

# **STUDIES ON CONDITIONAL GENE EXPRESSION**

Thesis Submitted for the Degree of Doctor of Philosophy by  
Scott K. Lyons

University of Edinburgh

1998



## **DECLARATION**

The work described within this thesis has been conducted by myself unless otherwise specifically stated.

S. K. Lyons, December 1998.

## ACKNOWLEDGEMENTS

This project was primarily funded by a Whyatt Postgraduate Scholarship from the Faculty of Medicine, University of Edinburgh and by the Faculty of Medicine Cancer Research Fund. I am most grateful to both sources for enabling this period of study.

I would also like to thank both Professor Martin Hooper and Dr Alan Clarke for their excellent supervision, guidance and inexhaustive support over the years I have spent at Teviot place.

This work was conducted in the Department of Pathology in the Faculty of Medicine and accordingly I would like to thank Professor Andrew Wyllie. I would also like to thank Dr Sandrine Prost for providing me with the benefit of her experience regarding the establishment of primary tumour cell lines and anti-p53 immunology. Further I would like to thank Professor David Harrison and Dr Alan Clarke for assisting with the histological analyses described within chapters 3 and 4, and Dr Sarah Howie and Eleanor Ritsou for their instruction regarding the use of the flow cytometer. Thanks also to Maria Wallace for all her hard work regarding the characterisation of transgene inducibility.

Finally I would like to thank everybody that has made my time spent living and working in Edinburgh such great fun. Thanks to everybody on the 5<sup>th</sup> floor at Teviot, most especially Jonny C, Damian Connaughton, Liz Lovejoy (the Vector NTI lifesaver!), Stefan Selbert, Tom Gardner, Rungtiva Kongkanuntn, Eleanor Duff, Dominic Rannie, Roger Malcomson, Jason Reed (for the great coffee!), Andrea Leitch, Chris Bellamy, Chris Armit, Neil Tofts and everybody else whose names I cannot fit on the page!

Thanks to Jean Steele for all her support and Jean Abbott for accomo in St Stephens Street at the start, also to the old boys from St A's for the beers and to 'da boYz' in Haarlemmermeer Straat for all their support towards the end. Thanks also to everybody at my new lab in the N.K.I. for all their support and patience whilst writing up.

HUGE thanks also going out to the whole Sturgeon Clan at both Eskbank and Stockbridge for their great friendship and support over the years. Big thanks also to Karen Young for her sense of fun and 'enduring' the write-up with me!

Finally though the LARGEST thanks goes to my folks (and sis!), without whose support this thesis would probably never have been completed.

# ABSTRACT OF THESIS

(Regulation  
3.5.13)

of Candidate ..... SCOTT KEITH LYONS .....

PhD

Date ..... 29/7/98 .....

Thesis ..... STUDIES ON CONDITIONAL GENE EXPRESSION .....

words in the main text of Thesis ..... 55 000 .....

p53 is an important gene in the control of cellular proliferation, apoptosis and DNA repair, and loss of wild-type p53 gene function has been demonstrated to be the most common type of event arising during the development of many human malignancies. My aim therefore, was to generate an experimental system in which it would be possible to externally control p53 gene expression and thus examine phenotypic consequence following introduction of wild-type p53 gene expression in an otherwise p53 null environment. Following recent advances in the field of inducible promoter technology, I attempted to render p53 expression inducible utilising several of the current promoter systems of choice via both targeting and transgenic approaches.

Using a double replacement targeting approach, I attempted to replace the p53 promoter region with inducible promoter sequence. Targeting constructs were designed to produce a mutant p53 locus, which would then subsequently allow the rapid and simple introduction of a variety of transcriptional regulatory sequences. Following the introduction of the first stage targeting vector into ES cells, southern analysis demonstrated that 1 clone from 508 HAT resistant clones possessed a recombinant p53 locus.

In a parallel approach, I used one of the candidate promoter systems to generate an interferon  $\alpha/\beta$  inducible p53 transgene. Sarcoma cells, derived from an engineered homozygous p53 deficient mouse, were established as lines *in vitro* and stably transfected with this construct. Analysis of resultant inducible clones demonstrated little significant transgene mediated effect upon tumour cell cycle, however, a clear transgene dependent apoptotic phenotype was observed following UV irradiation.

To test the possible extent of therapeutic benefit resulting from the introduction of p53 gene expression into p53 null tumour cells *in vivo*,  $5 \times 10^6$  inducible sarcoma cells were injected sub-cutaneously into the flanks of SCID animals. Tumours grew at the site of injection within 3 weeks, at which time the animals were injected intra-peritoneally with synthetic double stranded RNA (pI:pC) to induce transgene expression. No p53 protein could be detected in these tumour samples by either western or immuno-histochemical analyses. Subsequent re-establishment and analysis of cell lines derived from these tumours indicated that selection for a final presenting tumour cell with lost or silenced p53 transgene expression can occur during the clonal expansion of inoculated 1D cells growing *in vivo*.

Following pI:pC injection of SCID mice, an acute apoptotic phenotype was observed in the crypt compartments of the small intestine which resulted in the death of approximately 20% of all injected animals. It is known that levels of interferon  $\alpha/\beta$  are elevated during graft versus host response, but these cytokines had previously been described as exerting a proliferative effect upon colonic crypt cells. Observation of small intestine epithelia, 6 hours post-injection, clearly shows the underlying biological process responsible for this phenomenon is however apoptosis. Further experimentation would suggest that this intestinal phenotype is NK cell mediated and is most severe in T and B cell deficient animals.

# CONTENTS

	<b>PAGE</b>
<b>DECLARATION</b>	ii
<b>ACKNOWLEDGEMENTS</b>	iii
<b>ABSTRACT</b>	iv
<b>CONTENTS</b>	v
<b>CHAPTER 1 - Introduction</b>	<b>1</b>
<b>1.i.1</b> The Origins of the Laboratory Mouse	<b>2</b>
<b>1.i.2</b> Manipulation of the Murine Genome	<b>6</b>
<b>1.i.3</b> Transgene Expression	<b>10</b>
<b>1.i.4</b> The Problem with Transgenes	<b>11</b>
<b>1.i.5</b> ES Cells	<b>12</b>
<b>1.i.6</b> Basic Models of Homologous Recombination	<b>14</b>
<b>1.i.6a</b> Reciprocal Recombination	<b>15</b>
<b>1.i.6b</b> Meselson & Radding's Model of Homologous Recombination	<b>16</b>
<b>1.i.6c</b> The Double Strand Break Repair Model of HR	<b>17</b>
<b>1.i.7</b> Gene Targeting	<b>22</b>
<b>1.i.7a</b> Basic Targeting Strategy	<b>22</b>
<b>1.i.7b</b> Factors Affecting Targeting Efficiency	<b>25</b>
<b>1.i.7c</b> Selection of Complex Targeting Strategies	<b>26</b>
<b>1.i.8</b> Inducible Promoter Systems	<b>31</b>
<b>1.i.8a</b> Interferon Inducible Promoter Systems	<b>32</b>
<b>1.i.8b</b> The Molecular Basis of the Interferon $\alpha/\beta$ Response	<b>36</b>
<b>1.i.8c</b> Tetracycline Inducible Promoter System	<b>38</b>
<b>1.ii</b> The Molecular Basis of Cancer	<b>41</b>
<b>1.ii.1</b> p53	<b>45</b>
<b>1.ii.2</b> p53 Structure and Function	<b>46</b>
<b>1.ii.2a</b> The Core Domain	<b>47</b>
<b>1.ii.2b</b> N- Terminal Domain	<b>49</b>

<b>1.ii.2c</b> C- Terminal Domain	<b>51</b>
<b>1.ii.3</b> Activation of p53 Dependent Responses	<b>53</b>
<b>1.ii.4</b> Regulation of p53 Expression	<b>55</b>
<b>1.ii.4a</b> Transcriptional Regulation	<b>55</b>
<b>1.ii.4b</b> Post-Transcriptional Regulation	<b>58</b>
<b>1.ii.4c</b> Post-Translational Regulation	<b>59</b>
<b>1.ii.5</b> Mediation of Growth Arrest, DNA Repair and Apoptosis	<b>65</b>
<b>1.ii.5a</b> Growth Arrest and DNA Repair	<b>66</b>
<b>1.ii.5b</b> Apoptosis	<b>69</b>
<b>1.ii.6</b> p53 and Mutation Frequency	<b>73</b>
<b>1.ii.7</b> p53 and Tumourigenesis	<b>74</b>
<b>1.ii.8</b> p53 and Angiogenesis	<b>79</b>
<b>1.ii.9</b> The Requirement of p53 Expression during Embryogenesis	<b>80</b>
<b>1.ii.10</b> p53 and Gene Therapy	<b>81</b>
<b>CHAPTER 2</b>	<b>84</b>
<b>2.i</b> Inducible Transgene Approach	<b>84</b>
<b>2.i.1</b> Choice of Inducible Expression System	<b>86</b>
<b>2.i.2</b> Cloning Strategy of Inducible p53 Expression Constructs	<b>89</b>
<b>2.i.2a</b> Cloning Strategy of Interferon $\alpha/\beta$ Inducible p53 Constructs	<b>89</b>
<b>2.i.2b</b> Cloning Strategy of Tetracycline Inducible p53 Construct	<b>91</b>
<b>2.i.3</b> Derivation of p53 Deficient Sarcoma Cell Lines	<b>91</b>
<b>2.i.4</b> Derivation of p6-16 <sub>L</sub> p53-HYG Stably Transfected clones	<b>92</b>
<b>2.ii</b> Characterisation of Stably Transfected Clones	<b>92</b>
<b>2.ii.1</b> Optimisation of rtPCR Assay	<b>92</b>
<b>2.ii.2</b> p6-16 <sub>L</sub> p53-HYG Transgene is Inducible, but does not Splice	<b>95</b>
<b>2.ii.3</b> Optimisation of Quantitative rtPCR Assay	<b>98</b>
<b>2.ii.4</b> Further Analysis of Transgene Induction	<b>102</b>
<b>2.ii.5</b> Characterisation of Transgene Induction at the Protein Level	<b>105</b>
<b>2.ii.6</b> Kinetics of Transgene Translation	<b>107</b>
<b>2.iii</b> The Effect of p53 Expression upon Tumour Cell Cycle <i>In Vitro</i>	<b>109</b>

<b>2.iii.1</b>	p53 Induction and the Cell Cycle	<b>112</b>
<b>2.iii.2</b>	Effects of p53 Induction and DNA Damage upon Tumour Cell Cycle	<b>115</b>
<b>2.iii.3</b>	Conclusions from Section 2.iii	<b>119</b>
<b>2.iv</b>	The Effect of p53 Expression upon the Rate of Apoptosis <i>In Vitro</i>	<b>119</b>
<b>2.iv.1</b>	Effects of p53 Induction upon the Rate of Tumour Cell Apoptosis	<b>120</b>
<b>2.iv.2</b>	Conclusions from Section 2.iv	<b>122</b>
<b>2.v</b>	The Long Term Consequences of Transgene Dependent Effects <i>In Vitro</i>	<b>124</b>
<b>2.vi</b>	Further Research	<b>127</b>
<b>2.vi.1</b>	RGC-LacZ Reporter Assay	<b>128</b>
<b>2.vi.2</b>	Western Analysis of p6-16 <sub>L</sub> p53-HYG Translation Product	<b>129</b>
<b>CHAPTER 3</b>		<b>133</b>
<b>3.i</b>	The <i>In Vivo</i> Experimental System	<b>133</b>
<b>3.ii</b>	Optimisation of <i>In Vivo</i> Transgene Induction	<b>136</b>
<b>CHAPTER 4</b>		<b>149</b>
<b>4.i</b>	SCID Phenotype following Injection of pI:pC	<b>149</b>
<b>4.ii</b>	Interferon $\alpha/\beta$ and Morphology of the SCID Intestine	<b>153</b>
<b>CHAPTER 5</b>		<b>156</b>
<b>5.i</b>	Targeting Approach	<b>156</b>
<b>5.ii</b>	Targeting Vector Construction	<b>159</b>
<b>5.ii.1</b>	Vector Homology Requirements	<b>159</b>
<b>5.ii.2</b>	Derivation of the Upstream Arm of Homology	<b>160</b>
<b>5.ii.3</b>	Derivation of the Downstream Vector Arm	<b>171</b>
<b>5.ii.4</b>	Selection Marker Strategy and Construction of Targeting Vector	<b>175</b>
<b>5.iii</b>	Targeting Event Screening Strategies	<b>179</b>
<b>5.iii.1</b>	Development of PCR Screen to Detect HR at 5' Vector Arm	<b>179</b>
<b>5.iii.2</b>	Development of Screen to Detect HR at 3' Vector Arm	<b>183</b>
<b>5.iv</b>	Targeting Results using pFX $\Delta$ K-HPRT-F3K	<b>186</b>
<b>5.v</b>	Conclusions	<b>188</b>

<b>CHAPTER 6 - Discussion</b>	<b>193</b>
6.i A Brief Summary of Aims and Experimental Approaches	193
6.ii Discussion of Experimental Data	195
6.iii Future Perspectives and Concluding Remarks	206
<b>CHAPTER 7 - Materials and Methods</b>	<b>209</b>
<b>Section 1 – DNA Techniques</b>	<b>209</b>
MM1.1 Heat-Shock Transformation of CaCl <sub>2</sub> Competent <i>E. Coli</i> with Plasmid DNA	209
MM1.2 Preparation of Plasmid DNA from <i>E. Coli</i>	209
MM1.3 Restriction Digestion of DNA	212
MM1.4 Southern Analysis of Genomic DNA	213
MM1.5 Radiolabelling Probe with P <sup>32</sup> by Random Priming	217
MM1.6 Ligation of DNA Fragments	218
MM1.7 Construction of Vectorette Genomic DNA Libraries	221
MM1.8 PCR Amplification of DNA	222
MM1.9 Dideoxy-Sequencing of Plasmid DNA with Sequenase	223
<b>Section 2 – RNA Techniques</b>	<b>227</b>
MM2.0 Precautions	227
MM2.1 Extraction of RNA from Cultured Cells	227
MM2.2 Extraction of RNA from Fresh Tissue	228
MM2.3 Agarose Gel Analysis to Determine Integrity of Prepared RNA	228
MM2.4 Generation of cDNA from RNA Template with Reverse Transcriptase	230
<b>Section 3 – Protein and Immunological Techniques</b>	<b>231</b>
MM3.1 Western Analysis of Proteins	231
MM3.2 p53 Immuno-analysis of Cultured Cells on Chamber Slides	236
MM3.3 p53 Immunohistochemistry of Fixed Tissue on Microscope Slides	238
MM3.4 Immuno-flow Detection of p53	240
<b>Section 4 – Cell Culture Techniques</b>	<b>241</b>
MM4.1 ES Cell Culture	241
MM4.2 Sarcoma Cell Line (SL) Culture	245
MM4.3 Flow Cytometric DNA Analysis of Cultured SL Cells	248
MM4.4 Visualisation of Apoptosis by Acridine Orange Staining	249
<b>Section 5 – Whole Animal Techniques</b>	<b>251</b>
MM5.1 Subcutaneous Injection of SL Cells	251
MM5.2 <i>In Vivo</i> Induction of Type I Interferons with pI:pC	251
MM5.3 Apoptotic Analysis of Whole Length Small Intestine	251



<b>BIBLIOGRAPHY</b>	<b>253</b>
<b>APPENDIX – Plasmid Maps</b>	<b>280</b>
Inducible p53 Transgene Constructs	1-6
Targeting Vector Constructs	7-9

## CHAPTER 1

One of the greatest challenges to face biomedical science this century has been to develop the ability to effectively cure inherited and acquired human genetic disease. In order to understand fully the molecular nature underlying the biology of human genetic disease, a prerequisite before such a therapeutic goal can be considered feasible, scientists have found it necessary to employ the use of experimental animals as model systems. Observations recorded in the course of *in vitro* experimentation with mammalian cell lines, although certainly highly informative as regards cellular biology, cannot be directly extrapolated as being of relevance to the genetic disorder *in vivo*. The model species of choice in this field of experimental science is for many reasons the common house mouse, *Mus musculus*.

The biology of this species is of far more physiological relevance to human biology than that of the other “classical” genetic model systems, such as *E. coli* and *D. melanogaster*. The mouse’s small size, fertility and short gestation period make their practical handling and storage convenient for often necessary large scale breeding programmes. Moreover, the existence of many inbred laboratory mouse strains has generated a resource of greater than 1000 spontaneous, radiation or chemically induced mutant lines (Doolittle *et al*, 1996). This has enabled researchers in the past to map the genes implicated in disease to specific chromosomal loci, thus deriving the first genetic linkage maps. However, it is most certainly the technological advances made in the past two decades that have

been instrumental in fixing the mouse as the mammalian system of choice in the modelling of human disease.

Despite the complexity of the murine genome ( $\sim 3 \times 10^9$  base pairs (bp)/haploid genome), parallel breakthrough advances in Embryonal Stem cell (ES cell), gene targeting and gene cloning technologies mean that it is now possible to modify specifically the expression of any known gene and analyse phenotypic consequence on virtually any inbred or outbred genetic background. In addition, the ever-improving resolution of genetic linkage and physical maps of the murine genome is greatly facilitating the identification of candidate disease genes of interest. It is predicted that the mouse genome will be fully sequenced by the year 2003 (Jordan and Collins, 1996).

### **(1.i.1) The Origins of the Laboratory Mouse**

The origins of mouse genetics began formally at the turn of the 20<sup>th</sup> century. It was at this time that researchers first recognised the need for a higher homogeneity of genetic background in the experimental mouse. Strains of mice with inbred backgrounds were required to fix alleles and permit controlled scientific experimentation exploring the genetics of disease traits. In 1909, Little began breeding the first inbred mouse strain, DBA (*d*, dilute; *b*, brown; *a*, non-agouti), with the intention of investigating the genetics of cancer susceptibility loci.

Inbred strains of mice are created following approximately 20 consecutive rounds of brother x sister mating (Hogan *et al*, 1995). There now exist approximately 478 strains of inbred mice (Festing, 1998) and many of the lines found in the

laboratory today share their origins from the 1920's and 30's, from within the laboratories of Little, Strong and MacDowell.

In the 1940's, Snell pioneered the development of the congenic strain and these mice were initially bred in an attempt to identify loci influencing tissue transplantation. Congenic strains are constructed by backcrossing one inbred line carrying a locus, phenotypic trait or mutation of interest onto another inbred strain background. The desired trait is selected at each of approximately 10 backcrosses. After this breeding programme is complete, the resultant mouse differs from the parental mouse statistically by approximately 20cM of DNA, from within which, is encoded the trait of interest. These mice have proved invaluable in the mapping of polymorphic disease and marker loci (for review see Frankel, 1995).

