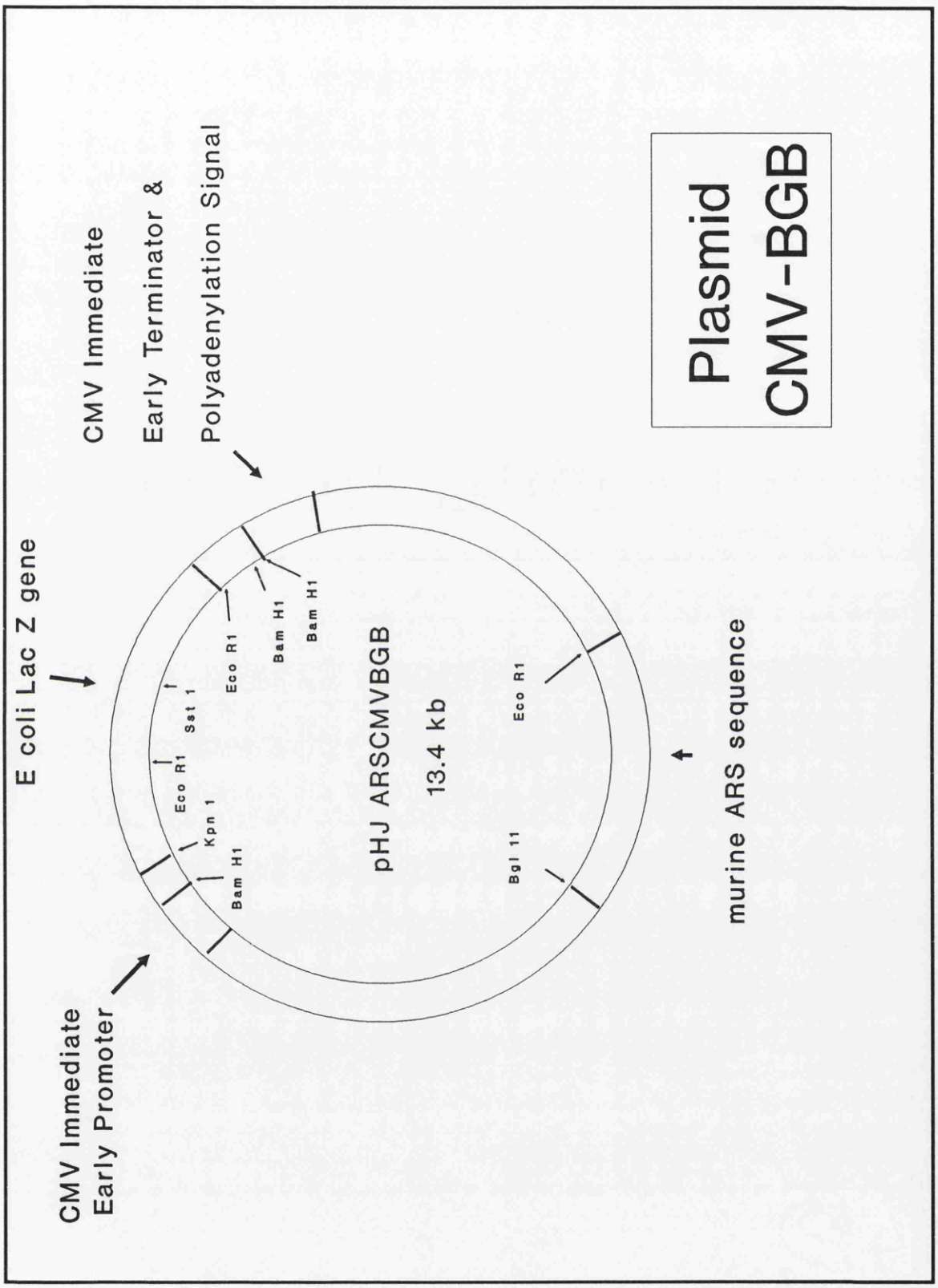


Figure 1.5.

Diagram of Plasmid CMV-BGB Showing Relevant Restriction Enzyme Sites.

1.12. Statistical Analysis

Where appropriate the results were analysed by standard deviation, students t-distribution or chi-square using a minitab programme (Minitab Statistical Software, Minitab Inc.). All chi-squares are based on a 2x2 contingency table with 1 degree of freedom (d.f.), except where specified in the text. The Yates correction factor was not applied but no significant result had an expected value of 5 or below (Campbell, 1967).

CHAPTER 2 - MURINE EMBRYO CULTURE

SECTION 2. 1. - INTRODUCTION

There are a number of different methods used to produce transgenic mice but murine embryo culture is an important component of each approach. The quality of the media and the suitability of the protocol employed are important in order to maximise the overall efficiency of transgenic animal production. Although murine embryo research extends over the past three decades it is still an area of active research as workers continue to endeavour to improve culture conditions. As a part of transgenic research it is important to optimise culture conditions as it not only affects the number of embryos suitable for transfer but can also affect subsequent *in vivo* development. The following brief literature review highlights the important findings in the development of murine embryo cuture media and culture conditions.

Early Work

In 1949 Hammond reported the successful development of preimplantation mouse embryos *in vitro*. The culture system designed by Hammond consisted of a simple salt solution supplemented with glucose and egg extract and with this media he was able to culture embryos from the four-cell stage to the blastocyst stage. However, he found that this system could not support the development of two-cell embryos beyond the four-cell stage.

Whitten (1956) extended these studies and showed it was possible to culture eight-cell embryos to the blastocyst stage in Krebs-Ringer bicarbonate solution supplemented with glucose and bovine albumin. The following year Whitten (1957) made a number of observations concerning the factors which affected the *in vitro* development of murine preimplantation embryos. This work showed that egg albumin or a mixture of amino acids and peptides could replace bovine serum albumin (BSA) as the nitrogen source but that the removal of a macromolecule made handling mouse embryos difficult. Further, he showed that embryo culture was successful when 5% CO_2 in air or 5% CO_2 in nitrogen was used but that embryos failed to develop in oxygen. In addition, Whitten identified those sugars which could support embryo development and those which could not. It was observed that development was superior when lactate was used and when CaCl was replaced with Ca lactate it was possible to culture a proportion of two-cell embryos to the blastocyst stage.

McLaren and Michie (1956) perfected the technique of embryo transfer in the mouse and by 1958 McLaren and Biggers were able to show that embryos cultured

using Whitten's technique were capable of developing into normal healthy adult mice after transfer to foster mothers.

In 1965 Brinster (Brinster 1965a, 1965b and 1965c) produced a series of reports which investigated and expanded upon many of Whitten's original observations. Brinster (1965a) examined the capacity of murine embryos to develop at different osmolarities and found that the range over which embryos could be cultured was from 216-339 milliosmoles with an optimum of 276. However, as NaCl made up such a large proportion of the salts present in the medium it was difficult to distinguish between the effects of varying the NaCl concentration and varying the osmolarity.

The hydrogen ion concentration is also a critical factor influencing the success of *in vitro* embryo culture. Whitten (1956) observed that eight-cell embryos only developed between pH 6.9-7.7. For two-cell embryo development, Brinster (1965a) found the optimum range to lie between 6.4-7.2 and reported that the greatest number of embryos developed at around a pH of 6.8. However, he noted that complex interactions between the effects of pH and energy source made it difficult to establish a response curve to pH alone.

Brinster (1965b) also examined the energy sources which could support development and the optimum concentrations of these compounds. He found that lactate, phosphoenolpyruvate, oxaloacetate and pyruvate could all support embryonic growth. However, glucose and malate which will support development of the eight-cell mouse embryo (Whitten, 1956) would not support that of the two-cell mouse embryo. Brinster (1965c) also established that murine embryos would not develop in the absence of a fixed nitrogen source but that serum, Bovine Serum Albumin (BSA) and free amino acids could all be used as a source of fixed nitrogen.

BSA appeared to have other beneficial effects. In addition to that of supplying a source of nitrogen, its absence from embryo culture media made the embryos sticky and difficult to handle (Brinster, 1965c). It has also been suggested that it may remove toxic factors as BSA is known to be a potent binder of small molecules (Kane, 1985 and references therein).

In 1968 Whitten and Biggers reported that it was possible to culture murine preimplantation embryos from the one-cell stage to the blastocyst stage, but found that this could only be done with certain hybrid strains and was not possible with randomly bred mice. Therefore, these workers were able to show, at least for the hybrid strain used, that a specific maternal factor was not required at this stage of development. A number of modifications were made to the media used in these experiments and it was, therefore, not easy to assess the importance of each. However, two changes which may have contributed to the improvement were the reduction in osmolarity and the increase of BSA concentration from 1mg/ml to 4mg/ml.

Factors Affecting The Successful Culture Of Murine Embryos

Since the time of these innovative experiments, numerous modifications have been made to the media originally formulated by Whitten and Brinster. The inability to culture certain hybrid strains and randomly bred mice, together, with the observation that the rate of cell division is slower *in vitro* than *in vivo* (Harlow and Quinn, 1982) has stimulated a large number of groups to explore the limitations of this technique. In addition, the growth of human IVF and embryo culture has expanded the use of murine embryos both as a quality control assay and as a model for assessing modifications to either the media or the protocol used in culturing embryos.

Murine embryo culture media are relatively simple solutions and all contain water, inorganic salts, an energy source and a source of fixed nitrogen. However, different modifications vary in the relative concentrations of these constituents.

In an attempt to optimise *in vitro* culture conditions Quinn and Harlow (1978) investigated the gaseous environment used for culturing mouse embryos. These workers noted that the greatest number of embryos developed when the oxygen concentration was between 2.5% and 5%, the balance consisting of CO2 (5%) and nitrogen. In addition, blastocysts grown in 5% O2 had a greater number of cells at the blastocyst stage than those grown in 20%.

In 1971 Whittingham reported that for embryo culture the use of triple distilled water was superior to that of water distilled once or twice. The importance of water quality was further emphasised by Fukuda *et al.* (1987) who compared five different classes of water quality and showed that optimum culturing results were achieved with water subjected to purification using the millipore system and that this was superior to deionised water or triple distilled water.

Quinn *et al.* (1985) compared modified Tyrodes solution (T6) with a media formulated on the composition of human tubal fluid (HTF) and found that HTF was better able to support murine embryo development. The main difference between the two media was the potassium concentration, the potassium concentration of HTF and T6 being 4.9 mM and 1.4 mM respectively. Reducing the potassium concentration of HTF to that of T6 resulted in a reduced capacity to support development causing these workers to suggest that higher potassium concentrations improved embryo culture rates. Conversely, Wiley *et al.* (1986) found that reducing the potassium concentration from 6 mM to 1.4 mM improved the capacity of T6 media to support the development of murine embryos. These authors also found that increasing the number of embryos cultured in each microdrop increased the rate of embryo

development, suggesting that embryos have the capacity to positively condition the media.

Those researchers primarily interested in mouse embryo culture have mainly used bovine serum albumin as a source of fixed nitrogen and most have found it satisfactory for the purpose. However, workers interested in human embryo culture and the culture of the embryos of domestic animals have been interested in assessing various serum supplements. A number of reports concerning the use of serum in embryo culture have been produced but it is difficult to establish a clear picture as some of the results are contradictory. Menino *et al.* (1985) found that, at certain concentrations, heat treated bovine serum gave better results than BSA and Bates *et al.* (1985) found that human foetal cord serum was superior to foetal calf serum. However, Shirley *et al.* (1985) reported that culturing in Hams F-10 with foetal cord serum did not improve culture results compared to Hams F-10 alone whilst Ogawa *et al.* (1987) found that embryo culture rates were markedly reduced by the use of foetal cord serum compared to using BSA. Kane (1987), in a review of embryo culture, emphasised the difficulties associated with comparing different types of serum or protein supplements pointing out that these are undefined substances which can vary widely from batch to batch. Kane's group has investigated these variations and has found a low molecular weight contaminant in a batch of BSA which is embryotrophic (Kane, 1985).

An additional problem in using the preimplantation embryo culture rate to assess culture media was outlined by Arny *et al.* (1987). These workers compared murine embryo culture rates using T6 with and without serum supplementation. The two groups of embryos cultured to the blastocyst stage at similar rates but post-implantation development of those embryos cultured with FCS was significantly poorer than those embryos cultured in T6 alone.

The inability of certain strains and randomly bred mouse embryos to develop *in vitro* beyond the two-cell stage was originally noted by Whitten and Biggers (1968) and these observations were extended by Shire and Whitten in 1980. Muggleton-Harris *et al.* (1982) demonstrated that substances present in the two-cell cytoplasm of certain non-blocking strains were responsible for the embryos ability to overcome the two-cell block. These workers transferred small quantities of cytoplasm from non-blocking to blocking embryos and found that embryos treated in this manner could continue development beyond the two-cell stage. Cytoplasm from the two-cell embryo was better able to support development than that of the one-cell embryo. Work by Goddard and Pratt (1983) revealed that it was the maternal genotype of the embryo which was the important factor in determining whether the embryo blocks or not and suggested that maternally inherited information played an important role in controlling early cleavage in the mouse embryo.

Recently, workers have reported that the two-cell block observed in certain strains of mice and in randomly bred mice could be overcome. In 1977 Abramczuk *et al.* found that the inclusion of small quantities of ethylenediamine tetraacetate (EDTA) could result in a proportion of embryos from outbred mice culturing from the one-cell stage through to the blastocyst stage but Poueymirou *et al.* (1989) found that the results achieved using EDTA were very variable. In 1989, Chatot *et al.* developed a medium (CZB) which could consistently support the development of one-cell mouse embryos, including those embryos which originated from strains of mice that normally exhibit the 2-cell block. This medium contained EDTA but three other modifications were also made. These authors found that the presence of glucose had a detrimental effect during early cleavage stages but was required for development from the eight-cell stage. In addition, they found that increasing the lactate to pyruvate ratio improved embryo culture results as did adding glutamine to the medium. Poueymirou *et al.* (1989) compared Whittens medium with CZB medium and found that embryos grown in CZB had a significantly higher rate of protein synthesis and that the synthesis of embryonic proteins was delayed when embryos were grown in Whittens medium. Chatot *et al.* (1990) extended their studies to other strains of mice which normally exhibit the two-cell block and showed that this media supported the development of embryos in all strains tested.

MURINE EMBRYO CULTURE

SECTION 2. 2. - RESULTS

2.2.1. Introduction

In the following experiments two different culture media, T6 (Howlett *et al.*, 1987) and M16 (Whittingham, 1971), were examined for their ability to support embryo development. In addition the effect of two different types of handling media, M2 (Fulton and Whittingham, 1978) and PB1 (Whittingham and Wales, 1969), on the embryos' subsequent ability to culture was examined. It was considered important to discover the optimal combination of handling and culturing media as a number of experiments necessitated holding the embryos outside the CO_2 incubator for extended periods of time. In this section the technical aspects of murine embryo culture were also examined. These included the effect of the length of media storage time on embryo development, the embryo toxicity of liquid paraffin and the batch variation of embryo culture medium. These results allowed an "in-house" protocol to be established for this aspect of transgenic mouse production.

Only those one-cell embryos which appeared healthy were incubated in the culture group. Those zygotes which were fragmented, shrunken or otherwise abnormal in appearance were excluded. However, the embryos were not examined closely to distinguish between fertilised one-cell embryos and unfertilised oocytes. For this reason, any given collection of embryos probably contains a proportion of unfertilised oocytes. Therefore, although all embryos were cultured from the one-cell stage, the success of the groups were evaluated by recording the number of two-cell embryos that developed to the expanded blastocyst stage. There are two potential sources of error in this approach 1) it assumes that all fertilised one-cell embryos will develop to the two-cell stage, obviously this is not the case. However, when assessing media or conditions of culture, the first cleavage division is not a particularly sensitive stage of development. 2) Activation of unfertilised oocytes or fragmentation of the oocytes may cause a number of oocytes to be mistakenly scored as two-cell embryos. Despite these problems most experiments involving embryo culture were of a comparative nature. Embryos were randomly distributed to the different groups and were examined at least once daily.

2.2.2. Comparison Of Two Different Embryo Culturing Media

M16, a modified Krebs-Ringers solution, and T6, a media originally based on Tyrodes solution, were compared for their ability to support the development of

mouse preimplantation embryos. When embryos were cultured in T6, 72.0% of two-cell embryos developed to the blastocyst stage whereas 74.2% of two-cell embryos developed when cultured in M16. The results of pooled experiments are shown in Table 2.2.1., there was no significant difference between the two media in their ability to support embryo development ($x^2 = 0.3$: $P > 0.05$).

Table 2.2.1. Preimplantation Embryo Development in M16 and T6.

Media	1-cell	2-cell	Blastocyst	2-cell/Blastocyst[a] (%)
T6	255	246	177	(72.0)
M16	269	252	187	(74.2)

a. refers to the proportion of two-cell embryos culturing to the blastocyst stage.

2.2.3. Effects Of Holding Embryos In M2 And Culturing In M16

The effect of holding embryos outside the CO_2 incubator in M2 medium for periods ranging from 4 to 6 hours was examined. In group A, embryos were collected, washed in M2 and cultured in M16 shortly after collection. In group B the embryos were held in M2 at 37°C for between 4 to 6 hours before being placed in culture. The results are pooled and presented in Table 2.2.2. Although the embryos in group A appear to have greater viability as assessed by their ability to develop from the two-cell stage, this difference is not significant ($x^2 = 3.6$: $P > 0.05$).

Table 2.2.2. Effect of Holding Embryos in Handling Media Before Culture

Group	1-cell	2-cell	Blastocyst	2-cell/Blastocyst[a] (%)
A	269	252	187	(74)
B	140	116	75	(65)

Measuring the viability of two-cell embryos may not be the best test for assessing an "insult" to one-cell embryos. It may be that those embryos which survive to the two-cell stage have fully recovered from the effect of extended holding in M2. A comparison of the cleavage rate from the one-cell to the two-cell stage reveals that significantly more embryos cleaved through to the two-cell stage when incubated shortly after collection than when held in handling media for 2-4 hours ($x^2 = 12.0$: $P < 0.01$).

2.2.4. Effect Of Holding Embryos In PB1 Or M2 And Culturing In T6

The effect of holding embryos in M2 or PB1 before culturing in T6 was examined by either placing the embryos directly in culture shortly after collection or storing the embryos for 4-6 hours in one of the two holding media before placing them in the culture media. The cleavage rate from the one to the two-cell stage differs significantly between those embryos which were held in handling media for a time before being cultured or those embryos that were cultured immediately. Of those embryos incubated immediately in T6, 96% cleaved to the two-cell stage. This is a significantly greater cleavage rate than those embryos held for 4-6 hours in either PB1 ($x^2 = 9.6$: $P < 0.01$) or M2 ($x^2 = 27.4$: $P < 0.01$), in these groups the percentage of embryos cleaving to the two-cell stage was 89% and 82% respectively. A greater number of embryos cleaved to the two-cell stage after being held in PB1 than in M2 ($x^2 = 4.7$: $P < 0.05$). However, there was no significant difference in the culturing rate from the two-cell to the blastocyst stage between those cultured immediately and those held in handling media ($x^2 = 0.3$: $P > 0.05$). In addition there was no difference in the culture rate to the blastocyst stage between the different holding media, PB1 and M2 ($x^2 = 0.4$: $P > 0.05$). These results are shown in Table 2.2.3.

Table 2.2.3. Effect of Holding Embryos in Different Types of Media.

Group	1-cell	2-cell	Blastocyst	1-cell/2-cell[a] (%)	2-cell/Blastocyst[b] (%)
T6 only	255	246	177	(96)	(72.0)
T6 + PB1	214	191	144	(89)	(75.4)
T6 + M2	308	254	185	(82)	(72.8)

a. refers to the proportion of one-cell embryos cleaving to the two-cell stage.
b. refers to the proportion of two-cell embryos culturing to the blastocyst stage.

2.2.5. Effect Of T6 Batch Variations On Embryo Development

In order to assess if there was significant variation between different batches of T6 media each batch of T6 was assessed shortly after preparation. The results, shown in Table 2.2.4., show that there were small differences between different batches of media. However, none of these differences were significant when the results were analysed using a 9x2 contingency table with 8 d.f. (x^2: = 11.5: P > 0.05). On average 84% of two-cell embryos developed to the blastocyst stage.

Table 2.2.4. Embryo Culture Results for Different Batches of T6.

Batch	1-cell	2-cell	Blastocyst	2-cell/Blastocyst (%)
1	34	33	29	(87.9)
2	48	37	34	(91.9)
3	74	61	46	(75.4)
4	58	56	50	(89.3)
5	141	103	88	(85.4)
6	45	41	30	(73.1)
7	130	69	55	(79.6)
8	41	34	28	(82.3)
9	141	121	104	(86.0)
Total	712	555	464	(84)

2.2.6. Effect Of Different Batches Of Liquid Paraffin On Embryo Culture

It has been reported (Fleming *et al.*, 1987) that liquid paraffin which is used to cover the media can occasionally contain impurities or toxic substances which can retard embryo development. For this reason two different liquid paraffin products, Boots liquid paraffin and BDH light paraffin oil were tested (Table 2.2.5.). Two different batches were examined for each product. No consistent difference was detected between the different products but analysing the results using a 4x2 contingency table with 3 d.f. showed that different batches could differ significantly ($x^2 = 11.7$: $P < 0.01$).

Table 2.2.5. Effect of Different Batches of Liquid Paraffin on Embryo Culture.

Batch	1-cell	2-cell	Total Blastocyst	2-cell/Blastocyst (%)
Batch A (Boots)	54	41	30	73.2
Batch B (BDH)	91	75	49	65.3
Batch C (Boots)	71	60	51	85.0
Batch D (BDH)	70	61	53	86.8

2.2.7. Effect Of *In Vitro* Fertilisation On Embryo Culture

On some occasions it is considered desirable to fertilise oocytes *in vitro* instead of *in vivo*. Usually this is done to obtain a more synchronous population of embryos, an important prerequiste in many studies concerned with temporal aspects of embryo development. In this study, however, *in vitro* fertilisation (IVF) was carried out as Lavitrano *et al.* (1989) had reported that it was possible to produce transgenic mice by incubating DNA with sperm and then using this solution to fertilise freshly collected oocytes. Experiments were carried out in an attempt to verify this work, the results of which are presented in Chapter 3. As a result of these experiments it was possible

to compare the embryo fertilisation and culture rates between those embryos which had been fertilised *in vivo* and those that had been fertilised *in vitro*.

A total of 641 oocytes were collected and fertilised *in vitro* and of these 395 cleaved through to the two-cell stage giving a cleavage rate of 62%. This is significantly lower than the cleavage rate obtained after *in vivo* fertilisation ($x^2 = 7.5$: $P < 0.01$). It is also significantly lower than the 1st cleavage rate of control embryos used throughout this study ($x^2 = 166.0$: $P < 0.01$).

A lower percentage of two-cell embryos developed to the four-cell stage or the blastocyst stage in the IVF group compared to the control group, however, in neither case was the difference between the two groups significant. This may be because the control group is small. If the culture rate to the four-cell and blastocyst stages of the IVF group is compared against *in vivo* fertilised control embryos (group B) cultured throughout the entire period of this study then the difference from the two-cell to the four-cell and from the two-cell to the blastocyst stages is significant ($x^2 = 8.5$ and 5.2 respectively: $P < 0.01$ and $P < 0.05$). These results are shown in Table 2.2.6.

Table 2.2.6. Embryo Development After *In Vitro* Fertilisation.

Group	1-cell	2-cell	4-cell	Blastocyst	1-cell/2-cell[1] (%)	2-cell/Blastocyst[2] (%)
A	41	34	28	28	(83)	(82.4)
B	3712	3101	2833	2353	(84)	(75.8)
C	183	93	68	61	(51)	(66.0)
D	458	302	260	-	(66)	-
C + D	641	395	328	-	(62)	-

A) - *In vivo* fertilised group run in parallel with IVF group.
B) - overall controls pooled throughout study.
C) - IVF embryos cultured to the blastocyst stage.
D) - IVF embryos cultured to the four-cell stage.
C+D) - Total number of IVF embryos cultured to the four-cell stage.

1. Proportion of one-cell embryos cleaving to the two-cell stage.
2. Proportion of blastocysts developing from the two-cell stage.

2.2.8. Media Storage

The T6 embryo culture media was made up at 1x concentration, divided into 50 ml aliquots and stored at between 0 - 4^0 C. BSA was not added to the medium until the aliquot was required. The effects of storing media were examined in two different batches of T6 media, see Table 2.2.7. Batch A was used over a 5 month period and Batch B was used for 3 months, no significant decrease in the capacity of this media to support development was observed in either Batch A ($x^2 = 8.3$: $P > 0.05$, using a 2x5 contingency table with 4 d.f.) or Batch B ($x^2 = 5.1$: $P > 0.05$, using a 2x3 contingency table with 2 d.f.). The quality appeared to decline after 4 months storage in Batch A, but this result is not significant, perhaps due to the relatively small sample size in this group. Therefore, it appears that this media can be stored for at least 4 months without any significant reduction in its ability to support embryo culture.

Table 2.2.7. Effect of Storage Time on Media Quality.

	1-cell	2-cell	Blastocyst	2 cell/Blastocyst (%)
Batch A				
0-1 month old	84	76	61	(80)
1-2 months old	171	170	117	(69)
2-3 months old	278	252	183	(73)
3-4 months old	134	124	94	(76)
4-5 months old	40	32	18	(56)
Batch B				
0-1 month old	213	121	92	(76)
1-2 months old	189	146	117	(80)
2-3 months old	289	210	180	(86)

It has been reported (Fleming *et al.*, 1987) that culturing embryos after the zona pellucida has been removed is a more critical test of media quality and more readily

identifies the presence of toxic substances in the media. To check that low level contaminants were not present in the media the zonae were removed from one-cell embryos. In this experiment 20 blastocysts developed from 23 two-cell embryos suggesting that toxic factors were not present in the media or the liquid paraffin.

In addition, Wiley *et al.* (1986) have reported that embryos cultured in groups have a faster rate of development than embryos cultured singly. Table 2.2.8. shows that a greater percentage of two-cell embryos cultured in groups reached the blastocyst stage than when they were cultured singly. However, the difference between the two results was not significant ($x^2 = 3.2$: $P > 0.05$).

Table 2.2.8. Group Size and Zona Removal - Effect on Development.

	2-cell stage	Blastocyst	2-cell/Blastocyst (%)
Singly, Without Zona.	23	20	(87)
Singly, With Zona	49	37	(76)
Groups (20-30), With Zona	86	76	(88)

2.2.9. Overall Culture Rate Using T6

Table 2.2.9. shows the overall culturing rate achieved with a number of batches of T6. Examination of embryo loss throughout preimplantation development reveals that 16.5% of one-cell embryos failed to cleave to the two-cell stage and there is significantly greater embryonic loss at this point than between any other two stages. Although obviously abnormal embryos were excluded from the culture group, a proportion of these one-cell embryos would have been unfertilised oocytes as a detailed examination of every embryo placed in culture was not carried out.

Excepting the loss incurred between the one and the two-cell stage, the greatest loss of embryos occurred between the morula and blastocyst stage. The loss at this stage was significantly greater than that between the four-cell and the morula stage ($x^2 = 20.9$: $P < 0.01$) and was greater than the loss incurred between the two-cell and the four-cell stage, the difference being significant at the 5% level but not at the 1%

level (x^2 = 6.3: P < 0.05). A greater proportion of two-cell embryos failed to develop to the four-cell stage compared to the proportion of four-cell embryos that failed to form morulae, the difference being significant at the 5% level (x^2 = 4.9: P < 0.05).

The morula and blastocyst stages are easily recognisable but the development from the morula stage to the expanded blastocyst requires two cleavage divisions, all other stages counted being separated by only one cleavage division. This may explain why apparently greater embryonic loss occurs at this point than at other stages of preimplantation development.

Table 2.2.9. Overall Culturing Rate Using T6.

1-cell	2-cell	4-cell	Morula	Blastocyst
3712	3101	2833	2632	2353
(119.7%)	(100%)	(91.4%)	(84.9%)	(75.9%)

MURINE EMBRYO CULTURE.

SECTION 2.3. - DISCUSSION

The major objective of this study was to examine those aspects of murine embryo culture which were relevant to transgenic mouse production.

Although a number of different procedures have been developed for producing transgenic mice each necessitates the *in vitro* holding and usually the *in vitro* culture of preimplantation embryos. It was considered important, therefore, to optimise the culture conditions in an attempt to maximise the number of manipulated embryos suitable for transfer. It has been shown (Arny *et al.*, 1987; Ryan *et al.*, 1990) that culture conditions can affect the subsequent development of transferred embryos and ultimately influence the number of offspring born.

There is no generally accepted optimal formula for murine embryo culture media and a number of different media have been used in different laboratories. These media are similar in that they all contain a chemically defined salt solution, bicarbonate, lactate, pyruvate and a macromolecule usually bovine serum albumin. Some workers, especially those primarily involved in human embryo culture, regularly use a serum component in the media.

In all of the culturing experiments described the embryos were cultured from the one-cell stage rather than the two-cell stage as it has been reported that culturing from the one-cell stage is a better indicator of culture conditions (Davidson *et al.*, 1988). However, the results are based on the number of two-cell embryos reaching the expanded blastocyst stage as it was not possible to accurately define the fertilisation status of the one-cell stage.

Two different culture media routinely used to culture mouse embryos were compared: M16 - a modified Krebs-Ringers solution very similar to Whitten's medium (Whitten, 1971; Whittingham, 1971) and T6 - a modification of tyrodes medium (Howlett *et al.*, 1987). Within the experimental design described there was no significant difference between these two media in their ability to support murine embryo development. There are a number of differences between these two media. The concentration of both sodium chloride and potassium is higher in M16 than in T6 but these differences do not appear to have significant effects on embryo culture.

T6 was adopted for general use and all subsequent culturing experiments were carried out using this media. During the course of this work a total of 3101 two-cell embryos were placed in culture and 2353 developed through to the expanded blastocyst stage. This gave a culturing rate of 76% which was deemed satisfactory.

It is extremely difficult to make direct comparisons between the culturing rates reported by different workers as a number of different genetic strains of mice are used

and small variations in procedure, water quality and ingredients are inevitable. For example, batch to batch variations in the quality of bovine serum albumin have been reported (Kane, 1987), some of this variation being ascribed to the presence of embryotrophic contaminant (Kane, 1985). However, Quinn *et al.* (1984) reported that up to 80% of one-cell embryos cultured through to the expanded blastocyst stage when T6 was used and Pomp *et al.* (1988) achieved a culturing rate of 98.5% using a low lactate modification of Whitten's medium. Whitten's medium itself supported a culturing rate of 89% using F1 hybrid females ICR x C57Bl/6J. It would appear, therefore, that given certain mouse strains and a high standard of media quality high rates of development can be achieved with mouse embryos.

Embryos were collected into handling media and held in this media outside the CO_2 incubator until they were microinjected. Microinjection usually took a number of hours to perform and this necessitated the holding of embryos outside the CO_2 incubator for extended periods of time. The effect of incubating one-cell embryos in handling media outside the CO_2 incubator for 4-6 hours was examined. Holding embryos outside the CO_2 incubator significantly reduced the number of one-cell embryos cleaving to the two-cell stage. This was observed both when embryos were held in M2 before being cultured in M16 and when embryos were held in M2 or PB1 before being cultured in T6. Fewer embryos cultured to the two-cell stage in T6 after being held in M2 than those held in PB1. Although embryos were randomly distributed to the different groups, an unequal allocation of unfertilised embryos to any one of the test groups could have affected the significance of the results. The intrinsic differences between the embryos from different donor females could be excluded from the experiment by culturing embryos within donor groups. It has been argued that an experimental design of this type would be a statistically more powerful approach (Pomp *et al.*, 1988). When the subsequent culture rate of two-cell embryos to the blastocyst stage was examined no significant difference was observed between the different groups.

There are three major reasons for culturing embryos in a controlled gaseous environment such as exists within a CO_2 incubator: (1) embryos require a source of CO_2/HCO_3 for normal development (Quinn and Wales, 1973), (2) a 5% level of CO_2 is required to interact with the bicarbonate in the medium to maintain an optimum pH - the importance of buffering and adequate equilibration of the media has been stressed by a number of authors (John and Kiessling, 1988) and (3) it has been shown that decreasing the oxygen concentration from 20% to 5% significantly improved culture conditions for preimplantation embryos (Quinn *et al.*, 1978). In this study, however, embryos were cultured in the presence of 20% oxygen.

The handling media is already buffered in atmospheric conditions, PB1 is a phosphate buffered medium and M2 is a HEPES buffered medium. Therefore, the

reduction in the cleavage rate in those embryos incubated outside the CO_2 incubator appears to be due either to the absence of an appropriate CO_2 concentration exerting a direct effect on the zygote stage or because the handling media is suboptimal for embryo culture.

Lactate is present in murine embryo culture but absent from the handling media PB1 and M2. The absence of lactate should not be detrimental to the one-cell stage as it has been reported that reducing the lactate concentration (and/or the osmolarity) improves the developmental capacity of one-cell embryos (Pomp *et al.*, 1988) and that lactate is actually detrimental to normal first cleavage (Cross and Brinster, 1973). However, in contrast to these findings, studies in recent years (Chatot *et al.*, 1989, 1990) have found that a high lactate:pyruvate ratio was beneficial for the development of early stages.

The effect of prolonged incubation of mouse embryos in HEPES containing medium has not been reported but Quinn *et al.* (1984) have reported that holding in HEPES buffered T6 for 30-60 minutes had no significant effect on subsequent development.

Batch Variations

After each batch of medium had been prepared its ability to support embryo development was tested. There were only small differences in media quality between different batches with only small variations in embryo culture rates being recorded. As the same batches of reagents were used throughout, this experiment was designed to test the repeatability of media preparation. It would appear that inevitable small variations in the preparation of media do not affect the quality of the media to any great extent. Batch variations in embryo culture media have been widely reported but these have usually been attributed to variations in the quality of the individual reagents such as liquid paraffin (Fleming *et al.*, 1987), BSA (Kane, 1987), serum (Shirley *et al.*, 1985) and water (Fukuda *et al.*, 1987). A significant difference in the success of human IVF has been attributed to different batches of T6 (Gianaroli *et al.*, 1986) but these authors did not identify the reasons for these differences. Batch variations based on inherent variations in procedure have not been reported and these results suggest that either small variations in protocol do not affect the final product greatly or that the procedure was repeated fairly accurately.

In this study different products and batches of liquid paraffin were examined for their ability to support embryo development. Two batches of liquid paraffin (Boots) and two batches of light paraffin oil (BDH) were tested and although there were no consistent differences between the products there was a significant difference between batches. It has been reported that toxicity effects associated with liquid paraffin can

be subtle (Fleming *et al.*, 1987) and the experimental design reported here may not be the most sensitive method for detecting less obvious toxic effects.

In vitro Fertilisation

Embryo fertilisation and culture rates were compared between those embryos which had been fertilised *in vivo* and those that had been fertilised *in vitro*. A difference might arise between these two groups for at least two reasons. Firstly, the oocytes were collected from the donors between 12 and 14 hours after hCG injection whereas the embryos were collected between 22 and 24 hours after hCG injection and therefore were cultured for twelve hours longer than the *in vivo* group. Secondly, the embryos fertilised *in vitro* would be exposed not only to T6 and PB1 but also to fertilising media for 6-8 hours.

In this study the fertilisation and/or one-cell cleavage rates of oocytes fertilised *in vitro* was significantly lower than those embryos fertilised *in vivo*. A report by Brinister *et al.* (1989) summarises the results of similar experiments undertaken by their group and a number of other workers in which fertilisation rates ranged from 49% to 86%. The average fertilisation rate quoted in Brinster's report was 64% which is similar to the fertilisation rate of 62% reported here.

Although the difference was not significant, fewer *in vitro* fertilised embryos cleaved through to the four-cell stage and developed to the blastocyst than in the small control group run in parallel. If, however, the two to the four-cell and the two to the blastocyst cleavage rates of the *in vitro* fertilised embryos are compared against the overall culture rate achieved during the course of this work, then the difference in subsequent development is significant. A direct comparison between the development rate of the *in vitro* group and this composite control group of *in vivo* fertilised embryos cannot be made as the experiments were carried out at different times using different batches of media. It is possible that if more embryos had been included in the control group run in parallel with these experiments the difference may have been significant.

If the proportion of embryos developing from the two-cell stage is reduced, this may be due to either a direct effect of *in vitro* fertilisation (eg polyspermy) or may reflect the extended period of time these embryos spent *in vitro* at a sensitive stage of development.

Storage Times

Due to the short half-life and instability of certain reagents used in embryo culture media, namely penicillin and pyruvate (Wiley *et al.*, 1986), it has been variously recommended that medium made up at 1 x concentration and stored at 0-4^{o}C should be used within 2 days (Wiley *et al.*, 1986), 1 week (Hogan *et al.*, 1986) or 2 weeks (Pratt, 1987) of preparation. Pyruvate is important because it is used as an energy substrate for the first cleavage (Whittingham and Biggers, 1967).

In this study, the length of time that two different batches of T6, made up at 1x concentration, could be stored at 0-4^{o} C was examined. There was no significant difference in the ability of Batch A to support embryo development until after 4 months storage at which time the culture rate declined. Similarly there was no significant decrease in the quality of Batch B with up to 3 months storage.

Therefore, it was found, in contrast to the above recommendations, that murine embryo culture could be stored for extended periods without any loss of culturing capacity. It may be that this experimental design was not sufficiently sensitive to detect a reduction in media quality and that other more sensitive tests of media quality would be required.

Wiley *et al.* (1986) reported that culturing embryos in large groups improved the rate of development, it being suggested that a large group of embryos conditions the media and that factors produced by the embryo improve the quality of the media. Although these authors noted that the rate of development improved, the total number of embryos reaching the blastocyst stage was unaffected. As a result of this work, many labs have followed this protocol by culturing embryos in groups of 20-30 per microdrop, a microdrop usually having a volume of 30-50 μl. In this study it was found that a greater proportion of embryos cultured through to the blastocyst stage when in groups of 20-30 than when cultured singly although the difference was not significant.

The success of producing transgenic mice is dependent upon the success of a number of different experimental procedures. Embryo culture is central to transgenic production for, if the handling or culturing media is substandard, it will limit the number of manipulated embryos suitable for transfer at the appropriate stage. Sub-optimal embryo media can also affect subsequent development *in utero* (Arny *et al.*, 1987). Therefore, to maximise the number of manipulated embryos resulting in viable offspring it is essential that media be of the highest quality.

In these experiments, assessment of different conditions was made by examining the number of embryos developing to the expanded blastocyst stage. There is evidence from a number of sources that the culture of embryos from the one-cell to the blastocyst stage is not the best available assessment of embryo viability/potential

or of the culture conditions. Fleming *et al.* (1987) has suggested that the sensitivity of this assay could be increased by removing the embryos' zonae pellucidae. They showed that embryos cultured after removal of the zona pellucida, were sensitive to a toxic batch of liquid paraffin. This protocol was followed to assay one batch of media and liquid paraffin but there was no significant difference between the group cultured after removal of the zona pellucida and the control group.

Therefore, removing the zonae pellucidae of cultured embryos or examining foetal development of embryos grown in culture have both highlighted deficiencies in culture medium not detected by the embryo culture results. Other workers (Harlow and Quinn, 1982) have preferred to measure the rate of development or the embryo cell number at a given stage rather than the total number reaching the expanded blastocyst stage. Ryan *et al.*, (1990) showed that the addition of Platelet Activating Factor (PAF) to embryo culture media did not result in a greater proportion of embryos reaching the blastocyst stage but blastocyst cell number was enhanced in the treated group and more of these embryos implanted than in the control group. More sophisticated techniques, such as measuring the glucose uptake of embryos, have also been applied in order to assess viability more accurately (Butler *et al.*, 1988).

In this study the conclusions were drawn after comparing the proportion of embryos that developed to a defined stage between two or more groups of embryos. Although this method is not the most sensitive assay of culture conditions available, it was possible, using this approach, to assess media quality and to develop a standardised approach to murine embryo culture. As shown in the next chapter, embryos cultured for varying periods of time using the system described here were capable of developing into viable offspring after transfer to pseudopregnant recipients.

CHAPTER 3. - TRANSGENIC PRODUCTION

SECTION 3.1. - INTRODUCTION

Early Work

The ability to transfer genetic material into the mammalian germline has only been achieved during the last decade and resulted from the pioneering work of Brinster, Gordon, Ruddle and others (Gordon *et al.*, 1980; Brinster *et al.*, 1981c; Costantini and Lacy, 1981; Wagner *et al.*, 1981a; Wagner *et al.*, 1981c). During this time, this technology has been used to address fundamental questions in many aspects of biomedical science including the areas of genetics, development, immunology and oncology (for reviews see: Palmiter and Brinster 1986; Jaenisch, 1988; Cuthbertson and Klintworth, 1988; Hanahan, 1989).

The first successful attempt to transfer genetic material into the mouse genome was reported in 1974 by Jaenisch and Mintz. These workers microinjected SV40 DNA sequences into the blastocoel cavity of mouse embryos and the subsequent analysis of adult offspring revealed the presence of SV40 sequences in at least a proportion of the adult tissues.

During the 1970s, researchers made increasing use of amphibian oocytes and eggs to investigate the expression of a wide variety of cloned genes (for review see Gurdon and Melton, 1981). It was shown that DNA could be transcribed into functional message (Mertz and Gurdon, 1977) and in some cases this message could be translated to produce the protein product (De Robertis and Mertz, 1977). Many of the features which characterise the introduction of genetic material into mammalian embryos were first observed in amphibian oocytes and embryos. These included the observation that circular DNA has greater stability in biological systems than linear DNA and that efficient ligase activity is present in the amphibian oocyte nucleus (Gurdon and Melton, 1981). In 1981 Rusconi and Schaffner produced adult frogs carrying rabbit globin sequences introduced into fertilised eggs. Analysis of these frogs revealed that the introduced sequences were present in the form of head to tail tandem arrays - a feature also observed after the injection of DNA in mammalian embryos (Brinster *et al.*, 1981c).

The technique of DNA microinjection in amphibian oocytes and eggs was adapted to investigate the regulation of both mRNA (Brinster *et al.*, 1980, 1981b) and DNA within mammalian embryos (Brinster *et al.*, 1981a). These studies laid the foundation and developed the techniques subsequently used for the production of transgenic mice. In 1980, Gordon *et al.* successfully introduced cloned DNA into the mouse genome using the technique of pronuclear microinjection. The following year

four other groups reported the successful production of transgenic mice using this approach (Brinster *et al.*, 1981c; Costantini and Lacy, 1981; Wagner *et al.*, 1981a; Wagner *et al.*, 1981c). It was apparent from these reports that the integrated transgene could be transmitted to subsequent generations and that expression was possible both in the founder mice and their transgenic progeny. It appeared that the introduced DNA integrated into the host chromosomes at a very early stage in development, usually in head to tail tandem arrays. After these initial innovative reports the use of transgenic mice increased rapidly and data quickly accumulated concerning both the expression of the introduced genes and the efficacy of producing transgenic mice.

In 1982, Palmiter *et al.* introduced rat growth hormone sequences into the germ line of mice and demonstrated the dramatic affect transgenes could exert on the phenotype of the animal. These workers fused the promoter of the mouse metallothionein-I (MT-I) gene to the rat growth hormone gene. Some of the resultant transgenic mice showed high levels of rat growth hormone and produced double the normal growth rate in a proportion of offspring. Such alterations in normal physiology dramatically demonstrated the potency of this technique and gave researchers an insight into the many biochemical interactions that control important characteristics such as growth.

Gene Expression in Transgenic Animals

It is possible to direct transgene expression to particular tissues by fusing tissue specific promoters to the gene of interest. Gene expression has been targeted to the pancreas using both the insulin gene (Bucchini *et al.*, 1986) and an elastase - human growth hormone gene (Ornitz *et al.*, 1985). Tissue specific expression was also recorded in the lens of the eye using the murine α-A - crystalline promoter (Overbeek *et al.*, 1985) and muscle using the rat myosin light-chain 2 gene (Shani, 1986).

Examples of temporal control of expression have also been reported with the α-foetoprotein gene being expressed in a manner analogous to the endogenous gene (Krumlauf *et al.*, 1985). Human foetal globin transgenes introduced into mice were also regulated in a temporal manner (Chada *et al.*, 1986).

As information accumulated, it became apparent that transgene expression could be unpredictable. Different lines of mice produced with the same DNA construct showed different levels of expression and although the transgene copy number was different for each line the level of expression did not necessarily correlate with the copy number (Palmiter and Brinster, 1986). In some cases transgenes carrying tissue specific regulatory signals were expressed in inappropriate tissues. Stewart *et al.* (1984) reported that the c-*myc* oncogene, targeted to the mammary gland using the

Murine Mammary Virus (MMTV) promoter, produced mammary adenocarcinomas in the transgenic lines created. In one line, however, transgene expression was present in a number of organs resulting in spontaneous tumour development in a wide range of different tissues (Leder *et al.*, 1986).

Most researchers ascribed the variable and unpredictable nature of transgene expression to the influence of the surrounding chromatin at the site of integration and this was demonstrated by Allen *et al.* in 1988 when they produced transgenic mice expressing the reporter gene lac Z under the influence of the Herpes Simplex Virus Thymidine Kinase (Tk HSV) promoter. Different lines of mice differed considerably in both spatial and temporal patterns of expression. Palmiter and Brinster (1986) had previously suggested that some promoters were much more sensitive to their site of integration. Cumulative data using the MT-I promoter, a promoter active in a number of different cell types, revealed that tissue expression varied widely between different transgenic lines. In 1990 Al-Shawi *et al.* confirmed that the site of chromosomal integration can have a major influence on transgene expression. These workers produced a transgenic line of mice in which aberrant transgene expression was detected, reisolated the transgene from its chromosomal position and reintroduced this construct to create a new transgenic line. The different chromosomal position in the new line altered the pattern of expression in the secondary transgenic mice.

It now appears that variable gene expression is a combination of both chromosomal site and the absence of sufficient regulatory signals within the transgene construct. In 1987 Grosveld *et al.* reported that elements, now termed dominant control regions (DCR), situated between 65 and 44 kilobases upstream of the β-haemoglobin gene could exert a high degree of control upon transgene expression. When these elements were included in the transgene construct, expression in the resulting transgenic mice was highly tissue specific, of a high level, copy number dependent and independent of the site of integration. Similar elements have now been found associated with the human α-haemoglobin gene (Ryan *et al.*, 1989) and the CD2 gene (Lang *et al.*, 1988).

In 1986 Palmiter and Brinster noted that levels of expression could vary within a transgenic line, both between siblings and between generations. In some cases animals with low levels of expression gave rise to offspring with high levels of expression and vice versa. Allen *et al.* (1990) showed that levels of expression, although not the general pattern of expression, can vary within a transgenic line depending on the genetic background of the transgenic mice. This observation may explain why expression can vary within a line as most founder animals are initially hybrids and subsequent breeding will result in gene segregation - possibly including those genes which modify transgene expression.

Significant differences in transgene expression between generations are a feature of a phenomenon known as imprinting described by Reik *et al.* (1987), Sapienza *et al.* (1987) and Swain *et al.* (1987). These workers independently identified transgenic lines in which the paternal genetic contribution differed in expression potential from that of the maternal contribution. The consequence of this was that certain transgenes (and presumably certain endogenous genes) have a different level of expression depending upon whether the individual receives the transgene from the sire or the dam.

Factors Affecting The Production Of Transgenic Mice By Pronuclear Microinjection

The application of pronuclear microinjection is associated with high levels of embryonic mortality. The number of offspring carrying integrants varies widely both between experiments conducted by the same group and by different groups, but usually ranges between 15-30% (Gordon and Ruddle, 1983). In 1985 Brinster *et al.* investigated the factors which affected the successful production of transgenic mice using pronuclear microinjection. These workers demonstrated that the efficiency of integration increased with increasing DNA concentration in the injection solution. Above 2 μg/ml no further increase in integration efficiency occurred and embryo mortality increased when concentrations of 10 μg/ml were injected. Obviously at a given DNA concentration the number of copies injected will depend on the size of the plasmid or linear construct. It is not clear from Brinster's work whether the toxic effect on embryos results directly from a high concentration or high copy number. The form of injected DNA was also important, linear molecules with 'sticky ends' integrating at a much higher efficiency than either supercoiled circular plasmid or blunt ended linear molecules. This group also showed that by using hybrid mice the efficiency was eight fold greater than when an inbred strain of mice was used. This difference was due to a number of factors, namely, a greater number of fertilised eggs was recovered from the hybrid mice, a greater proportion of hybrid embryos survived injection and developed to term, and a greater percentage of hybrid offspring carried the transgene in the hybrid group compared to the inbred group.

The mechanism of DNA integration following pronuclear microinjection is not fully understood. It was established at an early stage that a variable number of gene copies are found in the resulting transgenic animals, usually in long head to tail tandem arrays (Brinster *et al.*, 1981c). It is believed that DNA integrates at random chromosomal breaks, the number of breaks limiting the number of integration sites per cell (Brinster *et al.*, 1985). Microinjection into tissue culture cells has also been developed (Capecchi, 1980). The previously established and most commonly used

method of introducing DNA into tissue culture cells is by transfection, this method utilises the cell's ability to take up DNA when it is precipitated with calcium phosphate (CaPO4). However, the proportion of cells taking up DNA after microinjection was reported as being 10^3 to 10^6 greater than after transfection. As there are a number of differences between the two techniques it is difficult to identify the factors which affect the proportion of cells taking up DNA. The difference probably reflects the fact that microinjection results in the DNA being deposited in the nucleus of the cell (Capecchi, 1980). However, it is possible that with microinjection of both cells and embryos, the physical trauma sustained by the nucleus (or pronucleus) assists the integration of DNA.

Pronuclear microinjection is associated with embryonic mortality which can be divided into two types, those embryos dying shortly after microinjection due to severe and usually obvious damage and those dying later in development. Wilmut pointed out that in sheep this deferred loss continues throughout foetal development (Wilmut *et al.*, 1990).

The efficiency of producing transgenic mice varies between groups and between experiments with the same group. The optimal rate for embryo survival is roughly 25% and a similar figure is considered optimal for DNA integration (Brinster *et al.*, 1985). This means that even with the most efficient operators and the most optimal conditions, the number of transgenic animals is unlikely to exceed 6% of all successfully injected embryos transferred to recipients.

This chapter describes various aspects of transgenic mouse production, concentrating on the detrimental effect of pronuclear injection which is examined at various stages of development. The efficiency of the various component parts which make up the protocol for producing transgenic mice is examined in an attempt to optimise this procedure.

TRANSGENIC PRODUCTION

SECTION 3.2. - RESULTS

3.2.1. Introduction

During the course of this work transgenic mice were produced using three different gene constructs. Two different methods of gene insertion were used in an attempt to produce transgenic mice - pronuclear microinjection (Hogan *et al.*, 1986) and sperm mediated transfer of DNA (Lavitrano *et al.*, 1989).

The production of transgenic mice is dependent upon the success of a number of different techniques and procedures which include: the superovulation and mating of young female embryo donors; the collection of embryos and subsequent pronuclear microinjection of these embryos; overnight culture to the two-cell stage and transfer into the oviducts of pseudopregnant recipients. Finally, the resultant offspring are screened for the presence of the introduced DNA by Southern blotting.

The primary purpose of these experiments was to produce transgenic mice. Information pertaining to the efficacy of the procedure was recorded, however, as this was a secondary consideration the sample sizes in some of the results presented are not always well balanced.

3.2.2. Embryo Collection

In a series of experiments in which the collection rates were recorded, a total of 5802 embryos were collected from 214 three to six week old superovulated females, the average number of embryos collected per mouse being 27.1. A number of embryos collected were rejected because they appeared lysed, fragmented or otherwise morphologically abnormal but overall 85.3% were judged as having a good appearance and on average each donor supplied 23.1 good embryos.

There was a considerable range in the number of embryos collected from each mouse. On very few occasions no embryos could be recovered, the most likely reason being failure of ovulation. Excluding these mice, however, the number of embryos collected from each mouse ranged from 6 to 66 (standard deviation - 11.9). In a series of collections the number of embryos recovered from each donor was recorded, Figure 3.2.1 shows a distribution graph representing these results.

Those embryos which had an obviously abnormal morphology eg fragmented or lysed, were rejected. The remaining embryos were pooled and held in either HEPES

or phosphate buffered medium until they were transferred to the microinjection chamber. Only a proportion of the embryos placed in the microinjection chamber were considered suitable for injection, namely those in which at least one pronucleus could be easily identified.

A proportion of embryos were damaged by microinjection, the most obvious manifestation of this being the leakage of cytoplasmic contents and cell lysis. Of 3783 embryos placed in the microinjection chamber, microinjection was attempted in 2202 (58.2%) and 1583 (41.9%) were not obviously damaged as assessed by visual examination 1 hour after microinjection. Therefore, 28.1% of those injected were obviously damaged, usually lysed.

Figure 3.2.1. Number of Embryos Collected From Superovulated Mice.

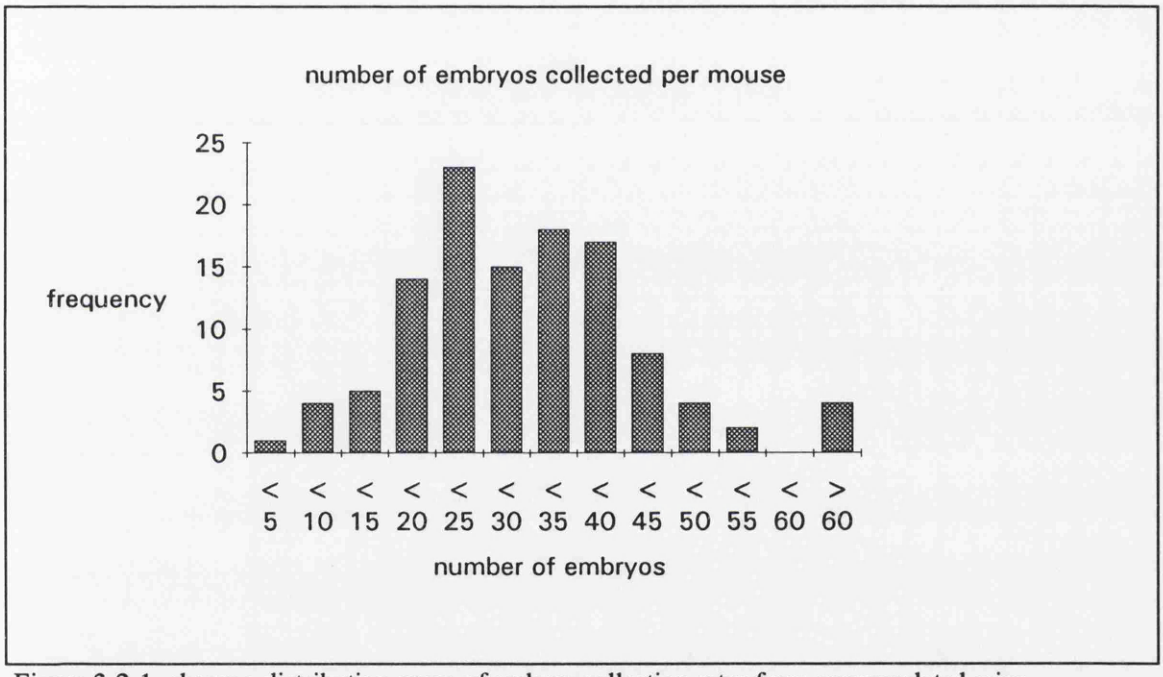


Figure 3.2.1. shows a distribution curve of embryo collection rates from superovulated mice.

The efficiency of microinjection was defined as the number of embryos surviving microinjection as a percentage of the total number of embryos in which injection was attempted. There was considerable day to day variation in the efficiency of microinjection and this was reflected in a similar variation in the proportion of embryos in which good visualisation of the pronuclei was observed. Good visualisation of the pronuclei was one of the factors which affected the number of embryos successfully injected, other factors included the quality of the microinjection needle and the quality of the medium although this was a subjective assessment.

The pronuclei swell progressively during the one-cell stage and are most easily injected when they are at their maximum size just before the nuclear membranes disappear (Hogan *et al.*, 1986). In an attempt to assess the optimal time for

microinjection, the efficiency of microinjection was recorded at different times of the day - these results are presented in Table 3.2.1. During this assessment, the day length in the animal house was automatically controlled and the time of lights-on and lights-off was kept constant. Microinjection was usually carried out between the hours of 2pm and 7pm but occasionally microinjection was started at 12 noon and on some occasions carried on until 8pm.

The efficiency of microinjection as measured by the number of embryos surviving microinjection did not vary significantly throughout the day. However, the proportion of injectable to uninjectable embryos was greatest between 2 and 4pm and declined later in the day (Table 3.2.1). The number of injectable embryos was significantly greater between 2-4 pm than between 4-6 pm ($x^2 = 26.1$: $P < 0.01$) and was significantly greater between 4-6 pm than 6-8 pm ($x^2 = 51.4$: $P < 0.01$).

Table 3.2.1. Time of Day - Effect on Efficiency of Microinjection.

Time of day. (P.M.)	No. of embryos placed in chamber (%)	No. of embryos microinjectable (%)[1]	No. of embryos surviving injection (%)[2]
12-2	77 (100)	52 (67.5)	36 (69.2)
2-4	1626 (100)	1074 (66.1)	787 (73.3)
4-6	1427 (100)	814 (57.0)	565 (69.4)
6-8	653 (100)	262 (40.1)	195 (74.5)

1. The percentage of the embryos placed in the chamber in which microinjection was attempted.
2. The percentage of embryos in which microinjection was attempted surviving injection.

Those embryos which did not appear obviously damaged were placed in culture overnight and the number developing to the two-cell stage recorded.

3.2.3. Cleavage Rate Of Zygotes To The Two-Cell Stage

A total of 3115 zygotes were microinjected with Tris-EDTA buffer containing DNA and then placed in culture and of these, 2532 embryos cleaved through to the two-cell stage giving an overall cleavage rate of 81.3%. These results include those embryos microinjected with a number of different constructs and different DNA concentrations (for a description of the different constructs see Chapter 1). The cleavage rate for zygotes injected with a solution which did not contain DNA - either

buffer alone or embryo culture medium being used - was 87.8%. Table 3.2.2. shows the cleavage rate for the different constructs and concentrations used.

Table 3.2.2. Cleavage Rate to the Two-Cell Stage After Microinjection.

Construct	[DNA]	1-cell	2-cell	(%)
Tk-env	2μg/ml	931	766	(82.3)
Tk-lacZ	2μg/ml	42	30	(71.4)
CMV-lacZ	2μg/ml	104	76	(73.0)
CMV-BGB	10μg/ml	688	540	(78.4)
Tk-BGB	10μg/ml	631	502	(79.6)
Tk-BGB/30	0.33μg/ml	719	618	(86.0)
Total Injected With DNA	-	3115	2532	(81.3)
Buffer Only	-	146	128	(87.7)
Medium 16	-	19	17	(89.5)
Medium PB1	-	16	14	(87.5)
Total Injected Without DNA	-	181	159	(87.8)
Overall Total No. Injected	-	3296	2691	(81.6)
Uninjected Controls		1023	855	(83.6)

The linear constructs; Tk-env, Tk-lacZ and CMV-lacZ were injected at a concentration of 2 μg/ml whereas the circular constructs Tk-BGB and CMV-BGB were injected at a concentration of 10 μg/ml and Tk-BGB/30 at a concentration of 0.33 μg/ml.

There was no significant difference between the two circular constructs injected at a concentration of 10 μg/ml. When the three linear constructs are compared it appears that significantly more embryos cleaved to the two-cell stage after injection with Tk-env than with the two linear lac Z constructs ($x^2 = 7.7$: $P < 0.01$). However, the number of embryos injected with the lac Z constructs was relatively small and although these experiments were conducted at different times this result may reflect differences in the quality of the DNA preparation.

The effect of DNA concentration can be examined by comparing the cleavage rate of those embryos microinjected with high and low concentrations of DNA and

comparing those embryos microinjected with DNA against those embryos injected with a DNA free solution. The circular construct Tk-BGB was injected at two different concentrations, 10 μg/ml and 0.33 μg/ml. The cleavage rate to the two-cell stage was significantly greater for those embryos microinjected with the lower concentration ($x^2 = 9.7$: $P < 0.01$).

In addition, a greater proportion of those embryos injected with either buffer or medium which did not contain DNA cleaved through to the two-cell stage than those injected with buffer carrying DNA. The difference was significant at the 5% level but not at the 1% level ($x^2 = 4.9$: $P < 0.05$).

A total of 1023 uninjected one-cell embryos were placed in culture after being held in the buffered medium for a period of time approximately equivalent to that of the injected group (embryos which were obviously abnormal were not included in either culture group). The cleavage rate in this group was 83.6%, there being no significant difference between this rate and that of the injected group. It is difficult to make a direct comparison between the two groups as those embryos in the injected group which were not considered suitable for microinjection were rejected. Therefore, embryos in the injected group were selected to a greater extent than those in the control group and this may have affected the result.

3.2.4. EMBRYO MORTALITY - *In Vitro* Preimplantation Development

In an experiment specifically designed to investigate the effect of pronuclear microinjection on the development of preimplantation embryos *in vitro*, embryos were injected with a number of different constructs and cultured through to the blastocyst stage in parallel with uninjected controls. The results are presented in Table 3.2.3. and Figure 3.2.2.

In this experiment 91% of controls developed from the one-cell to the two-cell stage and 86% of the resulting two-cell embryos developed to the expanded blastocyst stage when cultured *in vitro*. In the injected group 86% of microinjected embryos developed to the two-cell stage and 54% of these two-cells developed into expanded blastocysts. The cleavage rate from the one-cell to the two-cell stage did not significantly differ between the injected group and the control group ($x^2 = 2.4$: $P > 0.05$). However, a significantly greater number of embryos developed to the blastocyst stage from the two-cell stage in the control group when compared with the injected group ($x^2 = 57.7$: $P < 0.01$).

Examination of the cleavage rate from the one-cell stage to the two-cell stage revealed that there was no significant difference in the number developing to the two-cell stage between the DNA constructs and those embryos injected with buffer only

(x^2 = 0.3: P > 0.05). Similarly, there was no significant difference in the number of expanded blastocysts developing from the two-cell stage, between any of the groups of embryos injected with the different constructs nor the embryos injected with buffer only (results analysed using a 4x2 contingency table with 3 d.f. where x^2 = 2.0: P > 0.05).

Table 3.2.3. Cleavage Rate to the Blastocyst Stage After Microinjection.

Construct	1-cell	2-cell	4-cell	Morula	Blastocyst	1-cell/Blast. (%)	2-cell/Blast (%)
Controls	228	207	200	180	178	(78.0)	(86.0)
Buffer Injections	78	67	60	54	36	(46.2)	(53.7)
Tk-BGB/30	93	85	77	67	41	(44.0)	(48.2)
Tk-BGB	77	67	56	49	40	(52.0)	(59.7)
CMV-BGB	85	69	50	44	37	(43.5)	(53.6)
Total Injected	333	288	243	214	154	(46.2)	(53.5)

1-cell/Blast. and 2-cell/Blast. are the proportion of embryos developing from the 1-cell stage and the 2-cell stage to the blastocyst stage respectively.

In the control group a total of 228 embryos were placed in culture and of these a total of 50 embryos failed to develop. These consisted of 21 one-cell embryos which failed to cleave to the two-cell stage (42% of total loss), 7 two-cell embryos which failed to develop to the four-cell stage (14% of total loss), 20 four-cell embryos which did not form morulae (40% of total loss) and 2 morulae which failed to develop to the blastocyst stage (4% of total loss).

The pattern of embryo loss is quite different in the injected group. A total of 333 injected zygotes were placed in culture: of these 45 did not develop to the two-cell stage (25% of total loss), 45 two-cell embryos failed to develop (25% of total loss), 29 four-cells did not form morulae (16% of total loss) and 60 morulae failed to develop into blastocysts (33% of total loss).

Figure 3.2.2. Development of Microinjected Mouse Embryos

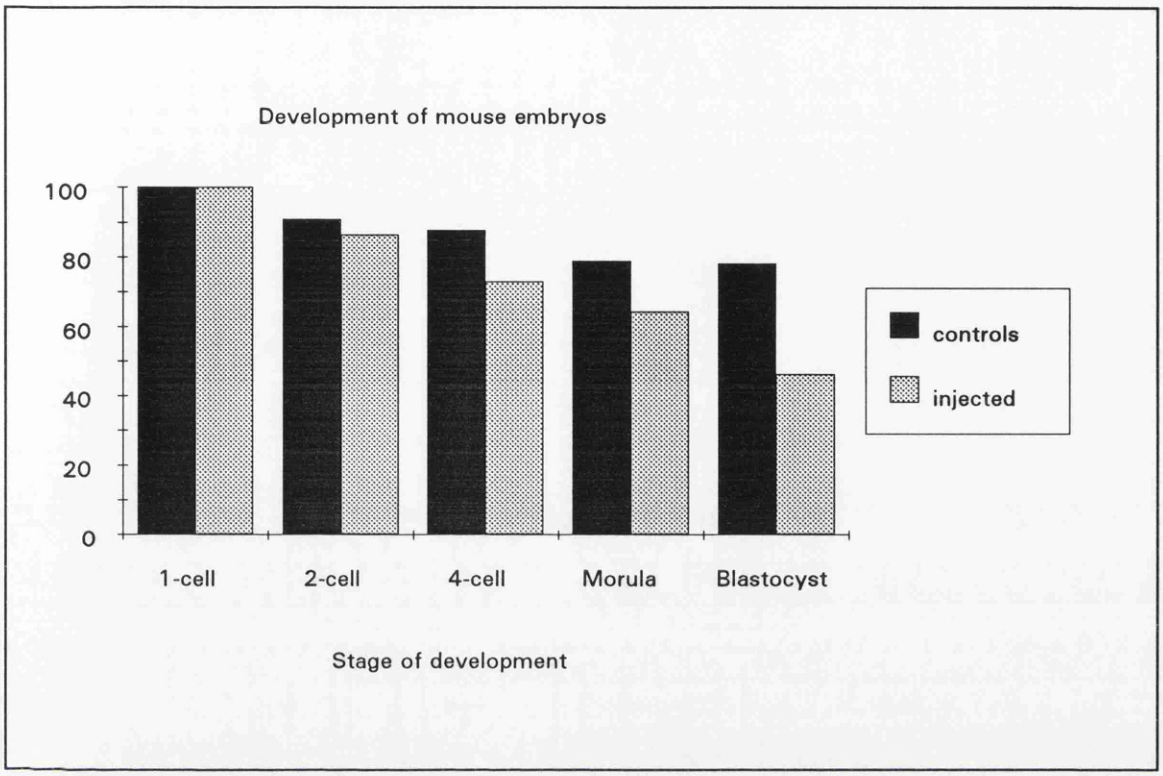


Figure 3.2.2. shows the difference between control and microinjected embryos in their ability to develop to the blastocyst stage.

Within the control group the embryonic loss from the one-cell to the two-cell stage is significantly greater than the loss at the two to the four-cell stage ($x^2 = 6.1$: $P < 0.01$) and the morula to the blastocyst stage ($x^2 = 12.4$: $P < 0.01$). In addition, the embryonic loss between the four-cell and morula stage is significantly greater than the loss at the two to the four-cell stage ($x^2 = 7.2$: $P < 0.01$) and the morula to the blastocyst stage ($x^2 = 13.7$: $P < 0.01$).

In the injected group the loss from the one-cell to the two-cell stage does not significantly differ from that between the two to the four-cell stage or between the four-cell to the morula stage. However, the loss between the morula to the blastocyst stage was significantly greater than the loss incurred between the one to the two-cell stage ($x^2 = 17.7$: $P < 0.01$), the two to the four-cell stage ($x^2 = 11.4$: $P < 0.01$) and the four-cell stage to the morula stage ($x^2 = 18.8$: $P < 0.01$).

3.2.5. EMBRYO MORTALITY - *In Vivo* Development Up To Midgestation

The difference in embryonic mortality between injected embryos and unmanipulated embryos during the first half of gestation was assessed by transferring

two-cell embryos into the oviducts of pseudopregnant recipients. Culling these recipients between 9-11 days post-coitus allowed their pregnancy status to be established.

In this experiment 13 recipients received unmanipulated control embryos, 3 recipients received embryos injected with Tk-BGB, 4 recipients received embryos injected with Tk-BGB and 2 recipients received embryos injected with CMV-BGB. Of the 13 recipients which received unmanipulated embryos 8 were pregnant and of the 9 recipients which received microinjected embryos 6 were pregnant. The results of this experiment are summarised in Tables 3.2.4. and 3.2.5.

Table 3.2.4. Embryo Development to Midgestation Stage After Microinjection.

	Controls Embryos	Injected Embryos
Total Number of Recipients.	13	9
Total Number of Pregnant Recipients.	8	6
Number of Embryos Transferred.	225	201
Number of Embryos Transferred Into P+.	147	151
Number of Uterine Swellings.	95	66
Number of Foetuses	83	47
Number of Viable Foetuses	79	43
Average Number of Embryos Transferred	17.3	22.3
*Average Number Embryos Transferred Into P+.	18.4	25.2
Average Number of Swellings	11.9	11.0
Average Number of Foetuses	10.4	7.8
Average Number of Viable Foetuses	9.9	7.2

*P+ represents those recipients which subsequently became pregnant.

The 8 recipients in the control group which were subsequently shown to be pregnant, received a total of 147 embryos. At day 9-11 65% of transferred embryos were represented as uterine swellings, 56% as identifiable foetuses with 54% of transferred embryos being judged as viable.

In the injected group 151 embryos were transferred into 6 recipients which subsequently became pregnant, of these 44% were represented as uterine swellings, 31% as foetuses with 28% of transferred embryos appearing viable at day 9-11.

Therefore, in those mice that became pregnant, significantly more transferred embryos resulted in swellings ($x^2 = 13.1$: $P < 0.01$), foetuses ($x^2 = 19.4$: $P < 0.01$) and viable foetuses ($x^2 = 19.7$: $P < 0.01$) in the control group when compared with the injected group.

A comparison between the number of viable foetuses recovered from the uterine swellings in the two groups reveals that a significantly lower number of viable foetuses were recovered from swellings in the injected group ($x^2 = 6.9$: $P < 0.01$). As embryos dying before implantation are unlikely to stimulate a uterine reaction this would appear to be an indication of the post-implantation foetal loss.

In those recipients which became pregnant, an average of 13.4 embryos were transferred into the control group and an average of 25.2 embryos were transferred in the injected group. Although embryonic loss was greater in the injected group, as calculated by the number of transferred embryos resulting in uterine swellings, there was no significant difference in the number of swellings found in each group. An average of 12 swellings was found in the control group and an average of 11 in the injected. Therefore, it was possible that the embryonic loss was influenced by the recipients' capacity to carry a large number of foetuses. However, it is unlikely that this factor had a major influence as in the control group one recipient had 19 swellings and yielded 19 foetuses and four other recipients had between 13 and 14 swellings.

Table 3.2.5. Embryo Development to Midgestation Stage After Microinjection - Different Constructs.

	Tk-BGB	Tk-BGB/30	CMV-BGB
Number of Recipients	3	4	2
Number of Pregnant Recipients	2	2	2
Number of Embryos Transferred	73	68	60
*Number of Embryos Transferred to P+.	51	40	60
Number of Swellings	19	22	25
Number of Foetuses	15	17	15
Number of Viable Foetuses.	12	16	15

*P+ represents those recipients which subsequently became pregnant.

Table 3.2.5. shows the comparison between the embryo transfer results of the 3 different constructs used. There is no significant difference in the embryonic mortality of those embryos receiving Tk-BGB and CMV-BGB. The sample size was

very small but a greater number of embryos survived to produce swellings and viable foetuses in the recipients receiving embryos microinjected with Tk-BGB/30 than either those receiving Tk-BGB or CMV-BGB injected embryos. The difference, however, is not significant (x^2 = 2.8 and 3.5 respectively: both $P > 0.05$).

3.2.6. EMBRYO MORTALITY - *In vivo* Development Up To Midgestation - Uterine Transfers

Control and microinjected embryos were cultured through to the blastocyst stage and transferred into the uterus of pseudopregnant recipients. These recipients were culled at between 9-11 days of pregnancy and embryo mortality assessed by examination of the uterus and its contents.

In the control group 48 transferred embryos were represented by 41 swellings (85%). This loss was not significantly different (x^2 = 0.7: $P > 0.05$) from the injected group in which 21 of the 27 transferred embryos were represented as swellings (77%). Therefore, the majority of transferred embryos implanted and initiated a uterine response.

However, in the injected group only 6 foetuses could be recovered from the 21 uterine swellings (29%) whereas in the control group 35 foetuses were recovered from 41 swellings (85%). Therefore, embryonic mortality between implantation and day 9-11 is significantly greater in the injected group (x^2 = 20.0: $P < 0.01$). These results are in agreement with the results described in the previous experiment and although the sample size is small it emphasises that there is significant embryonic loss beyond the blastocyst stage. These results are shown in Table 3.2.6.

Table 3.2.6. Embryo Development to Midgestation After Microinjection, *In Vitro* Culture and Uterine Transfer.

	Controls	Injected
Total Number of Recipients	3	3
Number of Pregnant Recipients	3	2
Total Number of Embryos Transferred	48	45
*Number Transferred into P+.	48	27
Number of Uterine Swellings.	41	21
Total Number of Foetuses Recovered.	35	6
Number of Viable Foetuses Recovered.	35	6

*P+ represents those recipients which subsequently became pregnant.

3.2.7. EMBRYO MORTALITY - *In Vivo* Development Up To Term

The great majority of microinjected and unmanipulated embryos transferred to pseudopregnant recipients were allowed to develop to term.

Forty-two recipients received unmanipulated control embryos and 71 recipients received injected embryos. The pregnancy rate in the control group was 40.5% and 45% in the injected group, the difference not being significant ($x^2 = 0.2$: $P > 0.05$).

In those recipients which became pregnant 35% of transferred embryos survived to term in the control group whereas only 25% of injected embryos survived to term. In those recipients which became pregnant the embryonic mortality in the injected group was significantly greater than that in the control group ($x^2 = 10.8$: $P < 0.01$). These results are summarised in Table 3.2.7.

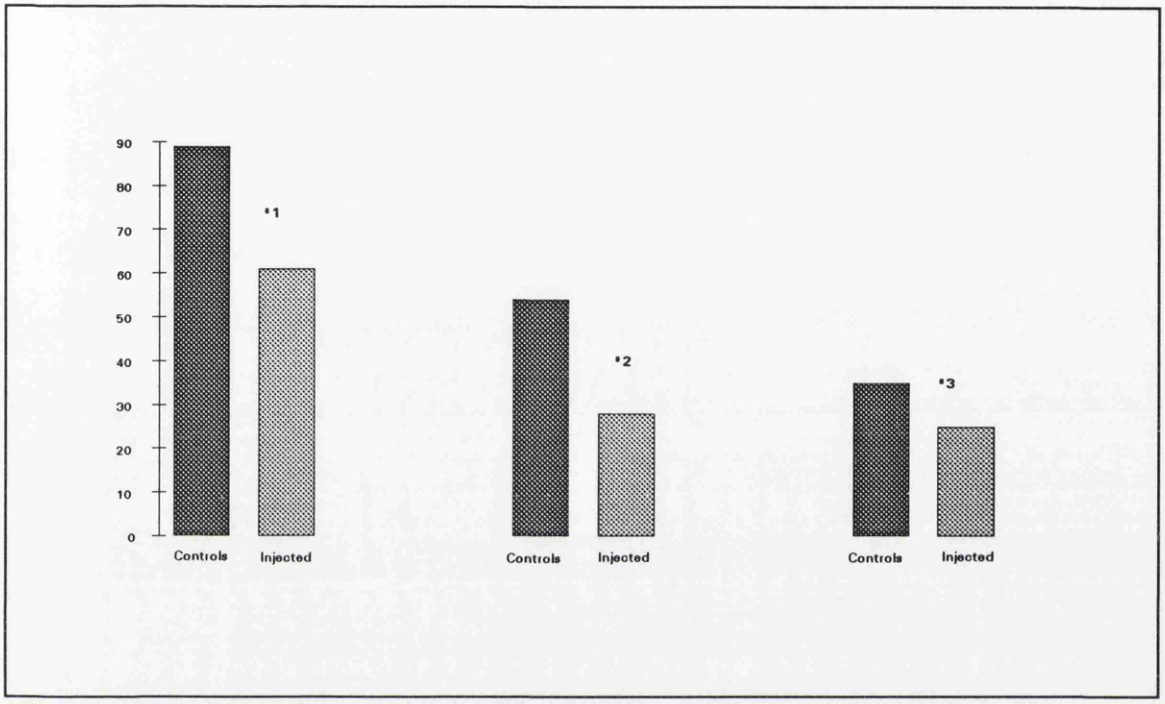
Table 3.2.7. Development to Term After Microinjection.

	Controls (oviduct)	Injected
Number of Recipients.	42	71
Number of Pregnant Recipients.	17	32
Number Transferred to Recipients	766	1446
*Number Transferred to P+	340	672
Number Born	119	169
Number Surviving	96	145
Number Transgenic	-	9
Percentage Pregnant	40.5%	45.0%
Average Number of Embryos transferred	18.2	20.4
*Average Number Transferred to P+	20.5	21.0
*Percentage Born/Transferred P+	35.0%	25.0%
Average Number Born	7.0	5.3
Percentage Born/Transferred to Recipients	15.5%	11.7%
Transgenic/Total Transferred.		0.62%
*Transgenic/Transferred P+		1.34%

*P+ represents those recipients which subsequently became pregnant.

Embryo survival results for *in vitro* culture to the blastocyst stage, embryo survival to midgestation and embryo survival to term after oviduct transfer are summarised in Figure 3.2.3.

Figure 3.2.3. Survival of Control and Injected Embryos to Three Different Stages of Development.



*1 *In vitro* embryo culture, percentage of 2-cell embryos developing to the blastocysts stage
*2 Percentage of embryos developing to mid-gestation
*3 Percentage of embryos developing to term.

Table 3.2.8. shows the embryo transfer results recorded for the different constructs. With the exception of those recipients which received embryos injected with Tk-BGB there was no significant difference in the pregnancy rate between the different constructs used: Tk-env, Tk-BGB/30, CMV-BGB and CD2-*myc* (based on a 2x4 contingency table with 3 d.f., $x^2 = 1.9$: $P > 0.05$). None of the twelve recipients receiving Tk-BGB injected embryos were pregnant.

From those recipients which were ultimately pregnant the following results were obtained. Of the recipients which received Tk-env injected embryos 61% were pregnant, the embryonic survival rate in these recipients being 14%. Embryos injected with CMV-BGB were transferred into 3 recipients, 2 of which became pregnant with 32% of embryos developing to term. In the Tk-BGB/30 group 54% of recipients were pregnant and 33% of embryos transferred to these pregnant recipients produced offspring. Finally, 43% of embryos transferred, after being injected with CD2-*myc*, went to term. In this last group 40% of mice were pregnant. Comparisons between the *in utero* mortality of embryos injected with the different constructs

reveals that the mortality of embryos injected with Tk-env is significantly greater than each of the other constructs, Tk-BGB/30, CMV-BGB and CD2-*myc* (x^2 = 24.5, 48.9 and 9.6 respectively, all $P < 0.01$).

These results are reflected in the average litter sizes born to each pregnant recipient. The Tk-env group with a high mortality *in utero* had an average litter size of 2.8 whereas the Tk-BGB/30, CMV-BGB and CD2-*myc* groups had average litter sizes of 7.1, 7.0 and 9.7 respectively.

Table 3.2.8. Transgenic Mouse Production - Different Constructs.

	Controls		Tk-env	Tk-BGB	Tk-BGB 30	CD2-*myc*	CMV-BGB
	Oviduct	Uterine					
Number of Recipients	42	17	28	12	13	15	3
*Number of P+	17	9	17	0	7	6	2
Number of Embryos Transferred	766	328	532	237	289	330	58
*Number of Embryos Transferred to P+	340	158	342	0	152	134	44
Number Born	119	84	47	0	50	58	14
Number Weaned	96	84	37	0	36	58	14
Number Transgenic	-	-	5	0	0	2	2
Percentage Pregnant	41%	53%	61%	0%	54%	40%	66%
Average Number of Embryos/Recipient	18.2	19.3	19.0	19.8	22.2	22.0	19.0
*Average Number of Embryos/P+	20.5	17.6	20.6	-	21.7	22.3	21.5
*Percentage of Transferred Embryos Born to P+	35%	53%	14%	0%	33%	43%	33%
Average Number Born	7	9.3	2.8	0	7.1	9.7	7.0

*P+ represents those recipients which subsequently became pregnant.

Table 3.2.8. also shows a comparison between those recipients in the control group receiving two-cell embryos transferred into the oviduct with those receiving either morula or blastocyst stages into the uterus.

Embryo transfer into the oviduct resulted in a pregnancy rate of 40.5% whereas transfer into the uterus resulted in a pregnancy rate of 53%. The difference was not significant (x^2 = 0.8: $P > 0.05$).

The embryonic survival rate in those recipients which became pregnant after oviductal transfer was 35%, whereas in those recipients that became pregnant after embryos were transferred into the uterus the survival rate was 53%. Therefore, there was significantly greater embryonic mortality in those recipients which received two-cell embryos transferred into the oviduct than in those which had embryos transferred into the uterus after *in vitro* culture ($x^2 = 14.7$: $P < 0.01$). This result does not, however, account for the embryonic loss sustained during *in vitro* culture.

3.2.8. Transgenic Production - Sperm Vector Method

An attempt was made to produce transgenic mice following the method described by Lavitrano *et al.* (1989). This involved collecting unfertilised oocytes, incubating them in the presence of freshly collected epididymal spermatozoa and 1μ g/ml Tk-BGB, moving the embryos into embryo culture medium and transferring the resulting cleavage stage embryos into suitably primed recipients. Therefore, those embryos transferred had not been microinjected but had been collected as oocytes and fertilised *in vitro*.

In this experiment 155 embryos were transferred into 8 recipients (results shown in Table 3.2.9.). Five of these recipients subsequently became pregnant after receiving a total of 101 embryos. Forty-two (42%) of these embryos developed through to term. Neither the pregnancy rate ($x^2 = 1.3$: $P > 0.05$) nor the embryonic mortality ($x^2 = 1.4$: $P > 0.05$) differed significantly from the control embryo transfer results shown in Tables 3.2.7. and 3.2.8. .

Table 3.2.9. Transgenic Production Using Sperm Vector Method.

Total Number of Recipients	8
Pregnant Recipients	5
Percentage Pregnant	63%
Total Number of Embryos Transferred	155
Percentage Born/Transferred	27%
*Number of Embryos Transferred to P+	101
*Percentage Born/ Transferred to P+	42%
Number Born.	42
Average Litter Size.	8.4
Number Surviving	41

*P+ represents those recipients which subsequently became pregnant.

The details of the pregnancy results were not recorded for another 5 recipients which received embryos fertilised in the presence of Tk-BGB. These recipients produced another 17 offspring. None of the offspring produced by this method were transgenic.

3.2.9. Integration of Exogenous DNA Following Pronuclear Microinjection

The numbers of transgenic animals produced using the different DNA constructs are shown in Table 3.2.10. Of the 37 offspring weaned after being microinjected with Tk-env, 5 (14.2%) proved to be transgenic.

From 14 mice microinjected with CMV-BGB only 2 were positive for the integrated transgene (14%). In addition, 15 day 9-11 foetuses were recovered after microinjection with CMV-BGB, 1 of which was positive for the transgene.

No offspring were born after microinjection with Tk-BGB. However, 12 foetuses (9-11 days) were recovered and of these 3 were positive for the transgene.

Thirty-six offspring and 5 foetuses were analysed by Southern blotting after being microinjected at the zygote stage with Tk-BGB/30. None of these proved to be positive for the transgene. This finding agrees with the work of Brinster *et al.*, (1985). These workers showed that if the injection concentration fell below 1 μg/ml the integration frequency declined. A total of 58 offspring which had been microinjected with CD2-*myc* were weaned. Two of these mice were positive for the transgene when analysed by Southern blotting.

Therefore in total 145 offspring were produced 9 (6.3%) of which were transgenic and of 27 midgestation foetuses recovered 4 (14.8%) were transgenic. The figures are remarkably consistent for three out of the four groups as 14% of offspring injected with Tk-env, 14% of offspring injected with CMV-BGB and 15% of foetuses injected with either CMV-BGB or Tk-BGB were transgenic. However, only 3.4% of offspring injected with CD2-*myc* were transgenic. It is possible that transgenic foetuses expressing this construct died in utero as offspring derived from these two lines of mice did not express this construct in any of the tissues analysed.

Based on the number of transferred embryos which ultimately resulted in transgenic mice the efficiency of production for Tk-env, Tk-BGB, Tk-BGB/30, CMV-BGB and CD2-*myc* was 0.93%, 0%, 0%, 3.4% and 0.6% respectively.

In summary, the overall results reveal that 75% of embryos did not survive to term and only 1.3% of transferred embryos resulted in live transgenic mice. The fate of embryos transferred to pregnant recipients is represented in Figure 3.2.4..

Table 3.2.10. Overall Efficiency of Transgenic Production.

	Tk-env (2 μg/ml)	Tk-BGB (10 μg/ml)	CMV-BGB (10 μg/ml)	Tk-BGB/30 (0.33 μg/ml)	CD2-*MYC* (2 μg/ml)
Number Injected Embryos Transferred	532	237	58	289	330
Number of Recipients	28	12	3	13	15
Number of Pregnant Recipients	17	0	2	7	6
Number of Offspring Weaned	37	0	14	36	58
Number of Offspring Transgenic	5	0	2	0	2
Percentage Transgenic	14%	0	14%	0	3%
Overall Efficiency (%)	0.93%	0	3.4%	0	0.6%

Figure 3.2.4. Embryos Transferred to Pregnant Recipients/Proportion Transgenic.

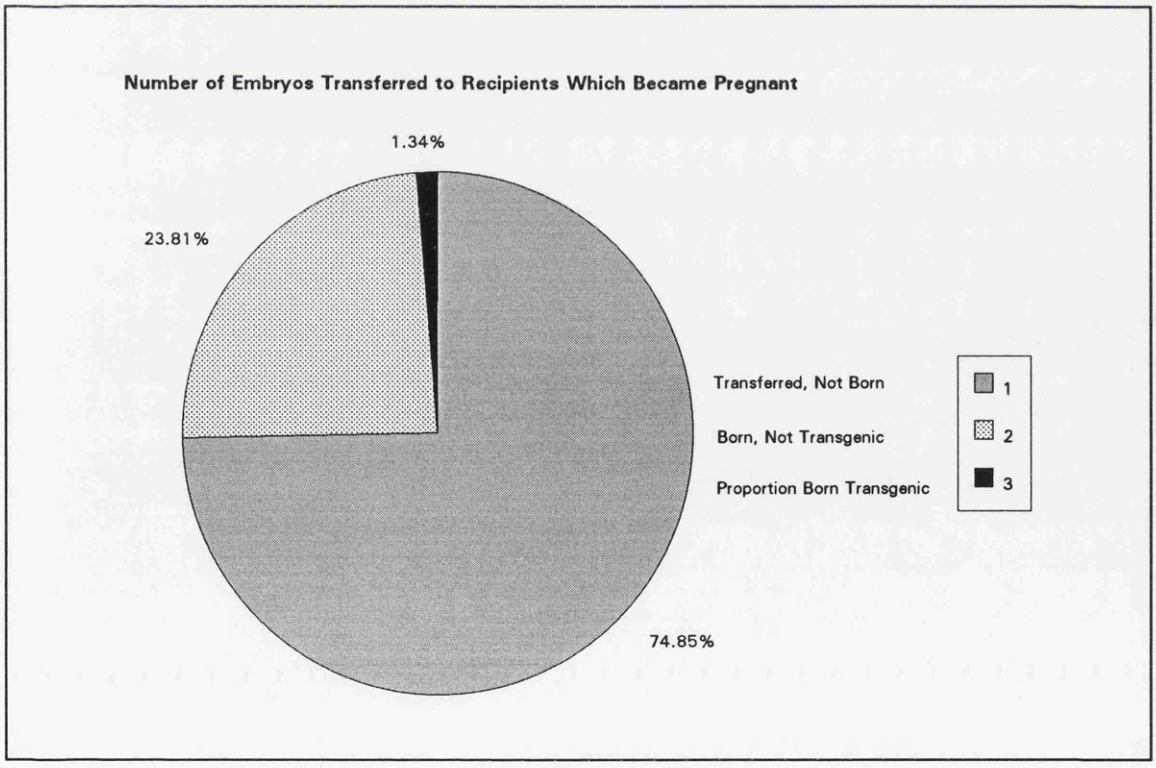


Figure 3.2.4. shows the fate of embryos transferred to those recipients which ultimately became pregnant.