Recombinant Inbred (RI) strains, originally derived by Taylor and Bailey during the 1970's, have also proved invaluable in the study of polygenic disease. These mice are created via the systematic inbreeding of randomly selected pairs from the F<sub>2</sub> generation resulting from an original cross of two different inbred mouse strains. Thus, RI strains comprise an overall 50:50 genetic contribution from both initial inbred parental lines, but all different strains originating from the F<sub>2</sub> intercrosses, exhibit a random combination and segregation of individual loci. These animal strains have been successfully employed to examine the complex genetics underlying the diseases of epilepsy (Frankel *et al*, 1994), atherosclerosis (Justice *et al*, 1992) and substance abuse (Crabbe *et al*, 1994).

A modification of the RI approach, termed the Recombinant Congenic (RC) approach was pioneered by Demant during the mid 80's {Demant and Hart,

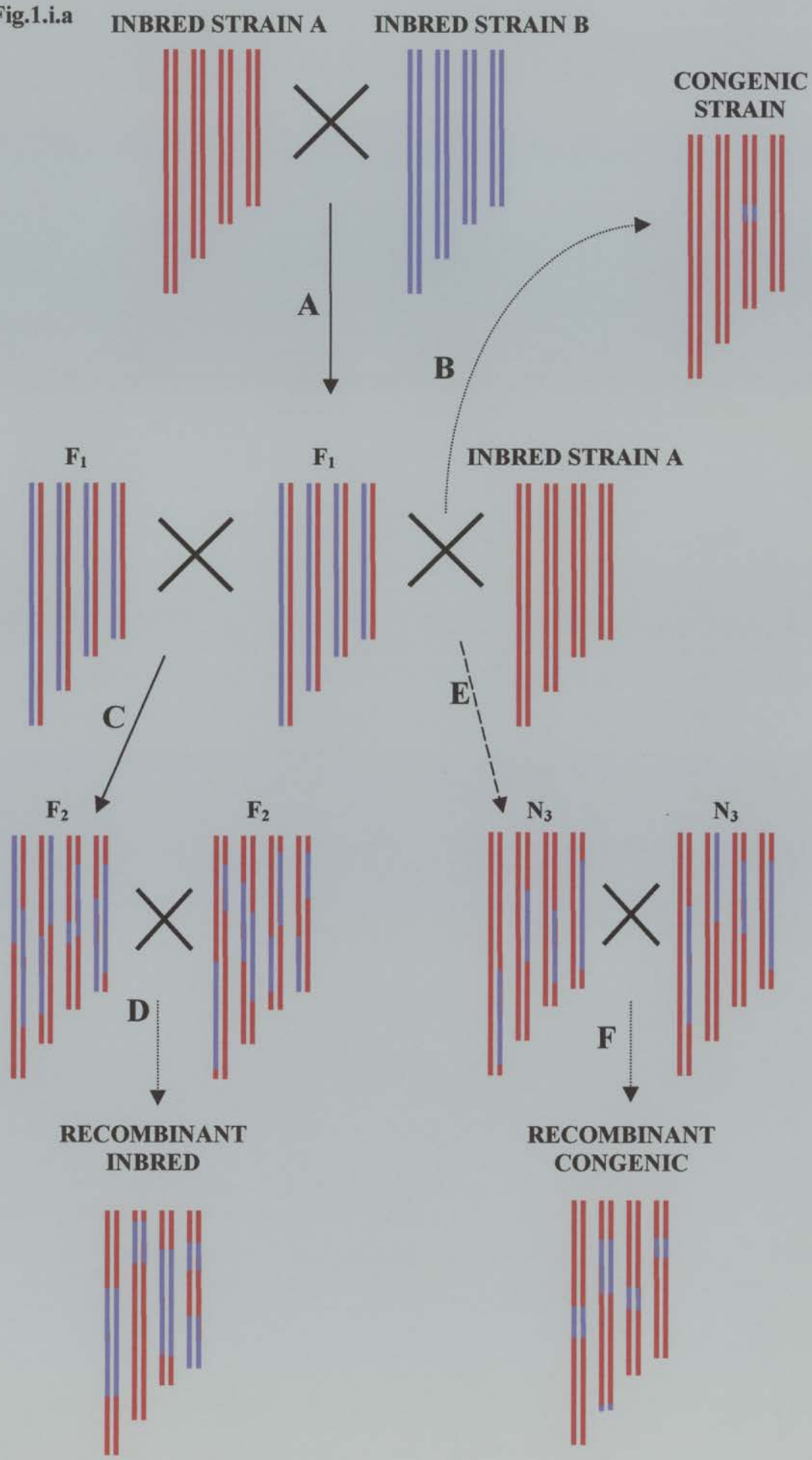
1986). Following the breeding of two inbred strains, RC mice are produced by initially backcrossing the resultant F<sub>1</sub> generation two times, followed by repeated brother x sister mating. The result of this breeding programme is that the RC strain is composed of approximately 12.5% of the donor genome, randomly distributed through 87.5% of the background genome. This increases the comparative probability of disease locus segregation in comparison to the RI strains and thus permits finer mapping of complex traits. The RC approach has been most extensively employed in mapping tumour susceptibility genes of colon (Moen *et al*, 1996) and lung cancer (Finjeman *et al*, 1995), and investigating the genetics underlying the T-cell response to selected cytokines (Lipoldova *et al*, 1995).

Figure 1.i.a on the next page illustrates the conventional breeding programmes used in the development of laboratory mouse strains.

### **KEY**

- A.** Single cross between two inbred laboratory strains.
- B.** 10 conventional backcrosses and phenotypic selections, onto one of the parental inbred genetic backgrounds to produce a congenic strain.
- C.** Production of the F<sub>2</sub> generation following F<sub>1</sub> (brother x sister) mating.
- D.** Randomly selected brother x sister mating originating at the F<sub>2</sub> generation, for more than 20 generations to produce a recombinant inbred strain.
- E.** 2 backcrosses onto one of the inbred parental backgrounds, producing the N<sub>3</sub> generation.
- F.** Randomly selected brother x sister mating between N<sub>3</sub> generation mice for more than 20 generations to produce a recombinant congenic strain.

Fig.1.i.a



### **(1.i.2) Manipulation of the Murine Genome**

The ability to modify the specific expression of a single allele on an inbred genetic background has rapidly accelerated our understanding of many diverse aspects of mammalian molecular biology, including the genetics of disease. The application of transgenic technology, originating in the late 70's and early 80's, has enabled researchers to introduce foreign DNA into the mammalian germline; thus permitting the passage of introduced DNA through generations of inbred genetic stocks.

Transgene technology has not been confined solely to the laboratory mouse and has been utilised to produce transgenic strains of other mammalian and domesticated species e.g. sheep, cow and chicken. These species however have been primarily developed within the biotechnology industry, with potentially massive commercial impact, in an attempt to improve the yield and quality of meat sold in the marketplace, or as 'bio-reactors' for the production of pharmacologically active peptides.

Transgenic technology has however been applied mostly in studies of phenotypic consequence following the introduction of foreign gene expression in mice. Further, this technology has proved useful in functional studies of the regulatory promoter sequences responsible for conferring either temporal or tissue specific patterns of reporter gene expression.

Pronuclear injection is the most commonly used approach to generate transgenic animals (Hogan *et al*, 1995). This method involves the direct microinjection of foreign DNA into the pronucleus of a fertilised egg (Gordon *et al*, 1980) and is

successful at producing stable transgenic mouse lines at a frequency of between 10-40% (i.e. between one and four transgenic mice are produced from approximately every ten pronuclear injections (Hogan *et al*, 1995)).

Transgenic animals produced in this manner characteristically possess multiple copies of the introduced DNA, arranged tandemly in a head-to-tail array, integrated randomly at a single chromosomal locus (Lacy *et al*, 1983). This appears to be the result of extra-chromosomal homologous recombination (ECR) arising between individual transgene copies immediately upon injection and prior to integration (reviewed in Bollag *et al*, 1989).

The efficiency of ECR does not appear to be affected by the concentration of injected transgenic DNA (it has been proposed that every transfected molecule may undergo at least one ECR event (Folger and Capecchi, 1985)) and can occur within very short lengths (<100bp) of homology (Rubnitz and Subramani, 1984). Further, rates of ECR can be demonstrably enhanced in yeast following the generation of a double-strand break within the sequence of introduced DNA (Orr-Weaver *et al*, 1981).

In light of these observations, it is highly likely that rates of ECR between transfected DNA transgenes are enhanced as a result of their preparation prior to introduction as opposed to the amounts introduced. Transgenic DNA is routinely linearised and removed from associated plasmid sequence prior to injection as prokaryotic sequence elements have been demonstrated to influence transgene expression negatively *in vivo* (Chada *et al*, 1985), (Hammer *et al*, 1987).



The integration of transgenic DNA into the host cell's genome occurs in most cases as a unique and rapid event following microinjection. It has been reported that up to 80% of transgenic mice produced by this technique possess the introduced DNA in all of their cells, i.e. integration occurs prior to pronuclear division. The remaining proportion of transgenic animals exhibit mosaicism of transgene expression resulting from delayed integration and require one round of breeding and selection before possessing the introduced DNA in every cell (Wilkie *et al*, 1986).

Infection of pre-implantation embryos with recombinant retrovirus is another common approach used to introduce foreign DNA into the animal germline. Rudolph Jaenisch produced the first transgenic animal in 1976 via this method, by directly exposing a pre-implantation stage mouse embryo to Moloney leukaemia virus (M-MuLV) (Jaenisch, 1976). In addition to simple exposure, another commonly used method of infecting animal embryos with retrovirus is to co-cultivate them in the presence of virus producing cells (Jahner *et al*, 1982). All resultant transgenic animals generated by these methods are mosaic for presence of the transgene (i.e. both somatic and germ cells from the same animal possess variable transgene copy number, ranging from zero to many copies).

Variation of titre and exposure time of retrovirus in contact with the animal embryo can influence resultant transgene copy number (Coffin *et al*, 1998). Long exposure times with high titre viral stocks can result in the super-infection of embryonal cells. Unlike the unique chromosomal integration event associated with multiple transgene copy number following pronuclear injection, retroviral

super-infection results in high copy numbers of transgene per cell, randomly integrated at independent chromosomal loci. The generation of single copy transgenic animals can be achieved relatively easily however, and so can enable the phenotypic characterisation of gene dosage effects *in vivo*.

The main advantage of recombinant retroviral gene delivery over pronuclear injection is that the introduction of foreign DNA is permissible with later stage embryos (between days 8 to 14 p.c.). This allows DNA to be introduced at different stages of embryonal development but, as a downside to this, viral integration occurs predominantly in somatic cell lineages as opposed to the germline (Soriano *et al*, 1986).

Unlike pronuclear injection however, retroviral gene delivery is limited by the maximum size of transgenic DNA the virus can effectively package and therefore introduce into the germline (maximum limit approximately 8Kb). In addition, as a result of producing a significantly higher frequency of mosaic animals, there is much greater variability in resultant transgene expression levels and pattern between animals produced in an identical manner.

The advent of ES cell technology has enabled further means of generating transgenic mice (see section 1.i.5). Transgenic DNA can randomly integrate within the ES cell genome following conventional *in vitro* transfection protocols. Stable recombinant cells can then be selected in antibiotic containing media and the relative levels of transgene expression can be determined *in vitro*, prior to blastocyst injection and whole animal generation.

### **(1.i.3) Transgene Expression**

The *in vivo* expression pattern of a transgene can be influenced by the choice of promoter sequence used to regulate transgene expression. Promoters of viral origin have been demonstrated to drive the overexpression of a construct in both a global (CMV) and tissue specific fashion (M-MuLV LTR in macrophages (Lang *et al*, 1987)). Promoter sequences derived from mammalian constitutively expressed (housekeeping) genes (e.g.  $\beta$ -actin), also direct transgene overexpression to all tissues.

Other commonly used promoter types confine transgene expression to specific tissue types (e.g.  $\alpha$ -crystallin to the lens (Goring *et al*, 1987),  $\beta$ -lactoglobulin to mammary epithelial cells (Simons *et al*, 1987)). These tissue specific promoters are especially useful in transgenic studies when the global expression of a transgene results in either a dominant deleterious phenotype or when phenotypic consequence is of interest only in a specific cell type *in vivo*.

Inducible promoters are a further class of promoter type now commonly used in transgenic experimentation. These promoter sequences have been derived from viral, prokaryotic and eukaryotic genes and conditionally direct transcription in the presence of an inducing molecule or following environmental stimulation. Although transgenic DNA is present within the genome at all times, this technology permits the induction (or ablation in conjunction with cre/loxP technology, for review see (Porter, 1998)) of transgene expression at any stage of embryonal or adult development.

The development of inducible gene expression technology is of obvious advantage regarding conventional transgenic experimentation. Conditional

transgenics are a powerful tool in the development of animal models of acquired genetic disease. The expression of many loci implicated in acquired genetic disease, in non-hereditary cancers for example, are proving to also be essential for embryonic development (for review see Copp, 1995). Thus, cancer research will benefit greatly from these promoter types, as it is now possible to model oncogene activation and tumour suppressor ablation in a highly specific manner in adult animals.

In addition, this technology has also greatly simplified the identification and validation of transgene dependent phenotype, both *in vitro* and *in vivo*. The nature of several of these promoters will be discussed in greater depth later in this chapter (section 1.i.8).

#### **(1.i.4) The Problem with Transgenes**

A common problem affecting the expression of foreign DNA in all transgenic animal lines generated by random integration is termed the position effect. As transgenic DNA randomly recombines within the host cell's genome, host DNA sequence and conformation in proximity to the site of transgene integration can influence transgene expression. Local chromatin structure and transcriptional interference from adjacent promoter sequence elements can both positively and negatively influence transgene expression. Position effect is often manifested by poor reproducibility in detected transgene expression level and pattern in transgenic animals generated in an identical fashion. To by-pass these problems, transgene expression patterns should be fully characterised in the initial founder lines, with desired levels selected and bred.

A relatively infrequent problem associated with the random integration of foreign DNA into the genome of a host cell is that of gene disruption. If the transgene integrates within a promoter/enhancer region of a host gene or within an open reading frame, the expression of that allele will be altered, even ablated. Random transgene mediated mutagenesis and gene disruptions have been reported to occur at a rate of approximately 5% of all integration events (Hogan *et al*, 1995). It is therefore important to make phenotypic comparisons between identically generated transgenic animals before determining whether any observable phenotypic modifications are transgene dependent.

The major limitation of transgene technology is that DNA can only be introduced, not removed from the animal germline. With the possible exception of introducing dominant-negative mutant transgenes (Harvey *et al*, 1995) or anti-sense expression constructs (Katsuki *et al*, 1988), which are both of highly variable efficiency, it is not possible to completely ablate specific wild-type gene expression using conventional transgenic approaches.

Parallel breakthroughs in ES cell and homologous recombination technologies however have made it practically possible to knock out gene expression routinely and have revolutionised mammalian genetic research.

#### **(1.i.5) ES Cells**

Embryonal Stem (ES) cells were first described by two independent groups in 1981, as permanent pluripotent cell lines derived from the inner cell mass (ICM) of 3.5 d.p.c. (Days Post Coitum) mouse embryos (Martin, 1981), (Evans and

Kaufman, 1981). Like Embryonal Carcinoma (EC) cells, ES cells can be maintained, essentially indefinitely, in an undifferentiated state in culture. To achieve this, ES cells were traditionally cultured on mitotically inactivated embryonic fibroblasts (Evans and Kaufman, 1981) or in media conditioned by EC or BRL (Buffalo Rat Liver) or bladder carcinoma cells (Martin, 1981), (Smith and Hooper, 1987), (Williams *et al*, 1988). ES cells have now also been demonstrated to retain their morphology and pluripotency when cultured in media supplemented with a single active peptide, e.g. Leukaemia Inhibitory Factor (LIF) (Smith and Hooper, 1988), (Williams *et al*, 1988), Ciliary Neurotrophic Factor (CNTF) (Wolf *et al*, 1994), or Human Oncostatin M (OSM) (Gearing and Bruce, 1992).

Upon withdrawal from these culture conditions *in vitro*, ES cells spontaneously differentiate into a wide variety of cell types. ES cells plated at low density characteristically differentiate into endoderm and mesoderm-like cells (Mummery *et al*, 1990), whereas cells grown at a higher density characteristically start to differentiate and form suspension aggregates termed embryoid bodies (similar to a 6 d.p.c. stage embryo). If these aggregates of cells re-attach to the tissue culture plate, further differentiation will occur, resulting in a wide range of cell types within a few weeks (reviewed Hooper, 1992).

The most important property of ES cells however, which is distinct from EC cells, is the ability to colonise both somatic and germline tissues at high efficiency following direct injection back into host blastocysts (Bradley *et al*, 1984). In practical terms, this enables the genomic manipulation of large numbers of cells, thereby permitting selection of relatively rare events, the phenotypic consequences of which can be analysed in many diverse cell and tissue types.

### **(1.i.6) Basic Models of Homologous Recombination**

Homologous recombination (HR) is defined as the exchange of genetic material between two DNA entities entirely upon the basis of their sharing lengths of homologous sequence. This process occurs naturally within eukaryotic organisms during meiosis, mitosis and DNA repair and its mechanisms have been exploited by researchers in a technique termed gene targeting (see section 1.i.7), which allows very precise modification of the genome (Hooper, 1992).

HR can occur in mammalian cells between introduced DNA molecules (extra-chromosomal recombination, ECR, reviewed in Bollag *et al*, 1989), between homologous sequences within the same chromosome (intra-chromosomal recombination) or on different chromosomes (inter-chromosomal recombination) or between introduced DNA sequences and host chromosomal sequences (reviewed in Hooper, 1992).

Different homology requirements have been reported (both in total length and in total un-interrupted length) for the ability of DNA molecules to homologously recombine in the various ways listed above (reviewed in Hooper, 1992). However, only the considerations and requirements for efficiently achieving HR between introduced DNA sequences and host chromosomal sequences will be discussed further in this chapter (see section 1.i.7).

Much of the research investigating the underlying mechanisms of homologous recombination have been carried out either in bacteria, fungi or yeast, primarily because of the low complexity of their genomes and in the latter cases, the ease through which they can be grown, both mitotically and meiotically. Many of the

principles of HR, derived from the use of these organisms, have also proven applicable to the observed HR occurring within mammalian cells.

In general, homologous DNA sequences can exchange information through either reciprocal or non-reciprocal recombination. Reciprocal recombination involves a balanced swap of nucleotides between sequences, whilst non-reciprocal recombination (or gene conversion) involves a unidirectional exchange of nucleotides. Both forms of HR are often observed to occur simultaneously at adjoining sequences and models have been proposed accordingly to attempt to explain their underlying molecular mechanisms.

#### **(1.i.6a) Reciprocal Recombination**

Figure 1.i.b illustrates the model proposed by Holliday to explain the mechanism of reciprocal homologous recombination (Holliday,1964). Although updated and modified, most notably in the models by Meselson & Radding (section 1.i.6b) and by Szostak & Stahl (section 1.i.6c), this model has been included to explain the concepts of heteroduplex DNA formation and the Holliday Junction, concepts which are universal to all of the above proposed models of HR.