TRANSGENIC PRODUCTION

SECTION 3.3. - DISCUSSION

The ability to incorporate exogenous DNA sequences into the mammalian germ line has proved to be an extremely fruitful technique for addressing questions in many areas of biomedical science (for reviews see: Gordon and Ruddle, 1983; Palmiter and Brinster, 1985; Palmiter and Brinster 1986; Jaenisch, 1988; Cuthbertson and Klintworth, 1988). The successful production of transgenic animals was first reported in 1980 by Gordon *et al.* and shortly afterwards five other groups reported the introduction of cloned DNA into mice (Wagner E. *et al.*, 1981a; Brinster *et al.*, 1981c; Costantini and Lacy, 1981; Wagner T. *et al.*, 1981c; Harbers *et al.*, 1981).

In this study nine transgenic lines were produced using three different DNA constructs. In 1985, Brinster *et al.* examined the factors which affected the efficiency of introducing foreign DNA into mice. Under optimal conditions of DNA form and concentration 24-31% of mice that developed contained integrated DNA sequences. This result is far superior to those reported in this study which range from 3.4% to 14%. Brinster's work demonstrated that the integration frequency of linear molecules was five times more efficient than circular plasmids. This finding was not reflected in this study in which the proportion of transgenic offspring born after microinjection with the circular plasmid was greater than that achieved with the linear DNA. However, in this study the sample size was comparatively small and the results achieved with linear DNA was considerably below that of Brinster's group.

Embryo Mortality After Pronuclear Injection

Embryo mortality following pronuclear microinjection was assessed at three different stages of development: at the blastocyst stage after *in vitro* culture, at mid-gestation (day 9 to 11) and at term. Pronuclear microinjection significantly reduced the number of embryos developing to the blastocyst stage *in vitro*, 86% of unmanipulated two-cell embryos reached the expanded blastocyst stage whereas only 54% of manipulated embryos developed to this stage. The mortality rate of embryos injected with buffer containing no DNA was comparable to those injected with DNA. It appears, therefore, that the presence of DNA in the microinjection solution does not significantly increase the mortality rate - at least during preimplantation development, although high concentrations of DNA did appear to significantly affect the number of embryos successfully completing the first cleavage division. In 1985 Brinster *et al.* examined preimplantation development to the morula and blastocyst stages. There was no difference in the culture rate to the morula and blastocyst stage between

embryos injected with buffer or low concentrations of DNA. However, at concentrations of 10 μg/ml or above the survival rate of cultured preimplantation embryos was further reduced. In the results presented here there was no significant difference in the embryo culture rate between embryos injected with buffer alone or DNA injected at either 0.33 μg/ml or 10 μg/ml although in this study only supercoiled plasmid DNA was injected at 10 μg/ml. The reduction in preimplantation embryo viability following pronuclear microinjection has also been observed in other species. Rexroad and Wall (1987) found that microinjection of sheep embryos significantly reduced development to the thirty-two cell stage.

King (1985) reviewed the intrinsic and extrinsic factors which affect embryo development and emphasised that despite appearing healthy the potential for development can be reduced by the *in vitro* handling of embryos. Therefore, a number of workers have used alternative methods to assess embryo viability including: the rate of embryo development (Harlow and Quinn, 1982), the ultrastructural appearance of the embryo (McReynolds and Hadek, 1972), metabolic parameters (Butler *et al.*, 1988) and by the degree of sister chromatid exchange (Saito *et al.*, 1984). However, the definitive test of success in experimental manipulations of mammalian embryos is the transfer to foster mothers and subsequent development to term (Whittingham, 1981).

When the development of microinjected embryos was examined at midgestation significantly more transferred embryos were represented as foetuses in the control group than in the injected group, 54% and 28% respectively developing to midgestation. Comparing these figures it appears that increased losses of injected embryos continue after the preimplantation stage. This is supported by the finding that only 65% of obvious uterine swellings yielded viable foetuses in the injected group compared with 83% in the control group.

The two groups also differed in the number of embryos transferred, on average 25 injected embryos were transferred to each recipient whereas only 18 control embryos were transferred to each recipient. If overcrowding at the time of implantation was an additional cause of embryo loss this would further reduce the proportion of embryos surviving in the injected group. However, the observation that significant embryo mortality occurred after the blastocyst stage was confirmed by transferring injected embryos at the blastocyst stage and assessing foetal loss at midgestation. In the injected group only 22% of transferred blastocysts were represented as foetuses at day 9-11 whereas 73% of control blastocysts developed to this stage. Wilmut *et al.* (1990) monitored foetal development using ultrasound scanning after the transfer of microinjected sheep embryos to recipient ewes and similarly showed that foetal mortality continued through development.

The results presented here also suggest that the efficiency of transgenic production may be slightly greater if embryos are transferred as soon as possible after collection. Only 12% of manipulated two-cell embryos survived to day 9 following culture to the blastocyst stage and transfer to a pseudopregnant recipient whereas when injected embryos were transferred at the two-cell stage 28% survived to mid-gestation. This pattern of increased loss after culture and transfer was not observed in the control group. However, Massip *et al.* (1984) have reported that the deleterious effects of handling preimplantation embryos are additive and Hahn and Schneider (1982) noted that the observed decrease in viability after transfer is related to the duration of *in vitro* culture.

The embryonic mortality in the injected group was significantly greater than in the control group. In those recipients which became pregnant 35% of unmanipulated embryos transferred at the two-cell stage developed to term whereas only 25% of manipulated embryos resulted in viable neonates. The 35% survival rate in the control group was probably an underestimate as most of the control embryo transfers were attempted at the start of the transgenic programme. Experiments conducted after more experience had been gained revealed that survival rates of 42% could be achieved after *in vitro* fertilisation, culture to the two-cell stage and transfer. In 1970 Bronson and McLaren explored the factors which affected the success of mouse oviduct embryo transfer. These authors showed that the size of fluid volume transferred was important and that under optimal conditions a pregnancy rate of 63% and an embryo survival rate of 71% could be achieved in the pregnant recipients.

The deferred loss of manipulated embryos observed in these results presumably results from genetic damage sustained at the time of microinjection. The stage of development at which individual embryos and foetuses die may reflect damage to genes which have a specific function to perform at some window of ontogeny. Chromosomal damage which involves genes required for important house keeping functions probably causes the loss of embryos at an early stage of development whereas genes required at a specific stage of development will cause embryonic mortality at that time or shortly after.

The nature and the extent of genetic changes that result from microinjection have not been investigated. Jaenisch (1988) has reported that the incidence of insertional mutagenesis in transgenic lines may be slightly greater for transgenic mice produced by pronuclear injection than by infection with retroviral vectors. However, creating transgenic mice (Van der Putten *et al.*, 1985) or sheep (Harvey *et al.*, 1990) by infecting early embryos with retroviral vectors or retroviruses does not appear to cause the high levels of embryo mortality which are associated with pronuclear microinjection. Therefore, insertional mutagenesis alone cannot account for the

extent of embryo and foetal loss observed and it seems likely that the physical disturbances associated with microinjection causes non-specific damage to chromatin.

For obvious reasons most attention has been directed at those mice which survive and carry the transgene. Estimates of the proportion of transgenic mice carrying recessive lethal mutations have varied from 7% (Palmiter and Brinster, 1986) to 15% (Covarrubias *et al.*, 1987). These are discovered when heterozygotes carrying the transgene at the same site of insertion are bred together in an attempt to produce homozygotes. In a proportion of cases, litters from these crosses are smaller than normal and homozygote animals are absent. More detailed reports have shown that a proportion of these embryos or foetuses die at various stages of development (Mark, Signorelli and Lacy, 1985; Wagner *et al.*, 1983). Mutagenesis can be caused by natural (Panthier *et al.*, 1988) or experimentally induced (Jaenisch *et al.*, 1983; Soriano *et al.*, 1987) infection of germ cells by murine retroviruses. In 1981 Jaenisch *et al.* infected embryos with MuLV. One of the transgenic lines resulting from this experiment contained a proviral insertion in the first intron of the α-1 (I) collagen gene and homozygotes for this integrated provirus failed to develop to term due to haemorrhage from a weakened vascular system (Jaenisch *et al.*, 1983; Schnieke *et al.*, 1983; Lohler *et al.*, 1984).

The chromosomal changes in some transgenic lines have been examined in detail. Wagner *et al.* (1983) were the first to report that insertional mutagenesis could be a sequel to pronuclear microinjection of one-cell embryos. These workers produced six lines of transgenic mice carrying human growth hormone sequences. Two of these six lines contained inserts that caused prenatal lethality in homozygotes for the transgenes. These transgenic lines were investigated further (Covarrubias *et al.*, 1986; Covarrubias *et al.*, 1987). Although these transgenes were inserted into different chromosomes there were a number of similarities between the two lines. Both caused embryonic mortality shortly after implantation (day 4-5) at the egg cylinder stage. In addition, there were extensive rearrangements of the flanking cellular DNA in both lines and a probable deletion in one of these two lines.

Southern blot data of transgenic lines reveals that insertion of transgene sequences is commonly associated with rearrangements of the flanking cellular DNA (Covarrubias *et al.*, 1987) although this does not necessarily cause mutations. A more unusual example of embryo mortality associated with a transgenic line was reported by Mahon *et al.* (1988). When heterozygotes carrying this transgene were crossed with non-transgenic mice the resulting litters were only 40% of the size expected, however, of those offspring born 50% were transgenic. Further analysis revealed that normal numbers of embryos implanted but around 60% failed to develop beyond day seven. Cytogenetic and molecular analysis showed that this line of mice

carried a reciprocal translocation between chromosomes 6 and 17 and that the transgene was sited at or close to the breakpoint of one the translocated chromosomes.

In addition to lethal mutations examples of non-lethal mutations have been described. Woychik *et al.* (1985) produced a line of transgenic mice with limb deformities associated with the site of insertion, a severe limb deformity in homozygote mice has also been reported by McNeish *et al.* (1988). Palmiter *et al.* (1983) produced a line of mice in which male transgenic mice were incapable of transmitting the transgene to their offspring although otherwise they appeared fertile and produced litters of normal size. These workers concluded that this was due to the disruption of a gene involved in spermatogenesis. In addition to recessive mutations it has been suggested that dominant mutations could also occur where the integrating transgene alters the regulation of an endogenous gene (Palmiter and Brinster, 1986).

Both retroviral infection and pronuclear microinjection can result in an approximately equal incidence of insertional mutagenesis. Whereas proviral integration appears to operate via an ordered and precisely controlled mechanism resulting in minimal disturbance to the endogenous genetic material, pronuclear microinjection can produce extensive rearrangements which include deletions, duplications and even translocations. These rearrangements may be promoted by the proposed unstable nature of the intermediate stages (Covarrubias *et al.*, 1987) or may be secondary to the pressure changes in the pronucleus caused by the injection process itself. It is thought that integration occurs at spontaneous breakpoints in the chromosomes but the role which microinjection plays in promoting these chromosomal breaks is unknown. However, it is likely that the physical disturbance associated with microinjection produces a large number of breaks in the chromosomes not necessarily associated with the presence of DNA and that this in itself leads to a number of rearrangements during the repair processes. This may explain the large proportion of deferred loss associated with microinjection.

Whatever the mechanism for producing the widespread genetic damage, inferred from the rate and timing of embryonic loss, it is clear that the application of transgenic technology will be a powerful method of generating mutagenesis. Naturally occurring mutations are comparatively rare in mammals and those agents (such as retroviruses) capable of inducing genetic damage in the germline of experimental animals have yielded a surprisingly diverse amount of useful information concerning the biology of these animals and mammalian biology in general. The attraction of this system is that the site of insertion is usually tagged by the transgene itself making the initial characterisation of the affected locus a little easier.

Spermatozoa As Vectors For DNA Transfer

In 1989 Lavitrano *et al.* reported that it was possible to produce transgenic mice using a very simple and previously untried technique. These workers incubated mature sperm cells with plasmid DNA and found that the exogenous DNA became intimately associated with the spermatozoa within 15 minutes. Sperm treated in this way were capable of carrying the foreign DNA into the eggs at fertilisation with the resultant production of transgenic mice carrying the integrated plasmid sequences.

Although three methods of producing transgenic mice had previously been developed most workers producing transgenic mice have chosen to use pronuclear microinjection as it is technically the simplest approach and the only one shown to be capable of producing transgenic lines of domestic animals. Nevertheless it is a painstaking procedure and is of low efficiency with the majority of injected embryos not developing to term.

In contrast, in the experiments described by Lavitrano *et al.*, 30% of offspring born contained plasmid sequences and embryonic mortality was not reported as being significantly greater than expected after *in vitro* fertilisation, culture to the two-cell stage and transfer. This procedure obviated the need for expensive equipment and the high degree of technical skill normally required for transgenic mouse production and promised to make the production of transgenic animals and particularly transgenic livestock, much more accessible and efficient

The results presented in this study represent an attempt to produce transgenic mice using the protocol described by Lavitrano *et al.*. The lac Z containing plasmid Tk-BGB was incubated with freshly collected spermatozoa. The sperm was then used to fertilise freshly collected oocytes and those embryos which developed to the two-cell stage were transferred. Five separate experiments resulted in 58 mice being born. These mice were screened for the presence of Lac Z sequences but none of these mice were transgenic.

Other attempts to verify this approach have also met with failure. Brinster *et al.* (1989) published results from their own group and also pooled results from eight other groups. A total of 1312 mice were produced using 18 different gene constructs some of which were linear and some supercoiled. None of the subsequent mice or foetuses produced showed evidence of DNA uptake in either an integrated or episomal form.

To date no other groups have successfully managed to repeat this method of producing transgenic mice. However, a subsequent report from the same group (Gandolfi *et al.*, 1989) details the generation of transgenic pigs using a method very similar to that described for mice. In this work Gandolfi *et al.* (1989) incubated boar spermatozoa with pSV2CAT plasmid DNA and the transformed sperm was then used to surgically inseminate oestrous sows by depositing the sperm in the uterine horns.

Twenty-one percent of resulting piglets were transgenic and all of them expressed the CAT gene.

The reasons underlying the inability of other research groups to repeat these results in mice and pigs remains to be elucidated. The ability to produce transgenic animals using this greatly simplified approach would significantly increase the accessibility of this area of research to many interested groups.

CHAPTER - 4

LAC Z EXPRESSION IN THE PREIMPLANTATION MOUSE EMBRYO

SECTION 4.1. - INTRODUCTION.

The primary objective of the experiments described in this chapter was to investigate the expression potential of the bacterial lac Z gene within the preimplantation embryo.

Initially this work was undertaken to compare the activity of two different promoters, HSV-Tk and CMV, both of which were linked to the indicator gene lac Z. The fate of exogenous DNA after microinjection was investigated by following the lac Z expression pattern through preimplantation development and an attempt was made to assess the role that methylation of the introduced DNA may have played in modulating expression.

As a background to these studies the following literature review summarises the work of those authors who introduced nucleic acid into the preimplantation embryo either to elucidate mechanisms of gene and message regulation in the early embryo or to yield information on the activity of the foreign DNA itself. This is followed by a brief description of the role of DNA methylation in the regulation of gene transcription.

The Introduction of Nucleic Acid into Preimplantation Embryos

The introduction of exogenous nucleic acid into oocytes and developing embryos can yield information on both the physiology of the tissue under study and the functional properties of the foreign nucleic acid. The use of murine oocytes and early embryos to explore the stability and regulation of exogenous nucleic acid was an extension of similar studies using the xenopus oocyte and the techniques developed in these experiments were quickly exploited to produce the first transgenic mice.

Microinjection with mRNA or DNA has provided information about the processing of nucleic acid by oocytes and embryos and has aided the understanding of the role which these cell types play in development.

Brinster *et al.* (1980, 1981b) demonstrated that mouse oocytes and fertilised ova were capable of translating foreign mRNA injected into the cytoplasm. Ebert *et al.* (1984) showed that there were marked differences in the stability of mRNA between

oocytes and fertilised ova. These workers injected ovalbumin and conalbumin mRNA into oocytes and fertilised one-cell embryos and demonstrated that the stability of these messages was 30 and 120 times greater respectively in oocytes than in fertilised ova. These observations reflect the different roles which these cell types play in development. Endogenous mRNA levels are relatively stable in unfertilised oocytes but after fertilisation certain species of maternal RNA are specifically degraded.

Injection of DNA into the nucleus also revealed differences in the nucleic acid metabolism at different stages of development. Genes transcribed by RNA polymerase III were expressed much more efficiently in oocytes than in fertilised one-cell embryos (Brinster *et al.*, 1981b) whereas the transcriptional activity of RNA polymerase type II genes was much greater in the fertilised egg than in the oocyte (Chen *et al.*, 1986). Further studies by Chen *et al.* (1986) demonstrated that the rate of protein synthesis and decay was similar for both oocytes and fertilised embryos.

In the mouse the embryonic genome is activated at the two-cell stage (Bolton *et al.*, 1984) and the transition from maternal to embryonic control is another area of early development which has been studied extensively. Microinjection of one-cell embryos has yielded information on this phenomenon. Viable one-cell embryos are unable to express genes introduced into the pronuclei (Chen *et al.*, 1986; Bonnerot *et al.*, 1987; Martinez-Salas *et al.*, 1989). It has been suggested that they are deficient in the transcriptional machinery which is necessary to carry out this function, thereby reflecting the quiescence of the genome at this stage of development (Martinez-Salas *et al.*, 1989). The time point at which embryos develop the capacity to express the foreign DNA coincides with activation of the embryonic genome (Martinez-Salas *et al.*, 1989). Expression is not dependent upon cleavage to the two-cell stage as spontaneously blocked one-cells and one-cell embryos arrested with aphidicolin can express exogenous DNA at high levels. A number of workers have reported that blocked one-cell embryos transcribe genes more effectively than developing two-cell embryos and that cleavage to the two-cell stage is associated with a reduction in transcriptional activity (Stuart *et al.*, 1984; Chen *et al.*, 1986; Ao *et al.*, 1988; Martinez-Salas *et al.*, 1989; W. Reik pers. comm.).

Martinez-Sales *et al.* (1989) have used the Luciferase gene (*Luc*) to measure expression in preimplantation embryos. Expression levels were greatly reduced at the two-cell stage and this group suggested that progression to the two-cell stage is associated with the appearance of a negative regulatory influence. Consistent with this view they found that inclusion of enhancer elements (sequences which up-regulate gene expression) to the gene construct increased expression at the two-cell stage but were not required at the one-cell stage (Martinez-Salas *et al.*, 1989).

Chen *et al.* (1986) and Ao *et al.* (1988) have assayed the transcriptional activity of injected exogenous DNA at different stages of preimplantation development.

Using the Thymidine Kinase gene, Chen *et al.* (1986) noted that enzyme activity did not appear until 12 hours after injection. Activity increased rapidly during the following 12 hours and remained level over the next 24 hours. These authors presented evidence to suggest that transcriptional activity declined markedly with cleavage and that it was possible that the plateau of activity observed represented a balance between residual synthesis and degradation. In addition, Ao *et al.* (1988) showed that expression of a gene construct coding for hypoxanthine phosphoribosyl transferase (HPRT) injected at the one-cell stage declined after the four-cell stage.

As well as using exogenous DNA to dissect the processes of the early embryo, the preimplantation embryo can be used as a vehicle to yield information concerning the DNA construct introduced. In 1982 Brinster *et al.* investigated the nature of the metallothionein (MT-I) promoter using preimplantation embryos. By fusing the MT-I promoter to the Thymidine Kinase (Tk) gene of herpes simplex virus (HSV), the enzyme product of which could be readily assayed, these authors were able to determine that the MT-I promoter was as effective as the normal HSV Tk promoter and that the activity of the MT-I promoter could be modulated by the presence of cadmium. Using this assay system these workers subsequently identified the elements within the MT-I promoter which were responsive to heavy metal stimulation (Stuart *et al.*, 1984).

Murine embryos have also been used to investigate the action of antisense RNA. This system has enabled researchers to measure the reduced activity of the target gene and the concentration of antisense DNA required to exert this effect (Ao *et al.*, 1988; Bevilacqua *et al.*, 1988).

Bonnerot *et al.* (1987) microinjected the lac Z gene of *E. coli* into fertilised ova and observed expression at the two-cell stage. These workers examined the activity of a number of promoters fused to the lac Z reporter gene. While the promoters of Rous Sarcoma Virus (RSV), Simian Virus 40 (SV40) and β-actin were equally capable of directing transcription in two-cell embryos, the long terminal repeat of Moloney Murine Leukaemia Virus (MoMuLV) was not functional even when high copy numbers of this construct were injected.

DNA Methylation

Mammalian DNA can be modified by the addition of a methyl group to the pyrimidine base, cytosine. This event occurs almost exclusively at the CpG dinucleotide. DNA methylation appears to be involved in the control of gene expression at several different levels including the transcriptional regulation of tissue specific and housekeeping genes (Yisraeli and Szyf, 1984; Stein *et al.*, 1983; Bird *et al.*, 1985; Bird, 1986), the formation of active and inactive chromatin (Keshet *et al.*,

1986; Michalowsky and Jones, 1989; Riggs, 1990), X-chromosome inactivation (Holliday and Pugh, 1975; Riggs, 1975; Monk, 1990) and in the differential modification of parental DNA (genomic imprinting) which has been reviewed by Surani *et al.* (1990).

In general, tissue specific genes tend to be methylated and inactive in all tissues except the tissue of expression where they are comparatively undermethylated (Naveh-Many and Cedar, 1981; Yisraeli and Szyf, 1984). Transfection studies have revealed that while unmethylated DNA transferred into tissue culture cells is active, methylated DNA constructs are not (Stein *et al.*, 1982; Busslinger *et al.*, 1983; Yisraeli *et al.*, 1986).

In contrast, housekeeping genes have a ubiquitous expression pattern. Many of these genes are characterised by clusters of CpG dinucleotides situated at the 5' end of the gene (Bird *et al.*, 1985). These areas known as CpG islands are not usually methylated (Stein *et al.*, 1983). The CpG islands associated with housekeeping genes on the inactive X-chromosome are, however, methylated (Toniolo *et al.*, 1988).

It is believed that methylation also plays a role in development acting as a general mechanism of gene control. Several workers have investigated the methylation status of DNA in different cell types involved in early embryonic development. Sanford *et al.* (1987) analysed the DNA methylation status of oocytes and spermatozoa. They examined both dispersed repeated sequences and specific genes and found that these DNA sequences were heavily methylated in sperm DNA and relatively undermethylated in oocyte DNA. Methylation levels in preimplantation embryos appeared to be intermediate, implying that the methylation status of the sperm and oocyte DNA was propagated through preimplantation development. Monk *et al.* (1990), who have also investigated the methylation status of preimplantation embryos, have reported a reduction in overall methylation levels between the morula and blastocyst stages.

Other workers have argued that *de novo* methylation can occur in the early embryo (Jahner and Jaenisch, 1984). This group introduced Murine Leukaemia Virus (MuLV) into preimplantation embryos, the resulting provirus integrated into the host genome but was not expressed rendering the virus incapable of replication. Analysis of the adult mice originating from the infected embryos revealed methylated viral sequences (Jahner *et al.*, 1982). These authors suggested that a methylation event occurred during preimplantation development and that this was responsible for the methylation of the viral genome. In addition, Surani *et al.* (1990) have recently reported that a *de novo* methylation event occurs during preimplantation development. These findings and the observation by Adams that methylase enzyme activity is high in early cleavage stages (Adams pers. comm.) argue that DNA methylation can occur in the early embryo and that the introduction of exogenous unmethylated DNA into a

system which has high DNA methylase activity may well result in rapid methylation of the introduced sequences.

In a series of experiments Buschhausen and co-workers (1987) examined the interaction between gene expression, DNA methylation and the assembly of gene sequences into chromatin. Using a tissue culture system these authors microinjected both methylated and unmethylated forms of the Herpes Simplex Virus Thymidine Kinase gene (HSV-Tk) and assayed the resulting Tk activity. They found that initially both forms of Tk were capable of being expressed. Eight hours after injection, however, transcription from the methylated gene had peaked and by sixteen hours post-injection Tk activity in these cells could not be detected. Tk activity from the unmethylated construct was not affected. The time taken to inhibit expression of the methylated Tk gene was similar to the time required for the assembly of introduced DNA into chromatin reported by Graessmann *et al.* (1985).

Buschhausen *et al.* (1987) provided direct evidence for the role of chromatin in gene regulation by assembling both methylated and unmethylated Tk sequences into chromatin *in vitro* and microinjecting the resultant chromatin into tissue culture cells. They found that assembly of the unmethylated DNA did not inhibit transcription but that the methylated HSV-Tk chromatin was not transcribed.

These authors demonstrated that for methylation to inhibit gene expression the DNA must be assembled into chromatin. In addition they showed, by examining a stably maintained episome, that extrachromosomal assembly of chromatin was sufficient to inhibit gene expression of methylated DNA and that integration of the gene construct into chromosomal DNA was not a prerequisite for transcriptional inhibition.

An important observation made by these authors was that *in vitro* assembly of HSV-Tk DNA did not reduce the expression of HSV-Tk unless the DNA is methylated. In contrast Martinez-Sales *et al.* (1989) argue that the reduction in transcriptional activity at the two-cell stage in preimplantation embryos is associated with the assembly of the introduced DNA into chromatin. Whether methylation is also involved in the reduction in transcription of constructs introduced into preimplantation embryos is not known.

The Lac Z Gene

The analysis of preimplantation embryos requires specialised approaches to overcome the problems encountered due to the small size of the tissue under study and the minute amounts of DNA, RNA and protein which can be recovered.

The lac Z gene of *E. coli* is frequently used for cloning genes in prokaryotic systems. In eukaryotic systems the use of the lac Z gene has provided a convenient

and sensitive biochemical (An *et al.*, 1982; Norton and Coffin, 1985) or cytochemical (Price *et al.*, 1987; Goring *et al.*, 1987; Allen *et al.*, 1988; Liu *et al.*, 1989) approach for studying gene expression.

The assay is rapid and simple to perform and enables gene expression to be directly visualised at the level of individual cells. The simplicity of the assay favours a high degree of repeatability and reduces the chances of experimental error caused by the employment of complex techniques.

The lac Z gene and its enzyme product β-galactosidase have been successfully used to monitor gene expression and gene regulation in tissue culture cells (An *et al.*, 1982; Norton and Coffin, 1985; Liu *et al.*, 1989), preimplantation embryos (Bonnerot *et al.*, 1987) and transgenic animals (Goring *et al.*, 1987; Allen *et al.*, 1988). In addition, the lac Z gene has also been used as a marker for lineage analysis (Price *et al.*, 1987).

In this study the lac Z gene was introduced into one-cell embryos and β-galactosidase activity assayed at different stages of preimplantation development.

LAC Z EXPRESSION IN THE PREIMPLANTATION MOUSE EMBRYO

SECTION 4.2. - MATERIALS AND METHODS

4.2.1. Plasmid Construction

(see general materials and methods)

4.2.2. Pronuclear Microinjection of Fertilised Mouse Eggs

(see general materials and methods)

4.2.3. Embryo Culture

(see general materials and methods)

4.2.4. Assay of β-galactosidase Activity in the Preimplantation Embryo

a) Materials

Fixative	0.2 % glutaraldehyde (Sigma) in 0.1 M phosphate buffered saline, pH 7.3 (Sigma)
Washing Solution.	0.05% bovine serum albumin (Sigma) in 0.1 M phosphate buffered saline, pH 7.3 (Sigma)
Staining Solution.	5mM potassium ferricyanide (Sigma) and 5mM potassium ferrocyanide (Sigma) in 0.1 M phosphate buffered saline, pH 7.3.

To 1 ml of this solution was added 30 μl of 2% X-gal (Sigma) in dimethyl formamide (Sigma)

Embryos were washed, fixed and stained in 35 mm petri dishes (Nunc)

b) Method

The lac Z gene of *E. coli* encodes the bacterial enzyme β-galactosidase. This assay is based on the ability of β-galactosidase to convert a colourless substrate, 5-bromo-4-chloro-3 indoyl β-D galactopyrinoside (X-gal, Sigma), into a blue product which acts as a histochemical stain.

Embryos microinjected with lac Z DNA and control embryos were assayed for β-galactosidase activity by incubating embryos in the presence of 5-bromo-4-chloro-3 indoyl β-D galactopyrinoside (X-gal, Sigma).

The embryos were removed from the culture medium and washed once before being fixed for five minutes at 4° C. Following this treatment the embryos were removed from the fixative solution and washed by moving them through six 100 μl drops of 0.1 M phosphate buffered saline, pH 7.3, (Sigma) containing 0.05% bovine serum albumin (Sigma).

Finally, the embryos were transferred into 1 ml of staining solution and incubated overnight before being examined. To prevent evaporation the petri dish containing the staining solution was placed in a humidified box. Embryos were examined using an inverted microscope (Diavert, Wild).

This protocol was communicated to me by Azim Surani, to whom I am most grateful.

4.2.5. *In vitro* DNA Methylation Using Mammalian Methylase.

a) Materials.

Buffer M	50 mM Tris HCL pH 7.8 (Trisma Base HCL - Sigma) 1 mM EDTA (Ethylenediaminetetraacetic acid - Sigma) 1 mM DTT (Dithiothreitol - Sigma) 0.01 % NaN3 (sodium azide - Sigma) 10 % glycerol (BDH)
To 100 ml of buffer M add 1 ml of PMSF (Phenylmethylsulfonyl fluoride) 6 mg/ml ethanol (Sigma)	
Stopper Solution	1 % SDS (Sodium dodecyl sulphate - Sigma) 2 mM EDTA (Sigma) 5 % butanol (BDH) 3 % p-amino salicylate, added just before use. (Sigma) 0.125 M NaCl Before use 100 μg/ml pronase is added (Sigma)
Enzyme	mammalian methylase is extracted from mouse ascities cells (Adams pers. comm.)
Reaction Mixture	5 μg of plasmid DNA 20-30 μl activated enzyme 3 μl SAM 1mM - (S-adensylmethionine - Sigma) 70 μl buffer M

b) Method.

The reaction mixture was incubated for 4-6 hours at 37° C. The reaction was stopped by the addition of 300 μl of stopper solution containing 300 μg of pronase which destroys the methylase enzyme. Finally, the DNA was recovered and prepared for microinjection as described in general material and methods. This procedure was communicated to me by Dr. Adams. I am also indebted to Dr. Adams for his kind gift of activated methylase enzyme.

4.2.6. *In vitro* DNA Methylation Using Sss I Methylase

a) Materials.

NE Buffer 2	50 mM NaCl 10 mM Tris HCl, pH 7.9 at 25° C (Trisma Base HCL - Sigma) 10 mM $MgCl_2$ 1 mM DTT (Dithiothreitol - Sigma)
Enzyme	Sss I methylase (New England Biolabs.)
Reaction Mixture	2 μg of plasmid DNA 2 μl enzyme 1 μl SAM at 160 μM (S-adensylmethionine - New England Biolabs.) 4 μl NE buffer 2

b) Method.

The procedure was essentially the same as that described for the mammalian methylase, the reaction mixture being incubated for 24 hours at 37° C. Pronase was again included at the end of the reaction. Mock methylated DNA was prepared in the same way but in this reaction the co-factor S-Adenosylmethionine was absent. Finally, the DNA was recovered and prepared for microinjection as described in general material and methods.

LAC Z EXPRESSION IN THE PREIMPLANTATION MOUSE EMBRYO

SECTION 4.3. - RESULTS

4.3.1. Lac Z Expression In The Preimplantation Embryo - Introduction.

The main objective of the experiments described in this section was to investigate the short-term expression of exogenous DNA within the murine preimplantation embryo. To this end the reporter gene lac Z was used, lac Z encodes a bacterial β-galactosidase enzyme, the presence of which can be determined using a simple colourimetric assay. Embryos were microinjected with a number of different constructs, all of which carried the lac Z gene. Analysis of embryo expression allowed a comparison to be made between the different constructs. Two of these constructs were used to examine the pattern of lac Z expression throughout preimplantation development following pronuclear microinjection.

A number of different events could affect the levels of β-galactosidase activity in these embryos. A change in the transcriptional rate or the translational efficiency of the exogenous gene could modify expression levels over a period of time. Equally, changes in the half-life of the mRNA protein product could affect the pattern of expression. The rationale for this experiment was to examine the fate of exogenous DNA introduced into the mouse zygote, albeit indirectly through changes in the pattern of expression of the exogenous DNA. The possible reasons for the observed modulation of β-galactosidase activity with time are discussed.

In these results stained embryos are defined as an embryo which has some discernible blue stain in at least one of the blastomeres. For the purposes of these results three-cell embryos are considered as two-cell embryos. In these experiments a number of embryos were assayed after the development of that embryo had ceased, such embryos are referred to as blocked embryos. Those that were assayed when they were at the expected stage of development are referred to as fresh or developing embryos. The majority of one-cell embryos examined in these results were blocked.

4.3.1.a) Preimplantation Embryo Expression With CMV-lacZ And Tk-lacZ.

CMV-lacZ (Figure 1.3.) is a linear construct in which expression of the E coli lac Z gene is directed by the Human Cytomegalovirus Immediate Early (CMV-IE) promoter.

Tk-lacZ is shown in Figure 1.2. and is essentially similar to CMV-lacZ but the CMV-EI promoter is replaced with the Herpes Simplex Virus Thymidine Kinase (Tk)

promoter. One-cell embryos were microinjected with CMV-lacZ (2 μg/ml) or Tk-lacZ (2 μg/ml) and placed in culture. After microinjection of CMV-lacZ 40% of blocked one-cell embyros showed some evidence of lacZ expression, a blue stain indicating the presence of β-galactosidase activity. This is significantly higher than the staining rate of 9.6% seen with two-cell embryos ($x^2 = 26.0$: $P < 0.01$). There is no difference in the staining ratio between blocked and fresh two-cell embryos. None of the 7 four-cells recovered showed any evidence of lac Z expression. Although of a much smaller sample size the results are similar for embryos injected with Tk-lacZ, 25% of blocked one-cell embryos were stained but only 4.3% of two-cell embryos stained blue. As with the CMV-lacZ injected embryos a greater proportion of blocked one-cell embryos stained blue than the two-cell embryos. These results are shown in Table 4.3.1..

A higher percentage of blocked one-cell embryos stained blue after injection with CMV-lacZ than with Tk-lacZ. Similarly, a slightly higher percentage of two-cell embryos stained blue with CMV-lacZ than with Tk-lacZ, however, in neither case was the difference significant ($x^2 = 0.9$ and 1.4 respectively: both $P > 0.05$).

Table 4.3.1. CMV-lacZ and Tk-lacZ Expression (Number Stained).

	CMV-lacZ			Tk-lacZ		
Stage	Number Stained	Total Number	(%)	Number Stained	Total Number	(%)
1-cell	15	37	(40)	3	12	(25)
2-cell	23	240	(9.6)	2	47	(4.3)
4-cell	0	7	(0)	-	-	

Figure 4.3.1. shows the number of embryos stained compared to the total number assayed at various stages of development.

4.3.1.b) Preimplantation Expression With CMV-BGB.

CMV-BGB is outlined in Figure 1.5 and is a plasmid in which the CMV-EI promoter directs expression of the lac Z gene. For most experiments CMV-BGB was used at an injection concentration of 10 μg/ml. In order to examine the expression of lac Z at different stages of preimplantation development one-cell embryos were microinjected with CMV-BGB and placed in embryo culture medium. At different times after microinjection the embryos which had developed to the expected stage and the accompanying blocked stages were removed and placed in the staining solution.

The number of responders at each stage of development, that is embryos showing some evidence of lac Z expression, is shown in Table 4.3.2. There is a close correlation between the embryo stage and the numbers of embryos responding. A high percentage of early stages (one and two-cell embryos) which were assayed responded whereas a significantly lower proportion of four-cells, morulae and blastocysts showed evidence of β-galactosidase activity. For example, significantly more two-cell embryos stained blue than did four-cell, morula or blastocyst stages (x^2 = 30.8, 10.0 and 29.2 respectively: all $P < 0.01$). Between the four-cell to the blastocyst stage there was no significant difference between the numbers responding ($x^2 = 0.2$: $P > 0.05$, based on a 2x3 contingency table with 2 d.f.). However, significantly more blocked one-cell embryos responded than two-cell embryos (x^2 = 5.8: $P < 0.05$).

The one-cell embryos described above were all assayed more than 16 hours after microinjection and had failed to cleave to the two-cell stage within the expected time. In addition, a total of 49 one-cell embryos were assayed within four hours of being microinjected. None of these embryos showed any evidence of β-galactosidase activity, this result is consistent with previous work which suggests that at this stage of development embryos are incapable of expressing exogenous DNA (Chen *et al.*, 1986; Bonnerot *et al.*, 1987; Martinez-Salas *et al.*, 1989).

Table 4.3.2. CMV-BGB Expression (Number Stained).

Development Stage At Staining	Number Stained Blue	Total Number Assayed	Assayed/Stained (%)
1-cell[a]	0	49	(0)
1-cell[b]	49	61	(80)
2-cell.	97	153	(63)
4-cell.	71	209	(34)
Morula	7	24	(29)
Blastocyst	66	193	(35)

a Assayed within 4 hours of microinjection.
b Assayed the following day, greater than 16 hours after microinjection.

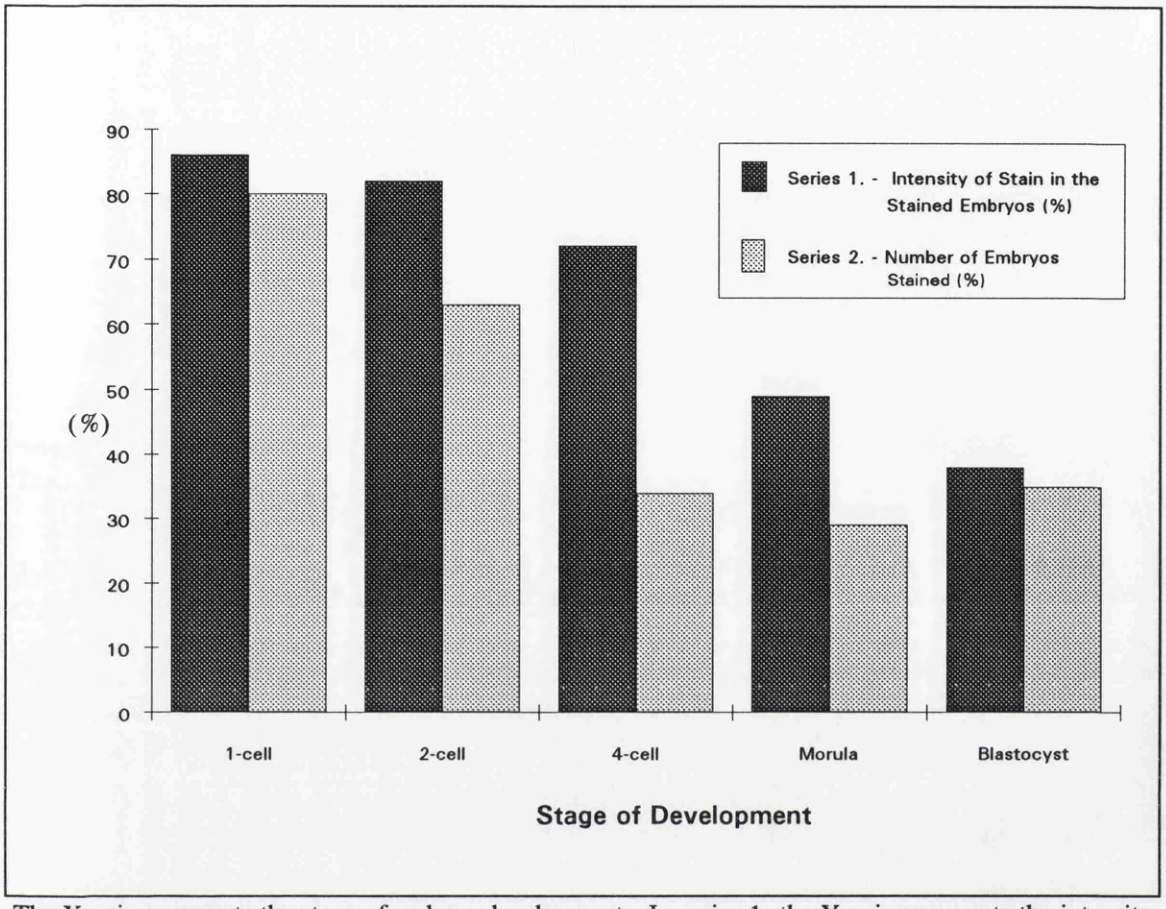
There is a good correlation between the number of embryos staining at a particular stage and the intensity of the stain at that stage. The average intensity of stain at a given stage of development was calculated by scoring individual embryos from 1-5 based on the intensity and extent of blue stain observed. Although subjective, this method gave a broad measure of β-galactosidase activity in individual embryos. The average activity at each stage was calculated by expressing the total score as a percentage of the maximum score possible (*i.e.* if every embryo scored a maximum five). In order to separate this analysis from the number of embryos responding, only those embryos showing evidence of lac Z expression were included. These results are shown in Table 4.3.3. and in Figure 4.3.1. In general, early embryos (one and two-cell stages) showed a high level of β-galactosidase activity with the majority showing a homogenous deep blue or indigo colour. The majority of four-cell embryos also showed a homogenous staining pattern although often the stain was of a lighter hue and a greater proportion of embryos displayed a mottled staining pattern. Later stages, namely morulae and blastocysts, displayed a much more heterogeneous pattern of expression and on average the level of evident β-galactosidase activity was significantly less than earlier stages ($x^2 = 237.0$: $P < 0.01$).

Using this method of analysis the four-cell stage appears to be intermediate for β -galactosidase activity. The average activity of the four-cell stage is significantly less than earlier stages ($x^2 = 20.7$: $P < 0.01$) and significantly greater than later stages ($x^2 = 86.8$: $P < 0.01$).

Table 4.3.3. CMV-BGB Expression (Intensity of Stain).

Development Stage	Actual Score	Maximum Possible Score	Actual/Maximum Possible (%)
1-cell	212	245	(86)
2-cell	397	485	(82)
4-cell	254	355	(72)
Morula	17	35	(49)
Blastocyst.	112	295	(38)

Figure 4.3.1. β-galactosidase Activity in the Preimplantation Embryo.



The X-axis represents the stage of embryo development. In series 1. the Y-axis represents the intensity of staining in that sub-population of cells which stained blue and in series 2. the Y-axis represents the percentage of embryos which stained blue.