In this schematic, the bold pairs of black and blue lines signify two double stranded molecules of homologous DNA. At point (A), both homologous sections of DNA pair to form a synaptic complex where both form single-strand nicks at homologous positions in strands of like polarity (B). Strand invasion occurs at the sites of DNA breakage (i.e. the free ends of both nicked DNA duplexes invade each other simultaneously) and become covalently sealed to form a structure termed the Holliday Junction (C). The Holliday junction can subsequently diffuse



along the length of DNA by a process termed branch migration, forming a stretch of heteroduplex DNA (i.e. a length of double stranded DNA composed from two different DNA molecules) as it proceeds (D). An alternate depiction of the Holliday junction following branch migration is shown in (E). Resolution of this structure can arise from diagonal cleavage at either points (F) or (G), resulting in the generation of 'non-crossover' or 'crossover' products respectively.

Since this model was proposed in the mid-60's, Holliday structures have been directly visualised in *E. Coli* using electron microscopy, thus providing part corroboration for the molecular mechanisms discussed here (Potter and Dressler, 1979).

#### **(1.i.6b) Meselson & Radding's Model of Homologous Recombination**

Meselson & Radding proposed this revised form of the Holliday model in 1975 {Meselson and Radding, 1975} in light of observations that HR rarely occurs in a reciprocal manner, and so to convincingly model the mechanisms underlying gene conversion.

As depicted in figure 1.i.c, at point (A), both homologous sections of DNA pair to form a synaptic complex where only one forms a single-strand nick (B), which subsequently invades the other intact homologous duplex, displacing a D-loop (C). The D-loop structure is degraded at (D), and an asymmetric heteroduplex is generated (i.e. only the recipient chromatid possesses heteroduplex DNA) as a result of DNA synthesis on the donor duplex and degradation on the recipient chromatid. Ligation occurs at (E) forming a Holliday junction which is resolved

as in the previous model, either without cross-over to generate the products at (F) or with crossover to generate the products at (G).

### **(1.i.6c) The Double Strand Break Repair Model of HR**

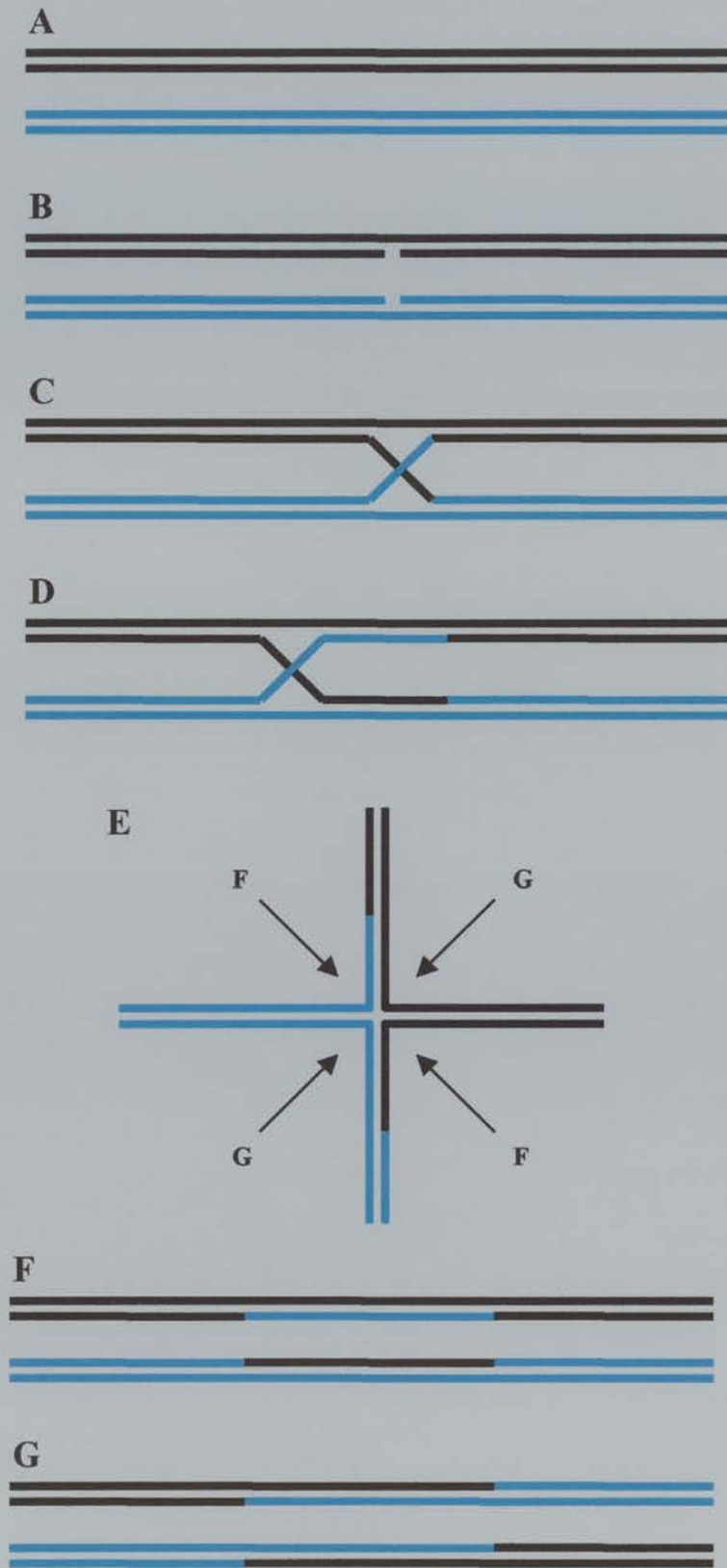
The Double-Strand Break-Repair (DSBR) model of HR was initially proposed by Szostak & Stahl to explain observable increases in recombination rates in yeast following the introduction of double-strand DNA breaks (Szostak *et al*, 1983). This model is similar to the other models mentioned in section 1.i.6, in that recombination is again initiated through the invasion of a single DNA strand into homologous recipient duplex DNA to produce heteroduplex DNA.

As depicted in figure 1.i.d, at point (A), both homologous sections of DNA pair to form a synaptic complex where one strand forms a double strand break, which is subsequently enlarged to a double strand gap as a result of exonuclease degradation (B). One of the free ends generated at point (B), invades the other duplex molecule to displace a D-loop structure on the recipient duplex (C), which extends as result of DNA synthesis primed from the initial invading strand (D). Also at point (D), the extended D-loop structure base pairs with homologous single-stranded DNA generated from the original degraded double strand break generated at point (B). Covalent linkage occurs at point (E) to form two Holliday junctions, both of which can diffuse by branch migration. In addition, the displaced D-loop, base-paired to the original donor duplex molecule, acts as a primer from which DNA synthesis can be initiated. Resolution of the two Holliday junctions generated at point (E) can result in the generation of four recombination products. (Fi) results from double non-crossover; (Fii) results from

crossover at the left, but non-crossover on the right; (Fiii) results from non-crossover on the right, but crossover on the left; and (Fiv) results from crossover at both junctions. Note that the gap resulting from endonuclease degradation of the original donor molecule at point (B) has been replaced by sequences derived from the recipient DNA molecule.

HR can also occur in mammalian cells in a non-conservative manner (i.e. two homologous DNA sequences recombine to generate a single species and a resultant net loss of DNA). A further model of HR, termed the single-strand annealing (SSA) model, was proposed by Lin in 1984 in an attempt to explain the generation of non-conservative extra-chromosomal HR products, arising from the introduction of double-strand DNA breaks within homologous sequences (Lin *et al*, 1984). The mechanisms of SSA are distinct from the models discussed above and are not included within this chapter as this model of HR does not appear to be able to explain the majority of recombination products that arise following HR between introduced DNA sequences and host chromosomal sequences i.e. products that arise during gene targeting (see section 1.i.7).

Fig. 1.i.b Reciprocal Homologous Recombination



**Fig. 1.i.c**      **Meselson and Radding's Model of HR**

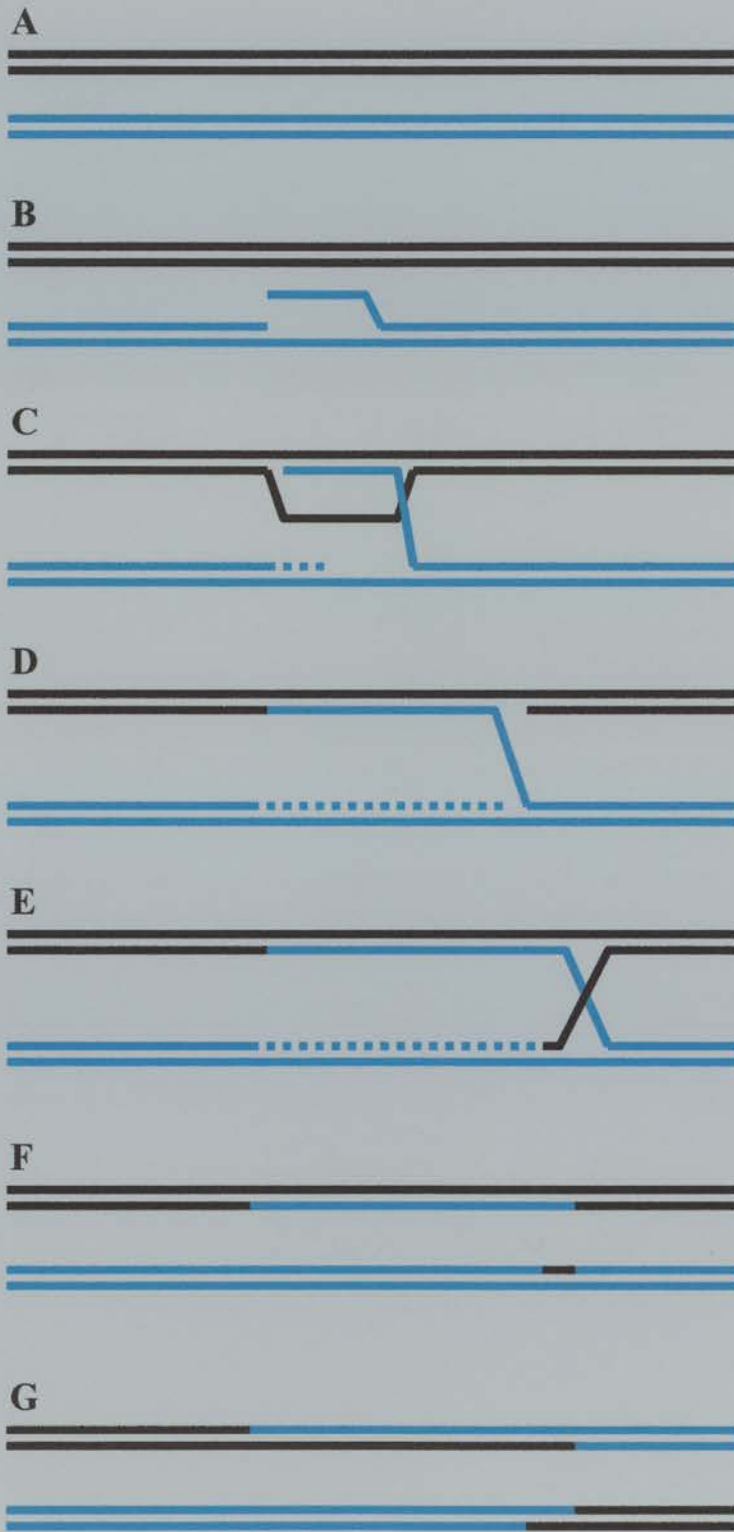
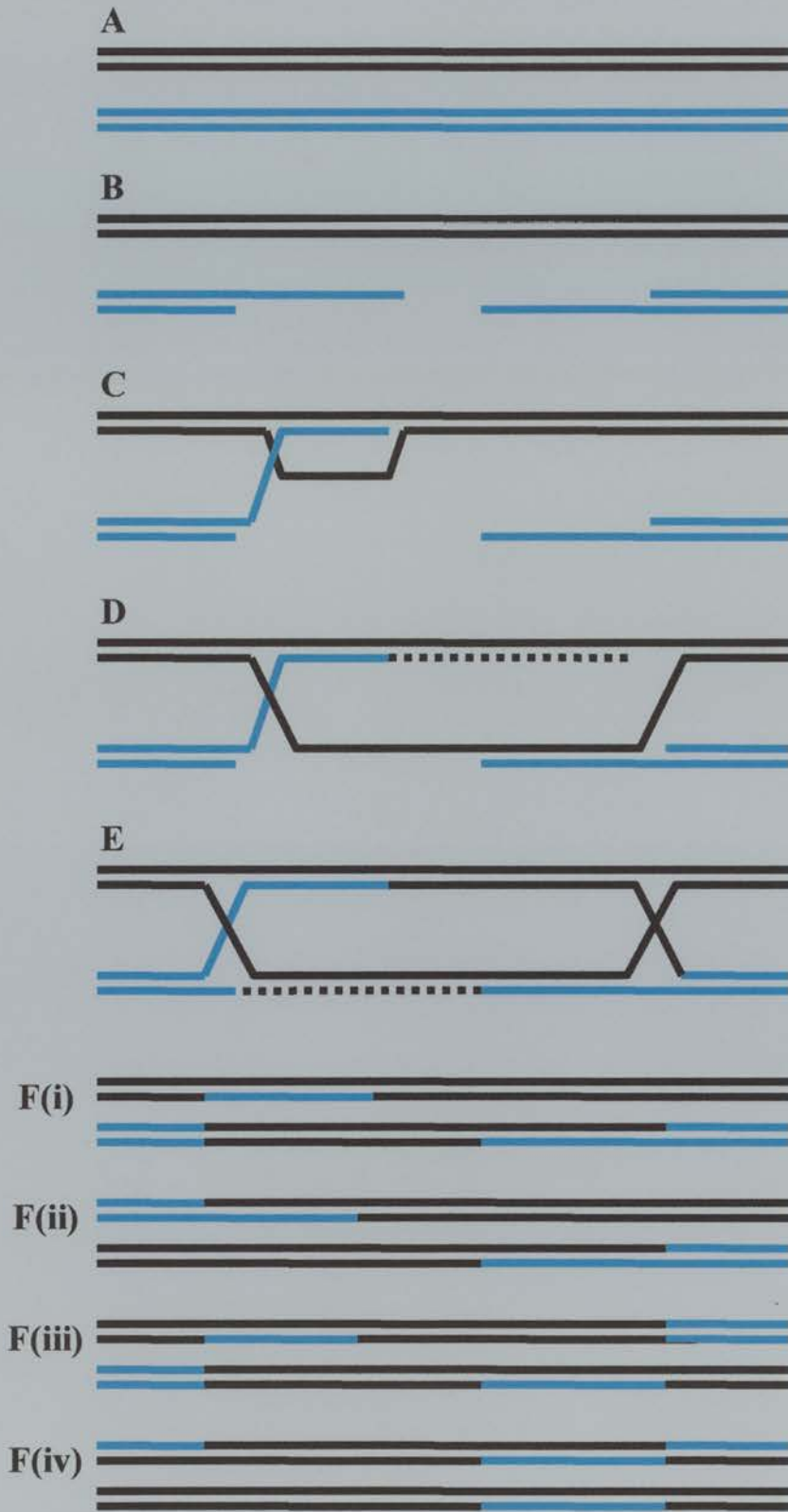


Fig. 1.i.d Double -Strand Break Repair Model of HR



### **(1.i.7) Gene Targeting**

Gene targeting is the term used to describe homologous recombination (HR) between DNA vector and host cell genomic sequence. Via application of ES cell and HR technology, it is now possible to introduce virtually any genetic modification into the animal germline, ranging from the subtle introduction of point mutations and small scale deletions (for review see Brandon *et al*, 1995, Bedell *et al*, 1997 (parts I and II)), through to a full spectrum of gross chromosomal alterations (Ramirez-Solis *et al*, 1996).

#### **(1.i.7a) Basic Targeting Strategy**

The simplest targeting strategy involves the inactivation of a target gene via the insertion of a positive selectable marker (e.g. a neomycin resistance cassette). Utilising an O-type (insertion type) targeting vector, linearised within the region of sequence homology, a single recombination event occurring at the vectors free ends will result in an insertion event at the target chromosomal locus. The result of such homologous recombination will be a duplication of the homologous sequence separated by plasmid vector sequence (see figure 1.i.e).

$\Omega$ -Type (replacement type) vectors are also commonly utilised to ablate gene expression and require two sections of homologous sequence. Homologous recombination between vector and host DNA, occurring within both sections of homology, will delete all host chromosomal DNA residing between crossover events and replace them with intervening vector sequence (also see fig. 1.i.e). It is possible to select for replacement type events if the targeting vector possesses a

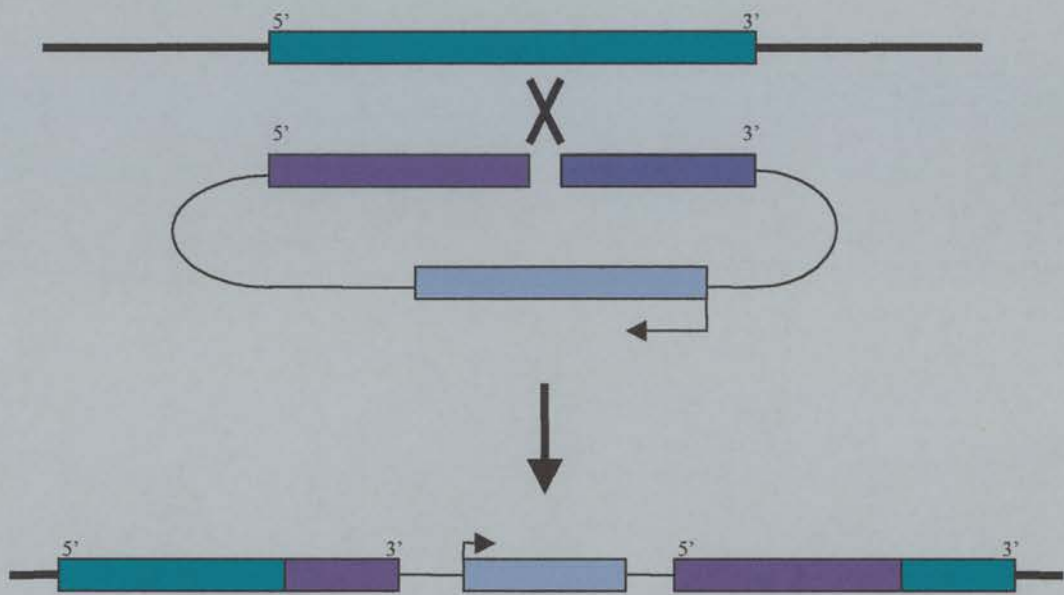
positive selectable marker residing between both portions of homologous vector sequence.

It has been observed that decreasing the length of one “arm” of homologous vector sequence does not significantly affect resultant replacement targeting frequency (Thomas and Capecchi, 1987) provided that the length of homology remains greater than 470bp (Hasty *et al*, 1991(A)). This evidence indicates that perhaps only one recombination event is required between host chromosomal and replacement type vector sequences to produce the desired recombination event. Subsequent branch migration of a single crossover event and resolution within the other region of homologous vector sequence could account for the observed similarity in targeting frequency of the resultant genetic modification.

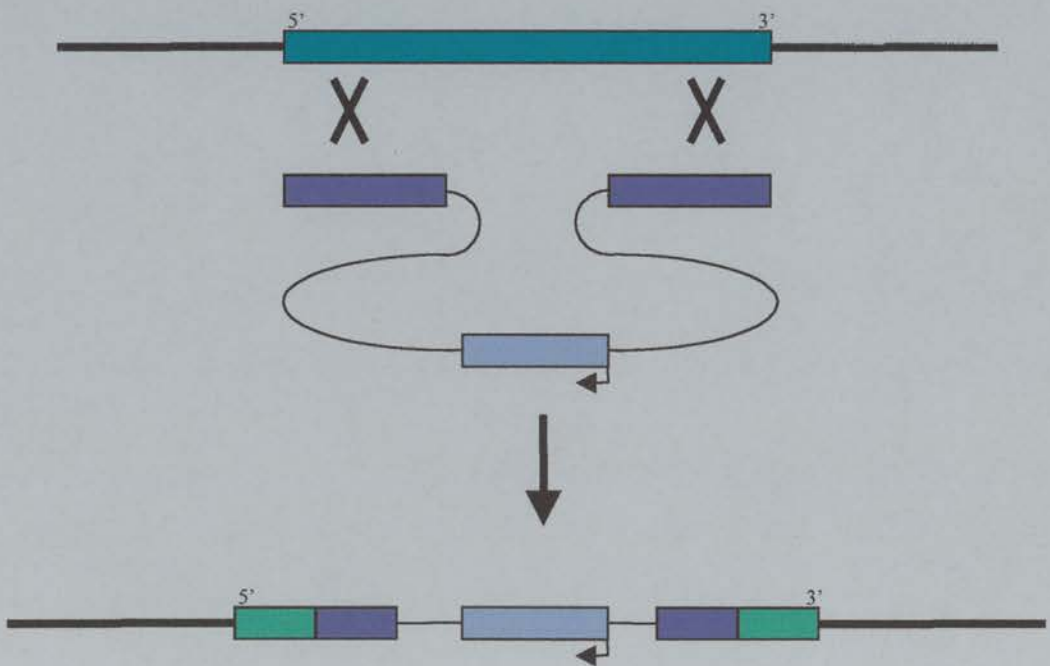


Figure 1.i.e

O-Type Insertion



$\Omega$ -Type Replacement



### **(1.i.7b) Factors Affecting Targeting Efficiency**

Compared to the frequency of random integration, the successful gene targeting of a locus in mammalian cells is a relatively rare event. Following the transfection of a targeting construct into ES cells, approximately  $10^{-3}$  mammalian cells exhibit random integration, whilst only around  $10^{-6}$  cells exhibit successful homologous recombination (Waldman, 1992). Further, large variability is often observed between the efficiencies of HR at independent loci following similar targeting experimental methodologies in mammalian cells.

The most likely explanation for this intrinsic variability of success in targeting experimentation is the complexity of the higher eukaryotic genome. The organisation of the genome creates variability in both chromatin composition and conformation, and therefore in loci accessibility. The frequency of HR is reportedly enhanced when targeting actively transcribing loci (Mansour *et al*, 1988), presumably as a result of a looser DNA: histone conformation, however this is by no means a prerequisite for gene targeting (Smithies *et al*, 1985), (Jeannotte *et al*, 1991).

Within practical limits, the greater the length of homology utilised in the targeting construct, the greater the frequency of observed HR although gene targeting has been achieved in mammalian cells with constructs containing as little as 1.3Kb overall homology (Hasty *et al*, 1991 (A)). Targeting experimentation at the *hprt* locus however, has demonstrated that as the length of homology increases, resultant targeting frequency also increases in either an exponential (Thomas and Capecchi, 1987), (Deng and Capecchi, 1992), or in a linear manner (Hasty *et al*, 1991 (A)). Whilst this may suggest the use of very large pieces of homology in

targeting vector construction, no further improvement in targeting frequency was observed once overall homology lengths exceeded 14Kb in length (Deng and Capecchi, 1992). The use of isogenic DNA has also enhanced HR frequency in targeting experimentation (te Riele *et al*, 1992). Incorporating homologous DNA from the same lab strain as that of the target ES cells, reduces the number of single base pair polymorphisms, thus maximising the length of uninterrupted sequence homology.

Linearisation of the targeting vector prior to transfection is also thought to enhance targeting efficiency following demonstrable enhancement of HR frequency in both fungi and extra-chromosomal recombination in mammalian cells (for review see Orr-Weaver, 1985).

#### **(1.i.7c) Selection of Complex Targeting Strategies**

The first gene targeting experiments using ES cells were aimed at modifying the hypoxanthine-guanine phosphoribosyltransferase locus (HPRT) (Thomas and Capecchi, 1987), (Doetschman *et al*, 1987). This gene located on the X-chromosome (therefore present only as a single copy in males), confers resistance to HAT (see section 5.ii.4) and so permitted the selection of desired targeting event above the predominant background of non- and illegitimately recombinant clones.

Since this time, strategies have evolved that permit the selection of increasingly complex HR events at other non-selectable loci. The transfection of DNA containing a positive selectable marker (e.g. *neo<sup>r</sup>* cassette, conferring resistance to G418 (Southern and Berg, 1982)), enables the selection of cells *in vitro* which

contain a stably integrated copy of that DNA. This comprises the basis of targeting experimentation at non-selectable loci.

The co-introduction of a negative marker gene (e.g. HSV $tk$ , or Diphtheria A toxin), enables enrichment of the targeting event by selecting for homologous over non-homologous stable recombination (Mansour *et al*, 1988), (Le Mouellic *et al*, 1992), (McCarrick *et al*, 1993). If positioned outwith the two portions of homology in a conventional replacement type vector, the majority of illegitimate recombinant clones possess both positive and negative selection markers and hence do not survive negative selection. Only clones that have undergone a double HR, or a single HR and branch migration event, will solely possess the positive selection marker gene and survive negative selection. PNS (positive-negative selection) is commonly reported to enrich for the desired targeting event by between 2 and 20 fold (Mombaerts *et al*, 1991).

Additional targeting strategies have been developed for when the desired genetic modification is subtler than complete ablation of gene expression.

The “Hit and Run” targeting strategy (see fig. 1.i.f) utilises a conventional insertion type vector and enables the introduction of subtle deletions or point mutations into the mammalian genome (Hasty *et al*, 1991(B)).

Both positive and negative selection cassettes are positioned adjacent to one another in a conventional insertion type vector, thus enabling both the conventional selection of stably recombinant clones, as well as the selection of clones that have undergone a second, intrachromosomal HR event. The resolution of this strategy, following the second round of HR, can result in either the removal

of duplicated and selective marker sequences leaving the target locus modified and intact, or in reversion back to the original wild-type allele.

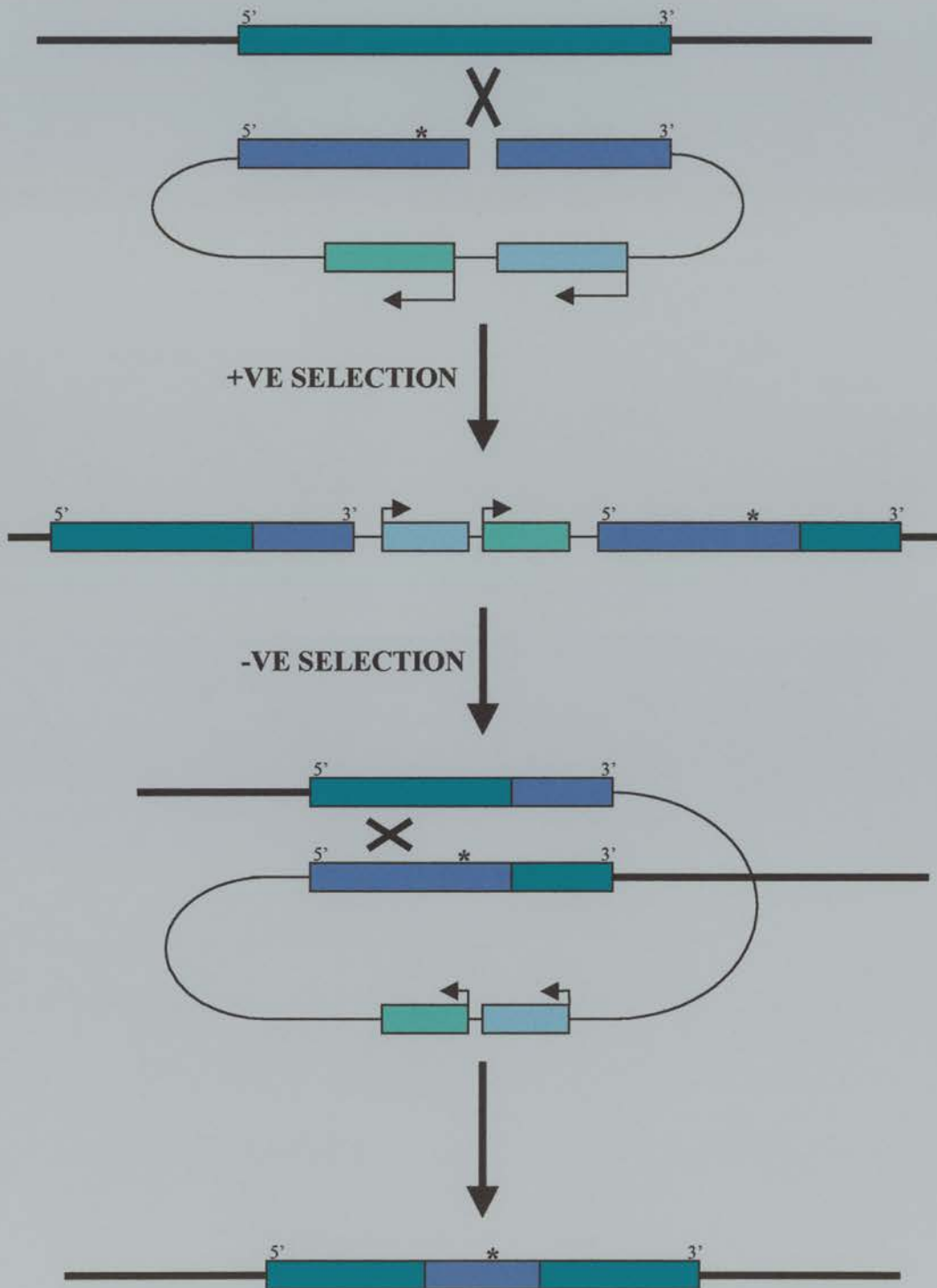
A further strategy, termed double replacement targeting (see fig. 1.i.g), requires the generation of two replacement type targeting vectors and sequential targeting (Stacey *et al*, 1994). As essentially any number of second round targeting vectors can be constructed, this strategy is especially useful in comparative studies when the generation of multiple clonal lines containing different modifications at the same locus is desirable.

In the initial vector, both positive and negative selection marker cassettes are positioned between both portions of homologous sequence in a replacement type vector. Successfully targeted cells from this stage are then re-targeted with the second construct. Typically, the second vector contains identical sequence homology as the first, but in place of the selectable cassettes is sequence containing the desired genetic modification. Cells successfully targeted for a second time can also be selected, as they will have replaced both positive and negative marker cassettes with the desired modifier sequence.

More recently, a modified gene targeting procedure was reported to be so efficient ( $10^{-1}$  cells are targeted) that selectable markers were not required to enrich for targeting events (Templeton *et al*, 1997). Through modification of ES cell electroporation conditions (standard method for transfecting targeting constructs) and plating methodology, the plating efficiency of electroporated ES cells has improved from 3% without selection (Reid *et al*, 1991), to between 94.2% and 99.2% efficiency. This data suggests that the intrinsic rate of HR in ES cells is in

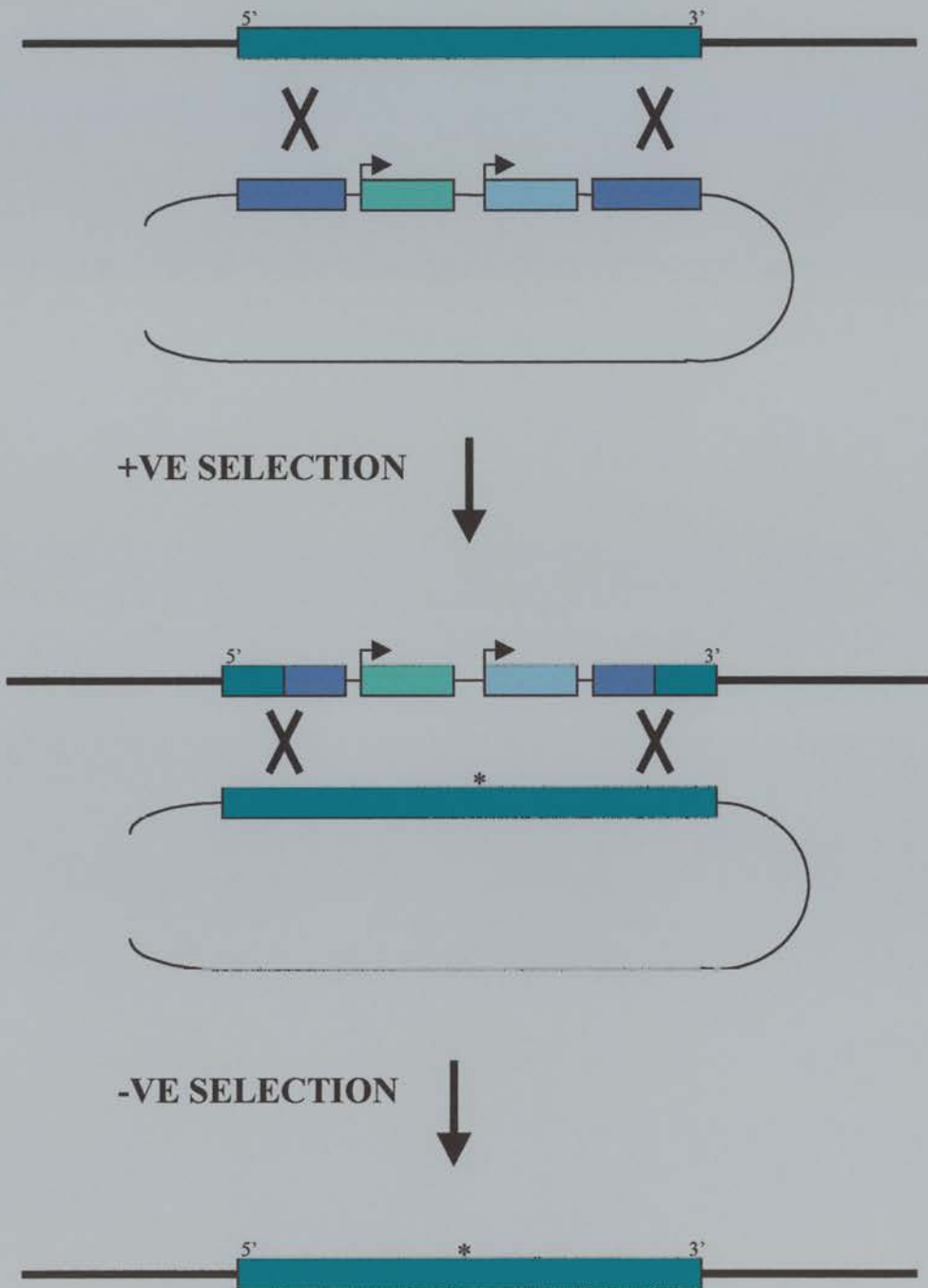
reality very high, but that existing methodology is inefficient at producing targeted clones.

**Fig 1.i.f** "HIT AND RUN" TARGETING STRATEGY



**Fig. 1.i.g**

**DOUBLE REPLACEMENT TARGETING STRATEGY**



### **(1.i.8) Inducible Promoter Systems**

Conditional gene expression has proved a valuable tool in the analysis of gene expression in mammalian experimental systems. In studies of oncogenicity, cell cycle or in the expression of dominant lethal alleles it is often undesirable to have continuous transgene expression, so the ability to introduce transgene expression rapidly and in a controlled fashion can be greatly beneficial in these experimental circumstances (see section 1.i.2).

The generation of recombinant transgene expressing adeno- and retroviral vectors does permit highly efficient gene transfer and expression *in vitro*, however their effectiveness at delivering efficient and stable transgene expression *in vivo* can be highly variable. In terms of reproducibility therefore, inducible promoter systems are superior to viral transduction as cells stably transfected with an inducible transgene can be fully characterised with regard to their expression patterns *in vitro*, prior to application *in vivo*.

The ideal conditional expression system should not permit gene expression in the absence of inducer, yet should enable high levels of gene expression upon stimulation in a rapid and dose-responsive manner. The means of induction should also be non-toxic and should exert no other pleiotropic effects upon the cell. Further, the expression system of choice should be easy to establish and rapid to optimise both *in vitro* and *in vivo*.

A number of inducible promoter systems have been derived and developed from viral, pro- and eukaryotic genes to achieve such goals (see table 1.i.a), but unfortunately as yet no developed conditional expression system meets all of the above criteria.



**Table 1.i.a Currently Available Inducible Promoter Systems**

Promoter	Inducer	Number of Components	Origin	Reference
MT	Heavy Metals	1	Human	McNeill <i>et al</i> , 1989
MMTV	Steroid Hormones	1	Virus	Lee <i>et al</i> , 1984
6-16	Interferon $\alpha/\beta$	1	Human	Kerr <i>et al</i> , 1991
Mx1	Interferon $\alpha/\beta$	1	Mouse	Hug <i>et al</i> , 1988
Lac operon	IPTG	2	E.Coli	Hu <i>et al</i> , 1987
Tet operon	Tetracycline	2	E.Coli	Gossen <i>et al</i> , 1993
Ecdysone	Muristerone A	2 or 3	Drosophila	No <i>et al</i> , 1996
Gal/ER <sup>TM</sup>	Tamoxifen	2	Yeast/Human	Webster <i>et al</i> , 1988

For the purposes of the experimental work described subsequently within this thesis, both the interferon  $\alpha/\beta$  responsive 6-16 promoter and the tetracycline inducible promoter were selected from the available systems detailed in table 1.i.a to regulate the expression of the tumour suppressor gene p53. These individual systems and the rationale underlying their selection are discussed in greater detail within sections 1.i.8a, 8b and 8c.

#### **(1.i.8a) Interferon Inducible Promoter Systems**

Interferon type I (interferon  $\alpha/\beta$ ) inducible promoters drive the expression of genes following interaction between secreted interferon  $\alpha/\beta$  (see section 1.i.8b) and its receptor. The application of these inducible promoters to drive transgene expression in mammalian cells are most likely universal as many mammals produce interferon  $\alpha/\beta$  in response to viral infection and the interferon  $\alpha/\beta$  receptor has so far been found to be present on all normal and malignant human

tissues, numbering between 100 to 2,000 functional receptors per cell (Merlin *et al*, 1985). Binding of type I interferons (interferon  $\alpha/\beta$ ) to this receptor results in the specific transcriptional upregulation of several gene products via activation of the ISGF-3 (Interferon Stimulated Gene Factor-3) protein complex.