4.3.1.c) Preimplantation Embryo Expression - Tk-BGB.

In order to compare the activity of the Tk promoter with the CMV promoter a small number of embryos were injected with Tk-BGB. Tk-BGB, shown in Figure 1.4., is a plasmid similar to CMV-BGB with the CMV promoter being replaced by the Tk promoter.

Eighty-five zygotes were successfully microinjected with TK-BGB at a concentration of 10 μg/ml, placed in embryo culture medium and cultured through to the blastocyst stage. Ninety-six hours after microinjection the resulting blastocysts and embryos blocked at different stages of development were assayed for β-galactosidase activity. These results are shown in Table 4.3.4..

Table 4.3.4. Tk-BGB Expression (Number Stained).

Stage of Development	Number Stained	Total Number	Stained (%)
Blocked 1-cell	8	13	(62)
Blocked 2-cell	10	16	(63)
Blocked 4 cell	1	3	(33)
Blocked morula	4	5	(80)
Blastocyst	12	48	(25)

Total Number = total number of embryos assayed

Although the numbers injected with Tk-BGB are relatively small there is no significant difference between the two plasmids in the number of embryos responding at any particular stage of development. Embryos injected with Tk-BGB show a similar pattern of lac Z expression to that of CMV-BGB. In general, early stages show a higher staining rate and are more extensively stained than later stages.

If the results of both transgenes are pooled then 77% of one and two-cell embryos are stained but only 33% of morulae and blastocysts show evidence of blue stain. Table 4.3.5. summarises the results of embryos injected with Tk-BGB and CMV-BGB.

Table 4.3.5. Summary of CMV-BGB and Tk-BGB Expression.

	CMV-BGB			Tk-BGB		
Stage of Development	Number Stained	Total Number	Stained (%)	Number Stained	Total Number	Stained (%)
1-cell	49	61	(80)	8	13	(62)
2-cell	97	153	(63)	10	16	(63)
4 cell	71	209	(34)	1	3	(33)
Morula and Blastocyst	73	217	(34)	16	53	(30)

In this table results obtained from blocked embryos have been pooled.

4.3.1.d) β-galactosidase Injection Of One-Cell Embryos.

In order to assess the stability of the lac Z product one-cell embryos were microinjected with β-galactosidase enzyme at a concentration of 2 mg/ml. Injected embryos were placed in culture and the β-galactosidase activity at different stages of development was assayed. One-cell embryos were assayed shortly after being microinjected.

The number of embryos displaying β-galactosidase activity at different stages of development is shown in Table 4.3.6.a) and the level of β-galactosidase activity in those embryos which did respond is shown in Table 4.3.6.b).

Table 4.3.6.a) β-galactosidase Enzyme Activity (Number Stained).

Stage of Development	Number Stained	Total Number	Stained (%)
One-cell	16	28	(57)
Two-cell	30	55	(55)
Four-cell	19	33	(58)
Morula	9	16	(56)
Blastocyst	11	16	(69)

Table 4.3.6.b) β-galactosidase Enzyme Activity (Intensity of Stain).

Stage of Development	Actual Score	Maximum Score Possible	Actual/Maximum Possible (%)
One-cell	74	80	(93)
Two-cell	131	150	(87)
Four-cell	88	95	(93)
Morula	36	45	(80)
Blastocyst	39	55	(71)

These results differ from the pattern observed after pronuclear injection with the lac Z gene. With β-galactosidase injection the proportion of embryos which responded remained constant through preimplantation development. The level of β-galactosidase activity of the responsive embryos is higher at all stages of development and only starts to decline at the morula stage.

4.3.2. Inhibitors Of Methylation

As can be seen from the previous section there is a general decline in β-galactosidase activity as preimplantation development proceeds following pronuclear injection at the one-cell stage.

In order to investigate the possibility that the introduced DNA may be subject to *de novo* methylation in the preimplantation embryo and that this was, at least in part, responsible for the reduction in β-galactosidase activity, microinjected embryos were cultured in the presence of known inhibitors of methylation.

Certain substances have been used to inhibit methylation in mammalian cells. It has been argued (Menezo *et al.*, 1989) that DL-homocysteine is one such substance which has this property and that it is of relatively low toxicity. DL-homocysteine was incorporated into the embryo culture medium supporting lac Z microinjected embryos in an attempt to ascertain if the pattern of lac Z expression could be modulated. In addition the effect of 5-azacytidine, a specific inhibitor of DNA methylation (Jones, 1985), on expression of the lac Z gene in preimplantation embryos was investigated.

4.3.2.a) Embryo Culture Rate With DL - Homocysteine

Initially, a number of embryos were cultured in various concentrations of DL-homocysteine in order to define the maximum concentration that could be used without significantly reducing the embryos capacity to develop. These results are presented in Table 4.3.7..

Table 4.3.7. Embryo Culture Rate - Varying Concentrations of DL - Homocysteine.

Concentration of DLH (μM)	1-cell	2-cell	4-cell	Morula	Blastocyst	1cell/blast. (%)	2cell/blast. (%)
50	16	12	12	10	11	(69)	(92)
100	45	40	22	21	23	(51)	(58)
250	46	42	40	35	37	(80)	(88)
400	174	107	79	65	69	(40)	(64)
500	169	93	86	74	73	(43)	(78)
750	33	32	30	29	27	(82)	(84)
1000	342	259	214	176	174	(51)	(67)
1500	20	17	8	6	6	(30)	(35)
2000	44	28	17	16	16	(36)	(57)
2500	22	6	0	0	0	(0)	(0)
5000	22	8	0	0	0	(0)	(0)
10,000	22	2	0	0	0	(0)	(0)
50,000	22	6	0	0	0	(0)	(0)
Controls	454	320	291	264	259	(57)	(81)

1cell/blast = percentage of one-cell embryos developing to the blastocyst stage.
2cell/blast = percentage of two-cell embryos developing to the blastocyst stage.

The results in Table 4.3.7. show that the embryo culture rate declined with increasing concentrations of DL-homocysteine (DLH). The development rate for control embryos which were cultured in parallel was 81%. The average culture rate of those embryos grown in the presence of 50-750 μM DLH was 74% whilst 67% of embryos developed when cultured in a concentration of 1000 μM. Those embryos cultured in the presence of 1500-2000 μM of DLH had a development rate of 49%.

The morphology of the embryos cultured in high concentrations of DLH was significantly altered. A number of embryos were placed in high concentrations of DLH (5000-50,000 μM) and when examined at the two-cell stage the blastomeres appeared much smaller and darker than normal.

A greater number of controls cultured from the two-cell stage through to the blastocyst stage than those embryos cultured in the presence of DLH at a concentration of between 50-750 μM ($x^2 = 5.1$: $P < 0.05$). The culture rate of embryos at a concentration of 1000 μM is significantly less than the controls ($x^2 = 14.4$: $P < 0.01$). At concentrations of 1500-2000 μM the culture rate declines still further and at concentrations in excess of 2000 μM embryos failed to culture.

4.3.2.b) Lac Z Expression In Embryos Cultured With DL-homocysteine.

Embryos were injected with CMV-BGB (10 μg/ml) and a proportion were grown in medium containing 500-1200 μM DLH in an attempt to ascertain if homocysteine influenced the expression pattern of the lac Z transgene. In this experiment one group of embryos was cultured for 48 hours at which point the resulting four-cell embryos and blocked embryos were placed in the staining solution. In another group, embryos were cultured for 96 hours and the resulting blastocysts and blocked stages were incubated in the staining solution at the end of the culture period. In pooled results 38% of microinjected embryos, cultured to the blastocyst stage in varying concentrations of DLH ranging from 500 μM to 1200 μM, showed evidence of lac Z expression. There was no difference in the proportion of embryos staining at any of the concentrations used. The staining rate of microinjected embryos cultured without DLH was 41%. These results and the accompanying blocked stages are shown in Table 4.3.8.a).

Table 4.3.8.b) shows the number of four-cell embryos staining blue in the test and control groups. DLH concentrations again ranged from 500-1200 μM in the test group, as there was no difference between the different concentrations used these results were again pooled. Twenty-one percent were stained blue in the test group and 21% were stained blue in the control group.

There is no significant difference in the number staining blue between those embryos grown in the presence of DLH and in culture medium only. This is true for both the blastocysts ($x^2 = 0.1$: $P > 0.05$) and the four-cell embryos ($x^2 = 0.01$: $P > 0.05$) collected and placed in the staining solution. There is also no significant difference between the number of blocked stages staining blue between the two groups.

Table 4.3.8.a) CMV-BGB Expression with DL-Homocysteine (Number Stained at the Blastocyst stage).

Culture medium with DLH				Culture medium without DLH			
Stage	Number stained	Total number	(%)	Stage	Number stained	Total number	(%)
B 1-cell	4	5	(80)	B 1-cell	5	8	(63)
B 2-cell	7	9	(77)	B 2-cell	10	17	(59)
B 4-cell	7	10	(70)	B 4-cell	9	10	(90)
B morula	2	11	(18)	B morula	5	12	(42)
blastocyst	24	63	(38)	blastocyst	18	44	(41)

B = blocked.

Table 4.3.8.b) CMV-BGB Expression with DL - Homocysteine (Number Stained at the 4-Cell Stage).

Culture medium with DLH				Culture medium without DLH			
Stage	Number stained	Total number	(%)	Stage	Number stained	Total number	(%)
B 1-cell	10	10	(100)	B 1-cell	5	5	(100)
B 2-cell	24	52	(46)	B 2-cell	10	32	(31)
4-cell	12	58	(21)	4-cell	15	70	(21)

B = blocked.

The staining results were similar to those described in 4.3.1. In both groups - the blastocyst and the four-cell group - a similar staining pattern is observed. In general a greater number of the early stages stain blue compared with later stages *i.e.* significantly more one and two-cell stages stain blue and more extensively than those embryos at the morula and blastocyst stage ($x^2 = 7.5$: $P < 0.01$). In total 24/28 (86%) of one-cells, 51/110 (46%) of two-cells, 43/148 (29%) of four-cells, 7/23 (30%) of morulae and 42/107 (39%) of blastocysts stained blue.

As mentioned earlier there was considerable variation in the degree of blue stain observed between individual embryos. This variation ranges from the identification of the occasional blue spot within a cell to all the cells staining blue with high levels of expression being represented by an intense indigo colour.

In general, intense staining was observed in the one-cell and two-cell embryos and good, even staining was observed in the majority of the four-cell embryos - the intensity of staining being slightly less in this group. In all three of these stages the staining in individual blastomeres tended to be homogeneous. In contrast, the majority of morulae and blastocysts stained showed only isolated blue patches. There was, however, a greater variation in the extent and intensity of stain observed in the morula and blastocyst stages with some embryos showing blue in most cells and some areas staining intensely. Figures 4.3.2.a-d. show examples of control and stained embryos.

In an attempt to quantify the levels of expression shown by each embryo the embryos were scored from 1-5 depending on: the number of blastomeres staining blue, the extent of the staining within the blastomeres and the intensity of the colour observed. Embryos reaching the blastocyst stage in either the DLH group or the control group were scored in this manner and the results are shown in Table 4.3.9.a).

Table 4.3.9.a) CMV-BGB Expression with DL - Homocysteine (Intensity of stain at Blastocyst Stage).

DLH Group				Control Group			
No. of Blastocysts.	Score	Total Score Possible	%	No. of Blastocysts.	Score	Total Score Possible	%
24	50	120	(42)	14	24	70	(34)

As can be seen from Table 4.3.9.a) there is no significant difference between the degree of staining in the blastocysts cultured in medium containing DLH and those cultured in medium lacking DLH ($x^2 = 1.0$: $P > 0.05$).

Those embryos cultured to the four-cell stage were also scored after staining and these results are presented in Table 4.3.9.b). The total score in the control group exceeded those cultured in the presence of DLH, however, the difference was not significant ($x^2 = 2.8$: $P > 0.05$).

Table 4.3.9.b) CMV-BGB Expression with DL - Homocysteine (Intensity of stain at the 4-cell Stage).

DLH Group				Control Group			
No. of 4 cells	Score	Total Score Possible	%	No. of 4 cells	Score	Total Score possible	%
12	35	60	(58)	17	61	85	(72)

In conclusion, therefore, the presence of homocysteine in the culture medium did not affect observed levels of lac Z expression - at least at the concentrations employed.

Figure 4.3.2.a. **Control Two-Cell Embryos**

Final magnification approximately 300x (Original in colour)

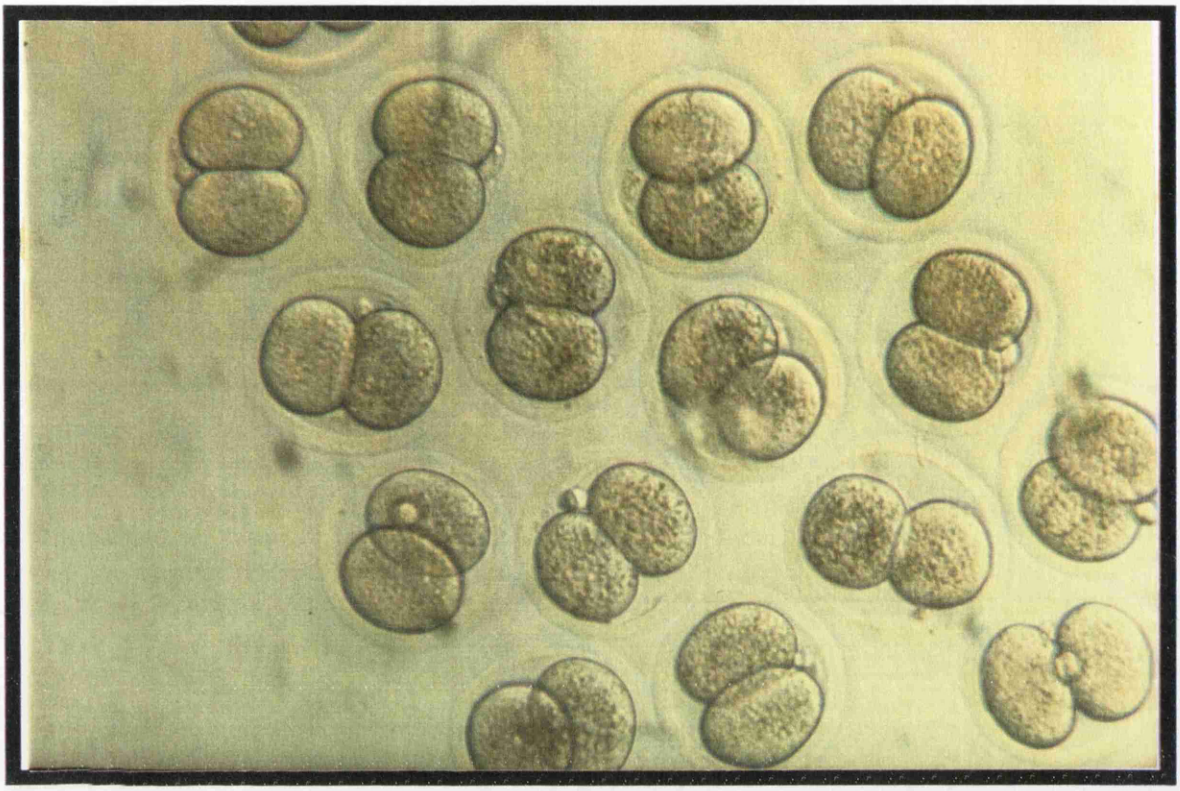


Figure 4.3.2.b. **Control Blastocysts and Developing Blastocysts**

Final magnification approximately 300x (original in colour)

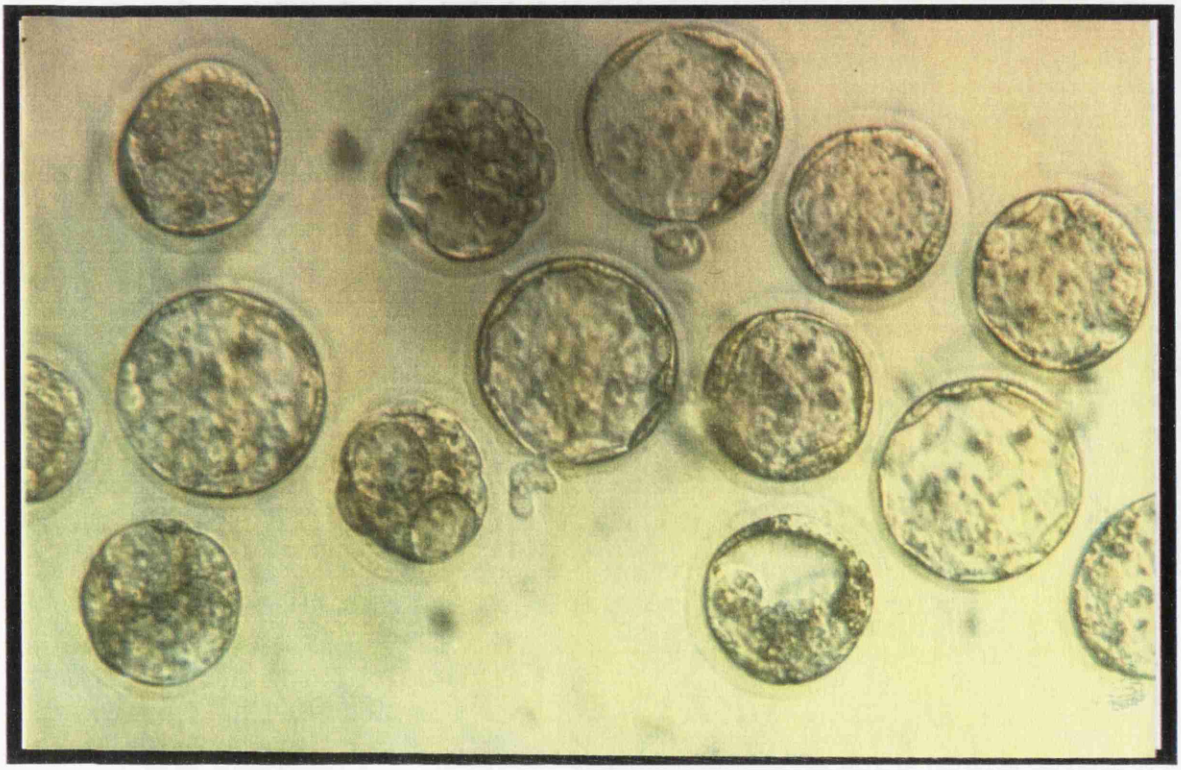


Figure 4.3.2.d. **A Two-Cell Embryo Showing β-galactosidase Activity**

Final magnification approximately 900x (original in colour)

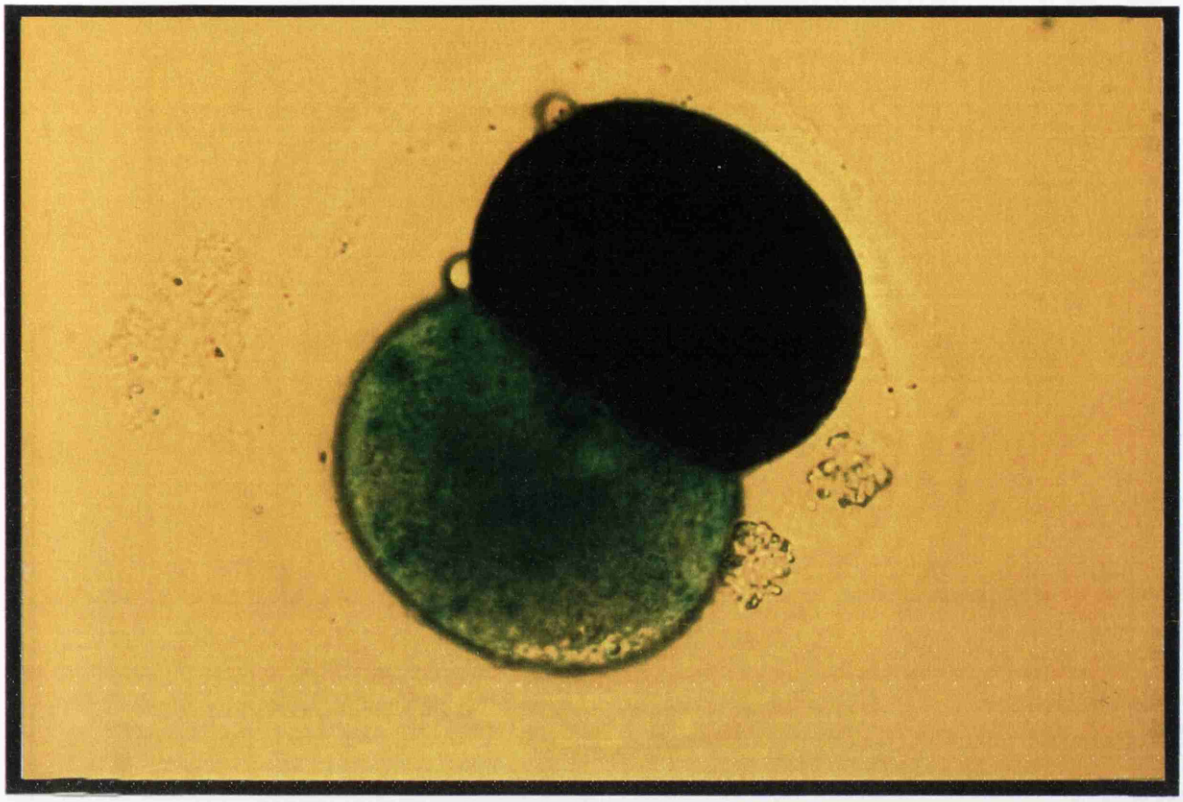
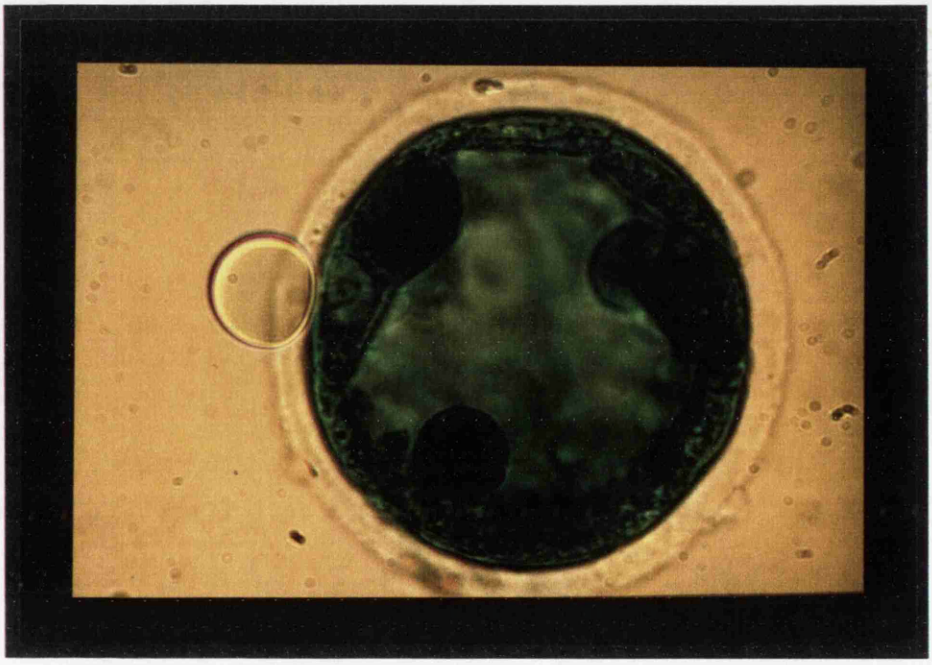


Figure 4.3.2.c. **A Blastocyst showing β-galactosidase Activity**

Final magnification approximately 700x (original in colour)



4.3.2.c) Embryo Culture Rate With 5-azacytidine.

5-azacytidine has been extensively used in tissue culture to inhibit methylation of the cytosine nucleotide in the DNA of mammalian cells (Jones, 1985). The ability of the embryos to develop in the presence of different concentrations of 5-azacytidine was examined. These results are shown in Table 4.3.10.

Table 4.3.10. Embryo Culture Rate - Varying Concentrations of 5-azacytidine.

Concentration of 5-aza (μg/ml)	1-cell	2-cell	4-cell	Morula	Blast.	1cell/Blast. (%)	2cell/Blast. (%)
0.0001	25	11	7	4	6	(24)	(55)
0.001	25	20	15	15	12	(48)	(60)
0.01	27	18	12	8	11	(41)	(61)
0.1	89	77	64	58	56	(63)	(73)
0.2	12	10	7	3	3	(25)	(33)
1.0	98	92	27	4	4	(4)	(4)
10	26	22	0	0	0	(0)	(0)
Controls	68	42	34	33	33	(48)	(79)

There is no difference in the ability of the embryos to develop when cultured in concentrations of 5-azacytidine varying from 0.0001 to 0.1 μg/ml. Fewer embryos developed in 0.2 μg/ml than in 0.1 μg/ml and only 4% cultured through to the blastocyst stage at a concentration of 1.0 μg/ml. No embryos developed when cultured at a concentration of 10 μg/ml. At all concentrations the embryo culture rate was less than that of the controls as assessed by the number of two-cell embryos developing into expanded blastocysts. A greater number of controls cultured from the 2-cell stage to the blastocyst stage than did embryos cultured in concentrations of 5-azacytidine ranging from 0.0001 to 0.1 μg/ml but the difference was not significant ($x^2 = 1.9$: $P > 0.05$). However, the development of 2-cell embryos was significantly reduced when they were cultured in the presence of 1.0 μg/ml 5-azacytidine ($x^2 = 79.4$: $P < 0.01$).

In addition 15, 14 and 18 morulae were cultured in medium containing 1 mg/ml, 10 μg/ml and 1 μg/ml respectively, all 14 morulae developed to the blastocyst stage in the control group but none of the morulae in the test groups developed.

Twenty-one one-cell embryos were cultured in medium containing 1 μg/ml and all developed to the two-cell stage. At this point the embryos were removed, washed

and transferred to fresh medium which did not contain 5-azacytidine and cultured. Only 4 (19%) of these embryos developed to the blastocyst stage. Similarly, 34 embryos were placed in culture at the one-cell stage in medium containing 1 μg/ml of 5-azacytidine. Only 12 embryos reached the four-cell stage and these were then washed and transferred to medium lacking 5-azacytidine. Only 2 of these embryos developed to the blastocyst stage. In the control group, which was run in parallel with these test groups, 20 one-cell embryos were placed in culture and 17 developed to the blastocyst stage.

Therefore, on the basis of these results it appears that concentrations in excess of 0.1 μg/ml of 5-azacytidine are deleterious to embryo development - although a proportion of embryos were capable of developing at higher concentrations.

4.3.2.d) Lac Z Expression With 5-azacytidine.

As a result of the above finding, microinjected embryos were cultured in medium containing 0.1 - 0.2 μg/ml of 5-azacytidine. Thirty-five embryos were microinjected with CMV-BGB and 25 of these embryos cultured through to the blastocyst stage. Twenty-one embryos microinjected with CMV-BGB were cultured in medium only, of these 13 cultured through to the blastocyst stage. The resulting blastocysts and accompanying blocked stages were assayed for β-galactosidase activity.

Of those embryos cultured to the blastocyst stage in the presence of 5-azacytidine 24% showed blue stain. These results were not significantly different from those embryos not brought into contact with 5-azacytidine ($x^2 = 0.4$: $P > 0.05$). In this group 15% of resulting blastocysts stained blue. These results are shown in Table 4.3.11..

Table 4.3.11. CMV-BGB Expression with 5-azacytidine (Blastocyst Stage).

Culture Medium with 5-azacytidine			Culture Medium without 5-azacytidine		
Stage	Number Stained	Total Number	Stage	Number Stained	Total Number
One-cell	3	5	One-cell	-	-
Two-cell	1	2	Two-cell	1	1
Four-cell	-	-	Four-cell	1	4
Morula	1	3	Morula	0	1
Blastocyst	6	25	Blastocyst	2	13

Therefore, no significant difference was observed in the degree of staining nor the number of embryos staining between the microinjected embryos cultured in the control group or in the presence of the methylation inhibitor 5-azacytidine.

4.3.3. Lac Z Expression Of Methylated CMV-BGB Plasmid.

In an attempt to investigate the factors which caused a decline in expression it was important to determine if the expression of the CMV-BGB plasmid could be altered by DNA methylation.

CMV-BGB was methylated *in vitro* using a mammalian methylase. One-cell embryos were then injected with the methylated CMV-BGB and subsequent lac Z expression was compared with embryos microinjected with the unmethylated plasmid, both plasmids being injected at a concentration of 30 μg/ml. Embryos were injected and cultured to the four-cell stage and in a separate experiment cultured to the two-cell stage. These embryos and the accompanying blocked stages were then assayed for β-galactosidase activity. The number of embryos showing β-galactosidase activity in both groups is shown in Table 4.3.12..

Table 4.3.12. Lac Z Expression of Methylated CMV-BGB - Experiment 1.

Unmethylated Plasmid (CMV-BGB)				Methylated Plasmid (mCMV-BGB)			
Stage	Number Stained	Total Number	(%)	Stage	Number Stained	Total Number	(%)
One-cell	10	12	83%	One-cell	4	11	36%
Two-cell	29	38	76%	Two-cell	7	29	24%
Four-cell	15	23	65%	Four-cell	0	36	0%

A greater number of one-cell embryos and significantly more two and four cell embryos (x^2 = 18.0 and 31.5 respectively: both $P < 0.01$) stained blue after being microinjected with CMV-BGB than with the methylated form of CMV-BGB.

This experiment was repeated with the intention of comparing the methylated plasmid with plasmid which was mock methylated, that is plasmid which was subjected to the methylation reaction but with the essential co-factor SAM (S-adenyl methionine) missing from the reaction mixture. As can be seen from Table 4.3.13. the results of this experiment appear to contradict those in Table 4.3.12.. In this experiment embryos were injected with both methylated and mock methylated CMV-BGB. In contrast to the results presented in Table 4.3.12. there was no significant

difference in the staining rate between the methylated and mock-methylated groups and a number of embryos injected with methylated CMV-BGB stained blue.

Table 4.3.13. Lac Z Expression of Methylated CMV-BGB - Experiment 2.

Mock methylated plasmid (CMV-BGB)				Methylated plasmid (mCMV-BGB)			
Stage	Number stained	Total number	(%)	Stage	Number stained	Total number	(%)
1-cell	0	1	0%	1-cell	0	0	0%
2-cell	7	17	41%	2-cell	6	14	43%
4-cell	4	15	27%	4-cell	5	15	33%

As these results were contradictory this experiment was repeated once more using Ss1 methylase (a methylase enzyme with greater activity than mammalian methylase, Adams, pers. comm.). In this experiment embryos were injected with both methylated and mock methylated CMV-BGB at a concentration of 20 μg/ml and cultured to the two-cell stage. The resulting two-cell and blocked one-cell embryos were assayed for β-galactosidase activity. As can be seen from Table 4.3.14. no expression was detected in those embryos injected with the methylated DNA whereas expression in the mock methylated group was relatively high.

Table 4.3.14. Lac Z Expression of Methylated CMV-BGB - Experiment 3.

Mock methylated plasmid (CMV-BGB)				Methylated plasmid (mCMV-BGB)			
Stage	Number Stained	Total Number	(%)	Stage	Number Stained	Total Number	(%)
1-cell	7	11	64%	1-cell	0	6	0%
2-cell	9	13	69%	2-cell	0	21	0%

These results appear to confirm those of the first experiment and together these imply that the expression potential of introduced methylated DNA is less than that of the unmethylated equivalent. The reasons for the conflicting results are not known but it was possibly due to a reduction in the efficacy of the mammalian methylase enzyme as it had been stored for some time before being used in the second experiment.

LAC Z EXPRESSION IN THE PREIMPLANTATION MOUSE EMBRYO

SECTION 4.4. - DISCUSSION

Introduction.

Introduction of the *E. coli* lac Z gene into mammalian cells allows the visual detection of gene expression at the level of the single cell using a simple assay system (Norton and Coffin, 1985). It is, therefore, suitable for examining expression in single preimplantation embryos (Bonnerot *et al.*, 1987). Using pronuclear microinjection this reporter gene was introduced into fertilised one-cell embryos and its expression at different stages of embryo development was investigated.

Two plasmid constructs were used to investigate expression in the preimplantation embryo. These plasmid were similar, the only difference being the promoter used to drive expression of the lac Z gene. In CMV-BGB the immediate early promoter of cytomegalovirus was used (Thomsen *et al.*, 1984) and the Herpes Simplex Virus Thymidine Kinase promoter (McKnight *et al.*, 1981) was used in Tk-BGB. Both plasmids were used at a concentration of 10 μg/ml for most experiments.

Comparison Of CMV And Tk Promoters.

The pattern of expression was similar for both plasmid constructs, however a greater number of embryos at the blocked one-cell and blastocyst stages stained blue with the CMV promoter than with the Tk promoter although the difference was not significant. Similar results were obtained when the linear constructs, Tk-lacZ and CMV-lacZ, were microinjected. A higher percentage of blocked one-cell and two-cell embryos stained blue with the CMV-lacZ construct than with the Tk-lacZ construct. Taken together these results suggest that the transcriptional activity of the CMV promoter is higher than the Tk promoter in preimplantation embryos. Although the TK promoter has been used to express a number of genes in preimplantation embryos (Brinster *et al.*, 1982; Ao *et al.*, 1988; Martinez-Salas *et al.*, 1989) it has been described as a weak promoter (Allen *et al.*, 1988). When the Tk-lacZ construct was used to produce a number of transgenic lines it was shown that the transcriptional activity of the Tk promoter was heavily influenced by the site of integration (Allen *et al.*, 1988). In contrast, the CMV regulatory sequence appears to direct widespread expression in a large number of organs and systems (Schmidt *et al.*, 1990).

Transcription At The One-Cell Stage.

A total of 49 one-cell embryos microinjected with CMV-BGB were assayed within 4 hours of microinjection, none of these embryos showed any evidence of β-galactosidase activity. However, blocked one-cell embryos generally showed a high level of activity. It would appear, therefore, that one-cell embryos are incapable of expressing the exogenous gene until a period of time has elapsed. The high level of expression in the blocked one-cell embryos shows that the ability to express the introduced gene is dependent not on cleavage to the two-cell stage but on a period of time elapsing. This situation is analogous to that described for endogenous genes in one-cell embryos. Although a low level of mRNA synthesis has been reported at the one cell stage it is generally accepted that activation of the embryonic genome occurs early in the two-cell cycle with a small number of mRNA species appearing 2-4 hours after cleavage (Bolton *et al.*, 1984). Treatment of one-cell embryos with aphidicolin prevents DNA synthesis and therefore DNA replication and as a result cleavage to the two-cell stage is blocked (Ikegami *et al.*, 1978). However, such treatment does not prevent the synthesis of a set of proteins indicative of the G1 phase of the two-cell stage (Bolton *et al.*, 1984; Poueymirou and Schultz, 1987).

The results reported here are consistent with similar results reported by other workers. Chen *et al.* (1986) demonstrated the existence of a lag phase between the injection of exogenous DNA and the onset of expression of that DNA and Bonnerot *et al.* (1987) reported that it is only at the two-cell stage that the embryo acquires the ability to express introduced DNA.

A more detailed investigation was carried out by Martinez-Salas *et al.* (1989). Using aphidicolin to prevent DNA replication of injected zygotes, this group showed that a time lag of 16-20 hours was required before the onset of expression in these artificially blocked one-cells. When the one-cell embryos were held in aphidicolin for 16 hours and then injected expression commenced immediately. They concluded that the onset of exogenous gene expression was dependent upon an embryo signal which was not related to DNA replication or cleavage and suggested that injected genes were being regulated by the same factors which determine the timing of zygote gene expression.

Staining Pattern In Early Embryos.

In the one-cell and two-cell embryo stages there was a clear distinction between those embryos which stained and those which did not. There were only a few one and two-cell embryos in which only a low level of expression was identified and which stained only partially or at a low intensity. It is possible that these results do

represent a normal distribution curve but that this pattern is distorted by the limited sensitivity of the assay at both ends of the spectrum.

Alternatively, these embryos might represent two distinct groups with a different capacity to respond to the introduction of the lac Z gene. It is possible that in a proportion of embryos the DNA solution was not correctly deposited into the pronuclei or that damage to the nuclear membrane caused leakage of most of the injected solution. Martinez-Salas (1988) reported that 30-80% of plasmid DNA is lost soon after injection and suggests this is caused by leakage to the cytoplasm where the DNA is rapidly degraded.

The embryo donors were C57BL6/CBA F1 hybrids. This is the genotype of the oocyte and as embryonic activation does not occur until the two-cell stage (Bolton *et al.*, 1984) it is also the genotype of the early embryo. Meiosis in the oocyte of an F1 hybrid will result in a number of different genes segregating and producing one-cell embryos with a unique mRNA and protein profile. It is possible that this may result in the presence of DNA binding proteins which could either enhance or suppress the expression of the introduced gene. Therefore, unlike embryos from an inbred line, the population used in this study is genetically heterogeneous which may partially explain the variable response observed. It has previously been shown, at least with integrated transgenes, that although the temporal and spatial pattern of expression remains the same the level of expression varies markedly depending on the genetic background of the transgenic mouse (Sapienza *et al.*, 1989; Allen *et al.*, 1990).

In this study, the pronuclei which appeared most accessible were injected and while the majority would probably be male pronuclei as they are larger and therefore easier to inject it is reasonable to assume that a significant proportion of female pronuclei were also injected. Martinez-Salas *et al.* (1989) report that expression of an introduced gene can vary considerably depending on which pronuclei are injected. Using the luciferase (*luc*) gene Martinez-Salas *et al.* found that expression after injection into the male pronuclei was twice that observed after microinjection of the female pronuclei.

A major problem in using the lac Z gene in this assay system is that it is difficult to quantify the level of expression - in these experiments this was achieved by subjective examination. If the intensity of blue stain is not a linear representation of transcriptional activity then it is possible to understand why relatively small changes in mRNA expression were not accurately reflected in the degree or intensity of blue stain observed and why some embryos do not stain at all while the majority stain intensely. However Brinster *et al.* (1982) showed, using the Herpes Thymidine Kinase (Tk) gene linked to the mouse Metallothionine promoter (MT-I), that there is a linear relationship between the concentration of the injected DNA and the resultant level of expression.

Description Of Staining Pattern

β-galactosidase activity declined with embryo development. The staining pattern was similar for both constructs CMV-BGB and Tk-BGB in that a high percentage of one and two-cell embryos stained blue but a significantly lower proportion of four-cells, morulae and blastocysts stained. As development proceeded not only did the number of embryos showing evidence of lac Z expression decline but the intensity and extent of stain in individual embryos also decreased. The majority of stained one-cell, two-cell and to a lesser extent four-cell embryos showed a homogenous staining pattern and high level of β-galactosidase activity. However, the staining pattern observed in later stages was much more heterogeneous with the majority of these stages displaying only a few blue spots.

Pooling the results of both plasmid constructs reveals that 77% of one cells and 63% of two cell embryos responded to the presence of the lac Z gene whilst only 34% of four-cells and 33% of morula and blastocyst stages showed any blue stain after microinjection at the one-cell stage. A responsive embryo was defined as an embryo showing any evidence of β-galactosidase activity. The level of expression of morula and blastocyst stages, however, was significantly below that of four cell embryos based on the intensity and extent of blue stain seen in individual embryos. With this exception there was a good correlation between the proportion of embryos responding and the degree of expression observed in individual embryos. This has been reported previously by Martinez-Salas *et al.* (1989). These workers showed that when expression of the reporter gene luciferase was increased by introducing an appropriate enhancer not only did the activity increase but the number of embryos responding also increased. There would, therefore, appear to be a close correlation between the level of gene expression and the number of responsive embryos.

Interpretation Of Staining Pattern

In order to compare the level of gene expression between different stages of preimplantation development over a relatively short time course, it is important to distinguish between expression and the evidence of that expression. The length of time β-galactosidase activity persists in preimplantation embryos is primarily dependent on two parameters - the initial level of enzyme introduced and the half-life of the enzyme in the embryo cells. β-galactosidase enzyme was introduced into one-cell embryos. The proportion of embryos staining blue did not decrease with preimplantation development and the intensity of stain decreasing only slightly at the morula stage. These results show that given a sufficiently high initial concentration of

enzyme, β-galactosidase activity can persist through preimplantation development and it is likely that β-galactosidase activity does not accurately reflect the time at which lac Z transcription is down-regulated.

The pattern of embryo staining observed at different stages of preimplantation development is consistent with a burst of transcriptional activity at the one or the two-cell stage and a decrease in transcriptional activity around the two to four-cell stage. Similar results for microinjection experiments have been reported by Chen *et al.* (1986) and Ao *et al.* (1988). Given the results achieved with β-galactosidase enzyme it is possible that the staining observed in morula and blastocyst stages resulted from residual β-galactosidase activity. These results do not, however, rule out some transcriptional activity at or after the four-cell stage. In the blastocyst group a wide variation in the intensity and extent of blue stain was observed. It is not possible, however, to distinguish if this represents residual β-galactosidase activity or continued transcription at the morula and blastocyst stages in a sub-population of embryos.

Possible Reasons For Decline In β-galactosidase Activity

One possibility for the observed decline in β-galactosidase activity is that the plasmid DNA is being degraded in the nucleus. Existing evidence does not favour this hypothesis as it has been reported that plasmid DNA, whether it replicates or not, is stable in the nucleus of murine blastomeres for up to 3 days (Martinez-Salas *et al.*, 1988 and Martinez-Salas *et al.*, 1989). Wirek *et al.* (1985) report that some reduction in the amount of introduced DNA is evident by 72 hours post-injection and suggest that the DNA is either degraded or no longer extractable because it integrates into cellular DNA or is recombined into larger oligomers. Indeed, Brinster *et al.* (1985) report that 24 hours after the introduction of supercoiled DNA the concentration of DNA remains the same but there were changes in the distribution of supercoiled, nicked circular and linear molecules. In 1988 Ao *et al.* introduced a supercoiled plasmid coding for the Tk gene but regulated by the MT-I promoter. They demonstrated that DNA transcription was capable of being stimulated 15 hours after microinjection by the addition of cadmium to the culture medium, indicating that the regulatory and coding sequences of the injected plasmid were still present at this time.

In conclusion, it would appear unlikely that significant degradation is responsible for the observed decline in β-galactosidase activity reported in this study.

The interpretation of the results is further complicated by the inclusion of the autonomous replicating sequence within the CMV-BGB and Tk-BGB plasmids (Ariga *et al.*, 1987). This sequence contains a putative origin of replication and the presence of this element may have resulted in extrachromosomal replication of the plasmid. If this were the case, it lends credence to the suggestion that the observed decline in β-

galactosidase activity was due to influences acting at the transcriptional or post-transcriptional level.

Although a good correlation between lac Z mRNA levels and enzyme activity has been reported (Norton and Coffin, 1985), changes in the stability of the β-galactosidase enzyme or the efficiency of translation of lac Z mRNA cannot be ruled out. Changes in the stability of endogenous stored maternal RNA are known to occur around this time in preparation for the onset of embryonic gene activation (Bachvarova and De Leon, 1980; Clegg and Piko, 1983 and Ebert *et al.* 1984). A more accurate assessment of the effect of developmental stage on transcription would have been gained by assaying mRNA levels directly.

A number of workers have reported that expression of introduced genes in spontaneously blocked and aphidicolin-arrested one-cell embryos is considerably greater than at the two-cell or later stages of development (Stuart *et al.*, 1984; Chen *et al.*, 1986; Ao *et al.*, 1988; Martinez-Salas *et al.*, 1989; W. Reik, pers. comm.). This finding is consistent with the results reported here in which the number of blocked one-cell embryos staining blue is considerably greater than the number of two-cell embryos staining and with the general finding of high transcription activity early in development.

Martinez-Salas *et al.* (1989) have examined this phenomenon using the Tk-*luc* gene and found that expression levels in blocked one-cell embryos were 21 fold greater than developing two-cell embryos. Although the addition of an enhancer sequence could increase the level of expression in two-cell embryos it was not required in one-cell embryos. These workers found a very high inverse correlation between Tk promoter activity and the rate of assembly of plasmid DNA into chromatin. At 45 hours post-injection when *luc* expression peaked in blocked one-cell embryos and developing two cell embryos, 70% of the DNA in two-cell embryos but only 10% of DNA in one-cell embryos was in the supercoiled form. It would appear, therefore, that injected DNA is not efficiently assembled into chromatin until the two-cell stage and that this allows the exogenous DNA to be expressed efficiently in the blocked one-cell embryo. Workman and Roeder (1987) have reported that sequestration of DNA into nucleosomes prevents transcription factors binding to the promoter.

This might imply that transcription is high in the blocked one-cell and early two-cell embryo resulting in high levels of lac Z mRNA and β-galactosidase accumulating and that expression is increasingly inhibited as more and more plasmid DNA is assembled into chromatin following the one-cell stage. If plasmid assembly is a progressive phenomenon through early embryo development this could result in high levels of expression initially (for example, the early two-cell stage) with a subsequent decline in transcriptional activity. Therefore, it is possible that the reduction in β-

galactosidase activity observed at the four-cell stage in these results is related to the assembly of the lac Z plasmids into chromatin.

As mentioned, Martinez-Salas *et al.* (1989) showed a correlation between sequestration of introduced plasmids into chromatin and a major reduction in transcriptional activity of the introduced plasmids. However, transcription of DNA within chromatin is dependent on the conformation of that chromatin, active or inactive, and not with assembly into nucleosomes *per se*. Buschhausen *et al.* (1987) microinjected plasmids expressing the Tk gene into the nuclei of mouse cells and found that both *in vitro* methylated and unmethylated constructs expressed the Tk gene initially. By 8 hours post-injection expression from the methylated but not the unmethylated plasmid had started to decline. Assembly of plasmid DNA into chromatin before microinjection did not inhibit transcription of the unmethylated Tk gene but no transcription occurred if the methylated DNA was assembled into chromatin and introduced into the cells. This work demonstrated that methylation of gene sequences did not reduce transcription unless those sequences are associated with chromatin.

Therefore, one possible contributory reason for the expression pattern described may be that the introduced sequences are being subjected to chromatin assembly and/or *de novo* methylation in the early embryo.

Staining Pattern Of Microinjected Embryos Cultured In The Presence Of Methylation Inhibitors

In an attempt to examine the role of DNA methylation in transcriptional regulation of foreign DNA in the preimplantation embryo, microinjected embryos were cultured in the presence of inhibitors of DNA methylation - DL-homocysteine and 5-azacytidine.

S-adenosylmethionine (SAM) is an important co-factor in the methylation of DNA, RNA and protein. It has been shown that depletion of the endogenous SAM pool can result in a reduction in methyl transferase activity involving nucleic acids (Kramer *et al.*, 1987). Menezo *et al.* (1989) incubated embryos with 500 μM of L-homocysteine and showed that endogenous levels of SAM were depleted and that the presence of L-homocysteine significantly reduced the number of embryos developing to the blastocyst stage. These authors suggested that development was compromised as a result of the induced inhibition of methylation activity. In this current study the rate of embryo development declined with increasing concentrations of DL-homocysteine but more than 50% of one-cell embryos cultured to the blastocyst stage when 1000 μM of DL-homocysteine was included in the culture media. In these

experiments a racemic mixture of D and L homocysteine was used, whereas Menezo *et al.* (1989) used only the active L-homocysteine. The reduced concentration of the active ingredient probably explains the difference in these results.

Those embryos which were microinjected and cultured in DL-homocysteine (500 - 1200 μM) were not significantly different from those cultured in the absence of this substance either in the numbers stained, the intensity of staining or the overall pattern of lac Z expression. In these experiments the embryos were cultured to the blastocyst stage for two reasons. Firstly, as the numbers which stain and the intensity of that stain were both much reduced compared to earlier cleavage stages, it might be easier to observe a difference between the two groups and secondly the longer the embryos were in contact with the methylation inhibitor the more the methylation pattern between the two groups was liable to diverge.

The nucleotide analogue 5-azacytidine is a strong inhibitor of DNA methylation and has been used extensively to induce transcription from methylated DNA (Jones, 1984; Jones, 1985; Jeggo and Holliday, 1986; Michalowsky and Jones, 1989). A small number of embryos were injected with CMV-BGB and cultured in the presence of 5-azacytidine. The β-galactosidase activity of these embryos was not significantly different from the control group.

In conclusion, co-culturing embryos in the presence of methylation inhibitors did not modify the observed pattern of β-galactosidase activity. These results do not provide evidence to support the hypothesis that the transcription activity of the exogenous DNA is being down-regulated by methylation of the DNA in early embryos.

Alternatively, it is possible that methylation is affecting transcription of the introduced plasmids but that methylation is not being sufficiently inhibited or reversed by the presence of the methylation inhibitors either because they are present at too low a concentration or their action is delayed. Treatment with homocysteine may require some time to reduce SAM levels sufficiently to interfere with methylase activity and by this stage the introduced DNA may already be extensively methylated. For 5-azacytidine to act as a methylation inhibitor it must be incorporated into the DNA. In tissue culture experiments generalised demethylation is usually accomplished by the use of relatively high concentrations of 5-azacytidine over a period of time often using multiple treatments (Flatau *et al.*, 1984; Michalowsky and Jones, 1989). In this study levels above 0.1 μg/ml significantly impaired embryo development whereas concentrations used in tissue culture experiments have ranged from 1-3 μg/ml (Jeggo and Holliday, 1986).

It is possible that *de novo* methylation is a progressive event which causes a quantitative reduction in expression over a period of time. *De novo* methylation of endogenous or exogenous DNA may be a gradual process *in vivo* requiring a number

of DNA replication cycles, indeed Monk *et al.* (1990) report that *de novo* methylation is a slow process occurring over several days. If this were the case in the early embryo, it is unlikely to have a major effect on transcription during the first 24-48 hours after fertilisation.

The evidence that DNA methylation within the preimplantation embryo is an important physiological event is contradictory. There is no direct evidence from the status of the embryos' endogenous genes to suggest that a significant *de novo* methylation event occurs during preimplantation development. Gametes show a marked difference in the extent of DNA methylation with oocytes being undermethylated and sperm DNA being heavily methylated. This appears to be true for both overall levels of methylation and for specific sequences (Monk *et al.*, 1987; Sanford *et al.*, 1987). Circumstantial evidence exists to suggest that this situation is propagated through early embryonic development as the degree of methylation in the preimplantation embryo is intermediate (Sanford *et al.*, 1987). There is a general reduction in DNA methylation during development from the morula to the blastocyst stage and a reduction in methylase activity at this time (Monk, 1988).

On the other hand Adams (pers. comm.) has reported that levels of the endogenous methylase are very high in the one-cell embryo and that these levels are maintained to the eight-cell stage at which point they start to decline. Certainly, the early embryo appears to have the capacity to methylate DNA as *de novo* methylation of specific sequences has been reported in the early preimplantation embryo (W. Reik and S.K Howlett, unpublished data cited by Surani *et al.*, 1990).

Further, for some genes methylation appears to be a secondary event which occurs after the inhibition of transcription and serves to enforce and perpetuate the original signal. Embryonal carcinoma cells serve as a model for undifferentiated embryonic cells such as exist within the eight-cell embryo. After infection of these cells with the retrovirus MuLV the provirus integrates but transcription of the provirus is blocked. However Gautsch and Wilson (1983) report that the provirus only becomes methylated much later and suggest that transcriptional inactivation serves as the signal for methylation and not vice versa. It appears, therefore, that methylation can be both the cause and effect of transcriptional inactivity.

In Vitro Methylation Of DNA

In order to ascertain if methylation of the lac Z plasmid CMV-BGB would reduce the transcriptional activity of the lac Z gene this plasmid was methylated *in vitro* and microinjected into one-cell embryos. Although the numbers are small the results of two separate experiments imply that pre-methylation of the DNA can reduce the transcriptional activity of constructs introduced into the early embryo.

This result shows that the transcriptional activity of the transgene is sensitive to the effects of methylation. Further, it suggests that the unmethylated construct was not methylated *in vivo* to the same extent as the methylated construct at the onset of gene expression. It does not, however, rule out methylation being involved in the observed reduction in transcriptional activity. A small number of embryos did display β-galactosidase activity after being microinjected with the methylated construct demonstrating that *in vitro* methylation of the construct did not completely block expression.

In a third experiment no difference was observed in β-galactosidase activity between the methylated and the mock methylated plasmid. One possible reason for this result is that the activity of the methylase enzyme used had declined by the time this experiment was conducted. However, this finding emphasises the need for further experiments to establish the effect of *in vitro* methylation on DNA transcription in early embryos.

Future Prospects

The preferred and most direct way of determining if exogenous DNA is being methylated in the early embryo would be to examine the introduced DNA for its methylation status at some time point after injection. This could be achieved by cutting DNA extracted from the blastocysts with methylation sensitive enzymes and amplifying the resulting fragments using the polymerase chain reaction (PCR). Properly designed this experiment should provide evidence of *in vivo* methylation of the transgene although a quantitative result would be more difficult to achieve.

A combination of reverse transcription and PCR is a possible method for identifying specific mRNA species in individual embryos. Such an approach would be a much more sensitive indicator of transcriptional activity of specific genes including introduced transgenes.

CHAPTER 5. - INFECTION OF OVINE EMBRYOS WITH FeLV

SECTION 5.1. - INTRODUCTION

Introduction

The primary purpose of the experiments described in this chapter was to examine the susceptibility of ovine preimplantation embryos to retroviral infection *in vitro*. Retroviral vectors have been used to produced transgenic mice and the work described here represents the initial step in a project designed to investigate the feasibility and suitability of adapting this approach to the production of transgenic sheep. The retrovirus selected for these experiments was the Type B Feline Leukaemia Virus (FeLV-B) and embryos were infected by injecting virus supernatant under the zona pellucida of the preimplantation embryo.

This experiment involved a moderately sized embryo transfer programme and this provided the opportunity to examine aspects of ovine embryo transfer. The results of both the embryo transfer work and the embryo infection are presented in this chapter.

Background

Retroviruses are a large family of viruses, the members of which can interact with many different species. They differ from other viruses in their mode of replication. These viruses have a single stranded RNA genome which replicates through a DNA intermediate, the provirus. This intermediate is an integral part of the retrovirus lifecycle and retroviruses carry an enzyme, reverse transcriptase (Temin and Mitzutani, 1970; Baltimore, 1970), which allows the RNA viral genome to be used as a template to produce the double stranded DNA copy. This copy is then efficiently inserted into the genome of the host cell. These viruses, therefore, have the capacity to transfer their viral genes into the host chromosome.

Transgenic animals have been broadly defined as those animals carrying, in the germ line, gene sequences of non-parental origin. Analysis of the mouse genome has revealed the presence of a large number of widely dispersed regions that show homology with retroviral sequences (Harel *et al.*, 1967; Jenkins *et al.*, 1982). These proviral sequences are present in the genome and are transmitted from one generation to the next according to mendelian expectations (Rowe, 1972). It is widely believed that these endogenous proviruses have arisen from the infection of the germ line with

ectopic murine retroviruses at some previous generation (Buckler *et al.*, 1982), indeed crossing certain inbred strains of mice can result in their progeny acquiring new proviral sequences. In these mice activation of the endogenous provirus produces a spontaneous viraemia which can result in the infection of very early embryos and the acquisition of new proviral copies in the germ line (Rowe and Kozak, 1980; Jenkins and Copeland, 1985). It would appear, therefore, that following the broadest definition, the creation of transgenic animals can be a naturally occurring phenomenon.

Germ Line Integration Of Viral DNA

In 1974 Jaenisch and Mintz showed that microinjection of Simian Virus (SV40) DNA into the mouse blastocyst could result in a percentage of the resulting offspring carrying SV40 specific DNA in at least a proportion of their tissues. Germ line transmission was not observed in these animals.

In 1975 Jaenisch *et al.* successfully infected mouse preimplantation embryos (four to eight cell stages) with Murine Leukaemia Virus (MuLV). Analysis of one viraemic offspring revealed that virtually all tissues of this animal carried similar amounts of viral DNA in each cell. These workers went on to show that infecting preimplantation embryos with exogenous MuLV could result in viral sequences being retained in the germ line with the proviral sequences being transmitted to the progeny of the infected offspring (Jaenisch, 1976). In these experiments incorporation of viral sequences into the germ line was accomplished by removing the zona pellucida and exposing the early embryo to concentrated stocks of retrovirus. In 1981 Jaenisch *et al.* showed that infecting embryos was also possible using blastocyst microinjection and that germ line integration of provirus was possible using this method albeit at a very low frequency.

The Design Of Retroviral Vectors

The intrinsic ability of retroviruses to efficiently and stably insert their viral genes into the chromosomes of host cells together with their capacity for non-lytic infection of a wide range of cell types makes these viruses ideal vehicles for gene transfer experiments.

Acutely transforming retroviruses differ from slowly transforming retroviruses due to the presence of additional genes within the viral genome (Duesberg and Vogt, 1970; Bishop, 1983; Bishop and Varmus, 1982). These additional sequences are

derived from cellular sequences and arise as a result of recombination events between the provirus and the adjacent cellular DNA (Varmus, 1982; Bishop, 1983). Acutely transforming retroviruses are in themselves usually replication defective, due to the displacement of viral protein coding sequences by the cellular sequences, but can be replicated when cells are co-infected with replication competent virus. Therefore, the recombinant viral genome can act as a vector for transferring cellular gene sequences from one cell to another and from one chromosomal position to another.

These observations have led to the development of retroviral vectors designed to deliver specific genes to target cells (for review see Donehower, 1987). There are two requirements which are necessary for these vectors to function. Certain non-coding sequences at the 5 and 3 prime ends of the genome must be retained whilst the viral proteins must be supplied by the complementary expression of the requisite viral genes within the cell (*i.e.* supplied *in trans*). Infecting the cell with replication competent viral sequences can provide the structural and functional proteins required but this approach results in a mixed population of vector and wild type virons being shed by the infected cell. The identification of the cis-acting sequences, which must be present in order to allow the viral genome to be packaged, led to the development of cell lines which could be used to produce helper free retroviral vectors. One of the first packaging cell lines was developed by Mann *et al.* (1983). These workers introduced, into the host chromosome, a retroviral genome which supplied all the necessary viral proteins but which could not itself be packaged into the virus particle due to a deletion of cis-acting sequences (the psi sequences) - these sequences being responsible for packaging the retroviral genome. The subsequent introduction of genes flanked by 5 and 3 prime retroviral sequences including the packaging signal allows these genes to be incorporated into the viral capsid.

It has been shown that recombination events can rescue replication competent virus from the helper cell lines (Hu *et al.*, 1987), therefore, a number of modifications have been made to packaging cell lines in order to reduce the likelihood of these events (Miller and Buttimore, 1986; Markowitz *et al.*, 1988; Dougherty *et al.*, 1989). Only those sequences absolutely necessary are retained within the retroviral vector and to further reduce the possibility of unwanted recombination events the viral coding gene sequences have been separated and introduced into different chromosomal sites.

Production Of Transgenic Mice Using Retroviral Vectors

Van der Putten and co-workers (1985) showed that it was possible to introduce foreign genes into the mouse germ line using a retroviral vector to infect

preimplantation embryos. In these experiments the retroviral vector carried a mutant dihydrate reductase gene. Embryos were infected using a mixed population of wild type helper virus and a recombinant vector but these workers also showed that it was possible to infect preimplantation embryos in the absence of helper virus. In experiments using a combination of wild type and recombinant virus 20-40% of embryos infected and transferred were born, up to 40% of offspring were positive for wild type virus sequences and 20% of offspring contained vector sequences. When embryos were infected with the retroviral vector exclusively 2% of offspring were positive for the presence of foreign gene sequences. Huszar *et al.* (1985) also showed that genes, in this case the bacterial *neo* gene, could be introduced into the mouse germ line using a retroviral vector. In these experiments embryos were infected by co-culturing the embryos with cell lines releasing either murine leukaemia virus vector or both vector and wild type MuLV. Slightly earlier embryonic stages were used in studies carried out by Rubenstein *et al.* (1986) who also used a retroviral vector to deliver the *neo* gene and assessed the genetic status of the resulting foetuses at day 17 of gestation. In these experiments it was shown that embryos cultured from both the two and four-cell to the morula stage were also susceptible to retroviral vector infection.

LTRs Can Interfere With Expression And Compromise Safety

Retroviruses carry sequences which direct the formation of the long terminal repeats (LTRs) situated at either end of the integrated provirus. These LTRs contain the endogenous viral enhancer and promoter sequences - the presence of which has a number of disadvantages in retroviral vectors.

The activity of the LTR is suppressed when retroviruses or retroviral vectors are introduced into preimplantation embryos or embryonal carcinoma cell lines (Stewart *et al.*, 1982; Jahner *et al.*, 1982). This block in expression is permanent and reactivation is a rare event occurring only in some adult tissues of the resultant offspring (Jaenisch *et al.*, 1981).

Soriano *et al.* (1986b) showed that it is possible to get both tissue specific and ectopic expression of genes introduced into the germ line with retroviral vectors. The β-globin gene was expressed in haematopoeic tissues by including β-globin's own promoter within the retroviral vector. Stewart *et al.* (1987) also showed that *neo* gene expression could be directed by an internal Thymidine Kinase promoter within a retroviral vector.

With an intact LTR there is still the possibility that endogenous retroviruses could supply helper functions and allow the spread of virus vector. In addition, the

transforming potential of retroviruses - even those that lack viral oncogenes - is well recognised. This occurs due to insertional mutagenesis, the promoter and enhancer elements contained within the LTR can locally activate cellular genes including proto-oncogenes (Teich *et al.*, 1982; Bishop, 1983).

These problems can be circumvented by designing a vector - the self inactivating vector (Yu *et al.*, 1986) - which contains a deletion in the 3' LTR. Due to the mode of retroviral replication the 3' LTR is used as a template for the formation of both the 5' and 3' LTR in the new integrating provirus. These deletions remove from the LTRs those regulatory elements which interfere with internal promoters and cause the induction of cellular genes.

Production Of Transgenic Domestic Animals

The first technique used to introduce selected DNA sequences into the mouse germ line was pronuclear microinjection (Gordon *et al.*, 1980; Brinster *et al.*, 1981c). This technique is now widely and successfully used to produce transgenic animals but it is not without some disadvantages as the technique is associated with embryo loss. In addition, pronuclear microinjection results in the integration of a varying number of copies of the DNA construct at a single site, usually arranged in head to tail tandem arrays.

The use of retroviral vectors is not associated with significant embryo mortality and the insertion of a single proviral copy into the host genome simplifies measurements of quantitative expression. Further, the integration of a single copy makes cloning of the integration site far easier (Schnieke, *et al.*, 1983; Soriano *et al.*, 1986b).

An alternative method of producing transgenic mice is to utilise embryonic stem cells (Bradley *et al.*, 1984). This approach has a number of advantages. Gene sequences can be introduced into these cell lines and those cells expressing the novel sequences selected. In addition, a consequence of this approach is that exogenous genes can be targeted to specific loci allowing the transgene to exploit the regulatory sequences of endogenous genes or to directly ablate the function of endogenous genes (Smithies *et al.*, 1985; Thomas *et al.*, 1986; Mansour *et al.*, 1988; Thompson *et al.*, 1989).

All three approaches have been successfully used to generate transgenic mice. Pronuclear microinjection, however, has proved to be a most accessible technology and is, therefore, the most commonly used method to produce transgenic animals. To date only this method has been exploited to introduce genes into the germ line of domestic animals although research to develop retroviral vectors (Ban *et al.*, 1989)

and embryonic stem cells suitable for use in domestic animals is continuing (Evans *et al.*, 1990).

There are a number of factors which make pronuclear microinjection more difficult in sheep (Wilmut *et al.*, 1990). Unlike the situation in mice it is not possible to collect large numbers of embryos all at the one-cell stage. This is partly due to the restricted availability of embryos and the greater variability in the stage collected. Cytoplasmic vesicles present in the early embryos of domestic animals obscure the pronuclei. In sheep this can be circumvented by differential interference contrast (DIC) microscopy and in pigs and cattle by a combination of centrifugation and DIC microscopy.

Despite these difficulties pronuclear injection is the only technique that has been successfully used to produce transgenic domestic animals. However, the efficiency of this procedure remains low. Wilmut *et al.* (1990) report that around 0.85% of injected ovine embryos are represented as transgenic lambs. It is possible that retroviral vectors could increase the efficiency of producing transgenic livestock partly due to the capacity of retroviruses to infect - at least in mice - a number of preimplantation stages but mainly due to their capacity to infect and integrate their genes with high efficiency in a manner which does not usually damage the target cell.

In this study Feline Leukaemia Virus (FeLV) was tested for its ability to infect sheep preimplantation embryos. FeLV was selected because it has been molecularly characterised and was known to infect ovine cells in culture (O. Jarrett, pers. comm.). As this study involved a moderately sized embryo transfer programme the factors affecting the efficiency of ovine embryo transfer were also examined.

INFECTION OF OVINE EMBRYOS WITH FeLV

SECTION 5.2. - MATERIALS AND METHODS

5.2.1. Experimental Design

This work had one primary objective: to determine the susceptibility of the ovine preimplantation embryo to retroviral infection. This is a requisite experiment in the development of a retroviral vector for use in sheep. In order to carry out this experiment it was necessary to collect the embryos, microinject virus stock under the zona pellucida and transfer the embryos into recipient ewes. After transfer the embryos were allowed to develop *in vivo* for a further 6-8 weeks and the resultant foetuses were collected at slaughter and analysed for the presence of integrated provirus. The ewes were synchronised using intravaginal progesterone sponges, the treatment lasting for between 11-14 days. Those ewes selected as embryo donors received a superovulatory dose of PMSG, whereas those ewes destined to be recipients received a small dose of PMSG to help synchronise ovulation. The donors were given access to an intact male ram wearing a raddled harness and time of oestrus onset was taken as the time the ewes were first seen marked. The recipients were placed with a vasectomised ram, similarly harnessed, in order to determine the approximate time of oestrus onset.

Embryo collection was timed so as to maximise the number of two-cell embryos gathered. After a number of collections had been carried out, the oestrus to operation interval was adjusted in an attempt to improve the yield of two-cell embryos. Following collection, embryos were exposed to concentrated stocks of FeLV by microinjecting virus containing supernatant under the zona pellucida. Embryos were transferred into the most synchronous recipients in order to maximise the viability of the embryos. After surgery both the donors and recipients were group housed with an intact ram. This had the duel benefit of checking both the current pregnancy status and assessing the ability of the ewes to establish and support a post-operative pregnancy. Six to eight weeks after embryo transfer the ewes were slaughtered and the contents of their uteri collected. The foetuses collected were analysed for the presence of proviral sequences derived from the infecting FeLV.

5.2.2. Synchronisation And Superovulation

Draft Scottish Blackface ewes were used in this experiment. Before entering the programme the health and condition of the ewes was assessed. In general those animals judged as being in good condition were selected as embryo donors and those in slightly poorer condition as the recipients.

Oestrus synchronisation of ewes was achieved by the use of progestagen (fluorogestone acetate) impregnated intravaginal sponges (Chronogest - Intervet). After 11-14 days treatment the sponges were removed. The donors were superovulated by an intramuscular injection of between 1200 and 1500 iu of Pregnant Mare Serum Gonadotrophin - PMSG (Folligon - Intervet). PMSG was administered to the donors between 0 and 24 hours before sponge removal. On average, sponges were removed 12 hours earlier in the recipients and 500 iu of PMSG was administered at this time or 8 hours before sponge removal. The donors were mated by natural service and the time of first mating was recorded. Oestrus onset in the recipients was assessed using a vasectomised ram.

5.2.3. Embryo Collection And Transfer

During the 24 hour period prior to surgery, food and water were withheld from the ewes. Anaesthetic induction was carried out using 6% Sodium Pentobarbitone (Sagatal - May and Baker) at a dosage rate of 0.44 ml/kg and delivered into the cephalic vein via a butterfly needle (Miniven - Laboratoire Portex).

The ewe was placed in dorsal recumbency and intubated with a Magills No. 11 endotracheal tube (Leyland) using direct visualisation with a 16 inch laryngoscope. The ewe was clipped and prepared for surgery by washing with Savlon (Hospital Concentrate, ICI) and swabbed with a pevidine/alcohol (70%) solution (Pevidine - Berk Pharmaceuticals)

A midline incision was made approximately 5-6 inches long just cranial to the mammary tissue. Entry to the abdomen was made by incising through the linea alba and the uterus was exteriorised through the incision. The ovaries were examined and the number of corpora lutea (CL) noted on each side.

5.2.3.a) The Donors

The oviducts were flushed in a retrograde direction, *i.e.* from utero-tubal junction towards the ampulla. The embryo collecting dish was covered with sterilised aluminium foil in an attempt to reduce airborne fungal contamination of the medium. The assistant collected the flush by inserting a glass U-shaped capillary tube (GC 100-

10 Clarks Electromedical Instruments) into the oviduct from the fimbrial end and directing the other end through the aluminium foil covering the collecting dish. Using a blunt butterfly needle attached to a 20 ml syringe the surgeon directed 12-16 ml of ovum culture medium (Flow) through the oviduct by injecting into the uterine tip - having clamped the uterus just caudal to the injection site between finger and thumb. This procedure was then repeated for the other side and any difficulty encountered when flushing (e.g. build up of pressure or leakage of medium) was recorded.

In a proportion of ewes the ovaries and uterus were irrigated with heparinised saline.

The abdominal wall was closed with simple interrupted sutures of 4 metric monofilament nylon (Monilon 4 metric, Arnolds Veterinary Products). The subcutaneous tissue was repaired with 3 metric catgut (Ethicon) using a continuous horizontal mattress suture. Finally the skin was closed with either 3 metric monofilament nylon, interrupted mattress sutures, or Michel clips using a Michel applicator (Martins).

2 ml of penicillin (Duphapen L.A. - Duphar Veterinary Ltd.) was administered by intramuscular injection to prevent post-operative sepsis. The ewes were observed until laryngeal function returned at which time they were extubated, placed in sternal recumbency and allowed to recover in a well bedded pen.

5.2.3.b) The Recipients

Surgical access for the recipients was as described for the donors. The fimbral end of the oviduct was held up and a fine glass pipette containing the eggs was inserted 3-4 cm into the proximal end of the oviduct. The eggs were then discharged by blowing gently down the attached plastic tubing. Throughout the procedure the tissues were liberally flushed with heparinised saline. Wound closure and aftercare was as described for the donors.

5.2.4. Virus Production

Feline Leukaemia Virus sub-group B (FeLV-B) was grown in feline embryo cells (FeA cells). This virus was selected because it was previously shown to successfully infect tissue culture lines derived from ovine tissues (O. Jarrett pers. comm.). The virus was harvested, concentrated and stored using standard techniques (Onions *et al.*, 1987). The titre of the final viral stock was in the range 5 - 7.2 x 10^6 focus forming units/ml (ffu/ml).

5.2.5. Embryo Manipulation

The ovine embryos were collected and handled in Ovum Culture Medium (Flow) supplemented with Foetal Calf Serum, penicillin and streptomycin (Gibco). Embryo manipulation was carried using a Leitz micromanipulator and a diavert microscope as described in general material and methods. An injection pipette with a tip diameter of approximately 5 μm was used to microinject virus-containing fluid into the extracellular space between the blastomeres and the zona pellucida of the embryo. Sufficient fluid was injected to cause the zona pellucida to expand markedly. After microinjection the embryos were held at 37^{o} C until they were transferred. Those embryos which had sustained obvious damage were discarded.

5.2.6. Post-operative Management of Ewes

In the period after collection/donation both the donors and recipients were housed and run with a harnessed ram. The ewes were examined daily to record the date of marking by the ram, and they were blood sampled in order to assay progesterone levels either 17 days after they were last in oestrus or the day of marking. In this way it was possible to assess the pregnancy status of both donors and recipients. In addition, the capacity of the ewes to become pregnant after surgery could be examined using this approach.

Progesterone levels were assessed using a commercial elisa kit (Farmkey).

5.2.7. Collection And Analysis of Foetal Material

Between 40 - 60 days after transfer the ewes were slaughtered and the number, position and approximate age of both healthy and degenerate foetuses was recorded. The foetal and placental tissue were separated and stored at -20^{o} C to await analysis. Preparation of DNA and analysis by Southern blotting were carried out by Dr. Hettle as described in general material and methods.

INFECTION OF OVINE EMBRYOS WITH FeLV

SECTION 5.3. - RESULTS

Introduction

The primary aim of this work was to discover if ovine preimplantation embryos were susceptible to infection with Feline Leukaemia Virus. This work constitutes the first step in the development of a FeLV based retroviral vector suitable for use in sheep.

Preimplantation embryos (one-cell - eight-cell stage) were collected by flushing the oviducts of superovulated donor ewes. Infection with FeLV was attempted by microinjecting virus stock under the zona pellucida of the embryo. After microinjection the embryos were held in media for a short time and those embryos which had sustained no obvious damage were placed into the oviduct of a synchronised recipient.

This work was carried out as two separate experiments with the first experiment involving the recovery of embryos from 38 embryo donors. A proportion of these embryos were subsequently transferred into a total of 16 recipients. This work took place from October to December and therefore fell into the first half of the breeding season for Scottish Blackface sheep. The second experiment was carried out in the following January and involved a total of 22 donors and 15 recipients.

A total of 60 ewes selected as embryo donors were synchronised and superovulated and of these, 59 showed signs of oestrus at which time they were mated to a fertile ram. All 59 had ovulated as assessed by ovarian examination at the time of collection. The ewe which was not observed as being in oestrus did not ovulate. All synchronised recipients showed signs of oestrus and ovulated.

5.3.1. The Interval Between Sponge Removal And Oestrus Onset

The mean interval between sponge removal and oestrus onset in the embryo donors was 25.2 hours with a range of between 20 and 34 hours (S.D. = 3.2). In the recipient group the mean interval was 40.2 hours with a range of between 28.5 and 49 hours (S.D. = 4.3), one ewe which took 80 hours to enter oestrus was excluded from the analysis. Therefore, the recipients took significantly longer (Student T test, $t = 15.2$ with 59 d.f.: $P < 0.01$) to enter oestrus after sponge removal than the donors.

Ewes were examined every 6 to 8 hours and oestrus taken as the time a ewe was first seen marked by the ram. Obviously there was a significant sampling error in this method as ewes could be marked shortly after being examined, however, even if this sampling error is accounted for (by assuming every recipient was marked shortly after being examined) the recipients still took significantly longer to enter oestrus after sponge removal (Student T test, $t = 7.1$ with 59 d.f.: $P < 0.01$).

The major difference in treatment between the two groups relates to the use of PMSG. These results demonstrate that the administration of PMSG, primarily used to superovulate the ewes, hastens the onset of oestrus. However, there are two distinct differences in the way PMSG was used between the donors and recipients - i) a significantly greater dosage of PMSG was administered to the donors (average 1400 iu) compared to that given to the recipients (average 350 iu) and ii) the length of time before sponge removal that PMSG was administered. On average PMSG was injected 20 hours before sponge removal in the donors and 6 hours before sponge removal in the recipients. In order to determine which of these two factors were important - the dosage or the timing of PMSG treatment - these variables were examined further.

5.3.1. a) The Relationship Between The Dosage Of PMSG And Time Taken To Enter Oestrus After Sponge Removal

In order to examine the influence of PMSG dosage on the sponge removal to oestrus interval, those ewes in which PMSG was given at a standard time before sponge removal but which received slightly different doses were compared. Two groups of donors were compared - those that received 1000-1250 units and those that received 1500 units. The results presented in Table 5.3.1. show that small differences in PMSG dosage between the donors are not reflected in a difference in the time taken to come into oestrus. Both groups, however, received a superovulatory dose of PMSG and the difference between the dosage the two groups received may be too small to allow a similar difference to be detected in the time taken to enter oestrus.

Table 5.3.1. Sponge Removal/Oestrus Interval - PMSG Dose.

Dose (iu) of PMSG	Sample Number	Average Sponge Removal/Oestrus Interval (hours.)
1000-1250	8	23.1 hours
1500	22	25.5 hours

The response to PMSG, as measured by the number of oocytes that ovulated, varied widely between individual ewes (see below). In order to exclude this large individual variation from the analysis the effects, rather than the dosage, of PMSG were compared with the time taken for the ewes to enter oestrus. This was achieved by grouping the donors on the basis of response and assessing if there was a relationship between these groups and the average time taken to enter oestrus after sponge removal. The results are presented in Table 5.3.2. and show that those classified as unresponsive (1-2 CL/ewe) had a slightly longer interval between sponge removal and oestrus onset. However, the difference between this group and those ewes ovulating between 3-6 CL was not significant (Students T-test, $t = 1.2$ with 13 d.f.: $P > 0.05$).

Table 5.3.2. Sponge Removal/Oestrus Interval - PMSG Response.

No. of CL*	Sample Size	Average Interval (hours.)
0-2	5	27.6 hours
3-6	10	23.9 hours
7-10	10	24.9 hours
10+	5	24.1 hours

* Corpora Lutea

As can be seen from Table 5.3.1. and Table 5.3.2. small differences in PMSG dosage or differences in response to PMSG do not correlate with differences between ewes in the time taken to enter oestrus after the withdrawal of progestagen treatment.

5.3.1. b) The Relationship Between The Timing Of Injection Of PMSG Before Sponge Removal And Oestrus Onset

Table 5.3.3. shows the average time taken for a ewe to enter oestrus after PMSG injection. These results exclude from the analysis both the dosage of PMSG given (within the donor and recipient groups) and the time of sponge removal and show that the PMSG to oestrus onset interval is remarkably similar between the donors and recipients.

Table 5.3.3. Interval Between PMSG Administration and Oestrus Onset.

Group	Average Interval*	Range	S.D.
Donors	47.5 hours	41.5 - 58	3.27
Recipients	46.5 hours	35 - 57	4.40

* Average interval from PMSG administration to onset of oestrus.

These results suggest that time of PMSG administration might be an important factor in determining the time of oestrus onset. However, it is not possible to draw a definite conclusion from these results as the relative importance of dosage, time of sponge removal and time of PMSG injection cannot be separated.

5.3.1. c) The Relationship Between Length Of Sponge Treatment And The Sponge Removal To Oestrus Interval

The length of time ewes were treated with progestagen sponges was either 12 or 14 days for the donors, or 11 or 13 days for the recipients. Those donors which had a 14 day treatment entered oestrus on average 24.9 hours after sponge removal and in those treated for 12 days this interval was on average 25.6 hours. The recipients treated for 13 days had an average interval of 40.0 hours and those treated for 11 days had an average interval of 40.5 hours. Therefore, the length of progestagen treatment does not appear to significantly affect the time taken to enter oestrus after sponge removal in either donors or recipients (Students T-test, $t = 0.7$ with 32 d.f. and 0.3 with 25 d.f. respectively: $P > 0.05$).

5.3.2. The Response To PMSG - Number of Corpora Lutea

The average response to a superovulatory dose of PMSG in the donors was 7.1 corpora lutea/ewe with a range of 1-24 and a standard deviation of 4.7. The ewes show a wide variation in their ovarian response to PMSG, these wide variations being well recognised in both sheep and cattle. The average response of the donors in Experiment 2 was slightly higher than that of Experiment 1, an average of 7.9 CLs compared with 6.6 but the difference was not significant (Students T-test, $t = 1.0$ with 57 d.f.: $P > 0.05$). Figure 5.3.1. shows the range and distribution of ovulation rates of PMSG treated ewes.

Figure 5.3.1. Ovulation Rate Of PMSG Treated Sheep

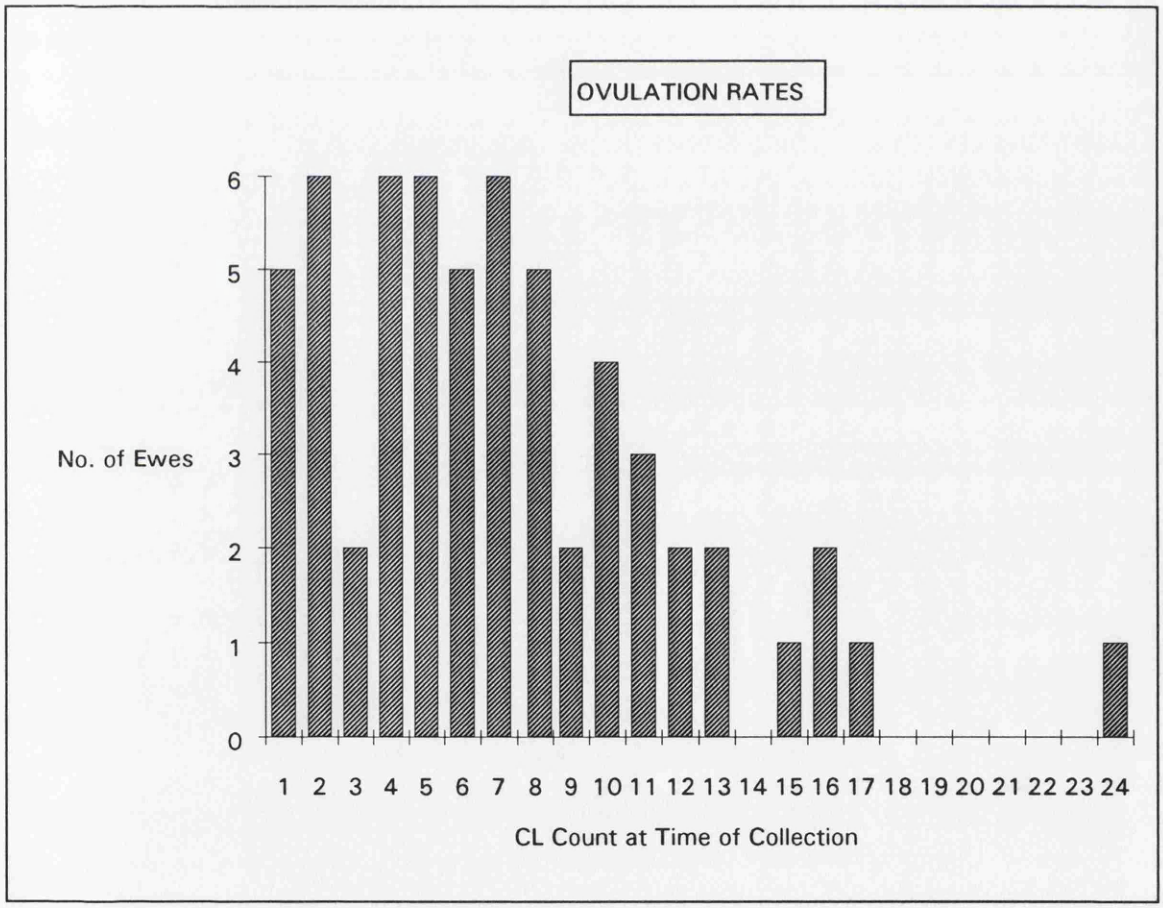


Figure 5.3.1. shows a distribution graph of ovulation rates in PMSG treated sheep.

5.3.2. a) Batch/Dose Variation Of PMSG - Response

A number of different batches and doses of PMSG were used throughout the course of this work the details of which are outlined in Table 5.3.4.a) and 5.3.4.b). There appeared to be some variation in the biological activity of the different batches of PMSG. However, because of the small sample size and the large variation in response of individual ewes it was not possible to definitely conclude if some batches differed significantly from the rest, but at least one batch, which produced a total of 6 CLs from 4 ewes, was suspected as having low biological activity and its use was discontinued.

In Experiment 1, 10 donors were superovulated with between 1200 and 1250 units of PMSG and 24 donors received 1500 units. There was no significant difference in the superovulation rate between the two groups (Student T-test, $t = 1.1$ with 32 d.f.: $P > 0.05$). These results, therefore, demonstrate that small differences in dosage were not reflected in significant differences in response.

Table 5.3.4.a) Ovarian Response to PMSG. Donors - Experiment 1

Batch	Dose	Sample Size	Average CL count	Range.
1	1000	2	1.5	1-2
1	1250	2	1.5	1-2
2	1250	8	8.2	2-11
2	1500	7	10.7	4-17
3	1500	7	6.0	1-7
4	1500	3	7.0	5-9
5	1500	7	7.1	4-11

Table 5.3.4.b) Ovarian Response to PMSG. Donors - Experiment 2

Batch	Dose	Sample size	Average CL count	Range
2	1600	12	8.3	1-24
6	1500	9	7.0	1-13
7	1500	1	10.0	10

5.3.2. b) Recipient Response To PMSG

A relatively small dosage of PMSG (between 250-700 iu) was administered to the recipients, the intention being to shorten the time taken for this group to enter oestrus after sponge removal but not to increase the ovulatory response of the ewes.

The average response in the 33 recipients synchronised was 2.6 CL/ewe with a range of 1 to 15 CL/ewe. Of the 16 recipients primed in Experiment 1 the average CL count was 1.6 and none of the ewes had more than 2 CLs. Two distinct groups could be identified in Experiment 2, 10 ewes which ovulated only 1 or 2 oocytes and 7 ewes which ovulated between 3 and 15 oocytes/ewe. The average response in this group was 7.7.CL/ewe, all the ewes in this group received the same batch and dose of PMSG. The most reasonable explanation for this response is that this batch had sufficient biological activity to produce a superovulatory effect at the dosage of 700 units/ewe.

5.3.3. Embryo Collection Rate

The average ovulation rate in the donor ewes was 7.1 with an overall collection rate of 58%. The ovulation rate in Experiment 2 was higher than that of Experiment 1 but the collection rate in Experiment 2 was significantly below that of Experiment 1 ($x^2 = 20.8$: $P < 0.01$). The collection rates are presented in Table 5.3.5.

Table 5.3.5. Embryo Collection Rate.

	No. Ewes	No. CLs	Average CL/ Ewe	No. Ova Collected	Average Ova/ Ewe	(%) Collected [a]
Exp 1	38	243	6.6	164	4.4	(67.5)
Exp 2	22	173	7.9	78	3.5	(45.0)

a. The number of ova collected expressed as a percentage of the number of CL counted.

5.3.3. a) The Effect Of Embryo Stage On Collection Rate

In Experiment 2 the embryo collections could be divided into two categories: those which resulted in only one-cell embryos being recovered and those in which more advanced stages were collected. These results are shown in Table 5.3.6. The collection rates in those flushes which yielded only one-cell embryos were significantly less than those flushes which only yielded more advanced stages (x^2 = 7.8: $P < 0.01$). The sample sizes are small, however, with only 9 flushes yielding one-cell embryos and 8 flushes yielding more advanced stages.

Table 5.3.6. Effect of Embryo Stage on Collection Rate.

Experiment 2	Number of Flushes	Number Collected.	Number Ovulated	Collection Rate (%)
Flushes containing only one-cells.	9	24	64	(38)
Flushes containing only advanced cells.	8	42	68	(62)

However, when the results of both Experiments 1 and 2 are pooled, this pattern is not observed. In collections which yielded only one-cell embryos, 67 out of a possible 122 embryos were collected giving a collection rate of 56% and in collections which yielded more advanced stages 175 embryos were collected from a possible 275 - a collection rate of 64%. Unlike the results of Experiment 2 the difference between the two groups was not significant ($x^2 = 2.7$: $P > 0.05$).

5.3.3. b) The Relationship Between The Level Of Superovulation And Collection Rate

To assess if the level of superovulation affected collection rates the efficiency of collecting embryos from ewes that had a CL count of below 7 was compared with those that had a collection rate of 7 and above. The results are presented in Table

5.3.7. and they show that the difference between the two groups is not significant ($x^2 = 0.9$: $P > 0.05$).