The ISGF-3 $\alpha$  component of this complex is initially located in the cytoplasm of the cell and is made up of three proteins, namely p84, p91 (STAT1 $\alpha/\beta$  heterodimer) and p113 (STAT2) (Fu *et al*, 1990 and Fu *et al*, 1992). Following the binding of ligand to the interferon  $\alpha/\beta$  receptor, the ISGF-3 $\alpha$  complex becomes activated following phosphorylation of specific tyrosine residues, mediated primarily through JAK1 and Tyk2 protein kinase activity. Activated ISGF-3 $\alpha$  then translocates to the nucleus where it associates with the DNA-binding protein ISGF-3 $\gamma$  (p48) to complete the formation of functional ISGF-3 protein complex (Schindler *et al*, 1992).

Activated ISGF-3 directly transactivates the expression of genes by binding to a sequence-specific DNA element termed the ISRE (Interferon Stimulable Response Element). This element is highly conserved among many species which respond to interferon  $\alpha/\beta$  and is found in the promoter sequences of all interferon  $\alpha/\beta$  stimulated genes (Witt *et al*, 1996).

Two other proteins, IRF-1 (Interferon Regulatory Factor-1) and IRF-2, operate antagonistically to modulate ISGF-3 mediated activation of gene expression by also binding to ISRE sequences. IRF-1 expression has been demonstrated to positively influence interferon  $\alpha/\beta$  mediated induction of gene expression, whilst expression of IRF-2 has been shown to repress the effects of IRF-1. A further negative regulator of interferon  $\alpha/\beta$  induced gene expression, which also operates

through binding to ISRE sequence, is the protein ICSBP (Interferon Consensus Sequence Binding Protein). The expression of this protein is induced by interferon  $\gamma$ , but has only been detected in macrophage and lymphocyte cell lineages (for review see Witt *et al*, 1996).

Two independent interferon inducible promoter systems have been developed and described to date, the murine Mx1 promoter and the human 6-16 promoter. The Mx1 gene was initially discovered as an interferon  $\alpha/\beta$  inducible transcript that inhibited the replication of influenza virus in cultured cells (for review see Arnheiter *et al*, 1990). The Mx1 promoter region has since been fully characterised (Hug *et al*, 1988) and has been used to drive the expression of foreign transgenes in an inducible manner both *in vitro* and *in vivo* (Kuhn *et al*, 1995).

The human 6-16 gene has been shown to be expressed in response to interferon  $\alpha/\beta$  in all human cell types analysed to date (Kelly *et al*, 1985), (Kelly *et al*, 1986). The function of 6-16 protein is currently unknown, but knockout analyses have shown that the expression of this gene is not essential for conferring the interferon type I induced “anti-viral” state upon the cell (Porter and Itzhaki, 1993 and section 1.i.8b). No known murine homologue exists.

The 6-16 promoter region consists of both TATA and GCAAT boxes, potential SP1 binding sites as well as a tandemly duplicated interferon (IFN) response element (IRE). Residing within each IRE is a 13bp consensus IRSE sequence, which confers interferon type I inducibility to the promoter (Whyatt *et al*, 1993).

Two variants of the 6-16 promoter exist, one long (6-16<sub>L</sub>) and one short (6-16<sub>S</sub>). The 6-16<sub>S</sub> version is reported to be less leaky than the 6-16<sub>L</sub> when in the non-induced state, but the 6-16<sub>L</sub> version has been demonstrated to induce higher levels of transgene expression upon induction (Whyatt *et al*, 1993).

One obvious advantage of utilising the 6-16 promoter as a means of achieving conditional transgene expression is that only one component is required. Transgene expression can be rendered inducible by simply positioning 6-16 promoter sequence in a 5' position relative to transgene ORF (open reading frame). This feature enables the researcher to rapidly generate and optimise inducible transgene expression both *in vitro* and *in vivo*. Further, levels of transgene expression have been shown to be dose-responsive with respect to the concentration of inducer (i.e. absolute levels of transgene expression positively correlate through a range of interferon  $\alpha/\beta$  concentrations). Whilst this is unlikely to be precisely manipulatable *in vivo*, this could potentially be useful when physiological levels of transgene expression are required, or in instances when it is desirable to modulate expression levels within the course of the same experiment in culture.

One of the major drawbacks from using this conditional expression system however is that in addition to inducing transgene expression, interferon  $\alpha/\beta$  confers other pleiotropic effects upon the cell (see section 1.i.8b). All transgene induction experiments utilising this system must therefore be tightly controlled in order to enable differentiation between interferon  $\alpha/\beta$ -mediated and transgene-mediated effects upon the cell.

### **(1.i.8b) The Molecular Basis of the Interferon $\alpha/\beta$ Response**

Interferon  $\alpha/\beta$  comprises a family of related peptides that are produced primarily in response to viral or microbial infection by all differentiated mammalian cell lines analysed to date (14 interferon  $\alpha$  proteins and 1 interferon  $\beta$  protein presently identified; reviewed by Witt *et al*, 1996).

The most potent *in vivo* inducer of type I interferons so far identified in the mouse is the synthetic double stranded RNA (dsRNA) molecule polyinosinic acid: polycytidylic acid (pI:pC) (Field *et al*, 1967). It is hypothesised that this reagent mimics a viral dsRNA genome or viral replication intermediate and so consequently induces a strong interferon  $\alpha/\beta$  cellular response.

Interferon  $\alpha/\beta$  can induce the expression of between 20 to 100 downstream genes (Sen and Ransohoff, 1993), many of uncharacterised function, but their cumulative effect results in the inhibition of general protein synthesis, a decrease in the rate of cellular proliferation and in a general systemic enhancement of cell-mediated cytotoxicity (Haines *et al*, 1991).

As many viruses utilise host cell machinery to replicate, it is proposed that interferon  $\alpha/\beta$ -mediated cellular responses serve to inhibit the expression of viral genes (and consequently viral replication) within infected cells. In addition, interferon  $\alpha/\beta$  can promote the removal of infected cells by signalling to and enhancing NK cell and macrophage activity (Reid *et al*, 1981), (Basham *et al*, 1984, (A) and (B)), thus further inhibiting the progression of viral infection within a tissue.

The antiviral and antiproliferative effects elicited directly through interferon  $\alpha/\beta$  and indirectly by dsRNA have been shown to be at least part modulated through

the induction and activation of three independent gene products; 2', 5' oligoadenylate synthetase (2-5A synthetase), dsRNA protein kinase (PKR) and dsRNA-specific adenosine deaminase (dsRAD).

Interferon  $\alpha/\beta$  can indiscriminately repress both viral and cellular gene expression (and consequently cell-cycle progression) through the induction of 2-5A synthetase expression. Once expressed, this enzyme converts free monomeric ATP into 2', 5' linked oligoadenylates (2-5A). 2-5A binds to and activates the otherwise latent endonuclease RNase L, which subsequently degrades both host and viral RNA within the cell. It should be noted that the cellular consequences associated with the presence of 2-5A in the cell are transient as a result of the inherent instability of this compound (for review see Player and Torrance, 1998).

PKR is a serine/threonine protein kinase implicated in both the transcriptional regulation of interferon  $\alpha/\beta$  induced genes via direct interaction with the transcription activator STAT 1, and in the inhibition of protein synthesis via phosphorylation of eIF-2 (eukaryotic Initiation Factor 2).

Inactive PKR binds to STAT1, inhibiting the ability of this protein to form the DNA-binding complex associated with the transactivation of those genes possessing the ISRE sequence element within their promoters. PKR becomes activated by autophosphorylation following binding to dsRNA. Once activated, PKR dissociates from STAT1 and phosphorylates the  $\alpha$  subunit of eIF-2, which in turn binds to the guanine nucleotide exchange factor eIF-2B, thus preventing further initiation of protein synthesis within the cell (Farrell *et al*, 1977).

A further gene induced by interferon  $\alpha/\beta$ , associated with mediation of the 'anti-viral' state but not anti-proliferative state, is an enzyme termed dsRNA-specific

adenosine deaminase (dsRAD) (Patterson *et al*, 1995). The activity of this enzyme catalyses the conversion of adenosine to inosine in dsRNA viral genomes through hydrolytic deamination (O'Connell, 1997). Upon reverse transcription of the viral genome, converted inosine residues base pair to cytidine and so dsRAD-mediated editing of the viral genome appears as a conversion from adenosine to guanosine, thus effectively mutagenising viral ORFs.

The cellular effects conferred by interferon  $\alpha/\beta$  as described, has resulted in the use of this reagent as a means of treating more than a dozen human malignancies (Witt *et al*, 1996). It could be hypothesised upon this basis therefore that utilisation of either Mx1 or 6-16 promoter sequences to drive the expression of tumour suppressor genes within a tumour cell environment may impart greater anti-tumour effect than either reagent in isolation (see section 2.i.1).

### **(1.i.8c) Tetracycline Inducible Promoter System**

The tetracycline inducible promoter system utilises components originating from the tetracycline-resistance (Tet) operon derived from the *E. coli* Tn10 transposon. Similar in modality to the Lac repression system, the tetracycline inducible system consists of a repressor molecule (tetR) which binds with high specificity to a DNA consensus operator sequence (tetO) (Kleinschmidt *et al*, 1988). In the absence of the anti-biotic tetracycline (Tc), the tetR molecule binds as a dimer with high affinity to tetO sequence, sterically inhibiting transcriptional initiation at the Tet operon. At very low concentration ( $0.1\mu\text{g ml}^{-1}$ ), Tc can bind to and

induce conformational change within the tetR molecule, in turn lowering the affinity between tetR and tetO, thus releasing transcriptional repression.

Initial applications of this prokaryotic repression system consisted of stably transfecting the tetR molecule into higher eukaryotic cells, along with a reporter transgene construct driven by a composite viral/ tetO promoter (for review see Gossen *et al*, 1993). This approach was unsuccessful at achieving transcriptional repression however, due to inefficient expression and resultant low cellular concentration of the tetR molecule. High concentrations of the tetR molecule have since been demonstrated to be toxic in mammalian cells (Shockett *et al*, 1995), therefore it is likely that these early studies were self selecting for poor tetR mediated repression.

The generation of a fusion protein with the C-terminal portion of VP16 (immediate early gene transactivator molecule derived from herpes simplex virus) and the tetR molecule coupled the properties of strong transcriptional transactivation with highly specific protein/DNA interaction (Gossen and Bujard, 1992). This protein, termed tTA (tetracycline responsive transactivator), binds to tetO sequences in the absence of tetracycline and activates gene expression.

Following random mutagenesis of the tTA molecule, a protein which differed from the wild-type by only 4 amino acids, termed the rtTA, was demonstrated to possess reverse characteristics and only bind to tetO sequences in the presence of tetracycline (Gossen *et al*, 1995). The development of both Tet-on (rtTA) and Tet-off (tTA) systems (Clontech) offer a greater degree of flexibility in the control of transgene expression in comparison with other inducible systems. Both



systems share a common inducer molecule, upon administration of which, will either switch on or off transgene expression.

Differential induction response kinetics have been reported through use of derivative tetracycline antibiotics as inducer molecules. The best to date (doxycycline) exhibited 100-fold greater efficiency in transgene induction than Tc (Gossen *et al*, 1995).

In comparison to the other systems listed in table 1.i.a, the Tet system currently enables the greatest degree of flexibility in terms of experimental design. Further, the relationship between Tc (and related derivatives) and the tetR molecule is highly specific and the effective concentration of inducer has not been reported to elicit any other gross pleiotropic effects within mammalian cells (see section 2.i.1).

### **(1.ii) The Molecular Basis of Cancer**

The dynamics of cellular flux within a tissue are basically modulated by two distinct classes of genes, namely the oncogenes and the tumour suppressor genes. The homeostatic maintenance of cell number within a tissue is finely balanced between the active processes of cellular proliferation and cell death, with conversion to malignancy typically associated with lesions affecting the genes intrinsic to both of these processes.

Oncogenes encode a class of proteins that act to promote cell growth through stimulation of the cell cycle or through inhibition of cell death. This class typically comprises of extracellular growth factors, cell surface receptors, intracellular signal transducing molecules and nuclear effectors (Weinberg, 1995). Inhibitors of apoptosis, such as Bcl-2, are also included in this classification group (Kroemer, 1997).

Conversely, tumour suppressor genes act to restrain the expansion of cell numbers within a tissue. Despite present debate surrounding what actually does and does not constitute a “real” tumour suppressor gene (Clurman and Groudine, 1997), it is generally accepted that this class of genes predominantly comprises of proteins that inhibit cell cycle progression. This class may possibly also include genes that enhance apoptosis and those that recognise and repair mismatched DNA.

In a normal healthy individual, these two classes of genes work in synchrony to preserve genomic integrity and regulate cell turnover within tissue. However, the relationship between the acquisition of lesions that affect these two gene classes and the malignant conversion of a cell is both complex and highly tissue specific.

The advent of both transgenic technology and novel molecular techniques within the past decade has enabled the specific investigation of oncogene and tumour suppressor gene loci within animal model systems, which in turn has served to rapidly advance our understanding of tumourigenic processes.

One could predict that a lesion arising within a single cell *in vivo* causing the over- or disregulated expression of an oncogene would result in an increased rate of cellular proliferation. The result of such a selective growth advantage over neighbouring wild-type cells would be the clonal expansion of affected cells within the tissue. Indeed, elevated expression levels of the oncogene c-Myc are observed at high frequency in many diverse tumour types (Spencer and Groudine, 1991) and therefore must be an important event in the malignant conversion of a cell.

C-Myc over-expression within cultured fibroblasts has been demonstrated to be sufficient to drive both normally cycling and quiescent cells into a continuous proliferative state, wherein they no longer respond to normal growth arrest signals (Eilers *et al*, 1989). The overexpression of c-Myc in otherwise wild-type fibroblast or haematopoietic cells however, does not result in an increase in cell number when growth factors are limiting (Evan *et al*, 1992), (Askew *et al*, 1991). Despite a marked increase in the rate of cellular proliferation, a correspondingly high rate of apoptosis is also observed.

The generation of a transgenic c-Myc overexpressing mouse, the E<sub>μ</sub>-Myc mouse (Harris *et al*, 1988), was designed to model the human B-cell malignancy

Burkitt's lymphoma and has been used to examine the *in vivo* consequences of c-Myc overexpression targeted to B cell lymphocyte lineages.

B-cell lymphomas were observed in 90% of these animals following a latency period of approximately 5 months. During this period, increasing numbers of large proliferating B lymphocyte lineages were detected in the blood, but so too were very high levels of apoptosis. Analysis of bone marrow at the time when the tumour had developed still exhibited high levels of apoptosis within the tissue.

This model supports the notion that the overexpression of an oncogene, in this instance c-Myc, drives tumourigenesis in combination with other genetic changes, and further demonstrates that these events occur within an environment characterised by high levels of proliferation and apoptosis.

Indeed, the tumourigenic effects conferred by c-Myc may be considered to arise as a direct consequence of increased cell turnover. Assuming that the mutation rate per cell cycle remains unaltered, hyperproliferating cells will accumulate more mutations over a set time period than normally proliferating cells.

In addition to exposing a given cell population to a greater range of mutations, c-Myc overexpression also places increased selective pressure for mutations that inhibit the elimination of cells through apoptosis (e.g. those resulting in the overexpression of Bcl-2). Failure to eliminate such cells through apoptosis would likely result in their rapid expansion and possibly lead to tumour formation.

This theory has been partly substantiated *in vivo* through the development of mice overexpressing both c-Myc and Bcl-2. Such animals were strongly predisposed to a rapidly emerging undifferentiated haematopoietic leukaemia (Strasser *et al*, 1990).

Genetic lesions resulting in loss of tumour suppressor gene function would also be predicted to confer selective growth advantage to the cell. Hypothetically, the selective advantage gained through loss of cellular proliferative restraint would result in the clonal expansion of affected cells within a tissue comprised of otherwise normal cells. These unrestrained proliferating cells could subsequently gain further selective advantage following the acquisition of genetic lesions that confer oncogene overexpression (i.e. those genes that enhance the rate of cellular proliferation).

In a normal diploid organism, whilst a single mutational event may be sufficient to confer gene overexpression, two mutational events are required to ablate gene expression in most instances. This “two-hit” tumour suppressor gene inactivation model was initially proposed by Knudson with regard to the retinoblastoma susceptibility gene, pRb (Knudson, 1971). This gene has since become the classical paradigm for tumour suppressor gene classification.

Human Rb heterozygotes develop retinoblastomas at almost 100% incidence and osteosarcomas to a lesser extent, predominantly during their early childhood. Further, loss of Rb gene function is frequently associated with tumours of the lung, breast, prostate and bladder in humans (Knudson, 1993).

Knudson proposed that two mutational events are required (one of which being inherited in cases of familial retinoblastoma) before the tumour suppressor function of pRb is ablated and individuals become predisposed to tumourigenesis. Thus, if ablation of tumour suppressor gene function is implicated as a key event in a proportion of tissue specific tumourigeneses, then it could be postulated that the re-introduction of tumour suppressor gene function into such tumour cells

could constitute an effective treatment. At the least, one could predict enhancement of conventional cancer treatment. An obvious candidate tumour suppressor gene for such replacement type gene therapy would be p53.

### **(1.ii.1) P53**

David Lane discovered the tumour suppressor gene p53 in 1979, as a gene coding for a protein tightly associated in a complex with the SV40 large T antigen (Lane and Crawford, 1979). Initial p53 expression studies showed that many immortalised and primary human tumour cell lines expressed elevated levels of p53 relative to the amounts expressed in normal wild-type cells (Zakut-Houri *et al*, 1983). The notion that p53 possessed cellular transformation associated properties was supported by further experimentation which demonstrated that elevated levels of p53 expression could co-operate with activated Ha-Ras and transform primary fibroblast cells in culture (Eliyahu *et al*, 1985).

Much evidence has since been accumulated however to firmly prove that the true physiological role of p53 is as an inhibitor of cellular transformation through the ability to preserve genomic integrity at times of cellular stress. Activated p53 suppresses cell transformation primarily through the ability to initiate cell cycle arrest or apoptosis in response to many stimuli including DNA damage, low oxygen conditions and inappropriate proliferative signals to mention but a few. Elegant animal models have also demonstrated that p53 dysfunction is commonly associated with either tumourigenesis or tumour progression in many different tissue and cell types. Indeed, lesions affecting wild-type p53 gene expression have been scored as being the most commonly detected across a wide spectrum of

human malignancies (Hollstein *et al*, 1994). All these aspects of p53 biology will be reviewed in the remaining sections of this chapter.