Table 5.3.7. Effect of Ovarian Response on Collection Rate.

No. of CLs.	Number of Ewes	Number Ovulated	Number Collected	Average/ Ewe [1]	Collection Rate (%)
less than 7	31	113	70	2.3	(62)
greater than 7	29	303	172	5.9	(57)

1. Average number of embryos collected per ewe.

5.3.4. Stage Of Embryo Collected

The experiment was designed to maximise the number of two-cell embryos collected. Infection at an early stage in development minimises mosaicism with respect to proviral integration. Infection at a later stage could possibly result in the cells of the developing foetus having a different number and pattern of proviral integrations. Although one-cell embryos were injected it was not the preferred stage as in most cases it was difficult to assess the fertilisation status of the embryos. For these reasons it was thought desirable to collect and use two-cell embryos, in addition the greater amount of space surrounding the blastomeres in the two-cell embryo made microinjection easier.

However, there was considerable variation in the embryo stages recovered at collection (see Figures 5.3.2 a,b,c). Of the 37 donors in Experiment 1, 23 yielded embryos which were all of the same stage, 13 ewes yielded two different stages of embryo - 7 of which consisted of one-cell embryos and a more advanced stage - and 1 ewe yielded 3 different stages. In Experiment 2, 14 ewes yielded one stage only, 4 ewes gave 2 different stages and 4 ewes gave no embryos.

The considerable overlap of different stages both from the same ewes and between different ewes made it difficult to collect a high percentage of two-cell embryos. In an attempt to establish the fertilisation status of the one-cell embryos a proportion of these embryos collected in Experiment 1 were placed in culture. A total of 62 one-cell embryos were cultured and 46 developed to more advanced stages (M. Waterson, pers. comm.). This indicates that the majority of one-cell embryos collected during Experiment 1 were probably fertilised.

Figure 5.3.2.a)

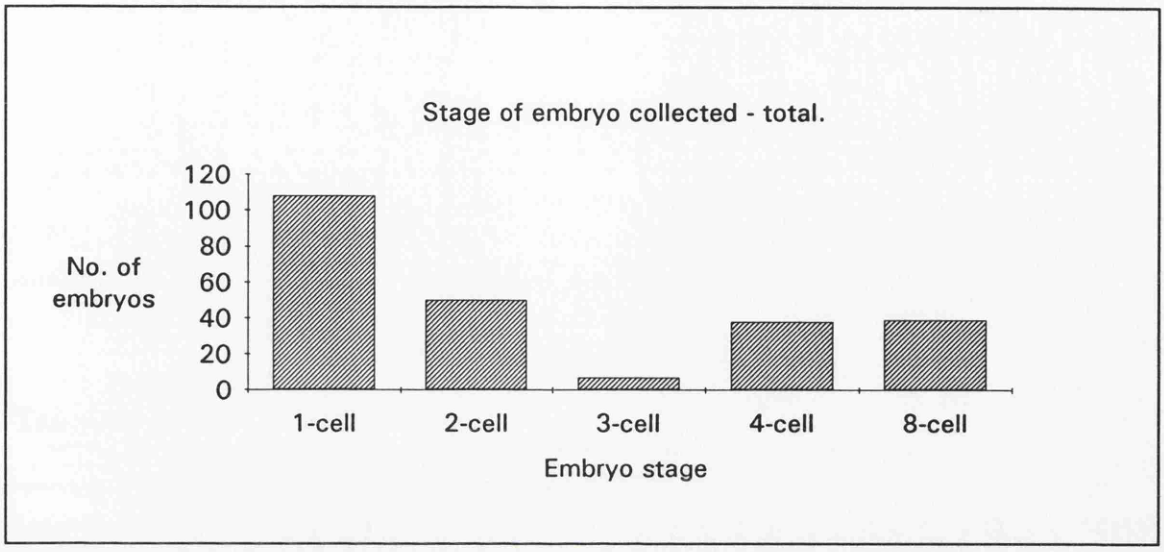


Figure 5.3.2.b)

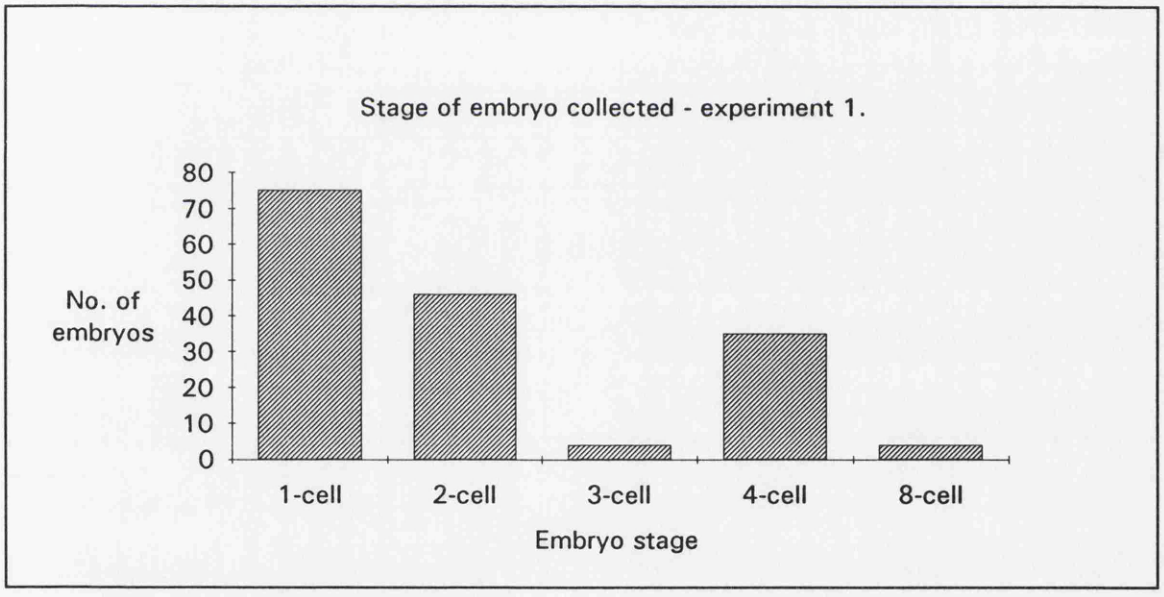
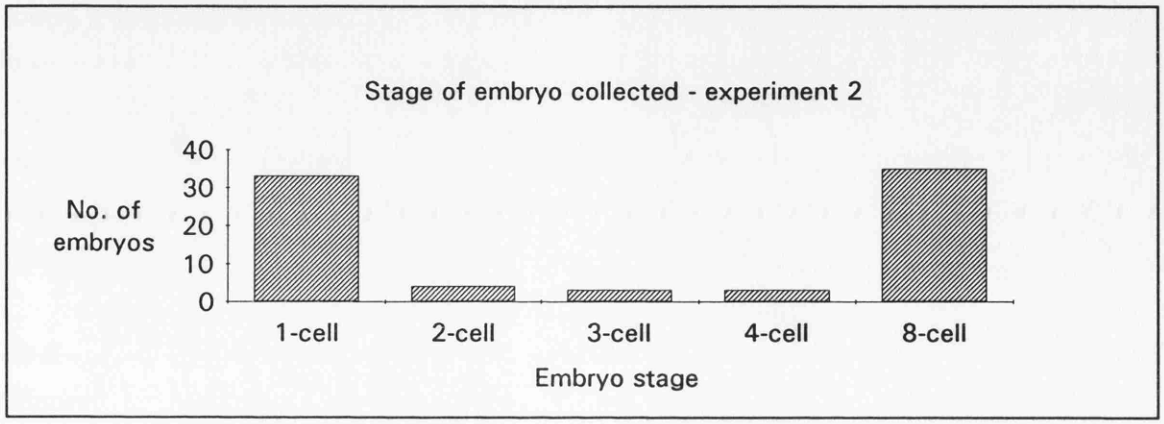


Figure 5.3.2.c)



5.3.4. a) Effect Of Sponge Removal To Collection Interval On Embryo Stage

The relationship between the time from sponge removal to operation and the stage collected is shown in Table 5.3.8. Within each experiment there was a poor correlation between the stage flushed and the interval between sponge removal and collection. Similarly the interval between the time the ewe was first noticed in oestrus and the time of collection did not correlate closely with the stage collected.

Table 5.3.8. Embryo Stage - Effect of Collection Time.

Average Embryo Stage Collected	Average Sponge Removal to Collection Interval (Hours).	
	Exp. 1	Exp. 2
1-1.9	75.2	90.8
2-2.9	75.4	91.0
3-3.9	73.8	-
4-4.9	75.5	-
5-5.9	78.0	-
6-6.9	-	96.5
7-7.9	-	97.5
8.0+	74.0	92.0

Early collections during Experiment 2 yielded only one-cell embryos and in order to determine if this was a reflection of the stage or the fertilisation status of the embryo the sponge removal to collection interval was increased. Therefore, the average sponge removal to collection interval was 75.6 hours in Experiment 1 and 91.8 hours in Experiment 2. As can be seen from Figures 5.3.2.b) and 5.3.2.c) this resulted in a greater percentage of more advanced stages being collected. Excluding one-cell embryos the average stage collected was 3.0 in Experiment 1 and 6.9 in Experiment 2.

In addition, the pattern of embryo stages collected shown in Figure 5.3.2.c) indicates that it is possible that the majority of one-cell embryos collected in Experiment 2 were unfertilised.

5.3.5. Subsequent Fertility In The Donors

After embryo collection the donors were run with an intact ram in order to assess subsequent reproductive performance. Forty-two percent became pregnant by natural service at the first oestrus after the operation (Table 5.3.9.). A pregnancy rate of 42% to first service is considerably below that expected for natural service in ewes, therefore, it is probable that post-operative inflammatory changes and subsequent adhesions affected the ability of the ewes to establish a pregnancy.

Table 5.3.9. Pregnancy Rate of Donors After Collection.

	Number of Ewes Put the Ram	Number Marked By Ram	Number Pregnant at *Post mortem*
Exp. 1	32	24	14 *
Exp. 2	13	12	2

* An additional 3 ewes were found to be pregnant from this time, however, these ewes were not marked.

5.3.5. a) Accuracy Of Pregnancy Diagnosis

Two methods of pregnancy diagnosis were employed for both donors and recipients. Harnessed rams were used to identify ewes in oestrus and in addition, ewes were blood sampled and their progesterone levels assayed. Slaughter of the ewes 6-8 weeks after they were first induced allowed a definitive diagnosis of their pregnancy status and it was, therefore, possible to examine the accuracy of pregnancy diagnosis using these two methods.

When blood progesterone samples were taken on either day 17 of the cycle or on the day of marking they proved to be accurate in 42 out of the 45 ewes sampled.

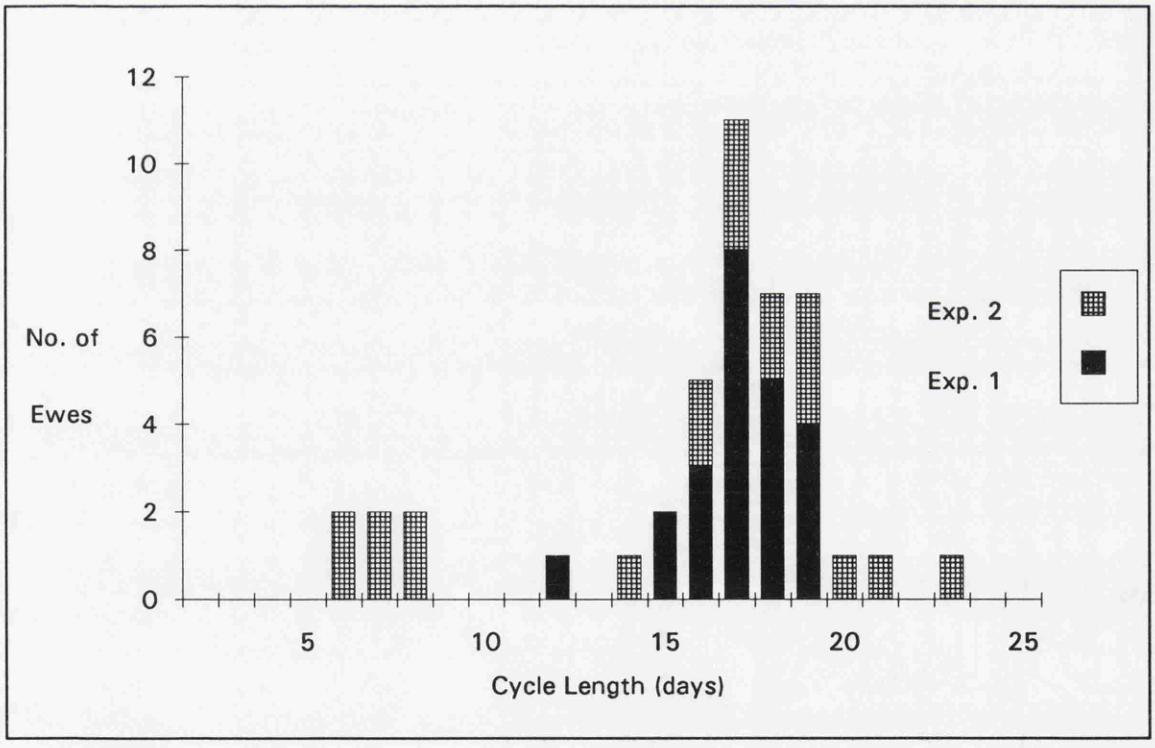
Using rams alone to identify those ewes in oestrus proved to be less accurate with only 35 out of 45 ewes being identified. However, using the ram as an indication of when to sample the ewes proved to be the most efficient system of pregnancy diagnosis.

5.3.6. Oestrus Cycle Length Of Donors After Synchronisation And Collection

The cycle length from the induced oestrus to the subsequent oestrus was recorded and the results shown in Figure 5.3.3. Fifty-one percent of ewes had a cycle length of between 16-18 days inclusive, 23% of ewes had a cycle length of greater than 18 days and 23% of ewes had a cycle length of less than 16 days. The overall average was a cycle length of 16.5 days. In Experiment 1 all ewes returned to oestrus after superovulation within 15 to 19 days inclusive.

The variation in cycle lengths is much greater in Experiment 2 where only 50% of ewes had a cycle length of between 15 and 19 days with 15% of ewes having a cycle length of longer than 20 days and 30% of ewes returning to oestrus 6 to 8 days after the induced oestrus.

Figure 5.3.3. Oestrus Cycle Length of Donors Following Induced Oestrus.



5.3.7. The Short Cycle Donors

Six donors in Experiment 2 had an abnormally short oestrous cycle ranging from 6 to 8 days. It was not possible to identify any factor or treatment which was unique to this group of ewes. These ewes responded successfully to synchronisation, were identified in oestrus after sponge removal, ovulated and yielded embryos. The average CL count of these ewes was 10.8 - the average for ewes having normal cycles

being 6.9. The majority of the ewes that were used had been previously primed at an earlier date and not used for surgical collection. However, the history of these ewes did not vary significantly from the other 14 donors in Experiment 2. It is possible that the stage of the breeding season might predispose ewes to these short oestrous cycles. All of the donors manifesting this anomaly were observed in the second half of the breeding season.

5.3.8. Recipients - Pregnancy Results

The pregnancy results are shown in Table 5.3.10. In total, 37% of transferred embryos were represented as foetuses when the uteri were examined at slaughter 40-60 days after transfer. 74% of recipients became pregnant and in these ewes 48% of transferred embryos were represented as foetuses. These ewes yielded a total of 35 healthy foetuses and 1 degenerating foetus.

Table 5.3.10. Recipient Pregnant Rate.

	Exp. 1	Exp. 2	Total.
Number of Recipients	16	15	31
*Number of P+ Recipients	13	10	23
Pregnancy Rate.	81%	66%	74%
Total Number of Embryos Transferred.	51	44	95
Average Number of Embryos Transferred.	3.2	2.9	3.1
*Total Number of Embryos in P+ Ewes.	43	30	73
Number of Foetuses Recovered.	19	16	35
Overall Embryo Survival Rate.	37%	36%	37%
*Embryo Survival Rate in P+ Ewes.	44%	53%	48%

*P+ represents those recipients which subsequently became pregnant.

5.3.8. a) Factors Affecting Recipient Pregnancy Rate - CL Count

The average CL count of the ewes which became pregnant was 1.5 CL/ewe and the average CL count of the non-pregnant ewes was 1.2 CL. These results exclude the recipients that showed a superovulatory response. In this group ewes with CL counts of 3, 7 and 15 all became pregnant but a ewe with a CL count of 8 did not become pregnant.

5.3.8. b) Factors Affecting Recipient Pregnancy Rate - Stage Of Embryo Transferred

In Experiment 2 four ewes received exclusively one-cell embryos, the fertilised status of which was not defined and none of these ewes became pregnant. No other stage was significantly associated with either pregnancy or non-pregnancy.

5.3.8. c) Factors Affecting Recipient Pregnancy Rate - Embryo Status

Seven ewes received 19 unmanipulated embryos which were transferred within 6 hours after collection. Five of these ewes were pregnant at slaughter yielding 7 foetuses. One ewe received 3 embryos (2 of which were mock injected) which were cultured for 24 hours, this ewe did not establish a pregnancy. In addition, 2 ewes received a mixture of control and injected embryos, both of these ewes were pregnant but the resulting foetuses were excluded from analysis.

Twenty-one ewes received 68 microinjected embryos, this resulted in 16 pregnancies yielding a total of 25 foetuses.

Therefore, 37% of unmanipulated embryos developed into foetuses and 37% of manipulated embryos developed and although the sample size is small it did not appear that microinjection significantly decreased the survival rate of the manipulated embryos ($x^2 = 0.02$: $P > 0.05$). Similarly, there was no significant difference between the survival rates of control and manipulated embryos in those ewes which subsequently became pregnant, with 50% of unmanipulated embryos and 46% of manipulated embryos being recovered as foetuses ($x^2 = 0.1$: $P > 0.05$). These results are shown in Table 5.3.11..

The pregnancy success results for the experimental group are improved if those ewes which received only one-cell embryos are excluded from the results. Of 17 ewes which received only cleaved, injected embryos, 16 ewes were pregnant. Therefore, it appears that sub-zonal microinjection did not significantly affect

subsequent viability although a small number of embryos (15%) were damaged at the time of microinjection and these embryos were not transferred.

Table 5.3.11. Injected Embryos - Recipient Pregnancy Rate.

	Control embryos	Injected embryos
Number of Recipients	7	21
*Number of P+ Recipients.	5	16
Pregnancy Rate.	71%	76%
Total Number of Embryos Transferred.	19	68
Average Number of Embryos Transferred.	2.7	3.2
*Total Number of Embryos in P+ Ewes.	14	54
Number of Foetuses Recovered.	7	25
Embryo Survival Rate.	37%	37%
*Embryo Survival Rate in P+ Ewes.	50%	46%

*P+ represents those recipients which subsequently became pregnant.

5.3.9. Efficiency Of Pregnancy Diagnosis In Recipients

None of the 23 ewes which were pregnant after embryo transfer were identified by the ram as being in oestrus and all of these ewes had progesterone (P4) levels consistent with pregnancy 17 days after transfer.

Of the 8 ewes which were ultimately not pregnant 7 were marked by the ram as being in oestrus and 7 had P4 levels consistent with oestrus.

One ewe which was not marked by the ram and was subsequently found to be not pregnant had a high P4 level - the most likely explanation being that this ewe was pregnant at the time of sampling and that pregnancy was subsequently lost.

All 7 ewes which were marked by the ram and were not pregnant had a cycle length of 16 to 19 days inclusive and had an average cycle length of 17.7 days.

5.3.10. Embryo Migration

Seventeen ewes received 2-4 embryos in one oviduct only. At slaughter it was found that 8 had only one foetus on the same side as transfer, 8 ewes had one foetus on each side and 1 had only one foetus on the contralateral side. Therefore, embryos migrated in at least 9 out of the 17 ewes and of the 25 foetuses found 9 had migrated to the contralateral side. This confirms the finding that embryo migration is the usual outcome when two ova are ovulated from the one side in sheep (Doney *et al.*, 1973).

5.3.11. The Effect Of CL Site On Pregnancy

Five recipients, which had 1-2 CL on one ovary, received 2 embryos on each side. Of the ten embryos transferred into each side 7 were found on the side with the CL and 3 were found in the side without the CL(s).

5.3.12. Necropsy Findings

In 51 out of the 59 donor ewes and in 29 out of the 31 recipients the reproductive tract was retrieved at slaughter. This allowed post-operative changes to be examined 6-8 weeks after the operation.

There was considerable variation between ewes in the degree of post-operative changes recorded at slaughter. Some ewes showed very extensive changes with many adhesions between uterine horns, between the uterine body and the horns, between ovarian bursa and the ovaries and/or the ovarian bursa and the uterus. In addition, adhesions were also seen between the reproductive tract and other tissues such as the broad ligament, omentum and bladder. Other ewes, however, showed very few adhesions with just the occasional strand of fibrous tissue being identified. A wide range between these two extremes was seen. In general there was significantly less evidence of post-operative adhesions in the recipients compared with the donors reflecting the considerably greater surgical handling the donors received. It also

appeared that a reduction in post-operative changes was correlated with an increase in surgical experience.

The oviducts of 32 donors were examined at *post mortem* in an attempt to assess their patency and/or the degree of bursal adhesions around the ovary. In 14 ewes it was possible to cannulate both oviducts, in 11, one oviduct could be cannulated and in 7 ewes neither oviduct could be cannulated as they were either not patent or due to ovarian bursal adhesions access to the oviducts was not possible. Of 14 recipients examined, both oviducts could be cannulated in 10 ewes, in 3 ewes one side could be cannulated and in 1 recipient both oviducts could not be cannulated.

Interestingly, in at least 7 donors a pregnancy was established from the side that had an oviduct which could not be cannulated. This pregnancy was established from the first natural service after surgery. In 4 of these donors neither oviduct could be cannulated at 6-8 weeks after surgery.

5.3.13. DNA Analysis Of Foetuses

Foetuses which developed from transferred embryos were recovered from the uteri of pregnant recipients at slaughter 6-8 weeks after the time of transfer. A total of 35 healthy foetuses and one degenerating foetus were recovered.

Of these, 7 foetuses had developed from unmanipulated embryos, 3 were recovered from ewes which had received both injected and uninjected embryos and 25 could only have developed from injected embryos. Seventeen of these 25 foetuses were analysed. DNA was prepared from both embryonic and placental tissue (where possible) and analysed by restriction enzyme digest and Southern blotting for the presence of the FeLV provirus, this work being carried out by S. Hettle.

Figure 5.3.4. is a diagrammatic representation of the FeLV provirus showing the relevant restriction sites. The integrated provirus should give a constant internal fragment of 1.2 Kb and one other fragment, greater than 0.5 Kb in length when completely digested with Kpn I enzyme. When digested with Hind III two variable fragments should be produced, one greater than 1.3 Kb and the other greater than 5.1 Kb.

A DNA probe corresponding to the U3 region of the provirus was used in this analysis. It can be seen from the autoradiograph shown in Figure 5.3.5. that the U3 probe used did not hybridise to DNA from the uninjected foetal material (control samples). In addition, Figure 5.3.5. shows samples isolated from foetus 146 which hybridise strongly to the probe. Foetus 146 has hybridisation bands of 1.2, 3.7 and 6.5 Kb in size when digested with Kpn I and bands of 1.85, 3.6 and 6.0 Kb when digested with Hind III.

Figure 5.3.4.

Diagram of the FeLV-B Provirus Showing the Relevant Restriction Sites

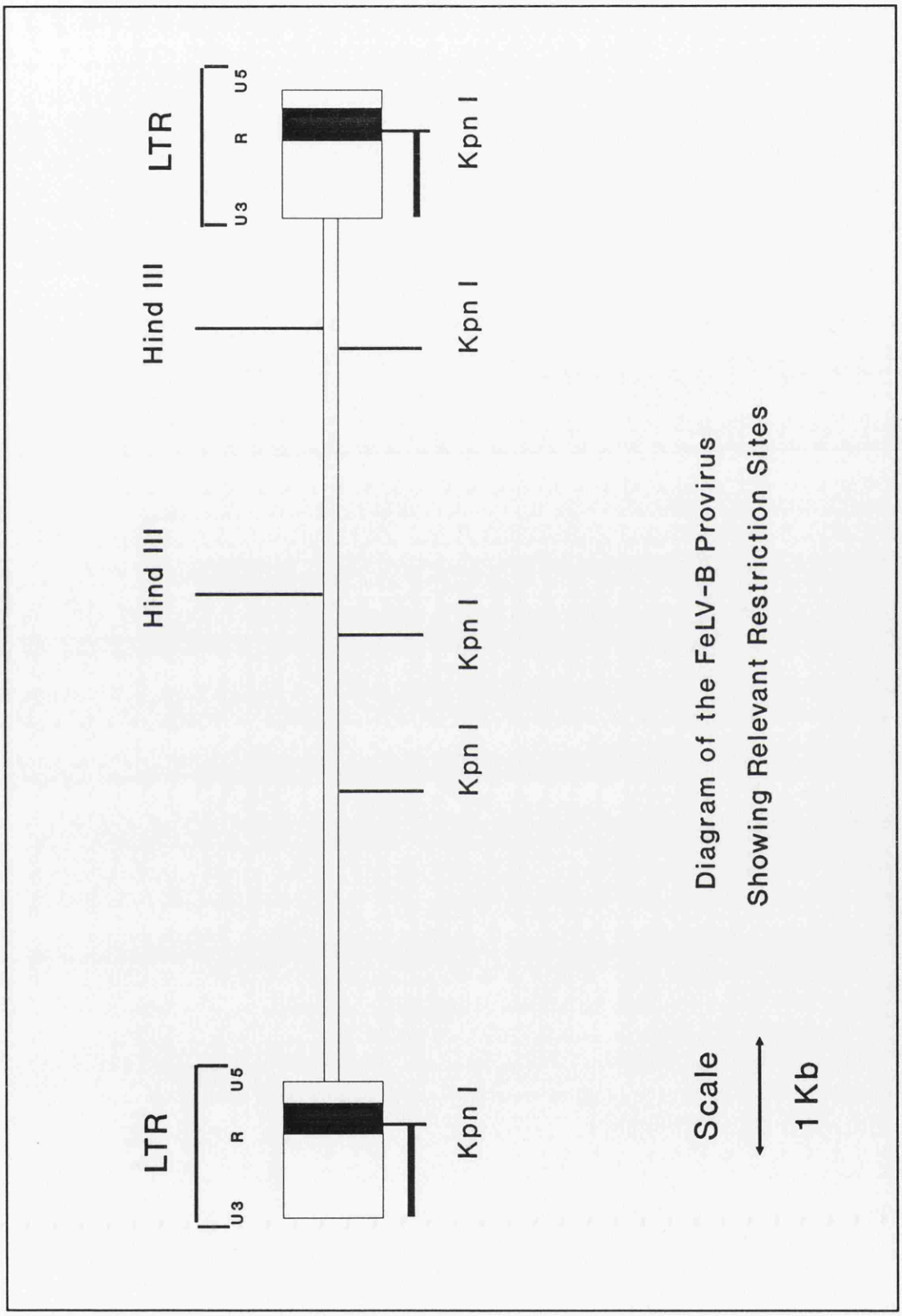


Figure 5.3.5. Southern Blot Analysis of Embryonic and Placental DNAs Isolated from Foetus 146 and Control Foetal Tissue

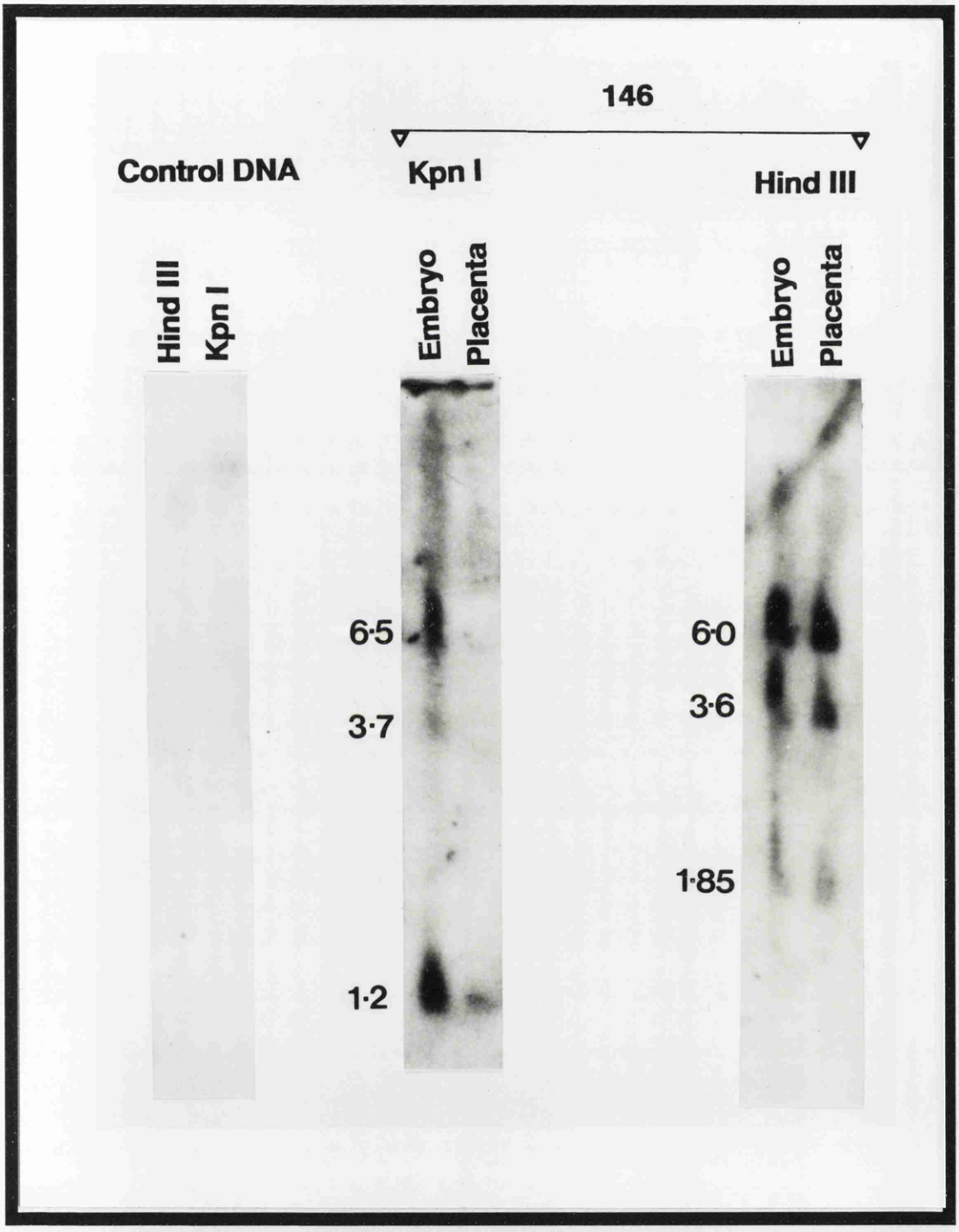


Figure 5.3.5. Southern blot analysis of embryonic and placental DNAs isolated from sub-zonally microinjected sheep foetus 146 and from control foetal tissue. DNAs isolated from these tissues were digested with restriction enzymes Kpn I and Hind III. Sizes of bands are shown in kilobase pairs (kb) alongside each band.

In samples isolated from another ewe (AK805) the following hybridisation bands were found - 1.2 Kb, 4.4 Kb and 7.0 Kb when digested with Kpn I and 4.3, 10 and 18.5 Kb when digested with Hind III.

Placental tissue from foetus 146 was digested with Kpn I and Hind III enzymes and placental tissue from AK805 was digested with Kpn 1. In all cases the results from the placental tissue paralleled that of the embryonic tissue.

The 1.2 Kb fragment resolved with the Kpn I digest from both foetuses, the 6.0 Kb band from foetus 146 and the 10.0 Kb band from foetus AK805 when digested with Hind III were approximately double the intensity of the other bands. This indicates that two copies of the bands were identified within the chromosomal DNA.

Therefore, out of 17 foetuses screened 2 proved to be transgenic. FeLV proviral sequences were found in both the embryonic and placental tissues of both foetuses. The pattern of hybridising fragments in the Southern blots indicates that both embryos may have undergone two separate integration events *i.e.* the cells of both foetuses contain 2 proviral copies of FeLV at different integration sites. These results are summarised in Table 5.3.12..

Table 5.3.12. Summary of Fragment Sizes from Foetuses 146 and AK805.

Restriction Enzyme Used	Expected Fragment Sizes	Fragment Sizes in Foetus 146	Fragment Sizes in Foetus AK805
Kpn	1.2	1.2	1.2
	> 0.5	3.7	4.4
		6.5	7.0
Hind III	> 1.3	1.85	4.3
	> 5.1	3.6	10.0
		6.0	18.5

INFECTION OF OVINE EMBRYOS WITH FeLV

SECTION 5.4 - DISCUSSION

The success of sheep multiple ovulation and embryo transfer (MOET) depends on a number of factors. In this experiment it was important to maximise the number of samples available for analysis, in this case day 40 to 60 foetuses which had been exposed to FeLV as preimplantation embryos. It was necessary, therefore, to optimise those parameters which could influence the final number of developing foetuses. This includes factors which affect both the number of good quality embryos available for injection and suitable for transfer in addition to those factors which affect the pregnancy rate and the number of transferred embryos which develop into foetuses.

Factors Affecting The Supply Of Embryos

A number of different criteria are believed to affect the ovarian response to gonadotrophins. These include the breed (Saumaude *et al.*, 1978; Armstrong and Evans, 1983), the age (Moore, 1982) and possibly the nutritional status (Moore, 1982). In general the more fecund breeds show a much greater response to the use of superovulatory drugs (Bindon *et al.*, 1971). The ovarian response in breeds of differing fecundity appears to be correlated with the ovarian sensitivity to gonadotrophins and not to the circulating level of gonadotrophins (Cahill *et al.*, 1979). Driancourt (1987) examined those aspects of the follicular population which are important in the response to gonadotrophins and found that follicles greater than 2 mm were negatively correlated and follicles of between 0.8 - 2 mm were positively correlated with the number of oocytes ovulated.

Sheep and other domestic species show a wide individual variation in their response to gonadotrophin treatment and the inability to induce a predictable and repeatable response remains a major constraint in embryo transfer programmes (Moore, 1982). This can result in either too many or too few embryos being available for transfer. Recipients can be reprimed if there are insufficient embryos available for transfer but embryo wastage can occur if large numbers of embryos are collected.

The type of gonadotrophin and the follicle stimulating hormone/luteinising hormone (FSH/LH) ratio of that gonadotrophin can influence both the average response and the range of the response (Chupin *et al.*, 1984; Armstrong and Evans, 1983). Armstrong and Evans (1983) also found that FSH produced a higher and less variable response than PMSG. A dose/response relationship has been established for

sheep, the optimal dosage varying with breed and weight of the ewes concerned (Moore, 1982). The use of PMSG is, at high doses, associated with an increased number of unovulated follicles and a corresponding decrease in the number of ova shed (Moore, 1982; Armstrong and Evans, 1983). The batch (Murphy *et al.*, 1984) and the timing (Moore and Shelton, 1964) of gonadotrophin treatment can also influence the response.

The average response in this study was 7.1 CL/ewe with a range of 1-24 CL/ewe. The ovulation rate of the untreated Blackface ewe is relatively low and this breed is not considered to be prolific. Moore (1982) has stated that the differences in ovulation rates between breeds are reflected by similar differences in the ovulatory response to exogenous gonadotrophins. The response to PMSG treatment observed here is compatible with results reported for breeds of similar fucundity and the considerable variation in the ovulatory response to PMSG previously reported was reflected in this study.

In 1979 Willadsen pointed out that the fertilisation rate after superovulation was significantly decreased in ewes with high ovulation rates. It appears that the use of PMSG can adversely affect both sperm transport (Evans and Armstrong, 1984) and sperm viability (Hawk *et al.*, 1987) both of which result in reduced fertilisation rates although this problem can be circumvented by intrauterine insemination (Trouson and Moore, 1974). The type of gonadotrophin used may also affect the number of oocytes fertilised. Schiewe *et al.* (1990) reported that the fertilisation rate is significantly higher with FSH or hMG (human Menopausal Gonadotrophin) treatment than with PMSG treatment. In the results reported here 45% (108/242) of all embryos collected were at the one-cell stage, however, it was not possible within the design of this current experiment to definitively establish the fertilisation status of these one-cell embryos. In Experiment 1, however, 62 one-cell embryos were placed in culture and 46 (74%) developed beyond the one-cell stage. In addition, a number of one-cell embryos were transferred with 4 recipients receiving a total of 11 one-cell embryos but none of these recipients became pregnant.

A number of factors can influence the developmental stage of embryos collected at a given time after synchronisation. These include the time of ovulation, the interval between ovulation and fertilisation and the rate of cleavage (reviewed by Wilmut *et al.*, 1985b). Betteridge (1977) observed that variation among embryos is greater in animals induced to superovulate. Further, there is even greater variation when embryos are collected from different donors, even if they come into oestrus on the same day (Wilmut *et al.*, 1985a).

The results presented here reflect these observations. Excluding one-cell embryos, 16% of ewes yielded more than one stage and variation between donors was

even greater. Some of this variation may also be due to morphologically normal but retarded embryos.

The collection rate in this study was 58% and there was considerable variation in recovery rates between ewes. No single reason could be identified to account for this variation as the procedure was essentially similar for all ewes. However, in Experiment 2 those ewes yielding only one-cell embryos had a significantly lower recovery rate. The overall rate of embryo recovery in this study was comparatively low. Gordon (1983) states that a collection rate of around 80% in embryo transfer programmes is common and Robinson *et al.* (1989) report recovery rates in excess of 90% when using retrograde flushing of the oviduct on day 3 (oestrus day = 0).

Premature Luteal Regression

A small number of donor ewes re-entered oestrus within 6-8 days of the induced oestrus. Wallace *et al.* (1989) have defined normal luteal function as an increase in progesterone commencing within 4 days of behavioural oestrus and rising to exceed 1 ng/ml for at least 8 days thereafter. These authors classified luteal function as inadequate when the CL either failed to secrete progesterone completely or caused a transient increase lasting only 12 to 36 hours. The ewes in this current study were not sampled daily for progesterone levels but samples taken on the day of behavioural oestrus were not consistent with levels found during the luteal phase. These ewes were not treated differently and did not appear to differ in response to treatment (based on ovulation counts) compared to those exhibiting normal luteal function. However, short cycling ewes were only observed in the second half of the breeding season.

Short luteal phases after exposure to the male are common during the transition period at the start of the breeding season (Knight *et al.*, 1981). In addition, induction of oestrus during anoestrus can result in abnormal luteal function but previous priming with progesterone can overcome these effects (McLeod *et al.*, 1982).

Premature luteal regression was a frequent observation when early *post partum* ewes (day 21) were induced to ovulate (Wallace *et al.*, 1989). The high incidence of inadequate luteal function was thought to be due to the high levels of prostaglandin in the involuting uterus - this effect was noted both during the breeding and non-breeding season. Luteal dysfunction was also noticed in an embryo transfer programme in which ewes were synchronised using the prostaglandin PGF2α. The abnormal luteal function in this study did not correlate with the superovulatory hormone used but was associated with the PGF2α treatment (Schiewe *et al.*, 1990). The association between prostaglandin treatment and premature luteal regression had

previously been reported by Willadsen in 1979. Willadsen also stated that premature luteal regression had also been observed in PMSG treated ewes which were not injected with prostaglandins. In cyclic goats superovulated with PMSG premature luteal regression occurs regardless of whether progesterone intravaginal pessaries (MAP - 6 alpha - methyl - 17 alpha - acetoxyprogesterone) or prostaglandin injections (PGF2α) were used for synchronisation (Armstrong *et al.*, 1982; Armstrong *et al.*, 1983a; Armstong *et al.*, 1983b; Stubbing *et al.*, 1986). Armstrong *et al.* (1983a) have suggested that in goats multiple ovulation induction can give rise to abnormal endocrine events which in turn cause release of endogenous PGF2α from follicles or uterus.

In 1991 Schiewe *et al.* specifically examined the phenomenon of premature luteal regression (PLR) and found that its occurrence was associated with the use of PGF2α although one the of eight ewes in the study, synchronised with medroxy progesterone acetate, also showed PLR. Further, these authors noted that in ewes undergoing premature luteal regression progesterone secretion was compromised within 60 hours of ovulation and that a reduction in the number of small luteal cells was evident three days after oestrus. Schiewe *et al.* (1990) have summarised the factors which may be contributory to abnormal luteal function as: inadequate hormone preparation of the preovulatory follicle; early release of a luteolysin and hypersensitivity of the luteal tissue to a luteolysin.

However, it is not clear what role these factors play in the short oestrus cycles observed in the present study as none of the predisposing factors previously described were relevant to the design of this study.

Factors Affecting Embryo Survival And Pregnancy Rate

An average of three embryos were transferred to each ewe and 74% of recipients were pregnant 40-60 days after transfer. Only 37% of transferred embryos were recovered as morphologically normal foetuses after day 40 of gestation. However, 48% of those embryos transferred to ewes which subsequently became pregnant developed beyond day 40.

In breeding ewes, maintained in good conditions, embryonic mortality has been estimated at 20-30% (Edey, 1979). Although chromosomal abnormalities, approximately 6% (Long and Williams, 1980), are responsible for a proportion of this mortality, all of the factors which contribute to this early embryonic loss have not been elucidated. Ashworth *et al.* (1989) have suggested that embryo survival can be influenced by periovulatory plasma progesterone concentrations and that variations in progesterone concentration could contribute to embryo loss. Williams and Long (1980) examined superovulated oocytes and embryos and found that the incidence of

chromosomal abnormalities associated with fertilisation was significantly increased in these embryos. The earlier in development such embryos are collected the greater the likelihood that they will be classified as normal and transferred.

The recovery and short term storage of embryos exposes them to a variety of potential insults which could adversely affect subsequent development. These include fluctuations in temperature and osmolarity of the handling media (Brinster, 1965a). Certain batches of serum have been shown to be toxic to embryos (Kane, 1987) and toxicity can also arise from other sources such as ethylene oxide residues present on equipment coming into contact with media (Schiewe *et al.*, 1984).

Effect Of Sub-Zonal Viral Injection On Embryo Development

There was no significant difference between the survival rates of manipulated and unmanipulated embryos or between the pregnancy rates of ewes receiving either unmanipulated or manipulated embryos.

Sub-zonal viral injection is less invasive than pronuclear microinjection as the integrity of the plasma and nuclear membranes is not breached in this technique. In this study 15% of embryos were assessed as being damaged by the procedure and were not transferred. Other workers report that pronuclear microinjection results in 49% (Ward *et al.*, 1986) to 30% (I. Wilmut, pers. comm.) of embryos being lysed.

Rexroad and Wall (1987) investigated the effects of pronuclear microinjection on early embryonic development. Twenty-nine percent of microinjected embryos and 86% of unmanipulated embryos transferred to synchronised recipients developed beyond the thirty-two cell stage. However, most published data on the effects of pronuclear injection on embryo development is based on the number of live lambs born (Hammer *et al.*, 1985; Murray *et al.*, 1988; Simons *et al.*, 1988).

Simons *et al.* (1988) also noted the number of midgestation foetuses. These workers transferred a total of 511 embryos. One hundred and nineteen foetuses (23%) were identified at midgestation by ultrasonic scanning and 92 live lambs and 17 dead foetuses were recovered at term. This group have observed, using ultrasonic scanning, that foetal death of injected eggs continues to occur throughout pregnancy (Wilmut *et al.*, 1990).