It was not discovered until years later that the early p53 overexpression experiments had been performed using mutated p53 gene sequences (Finlay *et al*, 1989). Therefore, in addition to simple abrogation of p53 expression, the nature of mutational event can also potentially confer a selective growth advantage to the cell. This perhaps goes some way to explaining why genetic alterations at this locus are so commonplace in malignancy and has further emphasised the importance of elucidating the nature of p53 mediated tumour suppression.

### **(1.ii.2) P53 Structure and Function**

P53 is a 53KDa phosphoprotein comprising of 393 amino acids that can be roughly subdivided into three separate functional domains. The amino- (N-) terminal portion (amino acids 1-43) comprises a transcriptional transactivation domain (Unger *et al*, 1992); the central core region (amino acids 102-292) comprises a sequence specific DNA binding domain (Wang *et al*, 1993); and the carboxy- (C-) terminal portion (amino acids 300-393) comprises both tetramerisation and non-sequence specific DNA binding domains connected by a short flexible linker sequence (Ko and Prives, 1996), (Jeffrey *et al*, 1995).

Functionally active p53 predominantly exists in a tetrameric conformation within the cell nucleus and has been ascribed a multitude of biochemical properties and interactions that can influence the cellular response to stimuli promoting genetic instability.

### **(1.ii.2a) The Core Domain**

One clear means by which activated p53 influences cellular fate is through both the transcriptional activation and suppression of a specific set of downstream target genes. X-ray crystallography has determined that p53 folds and oligomerises in such a way as to form a surface capable of binding DNA with contact at both major and minor grooves (Cho *et al*, 1994). P53 specifically recognises a tandem repeat of the 10-mer  $5' \text{RRRC}^{\text{A}}/\text{T}/\text{A} \text{GYYY} 3'$  (el-Diery *et al*, 1992), present as a motif in the promoter region of multiple genes, onto which it can bind with high affinity (for a more detailed review see Levine, 1997). Once bound to its recognition sequence, p53 exerts influence on the expression of downstream genes through interaction between its N- terminal domain and components of the basal transcriptional machinery (see section 1.ii.2b).

Point-mutation analyses of p53 alleles originating from human tumour samples have shown clustering, or mutational hotspots, affecting amino acid residues located within this sequence specific DNA binding domain. These studies also highlight that point mutations affecting either the N- or C- terminal domains are rare in comparison, implying that the integrity of the core domain is essential for tumour suppression functionality (Ko and Prives, 1996).

All p53 alleles possessing point-mutation(s) within this central core domain can be classified as either contact mutant alleles (as they possess altered amino-acid residues that directly contact DNA) or as conformational mutant alleles (as they possess alteration of amino-acid residues affecting protein folding; Cho *et al*, 1994).



Contrary to Knudson's "Two-Hit" tumour suppressor inactivation model, often cells possessing one point-mutated and one wild-type p53 allele possess attenuated p53 dependent responses. This dominant-negative effect, of mutant over wild-type capability, has been shown to be at least partly the result of altered tetrameric complex conformations. Hetero-oligomerisation between mutant and wild-type protein affects the ability of tetrameric p53 to bind specific DNA sequences and consequently to transactivate gene expression (Chene, 1998). Clearly therefore, certain point-mutated alleles confer potentially more serious tumourigenic consequences than others, and Harvey *et al* have proposed a grading classification system of p53 point-mutants accordingly (Harvey *et al*, 1995).

Several conformational mutant alleles have proven useful as experimental tools in the elucidation of p53 dependent cellular responses. Such an example is a mutant form of p53 that differs from wild-type only at position 135, where it possesses a valine residue instead of an alanine. The result of this single amino-acid substitution is a p53 mutant protein that adopts mutant conformation at 37°C, but assumes wild-type conformation at 32.5°C (Milner and Medcalf, 1990). Although certain differences must exist at the level of general cellular metabolism between these two temperatures, this temperature sensitive (ts) mutant p53 allele has proven useful in transgenic studies with regard to the elucidation of p53 dependent responses and in observing how modulation of these responses affects tumorigenic consequence (Harvey *et al*, 1995).

Further evidence arising from experimentation with SV40 large T-antigen stress the importance of p53's ability to bind sequence specific DNA in achieving tumour suppression. Expression of SV40 large T-antigen, within a wide range of

primary cell lines, results in cell immortalisation through the binding and functional inactivation of both p53 and Rb tumour suppressor genes. Functional inactivation of the former is achieved through binding of the large-T antigen to the central core domain of p53. One could therefore argue upon this basis that disruption of this region is a key event leading to the immortalisation of the cell (Holley and Lawlor, 1997).

### **(1.ii.2b) N- Terminal Domain**

The ability of activated p53 to initiate growth arrest, DNA repair and apoptosis have all been shown to be at least partly influenced by the ability of p53 to transactivate the expression of specific genes. The N- terminal, or transcriptional activator domain of p53 has been shown to bind to various proteins associated with the basal transcriptional machinery including TBP (TATA-binding protein) (Horikoshi *et al*, 1995), as well as TAF<sub>II</sub>40 and TAF<sub>II</sub>60 (TATA-associated factors; Thut *et al*, 1995) and p300/CBP (Gu and Roeder, 1997 and Lill *et al*, 1997). The nature of several of these transcriptionally activated genes has provided insight into many of the biochemical mechanisms underlying p53 mediated tumour suppression (see sections 1.ii.5a and 5b).

P53 is also known to repress the expression of certain genes, although the mechanisms through which this is achieved remain to be fully clarified. Further insight regarding how p53 inhibits tumourigenesis has however been gained through the identification of several genes that appear to be repressed in this manner. Amongst these are cellular oncogenes (c-fos, c-jun) and inhibitors of apoptosis (Bcl-2) (Donehower and Bradley, 1993 and Miyashita *et al*, 1994).

A distinction has previously been made between genes that utilise either TATA or Inr (initiator) elements to define their transcriptional initiation site, where gene members of the former group appear to have their transcription repressed in a p53 dependent manner, whilst those genes in the latter group do not (Mack *et al*, 1993).

The exact mechanism through which this distinction is made by p53 remains to be clarified, although a p53 mutant (Q22, S23) has been shown to no longer possess the ability to repress transcription (Sabbatini *et al*, 1995). This mutant retains the ability to bind TBP but is unable to bind the TAF proteins, thus implicating interaction between p53 and TAFs as essential for p53 dependent transcriptional repression.

Experimental evidence has also been recorded that describes efficient p53 dependent repression of TATA-less promoters (Iotsova *et al*, 1996), (Gopalkrishnan *et al*, 1998). These examples may be exceptions to the general rule, but it is more probable that our understanding of this aspect of p53 biology is incomplete.

The cellular oncogene *mdm2* has been demonstrated to inhibit p53 mediated growth arrest and apoptosis by binding to and blocking the transcriptional transactivating N- terminal domain of p53 (Oliner *et al*, 1993), (Chen J. *et al*, 1996). Indeed, so efficient is *mdm2* at ablating wild-type p53 gene function that analyses of *mdm2* overexpressing cell lines rarely exhibit evidence for further genetic alteration at the p53 locus (Leach *et al*, 1993).

p53 is known to transcriptionally activate the expression of mdm2, and as mdm2 can ablate the transactivational capability of p53, this creates an autoregulatory feedback situation whereby the expression of both gene products are mutually regulated. Mdm2 mediated control of p53 mediated transactivation has been shown to be of key importance during embryonic development as mdm2 deficient murine foetuses are aborted immediately after implantation in a p53-dependent fashion (Montes de Oca Luna *et al*, 1995), (Jones *et al*, 1995). All progeny foetus nullizygous for both mdm-2 and p53 were viable however, thus demonstrating that negative regulation of p53 is essential for normal embryonic development.

Further stressing the importance of the transactivation domain with regard to inhibiting cell immortalisation was the demonstration that adenoviral E1B-55Kd protein can block the p53 dependent apoptotic response observed in cultures of cells in an E1A conferred hyperproliferative state, through direct interaction with p53's N- terminal domain (Debbas and White, 1993). Binding of E1B-55Kd to p53 still permits sequence specific binding to DNA, but rather than just physically blocking interaction between p53 and basal transcription factors, the binding of E1B-55Kd is the equivalent of attaching a strong transcriptional repressor domain to the N- terminus of p53 (Yew *et al*, 1994). Consequently, cells co-expressing both adenoviral E1A and E1B-55Kd proteins exhibit a high frequency of transformation in culture (Debbas and White, 1993).

### **(1.ii.2c) C- Terminal Domain**

The C- terminal domain of p53 contains both the regions necessary for protein tetramerisation and for non-sequence specific DNA binding.



Monomer units of p53 protein initially form homodimer complexes through direct interaction between their respective tetramerisation domains (amino acids 320-360). Functional p53 tetramers subsequently form as a result of p53 homodimer dimerisation, again as a result of direct interaction within this region of the C-terminal domain (Jeffrey *et al*, 1995).

The non-sequence specific DNA binding region within the C-terminal domain of p53 (amino acids 363-393) confers ability to bind to both damaged DNA and denatured DNA templates, thus perhaps implicating a role for p53 in the detection or 'sensing' of DNA damage within the cell (Levine *et al*, 1997). This molecular sub-domain also enables p53 to re-anneal both complementary RNA and single stranded DNA (Wu *et al*, 1995).

A role for p53 in promoting the repair of damaged DNA template has already been established through demonstration that p53 can both directly interact with components of the DNA repair machinery and can positively influence the expression of genes which are involved in DNA repair (see section 1.ii.5a). Further, mapping to the central core domain, p53 has also been shown to possess 3' to 5' exonuclease activity (Mummenbrauer *et al*, 1996), thus perhaps implicating the direct involvement of p53 in excision repair.

In addition to these functional aspects, this portion of the C-terminal domain seems to be able to exert influence on the affinity of p53 for its target DNA sequences, as deletion of these 30 amino acid residues results in a molecule constitutively active for DNA binding (Hupp *et al*, 1993). Further, a naturally occurring splice variant of p53 has been identified in the mouse, which expresses an alternative C-terminus (Kulesz-Martin *et al*, 1994). This splice variant

accumulates in G2, can no longer reanneal single stranded DNA (Wu *et al*, 1995), but appears to be constitutively active for DNA binding (Wu *et al*, 1994).

A recently published body of work now suggests that the allosteric modification of specific residues within this domain can shift p53 from a latent, low-affinity DNA binding state to an active high-affinity DNA binding state (Muller-Tiemann *et al*, 1998). These modifications will be discussed in greater detail within section 1.ii.4c.

### **(1.ii.3) Activation of p53 Dependent Responses**

In the absence of cellular stress, p53 has a very short half-life ( $t_{1/2}$  between 5 and 40 minutes; Reihnsaus *et al*, 1990) and appears to be rapidly turned over in normal cells, predominantly through ubiquitin-dependent proteolysis (for review see Brown and Pagano, 1997).

Amounts of p53 protein accumulate within the cell in response to a multitude of stimuli including those that result in the physical damage, the inappropriate proliferation, or in the metabolic stress of the cell. Exposure to UV-C, ionising irradiation, (Maltzman and Czyzyk, 1984 and Kastan *et al*, 1991), selected chemotherapeutic drugs (Zhan *et al*, 1993), hypoxia, heat shock (Graeber *et al*, 1994), disorganisation of microtubules (Tishler *et al*, 1995), exposure to specific cytokines (Eizenberg *et al*, 1995), deprivation of cytokines (Canman *et al*, 1995), oncogene overexpression (Wagner *et al*, 1994) and depletion of rNTP's (Linke *et al*, 1996) have all been demonstrated to stabilise p53 and induce p53 dependent cellular responses in a variety of cell and tissue types.

Expression levels of p53 are barely detectable in normal cells *in vivo*, but rise rapidly in a tissue specific manner following stimulation (Midgely *et al*, 1995). Immunohistochemical analysis of mouse tissue following genotoxic insult showed a strong accumulation of p53 protein in spleen, thymus, bone, and small intestine (Midgely *et al*, 1995 and Clarke *et al*, 1994). P53 stabilisation has also been demonstrated in human skin following exposure to normal sunlight (Hall *et al*, 1993).

To test p53 transactivation functionality *in vivo*, transgenic animal models have been constructed to express a lacZ reporter gene in a p53 dependent manner (Gottlieb *et al*, 1997 and Komarova *et al*, 1997). Following  $\gamma$ -irradiation, sites of maximal reporter expression largely concurred with sites of maximal p53 stabilisation in adult mice. Interestingly however, although both red and white compartments of the spleen exhibiting high levels of p53 stabilisation in response to DNA damage, reporter gene expression was only detected in the red compartment. This observation perhaps indicates the involvement of a co-factor or allosteric modulator regulating p53 transactivation capability in a cell-type specific manner (e.g. p33<sup>ING1</sup>; Garkavtsev *et al*, 1998).

The exact mechanisms underlying the increase of p53 protein in response to stimulation are complex and remain only partially understood. It is currently held that post-translational modification of translated p53 protein is of the greatest significance in explaining how intracellular levels of p53 rise and become activated in response to stimulation. Comprehension of the mechanisms influencing p53 gene expression at all levels must be prerequisite however before

it is possible to fully appreciate how the amounts and activation-state of p53 protein are regulated in the cell.

#### **(1.ii.4) Regulation of p53 Expression**

The wide-ranging and potentially severe cellular consequences associated with p53 activation imply that levels of p53 protein must be strictly controlled within the cell. Yet, failure to rapidly promote p53 expression in response to genotoxic stress could result in the propagation of mutant cells, with potentially tumorigenic consequence.

The regulation of p53 gene expression has been demonstrated at multiple levels, presumably both as a means of ensuring against the inappropriate activation of p53 dependent pathways in the cell, but also to make provision for a rapid cellular response to DNA damage or cellular stress.

#### **(1.ii.4a) Transcriptional Regulation**

The promoter region of p53 lacks both TATA and GCAAT boxes (Bienz-Tadmor *et al*, 1985), which is a feature typically associated with constitutively expressed or “housekeeping” genes (Reynolds *et al*, 1984). Mapping to nucleotides –2 to +5bp (relative to the start of exon 1) is a consensus initiator (Inr) element, which has previously been demonstrated to position the transcriptional start site of other genes also lacking a TATA box (O’Shea-Greenfield and Smale, 1992).

The entire 216bp length of exon 1 is non-translated sequence and contains extensive dyad symmetry. Transcription of this sequence is putatively involved in the formation of a stable mRNA stem loop structure, implicated in the post-



transcriptional regulation of p53 expression (Mosner *et al*, 1995). The formation of this stable mRNA hairpin structure has complicated the accurate definition of p53 transcript initiation site. S1 nuclease protection and primer extension studies suggest three major transcriptional initiation sites at +1, +61 and at +105bp, the latter position being the most frequently used (see figure 1.ii.a) (Bienz *et al*, 1984 and Tuck and Crawford, 1989).

P53 transcription levels in murine fibroblasts have been shown to be upregulated following exposure to the phorbol ester TPA and serum stimulation. The former response is understood to be mediated by a novel AP-1 like transcription factor, termed PF1 (p53 Factor 1), which binds to a closely conserved variant of the AP-1 consensus sequence (at -64 to -57bp), to which AP-1 itself cannot bind (Ginsberg *et al*, 1990).

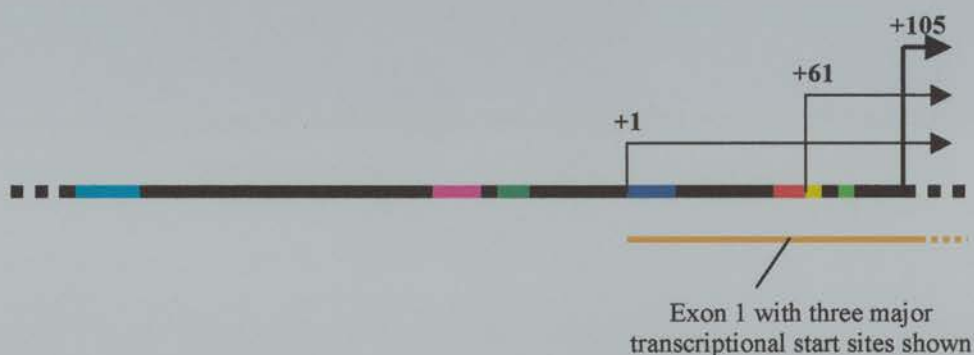
The latter response may be mediated through a helix-loop-helix binding motif, from +70 to +75bp, which once bound to the transcription factor USF, can enhance basal transcription 5-fold (Ronen *et al*, 1991). Transient overexpression of c-myc has also been demonstrated to enhance p53 expression via this promoter element (Reisman *et al*, 1993).

Expressed p53 protein has also been shown to exert positive feedback by influencing transcription from its own promoter (Deffie *et al*, 1993). The sequence element at nucleotides +55 to +66bp consists of a NF- $\kappa$ B motif, containing a one nucleotide variant of the p53 DNA binding consensus sequence as defined by el-Diery (el-Diery *et al*, 1992). Although p53 protein does not physically bind with this sequence, reporter studies indicate that following p53 overexpression transcriptional activity increases via this sequence element. This

implies the involvement of p53 as an interacting component of the stress-induced NF- $\kappa$ B-mediated transcriptional response.

Fig 1.ii.a

**PROMOTER REGION OF MURINE P53 GENE**



KEY	FACTOR	SITE	BINDING SEQUENCE (5' TO 3')
<span style="color: cyan;">■</span>	PF-2	-195 to -170bp	<b>GGTCCACTTACGATAAAAACTTAATT</b>
<span style="color: pink;">■</span>	PF-1	-64 to -57bp	<b>CTGACTCT</b>
<span style="color: green;">■</span>	SP-1	-49 to -44bp	<b>CCCGCC</b>
<span style="color: blue;">■</span>	NF-1	+1 to +14bp	<b>TGGCGACTATCCAG</b>
<span style="color: red;">■</span>	NF-_B	+55 to +66bp	<b>GGGACTTCCCC</b>
<span style="color: yellow;">■</span>	ETF	+63 to +66bp	<b>CCC</b>
<span style="color: lightgreen;">■</span>	HLH	+70 to +75bp	<b>CACGTG</b>

A sequence element identified at nucleotides -195 to -170bp has been demonstrated to be essential for p53 expression, binding to an as yet uncharacterised transcription factor, termed PF2, (p53 factor 2) (Hale and Braithewaite, 1995). In addition, an ETF binding site at +63 to +66bp (Hale and Braithewaite, 1995), and a NF-1 binding site present at nucleotides +1 to +14bp (Ginsberg *et al*, 1990), also influence p53 basal expression. A putative SP1 binding site at nucleotides -49 to -44bp has also been identified, but it remains

unresolved as to whether this transcription factor actually binds and influences p53 gene expression.

#### **(1.ii.4b) Post-Transcriptional Regulation**

Following serum stimulation of resting fibroblasts to re-enter cell cycle, p53 protein levels rise and reach maximal levels of expression significantly faster than corresponding levels of p53 mRNA or other housekeeping genes. In addition, when cultured in the presence of the transcriptional inhibitor actinomycin D, these cells exhibited a rapid rise in p53 protein levels (within 1 hour) following  $\gamma$ -irradiation. These results would implicate the involvement of some form of post-transcriptional mechanism(s) in the regulation of p53 expression (Mosner *et al*, 1995).

Kozak proposes that 5' stem loop structures in mRNA are important for the translational regulation of vertebrate mRNAs (Kozak, 1991). The fact therefore that the p53 transcript from man, rat and mouse all contain highly conserved dyad sequence symmetry at their 5' untranslated ends suggests that these sequences are of evolutionary relevance and further analysis has implicated them in the regulation of p53 translation (Mosner *et al*, 1995).

Initial studies of the 5' UTR (Un-Translated Region) of p53 predicted the formation of a stable stem loop structure between -216 and -108 base pairs (Bienz-Tadmor *et al*, 1984), possessing a free energy of -56 kcal/mol. Stem loop structures with a free energy of -50 kcal/mol require RNA helicase activity for dissociation and are sufficient to profoundly inhibit translation (Kozak, 1991). A

further putative stem loop structure was identified from between -216 and +284 base pairs with a predicted free energy of -170 kcal/mol (Mosner *et al*, 1995).

P53 can bind RNA, possesses RNA•RNA re-annealing activity and displays helicase inhibitory effects (Oberosler *et al*, 1993). P53 has also been demonstrated to preferentially bind to the 5' UTR of its own mRNA in competitive binding assays, directly implicating a role for the fully translated product in "masking" the translation of its own message (Mosner *et al*, 1995).

It is proposed that upon genotoxic stress, p53 protein can be synthesised quickly from the existing pool of masked mRNA, by-passing the requirement for additional transcription. In addition, p53 protein previously bound to and repressing the transcription of its own message can be immediately recruited to the nucleus without the requirement for additional translation, thus facilitating a rapid cellular response to DNA damage.

Additional sequences identified within the 3' UTR of human p53 mRNA have been demonstrated to increase the rate of translation from p53 mRNA following  $\gamma$ -irradiation (Fu and Benchimol, 1997).

#### **(1.ii.4c) Post-Translational Regulation**

From examination of the regulatory mechanisms discussed so far, p53 appears to be produced constitutively at low levels, to assume a latent (non-DNA binding) state and to be rapidly turned over within non-challenged cells. P53 dependent responses following challenge coincide with increased levels of p53 within the cell nucleus and in a conformational shift towards a more actively DNA binding state. Stabilisation and activation of p53 can be achieved through both the post-

translational modulation of p53 phosphorylation and acetylation states and additionally through specific protein: protein interactions.

Although levels of p53 protein increase in response to many stimuli (discussed in section 1.ii.3), the mechanisms through which stabilisation occurs appear to be partially shared, partially 'challenge' specific and by inference in certain contexts, cell-type specific.

Treatment of cells with  $\gamma$ -irradiation or topoisomerase inhibitors (e.g. etoposide), leads to the formation of double strand DNA breaks, which in turn results in increased intracellular levels of p53 and induction of p53 dependent cellular responses (Nelson and Kastan, 1994). Indeed, it has been proposed that a single double strand DNA break is sufficient to stabilise p53 in normal human fibroblasts (Di Leonardo *et al*, 1994).

A candidate molecule involved in linking the recognition of physical DNA breakage to p53 (and thus implicated in the stabilisation of p53) is the protein kinase ATM (ataxia-telangiectasia). Cells deficient in ATM exhibit a delayed p53 dependent response to  $\gamma$ -irradiation and double strand breaks, indeed MEF cells deficient in both ATM and p53 no longer arrest at G<sub>1</sub>-S in response to  $\gamma$ -irradiation (Kastan *et al*, 1992 and Xu *et al*, 1998).

ATM appears to both directly phosphorylate p53 at serine 15, and to indirectly dephosphorylate at serine 376, following exposure to  $\gamma$ -irradiation (Siliciano *et al*, 1997 and Waterman *et al*, 1998). Phosphorylation of serine 15 has previously been shown to increase both the stability and activity of p53, possibly through decreasing efficiency of mdm2 binding (Shieh *et al*, 1997), whilst

dephosphorylation at serine 376 has been implicated in the enhancement of sequence specific binding (Waterman *et al*, 1998).

Despite the ability of ATM *-/-* MEF cells to activate p53 dependent pathways in response to  $\gamma$ -irradiation, albeit with reduced efficiency (S15 can still be phosphorylated in ATM *-/-* cells; Siliciano *et al*, 1997), it remains clear that ATM kinase plays an important role in both the identification of DNA double strand breaks and in activating p53 correspondingly.

Further kinases implicated in signalling double stranded DNA breaks to p53 are DNA-PK and associated Ku autoantigen. These proteins have also been demonstrated to phosphorylate p53 at serines 15 and 37 in response to DNA double strand breaks (Shieh *et al*, 1997), but the relevance of their activity has yet to be demonstrated *in vivo*.

Exposure of cells to UV-C irradiation results in the generation of thymidine dimers, although the generation of these *per se* appears to be insufficient to stabilise p53. However, DNA strand breaks generated from excision repair or resulting from the replication of DNA through these genetic lesions appear to stabilise p53 (Nelson and Kastan, 1994). In addition, UV induced lesions can result in the stalling of transcription complexes, a molecular event that appears sufficient to stabilise p53 (Ljungman and Zhang, 1996).

Unlike the DNA lesions induced by  $\gamma$ -irradiation, ATM kinase does not signal UV induced damage to p53. Residue S15 is still phosphorylated in response to UV damage, although the UV-damage specific kinase responsible for this modification remains to be identified (Giaccia and Kastan, 1998).

In less specific terms, the phosphorylation of several amino-acid residues within the C- terminal portion of p53 has been proposed as a potential control mechanism regulating the switch from latent to active DNA binding form. Phosphorylation of amino acid residue S378 by Protein Kinase C (PKC) (Takenaka *et al*, 1995) or S392 by Casein Kinase II (CKII) (Hupp *et al*, 1992), have been demonstrated to stimulate the sequence specific DNA binding by p53. Microinjection of the C-terminal specific antibody pAb421 has also been demonstrated to activate p53 following binding to its epitope (Hupp *et al*, 1993).

A further residue phosphorylated by CKII (S386) was shown to stimulate p53 mediated repression of downstream target loci *in vitro* (Hall *et al*, 1996). A summary of established p53 modifications is listed in table 1.ii.a.

**Table 1.ii.a**

STIMULUS	MODIFIER PROTEIN	MODIFIED RESIDUES	TYPE OF MODIFICATION
Etoposide	CK I	S6 + S9	Phosphorylation
$\gamma$ -irradiation	DNA-PK	S15 + S37	Phosphorylation
$\gamma$ -irradiation	ATM +ATR	S15	Phosphorylation
$\gamma$ -irradiation	ATM	S376	De-phosphorylation
$\gamma$ -irradiation	CAK	S33	Phosphorylation
UV-C	?	S15 + S33	Phosphorylation
G <sub>1</sub> of cell cycle	cdk2 +cdc2	S315	Phosphorylation
Phorbol ester	PKC	S378	Phosphorylation
-	CK II	S386 + S392	Phosphorylation
$\gamma$ -irradiation and UV-C	p300	L382	Acetylation
$\gamma$ -irradiation and UV-C	PCAF	L320	Acetylation

**Table 1.ii.a** summarises the stimuli, effector kinase/acetylase molecules, p53 residues modified and the type of modification events demonstrated to effect either p53 conformation or stability. Where known, the context in which these modifications arise is discussed in the main text and the appropriate references are cited therein.

P300 and pCAF mediated acetylation of residues within the C- terminal domain of p53 have also been shown to enhance sequence specific binding of p53 in

response to both  $\gamma$ -irradiation and UV induced DNA damage (Gu and Roeder, 1997 and Lill *et al*, 1997). Evidence has also been presented which shows that p53 protein previously phosphorylated by DNA-PK is more efficiently acetylated than unmodified p53 protein (Sakaguchi *et al*, 1998). This would suggest that p53 phosphorylation and acetylation states might have combinatorial or additive effects with regard to the regulation of p53 specificity and action within the cell. Further research within this area may lead to the identification of the p53 modification states that 'decide' cellular outcome in response to activation, or indeed may lead to an explanation regarding the basis of certain tissue specific differences in p53 dependent outcome following activation.

In addition to stabilisation following sustenance of DNA damage, p53 protein is stabilised and activated in the cell following inappropriate overexpression of certain oncogenes. A recently published body of work has highlighted a key role for p19<sup>ARF</sup> in this context.

P19<sup>ARF</sup> is one of two alternative tumour suppressor transcripts encoded by the murine INK<sup>4A</sup> locus, and cellular levels are known to rise in response to the overexpression of certain oncogenes (e.g. c-myc, E1A, E2F) in the absence of corresponding levels of growth factor stimulation (Quelle *et al*, 1995). Induction of p19<sup>ARF</sup> in this manner has been demonstrated to stabilise levels of p53 *in vivo* and induce either growth arrest at both G<sub>1</sub> and G<sub>2</sub> stages of the cell cycle or apoptosis (Kamijo *et al*, 1997 and Zindy *et al*, 1998).

Overexpression of p19<sup>ARF</sup> cannot induce growth arrest or apoptosis in p53 <sup>-/-</sup> MEF cells however and so appears to be entirely dependent upon p53 to exert its



effect upon the cell (Kamijo *et al*, 1997). Further, the role played by p19<sup>ARF</sup>, as a ‘sensor’ of inappropriate hyperproliferative signals within the cell, appears to be a specific means of p53 activation as p19<sup>ARF</sup> -/- MEF cells respond as wild-type MEF cells when exposed to both ionising and UV-C irradiation (Kamijo *et al*, 1997) i.e. p19<sup>ARF</sup> does not influence the stabilisation of p53 in response to DNA damage.

The means by which the p19<sup>ARF</sup> gene product stabilises expression levels of p53 are now becoming clear. In addition to blocking p53 mediated gene transactivation (as mentioned in section 1.ii.2b), a further consequence of the interaction between mdm2 and p53 is an increase in the rate of proteasome mediated degradation of p53 (Kubbutat *et al*, 1997). The N- terminal portion of p19<sup>ARF</sup> can bind to the C- terminal portion of mdm2 and it appears that this interaction results in a reduction of the rate of proteosomal degradation of p53. Amounts of p53 rise within the cell as a consequence of this decrease in degradation rate (Zhang *et al*, 1998 and Pomerantz *et al*, 1998).

Several putative mechanisms have been proposed to explain how interaction between p19<sup>ARF</sup> and mdm2: p53 reduces the rate of p53 degradation. Some experimental evidence appears to suggest that binding of p19<sup>ARF</sup> to mdm2: p53 results in a modification of mdm2’s ability to poly-ubiquitinate p53. Also suggested is a p19<sup>ARF</sup> dependent mechanism whereby binding of p19<sup>ARF</sup> blocks nuclear export of the mdm2: p53 protein complex, thus ensuring that p53 protein remains localised in a separate sub-cellular compartment from the proteasome (Pomerantz *et al*, 1998 and Roth *et al*, 1998).

Finally, direct interaction with HIF- $\alpha$  (Hypoxia Induced Factor  $\alpha$ ) results in the stabilisation of p53 protein in cells growing under hypoxic conditions (An *et al*, 1998) and further, direct interaction with ref-1 in response to oxidative stress appears to activate sequence specific DNA binding of p53 (Jayaraman *et al*, 1997). The mechanisms underlying both these observations are presently not known.

#### **(1.ii.5) Mediation of Growth Arrest, DNA Repair and Apoptosis**

Although the general processes influencing both the stabilisation and activation of p53 are becoming apparent, little is currently known regarding how p53 activation can result in different cellular end-points in response to stimulation.

The extent of stimulation seems to influence the cellular response to activated p53 as several studies have highlighted that either growth arrest or DNA repair occur at low levels of DNA damage, whilst apoptosis seems to be the predominant response to sustenance of high levels of DNA damage (Chen X *et al*, 1996 and Li and Ho, 1998). This view is almost certainly too simplistic however, as even following exposure to equivalent doses of DNA damaging agents different cell types exhibit different p53 dependent responses. For example, human fibroblasts predominantly enter growth arrest at G<sub>1</sub>-S of the cell cycle following exposure to 5 Gy of  $\gamma$ -irradiation whilst intestinal crypt epithelia apoptose (Di Leonardo *et al*, 1994 and Clarke *et al*, 1994).

Further, the means through which p53 becomes stabilised and activated is also certainly of relevance with regard to cellular outcome. Depletion of intracellular rNTP level is known to induce p53 stabilisation, but these cells predominantly

exhibit a proliferative arrest rather than committing to apoptosis (Linke *et al*, 1996). Conversely, p53 activated in response to inappropriate oncogene overexpression predominantly initiate apoptosis (Hermeking and Eick, 1994).

As will be discussed in sections 1.ii.5a and 1.ii.5b, p53 mediated growth arrest requires the ability of p53 to transcriptionally activate the expression of downstream loci, whereas p53 mediated apoptosis can occur in the absence of downstream gene transactivation. Some experimental evidence exists to suggest that the ability of p53 to transactivate downstream gene expression requires the presence or 'assistance' of protein co-factors. In this respect, the coexpression of P33<sup>ING</sup> has been demonstrated to be essential in achieving p53 dependent growth arrest in response to DNA damage (Garkavtsev *et al*, 1997). Further, the addition of exogenous p19<sup>ARF</sup> into otherwise p19<sup>ARF</sup> deficient MEF cells proved to be essential for p53 mediated gene transactivation of a p53-reporter transgene (Kamijo *et al*, 1997). Certainly the hypothetical existence of tissue specific co-factors is an attractive means of explaining the observed tissue-specific differences in p53 dependent outcome resulting from identical challenge.

Finally, as discussed in section 1.ii.4c, allosteric modification p53's C- terminal domain can increase the affinity of p53 for its cognate target sequence. Although yet to be directly proven, it is highly probable that p53 dependent cellular outcomes can also be regulated at this level.

#### **(1.ii.5a) Growth Arrest and DNA Repair**

DNA damaged wild-type cells can 'choose' between p53 mediated growth arrest or death. Those that arrest at a cell cycle checkpoint, predominantly enter a

reversible growth arrest at G<sub>1</sub>-S of the cell cycle, but can also arrest at S or at G<sub>2</sub>-M phases in a p53 dependent manner (Agarwal *et al*, 1995 and Petrocelli *et al*, 1996). It is generally accepted that p53 dependent growth arrest is achieved almost exclusively via the transcriptional transactivation of a number of specific downstream genes, among which of particular physiological relevance is the cyclin-dependent-kinase (cdk) inhibitor, p21 (WAF1/CIP1).

The expression of p21 is induced in a p53 dependent manner following DNA damage (el-Diery *et al*, 1993). P21 is a potent inhibitor of the family of proteins responsible for increasing the phosphorylation state of pRb, the cyclin dependent kinases (Harper *et al*, 1993). Direct interaction between p21 and a number of cyclin/cdk complexes results in their functional suppression, one result of which being an inhibition of cdk-mediated phosphorylation of pRb. Hypophosphorylated pRb in turn remains associated with the E2F transcription factor complex, an interaction that inhibits initiation of S-phase and results in the cell being held in a reversible G<sub>1</sub> arrest (Sanchez and Elledge, 1995).

Initiation of p53 dependent cell cycle arrest also appears to create a cellular environment in which damaged DNA may be repaired with greater efficiency. P21 has also been demonstrated to interact with PCNA (Proliferating Cell Nuclear Antigen), a protein that facilitates DNA synthesis (Flores-Rozas *et al*, 1994 and Waga *et al*, 1994). PCNA is also involved in NER (Nucleotide Excision Repair), but interaction with p21 has been demonstrated to block DNA replication selectively without affecting PCNA dependent NER (Li *et al*, 1994). The relationship between p53 and p21 and mediation of the G<sub>1</sub>-S cell checkpoint is not

exclusive however, as fibroblasts derived from mice deficient in p21 are still partially able to arrest at G<sub>1</sub>-S following DNA damage (Deng *et al*, 1995).

Expression of GADD45 has also been shown to be upregulated in a p53 dependent fashion following exposure to ionising radiation in a wide variety of cell types (Kastan *et al*, 1992). This gene directly interacts with PCNA in response to DNA damage, in such a manner as to enhance DNA repair whilst inhibiting DNA replication. This interaction has also been associated with the induction of a growth arrest at G<sub>1</sub>-S (Hall *et al*, 1995).

In addition to inducing the expression of genes shown to affect the efficiency of DNA repair, p53 itself has been directly implicated in participating with the repair of damaged DNA template. In addition to possessing 3' to 5' exonuclease activity and the ability to bind recognise and bind damaged DNA template (see section 1.ii.2c), p53 can also directly bind to both the single-strand DNA binding protein RP-A and the DNA strand repair factor CsB (Dutta *et al*, 1993 and Wang *et al*, 1995). Further, the C- terminal domain of p53 is known to be able to directly interact with two helicase components of the TFIIH repair complex, XPD and XPB (Wang *et al*, 1995). The *in vivo* significance of these interactions has yet to be established however the *in vivo* consequences regarding p53 status and DNA mutation rate are presented and discussed in section 1.ii.6.

Recently published evidence has suggested a role for p53 mediated growth arrest in the suppression of cell transformation following attenuation of p53 independent apoptotic pathways in the cell.

The expression of a dominant negative FADD (Fas Associated Death Domain) transgene in T cells has shown that although still able to initiate apoptosis in response to UV light and dexamethasone, these cells are no longer responsive to Fas induced apoptosis (Zornig *et al*, 1998).

FADD has been demonstrated to directly mediate apoptotic signals generated in response to ligand interaction with the TNF (Tumour Necrosis Factor) receptor family to the effector molecules of apoptosis, the caspases (Boldin *et al*, 1996). Fas induced apoptosis signals through one such receptor (CD95) and is physiologically relevant with regard to mediating T cell cytotoxicity and in removing surplus numbers of mature B and T cell lymphocytes in the aftermath of infection, a process termed AICD (Activation-Induced Cell Death; Ekert and Vaux, 1997). One could predict therefore, that animals possessing T cells resistant to Fas induced apoptosis may be more likely to develop T cell lymphoma in comparison to wild-type animals. Rather than gaining selective growth advantage however, T cells expressing this mutant version of FADD exhibited growth arrest, which further experimentation proved to be p53 dependent (Zornig *et al*, 1998).

#### **(1.ii.5b) Apoptosis**

P53 status has been shown to be critical for the induction of a rapid apoptotic response in many tissue and cell types in response to the majority of stimuli listed in section 1.ii.3 (for review see Bellamy, 1997). Indeed, it is highly likely (but not directly proven) that the attenuated apoptotic responses exhibited by p53 deficient cells contribute to an overall increase in the probability of their malignant

conversion (see section 1.ii.7). As means of illustration, wild-type thymocytes commit to apoptosis in a p53 dependent manner following  $\gamma$ -irradiation, whilst p53  $-/-$  thymocytes do not. P53 deficient thymocytes still retain p53 independent apoptotic responses (e.g. in response to glucocorticoid exposure), yet p53 deficient animals are highly prone to the development of T- cell lymphomas early in life (Clarke *et al*, 1993 and Purdie *et al*, 1994).