Although recipients were culled at the end of the first trimester of pregnancy in this study, it appears that embryo survival rates of subzonally injected embryos were comparable with standard embryo transfer results but superior to survival rates observed after pronuclear microinjection.

Pregnancy rates and the proportion of embryos represented by foetuses increases if transfers involving one-cell embryos are excluded from the results. Of those

recipients receiving only injected cleaved stages 15 out of 16 were pregnant and 46 % of transferred embryos were recovered as foetuses.

Hammer *et al.* (1985) were the first to produce a transgenic sheep and these workers reported an integration frequency of 1.3%. This figure was considerably below the integration frequency quoted for mice leading some authors to suggest that there may be some intrinsic species differences limiting the efficiency of gene integration in sheep (Ward *et al.*, 1986) or that conditions for ovine microinjection were suboptimal (Pursel *et al.*, 1987). However, more recent reports suggest that integration frequencies in sheep comparable with the range reported in mice can be achieved. Simons *et al.*, (1988) have reported that 6 lambs out of 92 screened were transgenic, an integration frequency of 6.5% and Murray *et al.* (1988) reported 7 transgenic lambs from 93 screened (7.5%) although the overall efficiency was low with only 0.5% of injected embryos resulting in a transgenic lamb.

In this study proviral sequences were detected in 2 foetuses out of 17, an integration frequency of 11.8%. Although the numbers involved were small it does indicate that retroviral vectors may allow genes to be transferred into sheep embryos at a greater frequency than can be achieved by pronuclear injection. The overall efficiency reported by Wilmut *et al.* (1990), based on the number of injected embryos resulting in transgenic animals, is 0.84%. The overall efficiency of the work presented here is 3.7% although the results cannot be directly compared as the end point of this experiment was the day 40 to day 60 foetus.

The results presented here show that it is possible for FeLV to infect the early ovine embryo and that the resulting provirus can integrate into the ovine genome. Three different methods have been used to produce transgenic mice: pronuclear microinjection (Gordon *et al.*, 1980); the use of retroviral vectors to infect early embryos (Van der Putten *et al.*, 1985) and the introduction of gene sequences into embryonic stem cells which are then used to generate chimeric animals (Bradley *et al.*, 1984; Robertson *et al.*, 1986). However, only pronuclear injection has been successfully used to produce transgenic domestic animals. Three factors make the production of transgenic livestock by pronuclear microinjection more difficult than in mice (Wilmut *et al.*, 1990): fewer embryos are available; there is greater variability in the developmental stage recovered and the presence of cytoplasmic vesicles in the embryos of domestic animals obscures the pronuclei. In sheep, differential interference contrast microscopy (DIC) allows the pronuclei to be visualised and this problem has been overcome in cattle and pig embryos by the combined use of centrifugation and DIC (Pursel *et al.*, 1989). Despite these problems transgenic pigs, sheep (Hammer *et al.*, 1985) and cattle (Roshlau *et al.*, 1989) have been produced by a number of groups using pronuclear microinjection.

The use of retroviral vectors could circumvent some of these problems. The cell stage does not appear to be critical as there are no data to suggest that infectivity varies between different preimplantation stages although the more advanced the embryo stage, the more likely the resulting founder animal will be mosaic (Van der Putten, 1985; Husar *et al.*, 1985; Rubinstein *et al.*, 1986). The approach described here is technically less demanding and considerably less invasive than pronuclear microinjection.

The frequency of proviral integration in the foetuses analysed was 11.8% suggesting that the development of this approach may increase the efficiency of producing transgenic sheep.

Wild type retrovirus (Jaenisch, 1976; Jaenisch *et al.*, 1981; Soriano and Jaenisch, 1986a), retroviral vectors (Van der Putten, 1985; Husar *et al.*, 1985; Rubinstein *et al.*, 1986; Soriano *et al.*, 1986b) or a combination of both have been used to transfer gene sequences into the early embryos of mice. The overall efficiency of using retroviruses to produce transgenic mice appears to be highly dependent on the titre of virus used. At high titres greater than 50% of offspring carry proviral sequences (Jaenisch, 1976; Jaenisch *et al.*, 1981; Soriano and Jaenisch, 1986a).

Mouse embryos are usually infected by removing the zona pellucida and co-cultivating them with cells which actively release retroviral particles for between 12-24 hours (Van der Putten *et al.*, 1985). As retrovirons are unstable at 37° C this approach exposes the blastomeres to infecting particles for extended periods of time. This study demonstrates that for infection to occur, prolonged exposure to freshly released viral particles was not required.

As whole foetuses were analysed by Southern blotting it was not possible to estimate the degree of mosaicism and there is no direct evidence to suggest that integration of proviral sequences occurred before these cell lineages diverged. Differentiation of foetal tissue from placental tissue occurs before that of cell lineages within the foetus (Noden and DeLahunta, 1985). It appears likely, therefore, that all foetal cells, including the germ cells, contained the proviral sequences. Production of transgenic lines of mice using retroviruses or retroviral vectors is readily achieved despite the high levels of mosaicism that result from infecting early embryos. The embryos in this study were infected at the two-cell to four-cell stage of development, whereas in similar studies producing transgenic mice, embryos were usually infected at a slightly later stage of development (two-cell to sixteen-cell stage, Rubenstein *et al.*, 1986; Van der Putten *et al.*, 1985; Husar *et al.*, 1985). It is, therefore, likely that the mosaicism of these transgenic foetuses would be less than that seen in mouse studies.

Embryonic activation in mouse embryos occurs at the two-cell stage (Bolton *et al.*, 1984). As retroviral infection of mouse embryos has been carried out at or after this stage it is not known whether the cellular receptors utilised by the retrovirus were of maternal or embryonic origin. As embryonic activation does not occur until the eight to sixteen cell stage in the sheep embryo the viral receptors exploited in this experiment were likely to be of maternal origin. This raises the possibility that it may be feasible to infect sheep embryos earlier than the two to four cell stage such as one-cell embryos or even oocytes.

In conclusion, these results show that FeLV is capable of infecting ovine embryos and that the technique used to infect these embryos was not especially deleterious to subsequent development.

At present it is unlikely that this method will gain widespread acceptance for developing lines of transgenic sheep for release to the commercial sector. Experience with the mouse model has shown that complex interactions between endogenous and exogenous retroviral sequences are unpredictable and not uncommon. It is likely, therefore, that transgenic sheep produced by this method would be restricted to research usage unless long term work in this field reveals that such fears are unconfirmed.

CHAPTER 6 - A TRANSGENIC MOUSE MODEL OF T-CELL LYMPHOMA

SECTION 6.1. - INTRODUCTION

In this chapter the production of two lines of transgenic mice is described and the phenotypic changes caused by their altered genotype analysed. The action of the human c-*myc* proto-oncogene was targeted to the T-cell compartment by linking the c-*myc* gene to the dominant control region (DCR) of the human CD2 gene. The resulting transgenic lines showed a predisposition for T-cell lymphoma. The incidence of tumour development was relatively low with only 16% of mice succumbing over a wide age range. Significant pre-neoplastic changes in haematopoetic cell populations were not observed and transgene expression could not be detected in healthy lymphoid tissues. However, high levels of transgene expression were present in all of the resulting tumours. These results suggest that the presence of the CD2-*myc* transgene predisposed these mice to T-cell lymphomas but that random activation events were required to initiate tumourigenesis. This hypothesis is supported by the finding that MuLV infection of CD2-*myc* mice greatly accelerated tumour formation.

Using Transgenic Mice To Study The *In Vivo* Action Of Oncogenes

The evidence that genetic damage is intrinsic to tumour development comes from separate and distinct lines of oncology research. A fundamental observation is that cancer cells very often manifest chromosomal damage (Knudson, 1987; Knudson, 1986; Yunis, 1983). Such chromosomal aberrations can result in the loss of gene function due to coding and regulatory sequences being disrupted. The perturbation of transcriptional control can occur following the translocation of a gene when that gene comes under the influence of different regulatory regions (Adams *et al.*, 1983). Gene amplifications can also result in an alteration of the expression potential of genes (Collins and Groudine, 1982; Yokota *et al.*, 1986).

The long-standing observation that mutagenic agents can also be carcinogenic, that there appears to be a hereditary predisposition for some forms of cancer (Ponder, 1980) and that the susceptibility to tumour development is negatively correlated with the efficiency of DNA repair mechanisms (Hanawalt and Sarasin, 1986) all emphasise the importance of genetic damage in the process of oncogenesis.

Increasingly, knowledge is being acquired pertaining to those cellular genes which have a role to play in the control of growth and differentiation. The mutation or inappropriate expression of these genes can change the normal cellular gene (proto-

oncogene) into a gene which is capable of collaborating in a series of events which ultimately result in tumour development. The genetic targets of these changes broadly fall into two categories depending on whether regulatory or structural changes result in increased expression or biological efficiency (oncogenes) or whether the genes are down regulated or their functional competence inhibited (tumour suppressor genes). Whereas oncogenes exert their effect in a dominant fashion, due to the presence of the gene product, tumour suppressor genes are recessive as it is the absence of the gene product which results in the increased susceptibility to tumourigenesis.

Retroviruses play an important role in oncogenesis, particularly in the development of animal tumours. Research into retroviral induced tumourigenesis and retroviral biology in general have greatly extended our understanding of the genetic basis of cancer (for review see Tsichlis and Lazo, 1991). Retroviruses are self-perpetuating mutagenic agents as they have the ability to integrate into the host genome, this process being an essential part of the retroviral lifecycle.

Recombination events between viral and cellular sequences can result in the host gene being captured by the retrovirus and being moved from cell to cell, from chromosomal position to position and even from animal to animal. This process, known as transduction, has resulted in the identification of a large number of oncogenes (Varmus, 1982, Bishop, 1983). Retroviruses carrying cellular gene sequences with transforming potential are known as acutely transforming retroviruses and were identified as far back as 1911 when Rous discovered that chicken sarcomas could harbour an infectious tumourigenic agent. Initially, these sequences were considered to be viral in origin but later analysis showed them to be homologues of cellular genes and it was realised that host cells harboured genes with an oncogenic potential - the proto-oncogenes (Bishop and Varmus, 1985; Bishop, 1987). However, the transduction of cellular oncogenes is not a prerequisite for retroviral induced tumourigenesis as the process of proviral insertion can itself be mutagenic (for review see Tsichlis and Lazo, 1991). Insertion close to or within cellular genes involved in growth or differentiation can result in the activation of these genes because of the promoter and the enhancer sequences contained within the provirus. If this process results in the activation of a gene which confers a selective advantage on the cell then this event can contribute to neoplastic development. This was demonstrated in 1981 when proviral insertion was shown to activate the proto-oncogene *myc* (Neel *et al.*, 1981; Hayward *et al.*, 1981). The natural and experimental infection of animals with a variety of retroviruses has resulted in a host of oncogenes being discovered (Tsichlis and Lazo, 1991).

Oncogenes have the ability to assist in neoplastic development due to their involvement in the transduction of signals responsible for growth and differentiation but within this generic term these genes have different sites of action and perform

different functions. Genes coding for growth factors, growth factor receptors, membrane associated or cytoplasmic proteins involved in signal transduction and nuclear proteins can all participate in oncogenesis as a consequence of alterations in their structure or expression potential.

It would appear likely that increased or constitutive expression of growth factors will cause the pathological expansion of specific cell groups, a process which may contribute to tumour development *in vivo*. *In vitro*, the progression from growth factor dependent to growth factor independent proliferation is considered a definitive event in the multistep progression to the tumour phenotype.

It is not necessary for growth factors to be secreted from the cell in order to carry out their function. Dunbar *et al.* (1989) have shown that interleukin-3 (IL-3) retained within the cell can function in an autocrine manner to support growth. Examples of proviral insertion deregulating endogenous growth factors include: a myelomonocytic leukaemia cell line in which the IL-3 gene is constitutively expressed (Ymer *et al.*, 1985) and mutant haemopoeitic cells which are growth factor independent due to proviral induced deregulation of granulocyte-macrophage colony-stimulating factor (Stocking *et al.*, 1988). In addition, there is evidence to suggest that insertional mutagenesis of colony-stimulating factor-1 (CSF-1) may have co-operated with *myc* in inducing myelomonocytic tumours (Baumbach *et al.*, 1988).

Growth factor receptors also appear to be potential oncogenes. Analysis of murine myeloid tumours induced by F-MuLV revealed that in a proportion of the resultant tumours proviral insertion had activated the c-*fms* proto-oncogene (Sola *et al.*, 1988), the function of which is to act as a receptor for CSF-1. Examples of membrane associated proteins are coded for by the *ras* gene family, members of which can give rise to products with oncogenic properties when present in mutated forms.

The *ras* gene family is composed of three closely related members; c-Ha-*ras*, c-Ki-*ras* and N-*ras*. Ha-*ras* and Ki-*ras* are the cellular homologues of the oncogenes found in the Harvey and Kirsten strains of murine sarcoma retroviruses. The products of these genes are located in the inner aspect of the cell membrane and are involved in the transduction of signals associated with mitogenic stimuli. Mutations of these genes can result in an increase in the biological potency of the resultant products, due, at least in part, to an alteration of the capacity of the cells to regulate these proteins (Rayter *et al.*, 1989). The *ras* superfamily appears to be involved in a large number of cellular activities and is regulated in a complex manner which operates at a variety of different levels (for review see Evans *et al.*, 1991). One interaction which has recently been demonstrated is that which exists between platelet derived growth factor receptor and *ras*. The addition of platelet derived growth factor (PDGF) to quiescent cells results in increased phosphorylation of tyrosine residues of the *ras*-GAP

(GTPase-activating protein) protein complex (Molloy *et al.*, 1989). The *ras* gene product has been shown to be capable of complementing a number of nuclear oncogenes, including myc, in the development of the tumour phenotype (Langdon *et al.*, 1988). Other oncogene products which operate in the cytoplasm or associated with the cell membrane include the *pim*-1 product which is a serine/threonine kinase (Selten *et al.*, 1986; Domen *et al.*, 1987).

Nuclear Oncogenes

A number of oncogenes code for proteins which function within the nucleus. This group includes the *myc* oncogenes, a gene family comprised of three well characterised genes (c-*myc*, N-*myc* and L-*myc*) and three more poorly defined genes - R-*myc*, S-*myc*, and b-*myc* (DePinho *et al.*, 1987; Ingvarsson *et al.*, 1988). C-*myc*, N-*myc* and L-*myc* are functionally very similar, share extensive regions of homology and are functionally conserved across the vertebrate species (for reviews see Cole, 1986; Kelly and Siebenlist, 1986).

C-*myc* is expressed in a wide variety of tissues and is associated with cellular proliferation, in contrast, the expression pattern of N-*myc* and L-*myc* is restricted to specific cell lineages, usually at an early stage in that lineages differentiation (Mugrauer *et al.*, 1988; Downs *et al.*, 1989). Whereas N and L-*myc* expression is mainly confined to developing tissues c-*myc* expression is much more ubiquitous being found in both developing and mature tissues (Zimmerman *et al.*, 1986).

This expression pattern is consistent with the role these genes play in neoplastic development. C-*myc* was first identified in chicken sarcomas induced by Avian Leukosis Virus (Neel *et al.*, 1981; Hayward *et al.*, 1981). Since that time c-*myc* involvement has been detected in a wide variety of tumours and is the gene most frequently found to be activated by retroviral induced insertional mutagenesis (Tsichlis and Lazo, 1991). Activation of N and L-*myc* is associated with a more restricted tumour type, N-*myc* having been shown to be involved in tumours of primitive cell origin and T-cell tumours (Nisen *et al.*, 1986; van Lohuizen *et al.*, 1989a) whereas activated L-*myc* has been found in small cell lung carcinomas (Nau *et al.*, 1985). Unlike the *ras* oncogene, the product of which is a mutant form of the normal cellular protein, the c-*myc* proto-oncogene participates in tumourigenesis as a result of inappropriate expression of the normal cellular protein (Leder *et al.*, 1983; Cole, 1986).

There is strong circumstantial evidence that *myc* can function as a transcriptional factor but definitive proof for this role is still lacking. The c-*myc* product is thought to be involved in gene regulation due to its nuclear localisation and its ability to bind DNA. The protein contains structural domains, specifically the helix-loop-helix

(HLH) and leucine zipper (LZ) motifs, which are common to a number of transcriptional factors (Landschulz, *et al.*, 1988; Murre *et al.*, 1989). Further, c-*myc* can regulate its own expression, the deregulation of c-*myc* due to translocation as occurs in Burkitt's lymphoma results in the transcriptional suppression of the remaining allele (Dunnick *et al.*, 1983; Leder *et al.*, 1983). Similarly, targeted expression of *myc* transgenes results in the transcriptional activity of the endogenous gene being repressed (Adams *et al.*, 1985). Whether this acts directly or is mediated through other factors is not clear as Stewart *et al.* (1984) have described a situation where autoregulation of *myc* expression does not appear to occur. It is now known that the *myc* interacts with another cellular protein named *max*. *Myc* and *max* bind more strongly than *myc* does to itself and this heterodimer has been reported to bind a specific DNA sequence (Blackwood and Eisenman, 1991).

Myc may also play a role in DNA replication as the addition of anti-sense oligo-nucleotides to human peripheral T-cells results in inhibition of DNA synthesis (Heikkila *et al.*, 1987). Iguchi-Ariga *et al.* (1987) have shown that c-*myc* immunoprecipitates with a putative origin of replication and Classon *et al.* (1987) reported that levels of c-*myc* were correlated with extrachromosomal replication of SV40 DNA. However, Kaczmarek *et al.* (1986) reported that fibroblasts were capable of entering S-phase despite the presence of c-*myc* specific antibodies.

That *myc* is intimately involved in cellular self-renewal is beyond doubt. The introduction of *myc* into primary cell lines can result in proliferation (Palmieri *et al.*, 1983), growth factor independence (Rapp *et al.*, 1985) and immortalisation of the cell line (Mougneau *et al.*, 1984; Bartlett *et al.*, 1988; Land *et al.*, 1986) whereas, the introduction into a previously immortalised cell line can result in transformation (Kelekar and Cole, 1986; Keath *et al.*, 1984). This growth stimulatory affect *in vivo* manifests itself in hyperplasia of targeted tissues in transgenic mice (Langdon *et al.*, 1986) and by the involvement of *myc* in many different tumours.

Further endogenous *myc* levels seem to be positively correlated with proliferating tissue (Makino *et al.*, 1984) and suppressed in tissues approaching terminal differentiation (Lachman and Skoultchi, 1984; Griep and Deluca, 1986). The addition of specific growth factors to quiescent cells to induce growth is correlated with increased levels of c-*myc* expression (Kelly *et al.*, 1983; Coughlin *et al.*, 1985), whilst the overproduction of *myc* appears to block differentiation (Prochownik and Kukowsa, 1986) and the application of c-*myc* antisense RNA to cell lines accelerates terminal differentiation (Griep and Westphal, 1988). There is little evidence to suggest that *myc* alone is capable of transforming cells or tissues to the fully malignant phenotype. In primary cell lines the active presence of another oncogene, typically *ras*, is required for transformation (Land *et al.*, 1983) and in transgenic mice expressing exogenous c-*myc* further events as defined by clonality and

kinetics of tumour development appear to be required for the fully neoplasic phenotype to be expressed (Adams and Cory, 1991).

Tumour Suppressor Genes

The term oncogene describes those genes in which the mutation or deregulated expression of the gene increases the likelihood of a cell undergoing transformation. Tumour suppressor genes can be defined as those genes which express a protein, the presence of which makes transformation of a cell less likely.

Mutations abrogating the function of these genes are likely to be recessive as it is the absence of the gene product which contributes to tumour development and for this to occur, inactivation of both alleles is required. Two such genes have attracted considerable interest, the p53 gene and the Retinoblastoma Susceptibility gene (RB). The Li-Fraumeni syndrome of humans is a genetically inherited condition in which one of the two p53 alleles is non-functional due to a point mutation. Subsequent somatic mutations of the remaining alleles renders people with this genotype vulnerable to tumour formation (Srivastava *et al.*, 1990). The addition of the wild-type p53 gene to transformed cell lines reduces their growth rate (Baker *et al.*, 1990) and the wild type gene can inhibit transformation in cells expressing mutant p53 and *ras* genes (Finley *et al.*, 1989). Some indication of the importance of this gene in preventing tumour development is realised by a recent publication which revealed that mutations in the p53 gene are the most frequently observed genetic lesion in spontaneous human tumours (Vogelstein, 1990)

Retroviral induced insertional mutagenesis of the p53 allele has been reported. Wolf and Rotter (1984) have described an Abelson MuLV induced tumour cell line in which proviral insertion caused functional deletion of the p53 product, the presence of the normal allele could not be detected, presumably because it was also deleted. Hicks and Mowat (1988) have characterised a cell line derived from F-MuLV induced erythroleukaemias in which independent proviral integration in both alleles was detected.

Another tumour suppressor gene is the Retinoblastoma Susceptibility gene (RB). A predisposition for the childhood cancer retinoblastoma can be an inherited condition due to germline loss of one of the two alleles coding for the RB gene product. Subsequent somatic damage to the other allele leads to the tumour phenotype. Treatment and survival of patients with this condition revealed that they were prone to a large number of other tumours later in life.

Using Transgenic Mice To Study Oncogenesis

Transgenic mice have proved to be a valuable resource for the investigation of oncogene function and the tumourigenic process in general as these animals allow an investigation into the consequences of specific gene expression in specific tissues *in vivo* (for reviews see: Adams and Cory, 1991; Cardiff *et al.*, 1991; Pattengale *et al.*, 1989; Hanahan, 1988, Berns *et al.*, 1989). As well as complementing tissue culture studies, and other *in vivo* models, the transgenic system has a number of unique advantages.

The action of an oncogene or putative oncogene can be investigated by expressing the gene and characterising the resulting phenotypic changes. Expression can be either broad spectrum using constitutive regulatory sequences or targeted to a specific tissue. In this way the manner in which an oncogene functions within a particular tissue context can be better understood allowing the question of complementation between specific tissue types and oncogenes to be addressed. The presence of the transgene in the germline results in mice that have a hereditary predisposition to a particular tumour type and the generation of transgenic mice carrying oncogenes can therefore result in the establishment of a permanent resource. Indeed, the targeting of a specific tissue with an appropriate oncogene can be a means of generating particular cell lines which would not normally be available. In the transgenic model the ability to target oncogene expression to specific tissues is limited only by the availability of suitable regulatory signals, whereas *in vitro* studies are restricted by those cell lines which are amenable to study.

Transgenic studies have lent credence to the hypothesis that a series of events are required to bring about the truly malignant phenotype. The knowledge that an animal is likely to develop a specific tumour at a particular age range allows a detailed and wide ranging investigation of those crucial early events which precede the fully neoplastic phenotype. In addition, those secondary events which induce the manifestation of the fully malignant tumour can be examined using a series of tumour tissues all of which were subjected to common initiating events. Finally, transgenic mice allow the synergy between different oncogenes to be examined within the context of the living animal, this being achieved either by crossbreeding different transgenic lines or by infecting mice with retroviruses carrying viral oncogenes.

The Eμ-*Myc* Mice

The first study to use transgenic mice as a model system for leukaemic conditions utilised the Eμ-*myc* construct (Adams *et al.*, 1985) and since that time mice

bearing this construct have been studied extensively (Langdon *et al.*, 1986; Harris *et al.*, 1988b; Sidman *et al.*, 1988; Strasser *et al.*, 1990; Rosenbaum *et al.*, 1990; Adams and Cory, 1991; Haupt *et al.*, 1991). Translocations which place the c-*myc* gene under the influence of immunoglobin regulatory elements can result in the development of Burkitt's lymphoma in humans and mouse plasmacytomas (for review see Cory, 1986). In these transgenic mice, expression of the human c-*myc* gene is regulated by the immunoglobin heavy chain enhancer (Eμ) in order to mimic the situation which arises after these translocations.

A high incidence of lymphoma development was found in the resulting transgenic mice with 90% of offspring dying by 4 months of age. All of the tumours occurred within the B-cell lineage with the target cell being either the pre-B or B-cell. None of the tumours examined carried the T-cell marker Thy-1 (Adams *et al.*, 1985). The c-*myc* gene carrying its own regulatory elements was also introduced into the mouse germline in this study but this construct failed to produce any observable effect in the recipient mice.

Follow-up studies with these transgenic mice revealed that *myc* expression could induce preneoplastic changes within the B-cell lineage (Langdon *et al.*, 1986). Early stages had a greatly expanded population of pre-B cells with a reduced number of mature B-cells. These pre B-cells were large and blast like in appearance, a higher proportion than normal were proliferating and they were found in profusion outside the bone marrow instead of being restricted to this site. These results clearly showed that *myc* could increase cell cycle activity in this target cell. These cells did not, however, produce tumours when cells were transferred to histocompatible recipients (Langdon *et al.*, 1986) and were not autonomous in culture (Harris *et al.*, 1988a). The involvement of two cell types (pre-B and B-cells) in tumour development implies that *myc* induced tumourigenicity is not restricted to the one stage and/or that with some clones differentiation occurs after transformation. That the latter is possible was demonstrated when pre-B tumour cell lines were cultured *in vitro* and it was found that these cells could undergo maturation to the mature B-cell phenotype (Harris *et al.*, 1988b). It has been suggested that high levels of *myc* expression can block differentiation (Prochownik and Kukowsa, 1986) but these results imply that at least a degree of differentiation can occur in these mice. The ability of cells to differentiate while heavily under the influence of *myc* has also been reported by Spanopoulou *et al.* (1989). T-cells transformed by *myc* at the double positive stage (CD4+, CD8+) can progress to the more mature single positive stage.

The development of only monoclonal or oligoclonal tumours suggested that subsequent events are required for the c-*myc* expressing cell to progress to the fully transformed phenotype (Adams *et al.*, 1985). This observation appears to be true for most transgenic mice expressing oncogenes in both lymphoid (Moroy *et al.*, 1990;

Hariharan *et al.*, 1989; Spanopoulou *et al.*, 1989; Rosenbaum *et al.*, 1989; Yee *et al.*, 1989;) and non-lymphoid tissues (for review see Hanahan, 1988) with only a few exceptions being reported (Quaife *et al.*, 1987; Bautch *et al.*, 1987; Williams *et al.*, 1988; Muller *et al.*, 1988). Adams and Cory (1991) have postulated that the presence of a large population of progenitor cells susceptible to transformation made the likelihood of secondary events very high, ultimately leading to the development of fully malignant clones. This hypothesis is supported by the finding that on different genetic backgrounds the size of the expanded pre-B cell population, induced by the Eμ -*myc* transgene, varied and that this variation correlated with tumour incidence (Adams and Cory, 1991). However, in many oncogene-bearing transgenic mice pre-neoplastic, disturbances do not occur or are too subtle to be detected.

Influence Of Genetic Background On Oncogene Action

Working with the lac Z gene Allen *et al.* (1990) have compared mice of different genetic backgrounds carrying the same transgene. These authors found that the temporal and spatial pattern of the transgene was not altered but that the level of transgene expression differed markedly indicating that different genetic backgrounds could either permit or restrict transgene expression. Harris *et al.* (1988a) found that the genetic background could affect both the kinetics and the susceptibility of mice carrying the *myc* transgene. A low rate of tumour development (6% per week) and an average lifespan of 16 weeks was found in C57BL/6 mice whereas on an SJL background tumours developed at a linear rate of 36% per week and the average lifespan was reduced to 7 weeks. Balb/c mice represented an intermediate phenotype with these mice developing tumours early in life but the subsequent rate of onset being fairly low. Yukawa *et al.* (1989) compared the Eμ-*myc* transgene in pure-bred C57BL/6(B6) and C3H/HeJ mice and found that whilst B6 mice developed lymphomas of the B-cell lineage - like all previous descriptions of the Eμ-*myc* transgene - C3H/HeJ mice developed mainly T-cell lymphomas. It would appear, therefore, that the genetic background can affect not only the kinetics and rate of tumour onset but also the lineage. The reasons for the change in target cell are not fully understood as Yukawa *et al.* (1989) reported that *myc* expression occurs in both cell types and there is no preferential activation of either cell. These studies highlight the difficulty in extrapolating too much information from the effects of the transgene in one particular strain or hybrid of mouse as interactions between transgene and genetic background can alter the outcome.

Tissue Specificity Of Oncogenic Transgenes

Using the same regulatory unit allows the actions of different oncogenes to be compared within a particular tissue compartment. Adams and Cory (1991) and others (Rosenbaum *et al.*, 1990; Moroy *et al.*, 1990; Harris *et al.*, 1988a; Rosenbaum *et al.*, 1989; Harris *et al.*, 1988b; Harris *et al.*, 1990; van Lohuizen *et al.*, 1989b) have used the immunoglobin enhancer (Eμ) regulatory unit to pursue this approach and have compared a number of different oncogenes with this system. One caveat with this approach is that sequences associated with the transgene itself may affect the resultant expression pattern. A good example of this has been reported by Moroy *et al.*, (1990). These workers produced transgenic mice carrying the L-*myc* gene regulated by the immunoglobin heavy chain enhancer (Eμ) but unlike the analogous Eμ-N-*myc* mice which express the transgene in the B-cell compartment Eμ-L-*myc* mice preferentially express the transgene in the T-cell lineage. The result is that Eμ-N-*myc* display a B-cell disturbance and almost all develop tumours of the B-cell lineage (Rosenbaum *et al.*, 1989) whereas Eμ-L-*myc* mice have an expanded T-cell population and develop mainly T-cell tumours.

However, the targeting of different oncogenes to the same tissue can yield information on how tissue type complements oncogene action. For example the Eμ regulatory unit has been used to target both *myc* (Adams *et al.*, 1985) and v-*abl* (Rosenbaum *et al.*, 1990) expression. In both cases expression occurs at high levels in B-cells but whereas *myc* produces pre-B and B-cell tumours, only plasmacytomas arose in three out of the four Eμ-v-*abl* transgenic lines generated with the v-*abl* construct, implying that the plasma cell is especially vulnerable to *abl* transformation in these lines. The development of plasmacytomas in these mice often appeared to require spontaneous activation of the *myc* gene as rearrangement of the endogenous *myc* gene was found in over 80% of tumours analysed.

In v-*abl* mice the transgene appears to be active in both B and T-cell lineages, indeed, in one of the three lines expression was highest in the thymus. Despite this, only plasmacytomas arose in these three transgenic lines. To complicate the picture, the fourth transgenic line (v-*abl*-1) developed pre-B and T lymphomas but no plasmacytomas (Harris *et al.*, 1990). Therefore it would appear that the integration site altered the target cell, although the possibility that rearrangements of the transgene or its regulatory sequences cannot be excluded. In this line 70% of animals succumbed to tumour development within the first year of life, 18% of these mice developed pre-B cell malignancy and 82% developed T-cell malignancy but the susceptibility to these two different tumours was age restricted. Pre-B cell tumours were only seen in the first 10 weeks of life, after which only T-cell tumours arose.

Van Lohuizen *et al.* (1989b) introduced the Eμ-*pim*-1 construct into mice and although the *pim*-1 gene was expressed in both T and B-cells, almost all tumours in mice bearing the *pim*-1 gene were of the T-cell lineage. Whether this is due to the background strain of mouse used, the regulatory signals incorporated into the transgene, which include the Eμ enhancer and the MuLV long terminal repeat and enhancer, or was intrinsic to the action of *pim*-1 itself is not known.

Further evidence that different tissues respond differently to expression of the same oncogene is exemplified by the *fps* transgenic mice in which expression was observed in a large number of tissues not all of which became transformed (Yee *et al.*, 1989). In particular, no myocardial tumours arose despite high levels of expression in the heart. Other tissues which expressed the transgene (such as the brain, the testes and the lungs) developed either no tumours or only benign tumours. This oncogene is a member of a family of genes which code for non-receptor protein kinases and these mice showed a high incidence of thyomas and lymphomas causing the authors to suggest that these tissues are particularly sensitive to protein-tyrosine phosphatases.

Hariharan *et al.* (1989) have investigated the *bcr-abl* construct. The majority of lines failed to express the transgene suggesting that foetal expression may be lethal. Most *bcr-abl* lines showed a low tumour incidence with pre-B and T-cell lymphomas resulting. One mosaic founder gave rise to a subline which was highly tumourigenic, moreover, expression of the transgene in this line was subject to the phenomenon of imprinting (Adams and Cory, 1991). Transgene imprinting results in differential methylation of the transgene depending on whether it is passed via maternal or paternal inheritance (Reik *et al.*, 1987; Sapienza *et al.*, 1987; Hadchouel *et al.*, 1987). In this line of mice, animals inheriting the transgene through the maternal route showed a high rate of tumour incidence mainly in the B-cell lineage whereas in animals inheriting through the paternal route expression was not detected and a significantly lower rate of tumour onset, mainly in the T-cell lineage, was observed.

Thymic Lymphomas

Compared to B-cell malignancy there are fewer published reports describing the action of *myc* within the T-cell compartment. Spanopoulou *et al.* (1989) were the first group to deliberately target *myc* expression to T-cells and they achieved this by placing the murine *myc* proto-oncogene within the Thy-1 transcriptional unit. The Thy-1 gene is expressed in murine brain and thymus, being one of the earliest T-cell markers. Eight out of ten of the resulting founder mice and all of the offspring from two founders selected for breeding developed thymic tumours at between eight and

fourteen weeks of age. These tumours consisted of expanded populations of lymphocytes and epithelial cells both of which appeared to be completely transformed as defined by their ability to grow autonomously in culture. Cell lines established from the two cell types revealed that the lymphoid cells were double positive (CD4+, CD8+) T-cells. Although T-cell lines derived from tumour tissue expressed the transgene, the majority of epithelial cell lines did not appear to express the transgene. The authors concluded that either the epithelial cells expressed the *myc* transgene transiently inducing transformation independently of the lymphocytes or that the altered lymphocytes release signals which transformed the epithelial component without the direct involvement of *myc*.

The *pim*-1 gene is frequently activated in thymic lymphomas induced by infection of mice with the slowly transforming Murine Leukaemia Virus (MuLV) (Selten *et al.*, 1985). To test directly the oncogenic potential of the *pim*-1, gene van Lohuizen *et al.* (1989b) produced transgenic mice bearing this gene using the immunoglobin enhancer (Eμ) and the MuLV long terminal repeat (LTR) to regulate expression. The *pim*-1 gene was expressed at high levels in both B and T cells in the resulting transgenic mice. Despite this, no general disturbance of the haemopoeitic cells was observed. These mice showed a low incidence of tumour formation with 5%-10% of mice succumbing to lymphoma development at between 3 and 7 months of age. All of the tumours, except one, arose from the T-cell lineage and a comparison between tumours revealed that different T-cell sub-populations could be affected.

Synergy Between Different Oncogenes

In almost all studies describing the action of oncogenes in transgenic mice the resultant tumours have proven to be monoclonal or oligoclonal revealing that expression of the introduced oncogene is merely the first event in a multistep process which ultimately leads to the fully transformed phenotype.

In some of these transgenic mice the tumour cells have been screened for the presence of rearranged oncogenes. As mentioned above, Rosenbaum *et al.* (1990) introduced the v-*abl* gene linked to the Eμ enhancer into the mouse germline. Non-neoplastic haematopoetic tissues expressed the transgene but expression was always 20-40 fold greater in tumour tissue. This suggests that either enhanced levels of transgene expression are necessary for the progression to the fully transformed phenotype or that the process of transformation itself up-regulates transgene expression. A common secondary event associated with advancement of the tumour phenotype appears to be the deregulation of *myc* expression. 80% of tumours screened appeared to harbour a rearrangement of the endogenous *myc* gene leading the

authors to conclude that deregulated *myc* expression could complement v-*abl* in the induction of plasmacytomas. Whether these two steps were sufficient to transform cells to the malignant phenotype was unknown.

The complementary action of two oncogenes or putative oncogenes can be better understood by breeding two different transgenic lines. This results in a proportion of the offspring expressing two different oncogenes in the same tissue. To confirm that *myc* and v-*abl* were indeed complementary, Rosenbaum *et al.* (1990) created double transgenic mice by crossing transgenic lines carrying Eμ-v-*abl* and Eμ-*myc*. Compared to the single Eμ-v-*abl* transgenic lines, plasmacytoma development was greatly accelerated in these lines with all of the mice dying by 8 weeks of age. However the co-operation between these two oncogenes appears to be stage specific, as only plasmacytomas developed with no pre-B and B-cells tumours so characteristic of the Eμ-*myc* lines being detected. In the v-*abl* lines the main pathological finding was enlargement of the lymph nodes but the anatomical distribution of affected lymph nodes varied although the majority were mesenteric. By comparison, all of the tumours in the v-*abl/myc* lines developed gut associated plasmacytomas. The tumours arising from these double transgenic lines were oligoclonal rather than the monoclonal phenotype seen in the v-*abl* lines.

To investigate additional secondary events which transform pre-B and B-cells in the Eμ-*myc* mice Harris *et al.* (1988a) have screened tumour DNA for the presence of genes which can transform 3T3 fibroblasts. Analysis of one Eμ-*myc* tumour revealed the presence of a mutated *ras* gene. This mutated gene was cloned and was shown to be capable of transforming non-tumourigenic pre-B cells derived from Eμ-*myc* bone marrow.

To identify collaborative oncogenes Alexander *et al.* (1989) utilised a different approach. These workers infected cell lines derived from Eμ-*myc* mice with retroviruses bearing the v-H-*ras*, v-*raf* and the v-*abl* oncogenes. Both *ras* and *raf* appeared to transform cell lines to the tumour phenotype as shown by the development of tumours after the cells were transferred to nude mice. Infection of normal cells with v-*raf* did not produce tumours in recipient mice and tumours only rarely arose with v-*ras* infection of normal cells.

Independent studies carried out by van Lohuizen *et al.* (1989b) demonstrated that the Eμ-*pim*-1 transgene could also co-operate with *myc*. Infection of neonatal Eμ -*pim*-1 mice with MuLV resulted in a dramatic acceleration of tumour incidence and a decrease in the latent period. The resulting tumours were analysed and Southern blotting revealed that proviruses had integrated into the c-*myc* proto-oncogene in 21 out of 26 lymphomas, in the remaining tumours proviral integration involved activation of the N-*myc* oncogene. These authors concluded that there appeared to be co-operation between these two oncogenes but could not exclude the possibility that

expression of *pim*-1 may make the activation of c-*myc* more likely, perhaps by expanding a population of cells susceptible to transformation by c-*myc*. This work was extended when Verbeek *et al.* (1991) crossed transgenic mice bearing the Eμ-*myc* and Eμ-*pim*-1 transgenes to create doubly transgenic mice. The effects of the synergy between these two transgenes are the most potent to be described to date. Double-transgenic *myc/pim*-1 foetuses died *in utero*. The single-positive lines Eμ-*myc* and Eμ -*pim*-1 result in the development of B lymphoid and T lymphoid tumours respectively. Foetuses harbouring both oncogenes developed pre-B cell leukaemia at day 17-19 of gestation. The severity of disease in these foetuses varied, implying that stochastic events were involved in tumour progression. In addition, tumour cells were transplanted into nude mice resulting in the outgrowth of monoclonal tumours. Together these results suggested, despite the obvious potency of this oncogene collaboration, that additional genetic or epigenetic events were necessary for the development of truly malignant cells.

The one v-*abl* line which produced tumours in lymphoid cells also showed synergy with Eμ-*myc* (Adams and Cory, 1991). Offspring carrying both transgenes became ill at two weeks of age with a disseminated invasive lymphoblastic lymphoma and accompanying pre-B cell leukaemia. Despite the potency of this combination demonstrated by the age range at which these mice became ill, the tumours were not transplantable (Adams and Cory, 1991). Acceleration of tumour development also occurred when the Eμ-*myc* transgene was complemented with Eμ-N-*ras* transgene (Harris *et al.*, 1988a). All mice carrying both oncogenes rapidly developed B lymphoid tumours, in this case the tumours consisted of pre-B cells or an even earlier cell type defined as pro-B cells (Adams and Cory, 1991).

A common chromosomal translocation found in human follicular B-cell lymphomas brings together the *bcl*-2 gene and the immunoglobin heavy chain enhancer resulting in deregulation of *bcl*-2 expression (Cleary *et al.*, 1986). McDonnell *et al.* (1990) used an immunoglobin-*bcl*-2 minigene to express *bcl*-2 in both the B and T-cell lineages of transgenic mice. This appeared to have no effect on either T-cell numbers or on the relative proportions of T-cell subsets. However, these mice had an expanded population of small resting B-cells, these cells co-expressed IgM and IgD, and despite not actively proliferating, were responsive to lipopolysaccaride and anti-IgM stimulation. *In vitro* studies showed that these transgenic B-cells displayed extended survival times in culture and it seems likely that the expanded population of these cells arose not because of increased proliferation but rather due to an extended life span. These mice displayed no obvious increase in tumour incidence.

Strasser *et al.* (1990) bred Eμ-*myc* and Eμ-SV-*bcl*-2 mice together to produce doubly transgenic mice. Compared to the c-*myc* lines', tumour development was

accelerated with mice becoming ill at 5-6 weeks of age and with a greater degree of synchrony than observed in the Eμ-*myc* lines. These results showed that *bcl*-2 expression could complement the action of *myc*. Pre-B and B-cells are the target cell in the Eμ-*myc* mice (Adams *et al.*, 1985) and a population of B-cells are expanded in the Eμ-*bcl*-2 lines (McDonnel *et al.*, 1990) however, Eμ-*bcl*-2/*myc* mice all developed tumours which consisted of a novel, previously uncharacterised cell type (Strasser *et al.*, 1990). The phenotype of these cells was suggestive of a haematolymphoid stem or progenitor cell. These mice also had an expanded population of pre-B and B-cells characteristic of the Eμ-*myc* mice, but these cells did not appear to be malignant. It is unclear whether *bcl*-2/*myc* was responsible for full transformation of these primitive cells or whether a third step was necessary for their transformation.

Slowly Transforming Retroviruses Can Be Used To Identify Novel Oncogenes

Nusse and Varmus (1982), showed that slowly transforming retroviruses could be used to search for previously unknown proto-oncogenes. Murine retroviruses induce tumours by a process known as insertional mutagenesis. When the provirus integrates into the chromosomal DNA, enhancer and promoter elements within the provirus can cause the transcriptional activation of host proto-oncogenes. It is also possible that these mutational events could disrupt the regulatory or coding sequences of a tumour suppressor gene rendering the cell in which this happens more susceptible to transformation. Whatever mechanism operates, the resulting tumour may carry a provirus adjacent to or within a gene which has a role to play in tumourigenesis. The presence of the provirus can therefore act as a label with which to isolate and examine the surrounding DNA.

A number of groups have utilised this approach in mice (Mucenski *et al.*, 1988), rats (Tsichlis *et al.*, 1985) and cats (Fulton *et al.*, 1987). In mice the oncogenic process has been examined in different cells types by using different exogenous retroviruses and activated endogenous retroviral sequences such as Murine Leukaemia Virus (Cuypers *et al.*, 1984), Friend Murine Leukaemia virus (Bordereaux *et al.*, 1987), Mouse Mammary Tumour Viruses (Gallahan and Callahan, 1987), and Mink Cell Focus-Forming Viruses (Li *et al.*, 1984). These studies have yielded a large number of novel genes or insertion sites (for review see Kung *et al.*, 1991).

An alternative approach, outlined by Berns *et al.* (1989), is to infect transgenic animals already bearing an oncogene with a slowly transforming retrovirus. This inevitably shortens the latent period required for tumour development and as a result restricts the search to those host genes which complement the action of the transgene.

There are a number of advantages in adapting this approach to transgenic mice which already express a known oncogene. (1) The search for common insertion sites is narrowed by the underlying action of the transgene. (2) Transgene expression can widen the range of cells normally transformed by a specific virus species. (3) Complementation between a known oncogene and a novel one can be established.

Two independent publications (van Lohuizen *et al.*, 1991, Haupt *et al.*, 1991) have clearly demonstrated the value of infecting oncogene-bearing transgenic mice with slowly transforming retroviruses in order to identify novel host proto-oncogenes.

Both works involved infection of Eμ-*myc* transgenic lines with Moloney Murine Leukaemia Virus (Mo-MuLV) and in both cases infection of these lines resulted in a dramatic acceleration of tumour development. Van Lohiuzen *et al.* (1991) found that the average age of tumour development decreased from 150 days to 50 days and a similar reduction in the latent period was reported by Haupt *et al.* (1991). MuLV development normally results in the development of T-cell lymphomas, whereas, expression of the *myc* proto-oncogene is restricted to the B-cell lineage in Eμ-*myc* transgenic lines. Nearly all tumours developing in the MuLV infected *myc* lines were of B lymphoid origin, thus the use of transgenic mice has widened the tissue types that can be examined using this approach.

Van Lohuizen *et al.* (1991) examined proviral insertion patterns and immunoglobin heavy chain rearrangements and showed that the developing tumours were either monoclonal or oligoclonal. This implied that either only rare insertion events led to the advancement of the tumour phenotype or that in addition to integration in specific insertion sites additional events, unrelated to MuLV infection, were also necessary. Proviral integration sites were screened for the presence of known oncogenes or previously identified insertion sites. Van Lohuizen *et al.* (1991) found that in 30% of tumours the provirus had activated the *pim*-1 gene, in one tumour the provirus was detected within the *pim*-2 locus and in another within the ahi-1 locus. Proviral insertions were not found near c-*abl*, c-*raf*, *bcl*-2, c-*myb* nor the insertion site *pvt*-1 in any of the tumours analysed. Haupt *et al.* (1991) found no rearrangement of N-*ras*, H-*ras*, K-*ras*, *cbl*, *bcl*-2, *myb*, *raf* or *rel* proto-oncogenes in the tumours arising from their experiments, but the provirus altered the *pim*-1 gene in 18% of tumours analysed and was found within the *pim*-2 locus in 6% of tumours.

These groups independently discovered a previously unknown gene, the *bmi*-1 gene, which appears to co-operate with the *myc* gene in B lymphoid tumours. This locus was a common insertion site in 35% of the tumours examined by van Lohuizen *et al.* (1991) and in 47% tumours examined by Haupt *et al.* (1991). The *bmi*-1 gene appears to be expressed in the nucleus and has a sequence consistent with that of a transcriptional regulator. Two additional novel common insertion sites, *bla*-1 and

pal-1, were found by van Lohuizen *et al.* (1991) and another novel site, *emi*-1, was reported by Haupt *et al.* (1991).

Both groups reported that complementation existed between some of these different integration sites as a number of tumours carried proviral integrants in more than one site. This work forms the basis from which to establish groups of complementing gene products and examine their corporate role in transforming a specific tissue, in this case the B-cell lineage.

The results presented in this chapter describe an oncogene induced model of T-cell lymphoma. The human c-*myc* oncogene was targeted to the T-cell compartment using the CD2 locus control region. This has resulted in transgenic lines of mice which exclusively develop T-cell tumours. Experiments have been initiated to ascertain which host proto-oncogenes can synergise with *myc* within the T-cell lineage.

A TRANSGENIC MOUSE MODEL OF T-CELL LYMPHOMA

SECTION 6.2 - MATERIAL AND METHODS

6.2.1. Production Of Transgenic Mice

(see Chapter 1 - General Material and Methods.)

6.2.2. Southern Blotting

Preparation of high molecular weight DNA from mouse tissues was carried out as described in Chapter 1. DNA was digested with the appropriate restriction endonucleases, separated by agarose gel electrophoresis, transferred to nylon filters and hybridised to the appropriate radiolabelled probes at high stringency using the protocols described by Sambrook *et al.* (1989). Random priming (random priming kit, Amersham Ltd.) and radiolabelling of these probes was also carried out using methods described by Sambrook *et al.* (1989).

A total of three different probes were used. Transgene sequences were identified using a human c-*myc* exon 3 probe (1.38 Kb, ClaI-EcoRI). T-cell receptor β-chain (TCRβ) gene rearrangement patterns were determined using 1.2 Kb EcoRI fragment of the clone 86T5 (Hedrick *et al.*, 1984). Moloney Murine Leukaemia Virus (Mo-MuLV) proviral sequences were detected using a probe derived from the U3 domain of the long terminal repeat (Cuypers *et al.*, 1986). This work was carried out by M. Stewart, M. Campbell and R. McFarlane.

6.2.3. Northern Blotting

Cellular RNA was extracted from mouse tissues using the RNAzol B method (Biogenesis Ltd.). 20 mg samples were separated by electrophoresis on 1% agarose gels containing 2.2M formaldehyde, transferred on to nylon filters and hybridised using procedures described by Sambrook *et al.* (1989). The presence of human c-*myc* RNA was determined by probing with a 339 bp AvaI fragment of human c-*myc* exon 1 cloned into the AvaI site of plasmid pBR322 (the gift of Dr. G. Birnie, Beatson Institute for Cancer Research). This work was carried out by M. Stewart.

6.2.4. RNase Protection Assay

Plasmid pSPT-PIP2 was generated by subcloning a 2.54 kb HindIII/NaeI fragment containing 5' upstream sequences and the first 213 bp of the human c-*myc*

exon 1 into the plasmid PSPT18 (Boehringer Mannheim). Transcription of this plasmid by T7 polymerase after linearisation with SmaI generates a 314 nucleotide transcript.

For RNase protection analysis, 5 μg of total RNA and 7.5 x 10^4 - 1 x 10^5 cpm of labelled RNA antisense probe were prepared according to standard protocols for *in vitro* transcription from bacteriophage promoters (Melton, 1985).

RNA and probe were combined in a single eppendorf tube, dried down and the pellet resuspended in 20 μl of hybridisation buffer (80% formamide, 400mM sodium chloride, 40mM PIPES pH 6.4, 1mM EDTA). The solution was heated to 85°C for 5 minutes then allowed to cool slowly to 55°C and left at this temperature for 16 hours. RNase digestion (10mM Tris, pH 7.6, 5mM EDTA, 0.3 sodium acetate pH 7.0, 0.8 μg/ml RNase A, 3 units/ml RNase T1) was performed for 60 minutes at 30°C after which RNA-RNA hybrids were precipitated following addition of carrier yeast tRNA (20μg). The RNA pellets were dissolved in formamide denaturing buffer and analysed by electrophoresis on a 6% polyacrylamide 8M urea sequencing gel. Gels were fixed in 10% methanol, 10% acetic acid, dried down and exposed overnight at -70°C to Kodak XAR-5 film using an intensifying screen. Dr. M. Stewart carried out the RNase protection analysis and I am grateful to her for providing the above information.

6.2.5. Flow Cytometry

Thymus tissue was mixed with phosphate buffered saline and minced using sterile scissors and the dead cells removed by centrifuging the cells through a ficoll-hypaque gradient (2000xg for 10 minutes). Cells were directly labelled by resuspending them at 2.5 x 10^6 ml with a 1:40 dilution of pre-titrated antibody. Four hundred microlitre reaction mixtures were incubated at 4°C for 35 minutes before washing and analysis. Rat monoclonals FITC anti-CD8, RD anti-CD4 and FITC anti-macrph were obtained from Coulter Ltd. The analysis was carried out on a EPICS elite cytometer (Coulter Ltd.) This work was carried out by M Campbell, K Blyth and L. Keanie.

6.2.6. Histology And Immunocytochemistry

Material for histological examination was fixed using 10% neutral buffered formalin, paraffin embedded and stained with haematoxylin and eosin. Immunocytochemistry was carried out on snap frozen material sectioned in a cryostat and acetone fixed. Labelling of sections was carried out with monoclonal antibodies as described by Mason (1985). The monoclonal antibodies used in this work

included: Rabbit anti-rat immunoglobins (Dako Z455), Rat APAAP complex (Dako D488) and fast red substrate. Primary antibodies included rat monoclonal anti-CD4 (L3/T4 sera-lab clone YTA3.1.2), anti CD8 (Lyt 2 and Lyt 3 sera-lab clones YTS 169.4 and YTS 156.7), anti-Thy-1 (clone YTS 154.7) and anti-CD3. This work was carried out by D. Onions and S. Toth.

The work described in this chapter was a collaborative project involving a number of workers with different areas of responsibility. Dr. M. Stewart constructed the CD2-*myc* transgene and prepared it for microinjection. Southern blotting, Northern blotting and RNase protection assays were the primarily the responsibility of Dr. J. Neil, Dr. M. Stewart, Dr. M. Campbell and Mr. R. McFarlane. Histopathology and immunocytochemistry sections were prepared by Dr. S. Toth, and examined by Dr. Toth and Prof. D. Onions. Flow cytometry studies were primarily the responsibility of Prof. D. Onions and Dr. M. Campbell the samples usually being prepared by Miss L. Keanie and Mrs. K. Blyth.

A TRANSGENIC MOUSE MODEL OF T-CELL LYMPHOMA

SECTION 6.3 - RESULTS

6.3.1. Generation Of *myc* Transgenic Mice

In order to explore the pathogenesis of T-cell neoplasia, transgenic mice carrying the human proto-oncogene c-*myc* were produced. The human c-*myc* locus, a Hind III-EcoR1 fragment in plasmid pMC41 (Watson *et al.*, 1983), was fused to a 5.5kb BamHI-Xba fragment of the CD2 dominant control region (DCR). This transgene was designed to direct human c-*myc* expression to the T-cell compartment as the CD2 DCR has previously been shown to confer high level copy number dependent expression within the T-cell lineage (Lang *et al.*, 1988). The CD2-*myc* construct is shown in Figure 6.3.1.

Figure 6.3.1. The CD2-*myc* Transgene Construct.

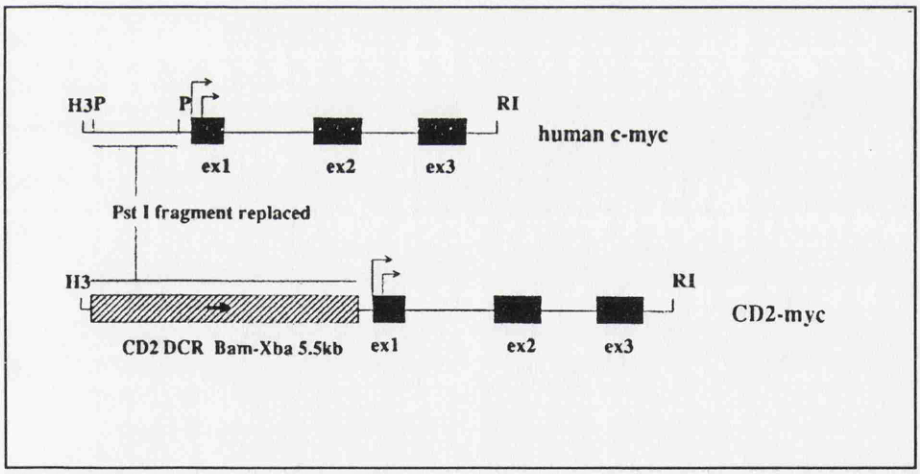


Figure 6.3.1.
This shows a map of the transgene construct used to generate the 800 and 900 lines. The Pst-1 fragment 5' to exon 1 of the human c-*myc* gene was replaced with a 5.5 Kb Bam-Xba fragment containing the dominant control region of the human CD2 gene.

A total of 330 embryos were successfully injected with the CD2-*myc* construct and transferred to 15 pseudopregnant recipients. Of these, 6 recipients gave birth to 58 pups all of which survived to weaning. All 58 were screened for the presence of human c-*myc* sequences and two mice, FO22 and FO51, were found to harbour the transgene. Southern blotting was carried out by M. Stewart. Compared to the integration frequencies reported by other workers (Brinster *et al.*, 1985) and the other

lines of transgenic mice produced in this study (see Chapter 2), these figures were low with only 3.4% of screened mice proving to be transgenic.

Both the transgenic mice and their non-transgenic littermates were C57Bl\6 (B6) x CBA\Ca F2 mice. In order to maintain this genotype each of the transgenic founders, F022 and F051, were bred to non-transgenic littermates to produce the 800 and 900 lines respectively, the 800 line being studied in detail. Therefore, the majority of mice in both lines are hybrids originally derived from the C57Bl\6 and the CBA\Ca inbred lines of mice.

6.3.2. Human c-*myc* Expression In Transgenic Lines

Transgene copy number was ascertained by Southern blotting and densitometry analysis after titrating genomic DNA and comparing it against known concentrations of plasmid DNA. Mice from the 800 line had approximately 14 copies of the transgene whereas mice from the 900 line had only 3 copies. Offspring from both transgenic founders were culled and total RNA was prepared from various lymphoid organs. A 339 base pair probe homologous to exon 1 of the transgene was used to determine if human c-*myc* transcripts were present in these tissues. However, in none of the organs analysed by Northern blot analysis was human c-*myc* mRNA detected. In order to exclude the possibility that the transgene was being expressed at levels below that of the sensitivity of Northern blot analysis or in a small sub-population of cells, RNase protection assays were carried out. Message specific for the human c-*myc* gene was not detected in any of the tissues analysed using this approach.

6.3.3. Thymic Lymphoma Development In *myc* Transgenic Mice

Despite the inability to detect transgene expression, a proportion of the 800 series and 900 series mice developed thymic tumours indictating that the presence of the trangene renders these mice susceptible to T-cell transformation. The incidence rate varied markedly between the two lines. These experiments are ongoing but at present 18 mice in the 800 series have spontaneously developed T-cell lymphoma, an incidence rate of 16%. To date, only 2 mice in the 900 series have developed lymphoma, an incidence rate of 3%. The age at which mice developed thymic lymphoma ranged from 10 weeks of age to 23 months. Mice that are less than 2 months of age have been excluded from the incidence analysis as no mice have succumbed to thymic lymphoma below this age. Thymic lymphoma has not been detected in non-transgenic mice kept throughout the duration of the study.

The majority of mice in the 800 series are of the C57Bl/6 x CBA/Ca genotype (hereafter referred to as B6/CBA), however 22 transgenic mice were derived from B6/CBA x Balb\c hybrids. The tumour incidence in mice of this genotype is 21% whereas the tumour rate in B6/CBA mice is 14%, although this difference is not significant ($x^2 = 2.2$: $P > 0.05$). As the experiment proceeds and more mice succumb, it will be possible to establish if crossing onto the Balb\c background renders animals more susceptible to transformation.

At present there is not enough data to determine whether or not homozygote animals have an increased risk of developing T-cell neoplasia. Of the 18 animals in the 800 series which have developed tumours, 12 are known to be heterozygote. The remaining 6 animals which succumbed could be either heterozygote or homozygote for the transgene and breeding studies suggest that the ratio of homozygote to heterozygote in this group is 40% to 60% respectively. Therefore, with the results presently available it does not appear that homozygosity increases the frequency of tumour development in these mice. The results of tumour incidence in the 800 series within the various groups is summarised in Table 6.3.1.

Table 6.3.1. Tumour Incidence in the 800 Line.

800 Series	(a) At Risk	Developing Tumour
Total Number of Transgenic Animals.	113	18
Number of B6/CBA Animals	85	12
Number of B6/CBA x Balb\c Animals	22	6
Number of Known Heterozygotes	63	12
Number of Homozygotes/Heterozygotes (40/60)	48	6
Number of Known Homozygotes	2	0

Table 6.3.1. Shows the tumour incidence in the 800 series maintained throughout the period of study. (a) "At Risk" describes the number of transgenic animals, over two months of age, which have been kept under observation through out the duration of this study.

To assess the incidence and age range at which tumour development occurs, groups of mice were monitored over a period of time. The age at which mice succumb to T-cell neoplasia ranges from 2 months to 23 months. The majority (88%) died between 2 and 9 months and 72% of mice died between 2 and 6 months of age. The experiment is still on-going and the results of the age incidence are at present preliminary as it is not yet clear how many mice will survive to old age. However, the results suggest that there is a broad age-range at which mice are more likely to develop T-cell lymphoma.

6.3.4. Tumour Phenotype

Affected mice showed loss of weight, deterioration of coat quality and an increased respiratory rate. The gait of ill mice was altered and they were often smaller than their litter mates. As soon as clinical signs became apparent, affected mice were culled. With the exception of one mouse the pathological changes were consistent with an enlarged thymus being found in every case, although the degree to which secondary organs were involved varied between mice. Two general pictures emerged. Those mice with grossly enlarged spleen and lymph nodes and those mice in which spread to secondary organs was not obvious with these organs retaining their normal size, shape and colour at *post mortem.*

The gross pathology of the thymus also varied. In some mice the thorax was filled with thymic tissue and obviously attached to the pleura and other thoracic organs. Occasionally, cords of thymic tissue extended from the main tumour mass giving a very irregular appearance. Other mice showed only an enlarged thymus which did not occupy the whole cavity, had a smooth surface and occupied a defined space. A large range of phenotypes was observed between these two extremes.

The size of the thymus did not appear to be related to the extent of secondary organ involvement. Similarly, the age of the affected animal was not related to the extent of thymic change, or the degree to which secondary organs were altered.

One mouse which became ill did not have a thymus that was greatly increased in size. This mouse was the founder animal of the 800 series and the oldest of all mice to develop T-cell lymphoma to date. The only obvious gross pathological feature found being a large abdominal mass which was of a similar colour, consistency and had a similar surface texture to that of the thymic tumours.

6.3.5. Histopathology

The pathology of all affected animals was similar. In most cases the normal thymic architecture was almost completely replaced by lymphoblasts. These cells showed some variation in size with ovoid or indented nuclei containing either single or multiple nucleoli. The cytoplasmic border of the lymphoblasts was fairly indistinct, eosinophilic and cells showed a high mitotic rate with many abnormal mitotic figures. In addition, marked lymphocytolysis and phagocytic activity resulted in "starry sky" appearance. In one mouse the cortex appeared to be the site of origin, the medulla being invaded from this site.

Other organs were altered to varying degrees. In every animal examined neoplastic changes were present in the spleen and extensive alteration of the spleen and lymph nodes was common, these organs being largely replaced with lymphoblasts again giving a "starry sky" appearance. In some cases areas of splenic red pulp were still recognisable. The bone marrow was diffusely infiltrated with lymphoblasts with only small numbers of residual haemopoetic cells present.

In the majority of cases, non-lymphoid organs were also heavily infiltrated with lymphoblasts. Changes were seen in the liver, the lungs, the kidneys and in one case the ovaries of affected animals. The degree of involvement in these organs varied.

The large abdominal mass recovered from the founder mouse consisted of lymphoblasts.

As most of the lymphoid organs were replaced by tumour cells the pathway of spread could not be established. However, with non-lymphoid organs a haematogenous route of spread was evident, indicating leukaemia. The histological examination of these tissues led to a diagnosis of lymphoblastic thymic lymphoma with associated lymphoblastic leukaemia.

6.3.6. RNA And DNA Analysis Of Tumour Tissue

In all tumour samples examined a moderate to high level of human c-*myc* expression was detected. Therefore, c-*myc* expression did not appear to be discernible in transgenic lymphoid cells until after they became transformed.

Transformed cells were also analysed to determine T-cell receptor beta (TCRβ) gene rearrangement. The presence of only one or two rearranged bands indicates that these tumours were either monoclonal or oligoclonal. The analysis may be complicated by the presence of non-transformed cells within the sample or rearrangement of the second allele, both of which would give an additional series of bands.

6.3.7. Phenotype Of Transformed Cells

Immunofluoresence staining revealed that transformed cells carried Thy-1, CD3, CD4 and CD8 cell surface markers, in none of the tumours examined was the B-cell specific marker evident, indicating that these tumours were of the T-cell lineage.

Cells were prepared from tumour tissue for analysis by flow cytometry on an EPICS Elite cytometer (Coulter Ltd.). Although only a small number of tumours have currently been analysed these results show that each tumour had a largely homogenous cell population. However, in different tumours the cell populations pathologically expanded differed. In two out of four thymic tumours analysed the major cell population was CD4+/CD8+ whereas in the other two tumours the cells were CD4-/CD8+. In the four tumours analysed there appeared to be a correlation between the cell type involved and the gross pathological picture. In the two CD8+ single positive tumours there was obvious metastasis with marked enlargement of secondary organs such as the spleen and lymph nodes whereas in the CD4+/CD8+ tumours the gross changes were confined to the thymus. Flow cytometry of healthy transgenic mice revealed that no abnormalities could be discerned when compared against control mice.

6.3.8. Induction Of Thymic Lymphomas Using MuLV And MuLV-supF

To determine if the presence of the transgene rendered these mice more susceptible to Murine Leukaemia Virus (MuLV) induced tumourigenesis animals, heterozygote for the transgene, were bred with Balb\c females and the resulting offspring infected with Moloney Murine Leukaemia Virus (Mo-MuLV) within the first 24 hours of life. Neonates were infected by injecting approximately 0.1ml of Mo-MuLV or Mo-MuLV-supF directly into the abdomen. These experiments were designed so that transgenic positive and transgenic negative littermates were infected with the same batch of virus on the same day. Animals were sacrificed when they were first noticed to be ill and samples taken at *post mortem.*

In total, three separate experiments were performed. In the initial preliminary experiment 9 offspring from the 800 line and 10 offspring from the 900 line were infected with Mo-MuLV. In the 800 series, 4 of the 9 infected mice carried the transgene and these mice succumbed on average 63 days after infection (range 53-70 days). The non-transgenic litter-mates survived significantly longer, dying on average 144 days after infection (range 103-203). A similar result was obtained with mice from the 900 series. Of the 10 infected mice, 4 proved to be transgenic and these 4 had an average lifespan of 67 days (range 61-72 days) whereas the control mice died on average 132 days post-infection (range 107-146 days).

The second experiment concentrated on the 800 series and on this occasion the Murine Leukaemia Virus carried the supF gene (Mo-MuLV-supF), this recombinant virus being used to facilitate the cloning of subsequent insertion sites (Reik *et al.*, 1985). Twenty-four mice were infected, subsequent analysis revealing that 12 of these mice were transgenic. The average lifespan for the transgenic group was 75 days (range 63-84). In the control group only 7 out of 12 mice developed lymphoma by 250 days at which point the experiment was concluded. The average lifespan of the control mice which succumbed before the end of the experiment was 191 days (range 135-250).

In the third experiment, all 8 of the Mo-MuLV-supF infected mice were transgenic as they were the offspring from an animal homozygote for the transgene and a Balb\c female. These mice died on average 70 days after infection (range 66-70) with 7 out of 8 mice being found ill on the same day. These results are summarised in Table 6.3.2 and Figure 6.3.2.

The results show that infection with MuLV increases tumour incidence and results in a dramatic acceleration of tumour development. Infection of CD2-*myc* mice changes the pattern of tumour development in three ways. 1) The incidence rate of spontaneous tumours is only 16% but all of the infected mice die. 2) Infected mice succumb to lymphoma development much earlier in life than the uninfected mice and 3) tumour development in the infected mice is much more synchronous than in the uninfected mice. Altogether 24 transgenic mice were infected in these experiments and only 31 days separated the ages of mice succumbing to thymic lymphoma.

Table 6.3.2. Lifespan of Infected Transgenic Mice and Infected Control Littermates.

Group	Group Size	Average Lifespan (days)	Range (days)	Standard Deviation
MuLV				
800 series	4	63	53-70	7.1
900 series	4	67	61-72	5.6
Controls	11	137	103-177	33.8
MuLV-supF				
800 Series	20	73	63-84	5.1
Controls	7	191	135-250	42.6

A summary of three experiments in which CD2-*myc* transgenic mice were infected with wild type MuLV or the recombinant MuLV-supF showing the average lifespan, range and standard deviation of affected animals.

There is no significant difference between the age at which transgenic mice develop lymphoma when infected with MuLV as opposed to those infected with MuLV-supF, although those infected with wild type MuLV died on average 10 days earlier than those infected with the recombinant virus. The recombinant retrovirus may be slightly less potent than the wild type or this may just reflect a difference in titre between the two virus batches.

The large difference in the latent period between transgenic and control mice clearly shows that the transgene collaborates with MuLV in the development of T-cell lymphoma. Although the numbers involved in Experiment 1 were small, these results indicate that the 800 and 900 lines are equally susceptible to MuLV induced tumourigenesis.

Figure 6.3.2. Survival Curves for Infected Transgenic Mice and Infected Control Littermates.

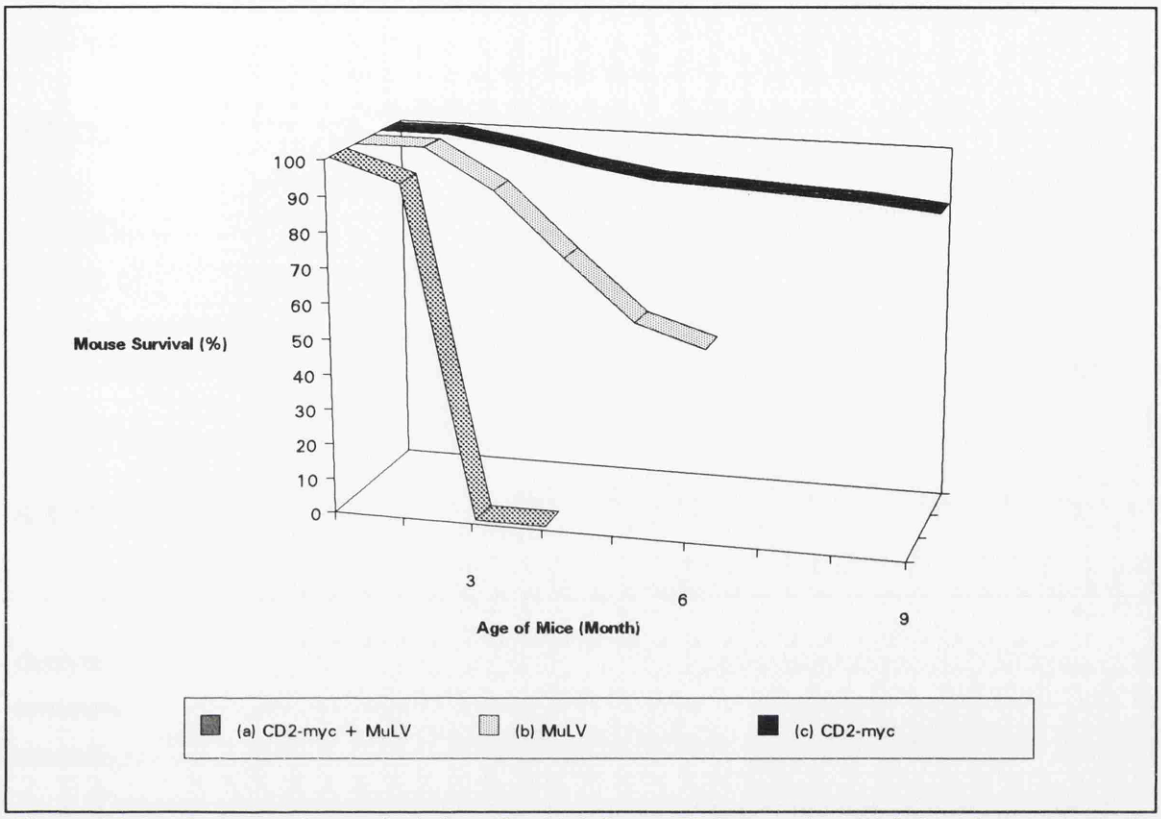


Figure 6.3.2. Compares the survival times for (a) infected CD2-*myc* mice, (b) infected non-transgenic mice and (c) uninfected CD2-*myc* mice.

6.3.9. Pathology Of MuLV Induced Tumours

There were no obvious differences in the profile of pathological changes between those mice spontaneously developing thymic lymphoma and those mice developing it after infection. In MuLV induced tumours the thymus was always markedly enlarged, often filling the entire thoracic cavity. Evidence of metastasis on gross pathology was variable but the spleen was frequently involved.

Histopathological findings were indistinguishable from those already described for spontaneous tumours. On histopathological examination evidence of tumour infiltration was found in all spleens examined.

6.3.10. Immunocytochemistry Of MuLV Induced Tumours

Tumour tissue recovered from CD2-*myc* animals infected with MuLV was tested for the presence of cell surface markers. The staining pattern observed was very

similar to that described for spontaneous tumours. All tumours examined were positive for Thy-1, CD3, CD4 and CD8 whereas none proved to be positive for the B-cell specific Ig marker. Therefore, in all tumours examined only cells displaying the T-cell phenotype were found.

6.3.11. Flow Cytometry Analysis Of Tumour Cells From Infected Mice

The cell types found in tumours arising from infected transgenic mice are similar to those seen in the spontaneous *myc* mice. To date, four tumours from this group have been analysed, in two of these tumours the major cell type was the CD4+/CD8+ cell whereas the other tumours showed a CD8+ singly positive phenotype.

6.3.12. Transgene Expression In The MuLV Induced Tumours

Southern blotting of T-cell receptor β chain (TCRβ) genes with a 1.2 Kb probe, derived from clone 86T5 (Hedrick *et al.*, 1984), revealed that the MuLV induced tumours were monoclonal or oligoclonal with only one or two TCRβ chain rearrangements being detected within each tumour.

The induced tumours were also clonal with respect to proviral integration, this was confirmed by the relatively simple proviral integration pattern observed after hybridisation with a probe specific for the U3 region of the viral LTR.

Total RNA was isolated from tumour tissue of MuLV infected CD2-*myc* strains and hybridised with a 339 bp probe recognising the transcript encoded by the human c-*myc*. As with spontaneous tumours, virally induced tumours in CD2-*myc* all showed high levels of transgene expression.

Although high levels of expression were found in both spontaneous and MuLV induced tumours, no expression was found in young, healthy animals when thymi were examined by either Northern blotting or RNase protection assays. To investigate the possibility that proviral integration occurred within the transgene tandem arrays, thereby causing activation of the transgene, the tumours were analysed for the presence of the transgene rearrangements using the human c-*myc* exon 3 probe. Southern blotting was used to compare the integrated transgene within the tumour cells with the germline pattern. Tumours from 6 mice were analysed but no obvious rearrangement of the transgene was evident in any of these tissues.

Therefore, there is no evidence of insertional mutagenesis occurring within any of the multiple transgene copies in any of the tumours analysed. These results do not, however, exclude the possibility that proviral sequences integrated at some distance either 5' or 3' to the transgene insertion site.

A TRANSGENIC MOUSE MODEL OF T-CELL LYMPHOMA

SECTION 6.4 - DISCUSSION

To explore the effects of the *myc* oncogene within the T-cell compartment, the human c-*myc* gene, coupled to the regulatory sequences of the CD2 dominant control region (CDR), was introduced into the mouse germline. This resulted in two transgenic lines being created both of which developed thymic lymphomas, albeit at markedly different rates. In the most intensively studied line (CD2-*myc*-800), 16% of mice succumbed, whereas in the CD2-*myc*-900 line only 3% of mice developed tumours.

The pathology and histopathology were indicative of T-cell tumours with the primary site of tumour involvement almost always being the thymus. The T-cell origin of these tumours was confirmed by examination of cell surface markers. The presence of the T-cell markers; Thy-1, CD3, CD4, and CD8 and the absence of Ig antigens shows that in every tumour examined thymocytes were the target cell. No tumours have arisen from any other tissue compartment.

These results show that the presence of the *myc* transgene predisposes these mice to tumour formation and that this is restricted to the T-cell phenotype by the CD2 dominant control region. This is the first work to directly target the oncogenic effects of the *myc* transgene exclusively to the T-cell compartment and we believe that the CD2-*myc* mice described here will be a valuable model with which to study the biology of T-cell lymphoma and leukaemia.

Examination of T-cell receptor (TCR) gene rearrangements revealed that these tumours were either monoclonal or oligoclonal. This, together with the variable latency and the relatively small proportion of mice affected, indicates that rare genetic events are probably responsible for the progression to the tumour phenotype.

The hypothesis that tumourigenesis is a multistep process has become well established in recent years and the use of transgenic mice to pursue the study of oncogenesis has played a major role in lending credence to this theory (Adams and Cory, 1991). The results in this study add weight to this hypothesis as they suggest that one or more additional genetic or epigenetic events are contributing to the tumour phenotype.

High levels of human c-*myc* expression were found in every tumour analysed but despite the increased incidence of thymic lymphoma in these mice, it has not been possible to detect transgene expression in the tissues of healthy CD2-*myc* mice using both Northern blotting and RNase protection assays. That the transgene does not appear to be expressed within preneoplastic T-cells is somewhat surprising as all

previous reports of transgene constructs employing the CD2 regulatory sequences describe high level, copy number dependent expression in the T-cell lineage. The low frequency of transgenic founders and the finding that the CD2-*myc* transgene was not expressed in the only two founder mice which resulted from this work, raises the possibility that foetuses expressing the transgene died *in utero* during early thymic development. Therefore, it is possible that over expression of *myc* in the T-cell compartment of the developing foetuses is lethal. Alternatively, the presence of plasmid sequences in the CD2-*myc* construct may have suppressed expression as it has previously been shown that the inclusion of plasmid sequences can inhibit transgene expression (Townes *et al.*, 1985).

There are a number of possibilities which could explain the role of the apparently quiescent transgene in the development of thymic lymphomas. Any rare genetic event leading to activation of the transgene may be the first step in tumourigenesis, that this event ultimately leads only to T-cell tumours is probably due to the presence of the CD2 regulatory sequences. This implies that either the CD2 element is functional, but cannot overcome the repression of the down-regulated *myc* gene, or that the action of the CD2 unit is itself compromised and that activation of the transgene involves a process which returns function to this regulatory unit. None of these putative activation events have resulted in expression being detected in a tissue other than the T-cell lineage and similarly, no tumours have been found outside this tissue type.

At present it is not possible to say whether activation of the transgene is the only event necessary for tumour development or whether subsequent random events are also required after transgene expression is initiated. A considerable amount of evidence supports the latter hypothesis, as expression of oncogenes (Hariharan *et al.*, 1989; Yee *et al.*, 1989; Mc Donnell *et al.*, 1990; van Lohuizen *et al.*, 1989b) including c-*myc* (Adams *et al.*, 1985) within transgenic animals does not appear to be sufficient to induce tumour formation in the majority of cases. Even in transgenic mice expressing two complementary oncogenes, additional events appear to be necessary for transformation to the fully malignant phenotype (Rosenbaum *et al.*, 1990; Verbeek *et al.*, 1991).

Alternatively, it is possible that the transgene is being expressed in a small sub-population of cells, with the total level of messenger RNA being below the sensitivity of our assays. Expression within these cells would render them more vulnerable to additional transforming events, which in turn could allow this population to become dominant. However, this hypothesis still fails to explain why only a very small group of T-cells should express the transgene given the reported dominance of the CD2 regulatory region. In addition, the phenotypic features of the transformed cells do not suggest that these cells are a specialised or unusual sub-population of T-cells.

Another possibility which cannot formally be excluded is that a small number of mice do express the transgene. The mice in this experiment are a genetically heterogeneous group of individuals and it is possible that inheriting a specific combination of alleles permits transgene expression rendering individual mice much more susceptible to neoplastic disease. Such a hypothesis might be investigated by repeatedly crossing transgenic mice into a variety of different genetic backgrounds, or alternatively, it may be possible, with long term breeding studies, to identify sub-lines of mice which are either susceptible or resistant to T-cell neoplasm.

There is a precedent for the situation described here. Hariharan *et al.* (1989) introduced the *bcr*-v-*abl* gene into the germline of mice and despite being unable to detect transgene expression in tissues of healthy mice, these animals showed an increased incidence of lymphoma development. Two constructs were used, in one the transgene was coupled to the immunoglobin enhancer (Eμ) and in the other, part of the long terminal repeat (LTR) of the Myeloproliferative Sarcoma Virus (MPSV) was used. The Eμ enhancer has been reported to produce expression in both B and T-cells, whereas, the LTR produces expression in myeloid as well as lymphoid cells (Bowtell *et al.*, 1988). It is interesting to note that 75% of tumours in the Eμ-*bcr*-v-*abl* mice were of T-cell origin and all of the tumours arising in LTR-*bcr*-v-*abl* mice were of T-cell origin. This may suggest that events which activate non-expressing transgenes are more likely to occur in the T-cell lineage than in the B-cell compartment.

Preneoplastic Changes

Consistent with the apparent lack of transgene expression, the haematopoietic populations in healthy transgenic mice did not appear to be perturbed. It is not known whether obvious preneoplastic changes would be expected if the *myc* transgene was being expressed. Constitutive expression of the *myc* proto-oncogene within the B-cell lineage causes disturbances in the B-cell population with expansion of pre-B cells and a subsequent reduction in the proportion of mature B-cells (Langdon *et al.*, 1986). Changes in B-cell populations in *myc* bearing transgenic mice have also been reported by Yukawa *et al.* (1989). These workers compared the action of the Eμ-*myc* transgene on two different genetic backgrounds and found that B6 mice developed mainly B-cell tumours, whereas C3H/HeJ mice developed mainly T-cell tumours. However, the transgene was expressed in both T-cells and B-cells in both strains of mice. Expression within the B-cell compartment produced changes in the preneoplastic B-cell population but no change in the characteristics of the T-cell population could be detected - even in the mouse strain which developed mainly T-cell

tumours. Therefore, in the C3H/HeJ strain, expression of the *myc* gene in the T-cell compartment was associated with the development of T-cell tumours but not with an obvious alteration to the preneoplastic T-cell population.

Further evidence for the action of *myc* on T-cells was reported by Spanopoulou *et al.* (1989). In this case, thymic expression of *myc* was achieved using the Thy-1 regulatory unit. These transgenic mice developed tumours in the thymus which consisted of lymphoid and epithelial cells, thymic expression of the *myc* gene in healthy tissue was comparable to that found in tumour tissue but expression of CD3, CD4, CD8 and heat stable antigen (HSA) in the thymocytes of young healthy mice was normal.

However, the L-*myc* gene does appear to affect T-cell populations. Moroy *et al.* (1990) found that the L-*myc* gene is preferentially expressed in T-cells when under the regulation of the immunoglobin enhancer. Expression of this gene caused disturbances in T-cell development with expanded thymic cortices, enlargement of cortical lymphocytes and spleens which contained enlarged and irregular follicles. The relative populations of T-cell subsets were, however, unaltered.

The Incidence Of Tumour Formation In CD2-*myc* Transgenic Mice

In this present study the two transgenic lines showed markedly different rates of tumour formation with the incidence rate in the 800 and 900 lines being 16% and 3% respectively. Transgene integration being essentially a random process means that the integration sites in the two transgenic lines differ. The two lines also differ with respect to transgene copy number with the 800 line carrying approximately 14 copies and the 900 line carrying only 3 copies. Although the great majority of transgene constructs are heavily influenced by their integration site, the CD2 dominant control region has been reported to confer on the transgene independence from the integration site influence and allow high levels of transgene expression (Lang *et al.*, 1988). If expression were present it would be expected to be directly correlated with the number of integrated copies. It is possible, therefore, that if expression is occurring in a sub-population of T-cells that the transgene copy number and resultant level of expression is directly influencing the tumour incidence.

If the transgene is being repressed and activation events are required to induce expression, then it may be that the transgene in the 800 line occupies a position within the host chromatin which is more likely to be activated than in the 900 line. In this case tumour incidence would be independent of transgene copy number. Copy number may influence the rate of tumour formation indirectly, the larger size of the

transgene integration site in the 800 line making random activation events much more likely to influence transgene expression as a consequence of target size.

Alternatively, some combination of these may be possible. The initial rate limiting event may be activation of the quiescent transgene. After this has occurred the next limiting event may be the level of expression which in turn is copy number dependent.

Genetic Background

Preliminary results suggest that the offspring of B6\CBA x Balb\c CD2-*myc* mice may have an increased tumour incidence. That genetic background can influence the incidence and pattern of tumour formation in transgenic mice bearing oncogenes has previously been reported by Sidman *et al.* (1988). These workers found that the oncogene potential of the Eμ-*myc* transgene varied on different genetic backgrounds. Tumours developed relatively slowly in B6 mice in sharp contrast to SJL mice which developed lymphomas rapidly. Balb\c mice represented an intermediate situation with lymphomas developing at an early age but the subsequent incidence rate flattening off. To investigate the effect of genetic background on the CD2-*myc* mice further would require moving the transgene onto different genetic backgrounds, including the Balb\c strain, and then assessing the tumour incidence.

MuLV Infection Of CD2-*myc* Mice

The presence of the transgene greatly accelerated MuLV induced tumourigenesis. CD2-*myc* mice all develop thymic lymphoma at between 9-12 weeks of age when infected with MuLV, whereas infected control mice had an average lifespan of 23 weeks. Further, infection of CD2-*myc* neonates increases the incidence and synchrony of tumour development when compared with spontaneous tumours arising in non-infected transgenic animals. Infection increases the incidence from 16 to 100% and whereas mice developed spontaneous tumours over a wide age range (2-23 months), all MuLV infected mice died at around 3 months. Taken together, these results suggest that MuLV infection and the CD2-*myc* transgene collaborate in the development of thymic lymphoma. That the infective oncogenesis does synergise with the CD2-*myc* transgene is confirmed by finding that all tumours examined in these mice strongly express the transgene.

The pathological and histopathological features of the MuLV induced tumours are very similar to those found in non-infected CD2-*myc* mice. Again, there is primary involvement of the thymus, with metastasis to other organs, both lymphoid

and non-lymphoid. In addition, a similar cell population is affected, the main target cell appearing to be either the CD4+/CD8+ double positive or the CD8+ single positive cell. That virally induced tumours are also clonal is shown by T-cell receptor (TCR) gene rearrangement patterns and the relatively simple pattern of proviral integration sites in individual tumours. In conclusion, it appears that although viral induction affects the kinetics of tumour development it does not appear to alter the characteristics of the resultant tumours.

Preliminary results suggest that although the incidence rate of spontaneous tumours is markedly different between the two transgenic lines, these lines are equally susceptible to MuLV induced tumourigenesis. This finding does not support the suggestion that the target size of the transgene integration site is an important factor in influencing the likelihood of spontaneous activation events.

Tumour tissue from infected mice was examined for transgene rearrangements in order to better understand the mechanism of tumour formation in infected mice and the nature of the interaction between the integrating proviral sequences and the transgene. No obvious rearrangement of the transgene was detected, indicating that integration had not occurred within the tandem arrays of the transgene. Despite this result, the simplest and most attractive explanation is that integrating proviral sequences activate the quiescent transgene with the responsible proviral sequences being situated 5' or 3' of the transgene insertion site. In this hypothesis, the integrating proviral sequences would substitute for the unknown initial event which induces activation of the transgene - the possible first step in the development of thyomas in the non-infected transgenic mice.

Future Prospects

The CD2-*myc* mice represent a powerful model with which to extend our understanding of the mechanism underlying T-cell lymphoma development. The CD2-*myc* lines are unique as they are the only mice in which the c-*myc* oncogene is exclusively targeted to the T-cell lineage and as such they will allow an investigation into those events which can collaborate with *myc* in the development of T-cell tumours.

Future research will attempt to identify those genes and those events that together with *myc* contribute to the induction of T-cell tumours. Infecting the CD2-*myc* mice with MuLV should identify those genes which can co-operate with myc in T-cell transformation and these studies may also result in the identification of previously uncharacterised genes and loci. This approach has been successfully used to identify those genes which complement one another in transforming B-cells (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991). These studies revealed that different oncogenes (or putative oncogenes) can be grouped on the basis of mutual complementation.

Parallel studies will investigate those functional characteristics, specific to T-cells, which render them vulnerable to transformation. T-cell function is based on the ability of these cells to interact with antigens via the T-cell receptor. This process involves recognition, activation and proliferation. In the case of self antigen, activation appears to be followed by programmed cell death - apoptosis.

We intend to investigate the relationship between antigen recognition and tumour development by deliberately stimulating the immune system with both endogenous and exogenous antigens. The attraction of this approach is that any transformed population which subsequently arises can be screened for its specificity to the original antigen.

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