P53 can induce apoptosis in response to DNA damage through both transactivation dependent and independent mechanisms. Experimental evidence exists to demonstrate that p53 can induce apoptosis in cells cultured in the presence of both actinomycin D and cyclohexamide (inhibitors of RNA and protein synthesis respectively) in response to DNA damage or c-myc overexpression (Caelles *et al*, 1994 and Wagner *et al*, 1994). Further, a C-terminal deficient and consequently transactivation deficient p53 molecule was shown to retain the ability to induce HeLa cell apoptosis in response to inappropriate oncogene expression (Haupt *et al*, 1995). An apoptotic domain, putatively essential for p53 mediated cell death has recently been mapped to amino acids 43-63 (Zhu *et al*, 1998).

The mechanisms underlying p53 dependent apoptosis in the absence of downstream gene transactivation remain to be elucidated, however direct interaction between p53's C- terminal domain and both XPB and XPD helicases seems to be essential in certain cellular contexts (Wang *et al*, 1996). Further, a recent publication has described the ability of activated p53 to directly mediate translocation of the Fas receptor from Golgi apparatus to cell surface. Activated

p53 also induced interaction between FADD and the Fas receptor and so can transiently sensitise cells to Fas-induced apoptosis (Bennett *et al*, 1998).

Examples also exist in the literature demonstrating an absolute requirement for p53 mediated transactivation or repression of downstream targets to induce apoptosis (Sabbatini *et al*, 1995 and Yonish-Rouach *et al*, 1995). The identity of several of these transactivated and repressed loci would theoretically appear to increase the likelihood of an apoptotic cellular outcome in response to cellular stress.

Activated p53 has been shown to both transactivate the expression of Bax in response to  $\gamma$ -irradiation and to repress the transcription of Bcl-2 (Miyashita and Reed, 1995 and Miyashita *et al*, 1994). Overexpression of Bcl-2 has been demonstrated to block p53-dependent apoptosis (Chiou *et al*, 1994), however cells become predisposed to apoptose when the relative levels of Bax predominate over the levels of Bcl-2 within the cell. In this respect, p53 influences the expression of both target genes to promote apoptosis. Indeed, a recently published study demonstrated that p53 dependent induction of Bax was essential in slowing the rate of neural tumour growth and progression *in vivo* (Yin *et al*, 1997).

P53 can also induce the expression of IGF-BP3 (insulin-like growth factor binding protein 3), a protein that appears to block mitogenic signalling resulting from stimulation of the IGF receptor (Buckbinder *et al*, 1995). P53 has been further implicated as a direct regulator of cellular IGF receptor levels (Neuberg *et al*, 1997) and so in this manner, p53 may either retard cellular proliferation rate or induce apoptosis through deprivation of growth signals.



In an attempt to further elucidate the mechanisms underlying p53 transactivation mediated apoptosis, the group of Bert Vogelstein analysed the number of gene transcripts influenced by activated p53 in a human colorectal cancer cell line prior to apoptosis (Polyak *et al*, 1997). Using SAGE (serial analysis of gene expression) they identified 14 transcripts (0.19%) that appeared to be induced greater than 10-fold and 20 transcripts (0.28%) repressed greater than 10-fold in a p53-dependent manner. Further analysis of the induced transcripts identified them as being either capable of generating, or responding to oxidative stress within the cell. Accordingly, they have proposed that p53 transactivation mediated apoptosis is initiated by induction of redox-related genes, which in turn results in the formation of reactive oxygen species, which in turn leads to mitochondrial degradation and cell death.

Interestingly however, this study reported no observable p53 dependent difference in the expression levels of Bax. Therefore, whilst Bax expression appears to be relevant regarding the suppression of tumours of neural origin, less reliance (if any) appears to be placed upon this apoptotic pathway regarding the suppression of colorectal tumours.

In summary then, this data would suggest that p53 can induce apoptosis through several pathways, but apoptotic outcome as yet is not predictable and appears to be highly dependent upon context *in vivo*.

From the evidence presented in each of the preceding sections, it is possible to make several predictions about the effects of p53 deficiency upon both mutation

rate and tumorigenesis. The availability of strains of p53 deficient mice has permitted a wide-range of experiments addressing both of these endpoints.

#### **(1.ii.6) P53 and Mutation Frequency**

Abrogation of wild-type p53 function is predicted to lead to an increase in the number of cells bearing DNA damage, as a result of altered apoptotic and DNA repair profiles. Experiments designed to test this prediction suggest that p53 dependent differences may only become apparent following exogenous damage. Two different groups have used a transgene target to monitor spontaneous mutation frequency, but in both cases no p53 dependent differences were observed (Sands *et al*, 1995 and Nishino *et al*, 1995). By contrast, experiments using either short pre-B cell cultures (Griffiths *et al*, 1997) or a transfected fibroblast line (Yuan *et al*, 1995) have now provided data in support of a p53 dependent decrease in the number of mutation bearing cells in response to genotoxic stress. *In vivo* experiments using the Dlb-1 locus as a marker for mutation, have also shown a p53 dependent decrease in mutation frequency in the murine small intestine (Clarke *et al*, 1997), although this was only observed at high levels of DNA damage. Taken together, these different experiments do support a role for p53 in preventing the acquisition of mutation, but it is clear that it is not the sole mechanism, particularly at low levels of DNA damage.

A further difficulty exists in interpreting the relevance of p53 dependent apoptosis because of the diversity of roles played by p53. However, evidence does exist to show that, at least in certain cell systems, the induction of apoptosis is the critical response to DNA damage. Thus, where mutation frequency and clonogenic

survival have been scored independently (Griffiths *et al*, 1997), it can be shown that mutation frequencies amongst surviving clones are largely unaffected by p53 status, but that the effect of p53 deficiency is to increase the number of mutation bearing progeny by virtue of increased cell survival. That is, the failure to initiate p53 dependent apoptosis in response to DNA damage is perhaps more relevant to the increase in DNA damage bearing cells than an actual DNA repair deficit.

#### **(1.ii.7) P53 and Tumourigenesis**

The majority of p53  $-/-$  mice develop normally to birth, but then rapidly succumb to tumourigenesis, predominantly of the thymic lineages. P53  $+/-$  mice develop both lymphomas and sarcomas at an approximately equal rate, but  $+/-$  mice develop a broader spectrum of tumours at low incidence in comparison to  $-/-$  mice and also exhibit a longer latency period before tumour appearance (Purdie *et al*, 1994 and Jacks *et al*, 1994).

Transgenic experiments using a dominant negative p53 transgene in combination with the p53 heterozygous and null animals (Harvey *et al*, 1995) have strongly supported the idea that mutation of p53 predisposes to tumourigenesis by blocking wild type p53 function. *In vivo* analysis of these strains has been used to address the relevance of p53 gene status at different stages of tumourigenesis.

A critical role for wild-type p53 in the suppression of radiation-induced malignancy was identified following exposure of mice mutant for p53 to  $\gamma$ -irradiation. Treated p53  $+/-$  and  $-/-$  mice developed tumours with an identical spectrum to non-irradiated counterparts, but exhibited a comparatively much reduced latency period (Kemp *et al*, 1994). Similarly, transgenic mice expressing

a dominant negative mutant p53 allele, treated in a similar fashion, displayed a higher incidence rate of sarcoma and lymphoma occurrence, and also demonstrated an increase in the incidence of double strand chromosomal breaks, and therefore exhibited increased genomic instability (Lee *et al*, 1994). Analysis of the tumours derived from irradiated +/- mice revealed loss of the remaining wild type p53 allele in 96% of all cases, a significantly higher level of loss than in the mock irradiated +/- mice.

Loss of p53 function therefore confers a strong selective growth advantage during the processes of  $\gamma$ -irradiation induced tumourigenesis, however p53 status is not the sole rate determinant. If this were the case, one would predict p53 -/- mice to develop large numbers of primary tumours simultaneously following irradiation.

The protective role of p53 is not limited to the prevention of  $\gamma$ -irradiation induced tumourigenesis. For example, treatment of p53 +/- animals with the carcinogen dimethylnitrosamine leads to a reduction in latency (in comparison with wild type) of tumour development within the liver (Harvey *et al*, 1993).

P53 status has also been shown to influence both initiation and progression of UV induced skin cancer (Zeigler *et al*, 1994). In addition to demonstrating that the majority of actinic keratoses (first clinically apparent lesion in skin cancer development) have at least one mutated p53 allele, p53 +/- and -/- cells possess a selective survival advantage over p53 +/+ cells following repeated re-exposure to sunlight.

Following the exposure of mice +/+, +/- and -/- for p53 to UV B irradiation, +/+ mice exhibited extensive sunburn, characterised by high levels of apoptosis, within 24 hours. Significantly, -/- skin tissue presented far fewer sunburn cells

and correspondingly less apoptosis and +/- skin tissue exhibited an intermediate phenotype.

The ability to become sunburn cells and enter apoptosis is clearly p53 gene dose dependent. DNA damaged cells surviving UV irradiation by virtue of compromised p53 activity would subsequently select for lesions positively influencing growth or negatively affecting death-promoting genes, and thus be predisposed to malignant conversion.

Skin swabbed with the carcinogen DMBA initiates tumourigenesis in the mouse. Promotion of tumourigenesis can be achieved by exposing the initiated skin to TPA, the result of which is the appearance of multiple papillomas (benign hyperplastic regions). Within two months, a small proportion of these papillomas become carcinomas, which vary in their state of differentiation and invasiveness. Kemp *et al*, utilised this well characterised model of chemically induced multi-stage tumourigenesis to determine the effects of p53 status upon these different stages (Kemp *et al*, 1993).

In the absence of DMBA treatment, no papillomas arose on the skin of p53 -/- mice swabbed with the tumour promoter TPA, demonstrating that loss of p53 function is insufficient for tumour initiation. Indeed, this experiment demonstrated that a p53 null phenotype is refractory to papilloma formation.

The effect of p53 status on the latency of papilloma development was also investigated and no difference was observed between mice +/+, +/- and -/- for p53, again implying a role for other genetic lesions at this stage of tumour development.

The conversion rate from benign papillomas to malignant carcinomas was, however, shown to be highly dependent upon p53 status. In p53 null mice, conversion occurred at higher penetrance and significantly faster than in +/+ animals.

These somewhat confusing results are supported by experiments using transgenes epidermally targeted to express v-rasHa, v-fos and human growth factor  $\alpha$ . These transgenic strains are predisposed to papilloma development, but this process is again blocked following breeding onto a p53 null background (Greenhalgh *et al*, 1996).

Taken together, these experiments argue that failure to initiate p53-dependent pathways leads to increased genomic instability and therefore increased rate of malignant progression, but only beyond the point of papilloma development. Prior to this point it is possible that cells possessing functional p53 can tolerate a higher level of DNA damage by virtue of attempted repair by p53 dependent pathways, whereas damaged p53 null cells are deleted by p53 independent apoptotic mechanisms. These experiments highlight the potential difficulty in over-interpreting the role of apoptosis in the prevention of tumourigenesis, as in this system failure to invoke p53 dependent pathways actually confers protection, at least against the benign lesion.

P53 deficiency clearly plays an important role in malignant development, yet as has been shown above, it is not the sole determinant of this process. The nature of the complex inter-relationship between p53 and other tumour-related genes has been investigated through a range of transgenic intercrosses. These experiments

have produced results which, while strongly supporting a tumour suppressor role for p53, clearly underline the cell specific nature of this function. For example, mice transgenic for an MMTV-driven c-Myc transgene predominantly develop mammary carcinoma, yet crossing onto a p53 null background reveals synergy in tumour development within the haematopoietic system, with no effect upon mammary tumourigenesis (Elson *et al*, 1995). Further evidence is available for synergy between p53 and c-Myc within the lymphoid system, as mice null for p53 and transgenic for a CD2 driven c-Myc construct show accelerated lymphoma development (Blyth *et al*, 1995).

Intercrossing p53 null mice with animals heterozygous for the APC tumour suppressor gene has yielded similarly contradictory results. Mice heterozygous for APC normally develop adenomas of the small and large bowel. When crossed onto a p53 null background, no difference in tumour formation or progression was observed even though loss of wild-type p53 function is strongly implicated in intestinal neoplasia (Clarke *et al*, 1995). Synergy between these mutations was however observed within the pancreatic tissues in the intercrossed animals, with almost 100% of doubly mutant mice developing neoplasia.

In summary, the experiments discussed above argue strongly for complex reliance upon p53-dependent pathways, largely determined by cell type. However, this may again be too simplistic a view of p53 function, as even within a given tissue type, deficiency of p53 can lead to different endpoints. For example, as stated above, p53 fails to synergise with c-Myc in mammary carcinoma development,

yet it does accelerate mammary adenocarcinoma formation in conjunction with a Wnt-1 transgene (Donehower *et al*, 1995).

These differences presumably arise as a consequence of other genes modulating apoptosis. For example, data from intercrosses of Bcl-2 transgenic animals and p53 null mice strongly argue that p53 deficiency may be substituted by Bcl-2 gene deregulation (Marin *et al*, 1994).

One alternative explanation for the variable reliance upon p53 derives from the multiple functions of this gene. If one presupposes that tissue types vary in their reliance upon apoptosis or DNA repair to eliminate mutation, it is inevitable that there will be a tissue specific pattern of reliance upon p53 and indeed, any other genes that regulate these processes.

Finally, it should again be stressed that although it is possible to detect a p53-dependent apoptotic phenotype within many of the systems discussed above, it is currently not possible to determine if this process is the sole mediator of phenotype. The future analysis of p53 point mutants may enable the dissection and analysis of specific p53 dysfunction and their phenotypic consequences. It seems more likely however, that it is the lack of a combination of p53 functions which ultimately determines predisposition to malignancy.

#### **(1.ii.8) P53 and Angiogenesis**

P53 has also been implicated in exerting influence over the process of neo-vascularisation of tumours *in vivo*. Hypoxic conditions, at the centre of poorly vascularised solid tumours, have been demonstrated to induce p53 dependent apoptosis *in vivo* (Graeber *et al*, 1996). P53 can be induced to bind sequence



specific DNA in response to the generation of reactive oxygen species (ROS) within the cell. These highly reactive molecules have been implicated in the physical damage of DNA but as such, remain to be established as being sufficient to stabilise p53 in the absence of further associated damage.

Certain cell lines have been demonstrated to secrete increased amounts of the antiangiogenesis factor, thrombospondin, in a p53 dependent fashion *in vitro* (Dameron *et al*, 1994), but again it remains to be determined whether this observation is of physiological relevance regarding *in vivo* tumour suppression (for review see Bouck, 1996).

#### **(1.ii.9) Requirement of p53 Expression during Embryogenesis**

P53 expression does not appear to be essential for normal murine embryogenesis (Donehower *et al*, 1992). However, a small proportion of p53 *-/-* male animals are affected with defects in spermatogenesis (Rotter *et al*, 1993), and a small subset of p53 *-/-* female embryos are affected by exencephaly (defect in neural tube closure) (Sah *et al*, 1995). These developmental phenotypes seem to be dependent upon the genetic background of the experimental mouse, as they were not observed in the initial knockout animals (Donehower *et al*, 1992).

P53 dependent gene transactivation is mostly detectable in the developing nervous system of developing embryo's and newborn animals (Komarova *et al*, 1997). It is therefore tempting to speculate that the observed overgrowth of neural tissue observed in female exencephalics result from the failed induction of a p53 dependent, embryonal stage specific, wave of apoptosis.

Whole body  $\gamma$ -irradiation of pregnant mice has shown a p53 dependent hypersensitivity to DNA damage in the early embryo (between days 8-13), characterised by high levels of apoptosis in virtually all developing tissue (Komarova *et al*, 1997).

P53 has also been demonstrated to remove mutant cells from developing embryos in response to low levels of  $\gamma$ -irradiation, thus reducing numbers of resultant teratogenic offspring (Norimura *et al*, 1996). At high levels of damage however, the opposite phenomenon occurs and a p53 dependent increase in teratogenic offspring is observed (Wubah *et al*, 1996). It is proposed that the high rates of p53 dependent apoptosis within developing tissue in response to extensive DNA damage cannot be compensated for by the proliferation rate of cycling non-damaged cells.

Inappropriate expression of wild-type p53 during murine embryogenesis has been shown to give rise to malformations of the developing kidney, resulting in progressive kidney disease and failure (Godley *et al*, 1996), and in the absence of accompanying mdm-2 expression, to be lethal (Montes de Oca Luna *et al*, 1995), (Jones *et al*, 1995).

#### **(1.ii.10) P53 and Gene Therapy**

In summary, the p53 tumour suppressor gene has been demonstrated to preserve genomic integrity by being a critical regulator of both cell cycle and apoptosis in response to stress. Further, loss of wild-type p53 gene expression is one of the most common events to arise during the development and progression of many human and murine malignancies.

Upon this basis and observations made through extensive *in vitro* overexpression experimentation (e.g. Baker *et al*, 1990; Yonish-Rouach *et al*, 1991), it is proposed that the reintroduction of p53 expression into p53 deficient and malignant cells *in vivo* may restore p53 dependent cellular pathways and thus confer positive therapeutic effect. Indeed, the cytotoxicity conferred by many conventional chemotherapeutic drug treatments is potentiated in tumour cells retaining some functional wild-type p53 expression (Lowe *et al*, 1993). Therefore even if the re-introduction of wild-type p53 gene expression into mutant tumour cells proved insufficient to revert tumourigenic state, then at the least one could expect an enhanced anti-tumour response to conventional chemotherapeutic treatment.

Several research groups have taken this initiative and have started human gene therapy trials testing both adeno- and retroviral p53 gene replacement vectors as a means of correcting otherwise untreatable malignancies (Clayman *et al*, 1996 and Roth *et al*, 1996). The current failing of conventional gene replacement style therapy however is the inability to deliver stable gene expression at high efficiency to the desired target cells (Blau, 1997). These problems are especially pertinent with regard to the application of replacement gene therapy in the treatment of malignant disease. If one considers the manner in which tumourigenic cells clonally evolve to progressively more aggressive and invasive phenotypes, then theoretically near 100% efficiency in gene delivery will be required before significant therapeutic benefit is conferred to the patient. Indeed, these predications regarding the success of replacement gene therapy in the treatment of cancer are being made without formal proof that re-introduction of

tumour suppressor gene function is capable of significantly modulating tumour phenotype *in vivo*.

The advent of inducible promoter technology and the comparative ease at which it is now possible to generate precisely modified mice on controlled genetic backgrounds has provided an opportunity to produce model systems with which to evaluate the efficacy of replacement gene therapy as a means of treating cancer *in vivo*.

## CHAPTER 2

The aim of this chapter was to apply currently available inducible promoter technology to develop a tumour cell-line based system in which it would be possible to examine the phenotypic consequences resulting from the introduction of p53 expression within an otherwise p53 deficient genetic background.

### **(2.i) Inducible Transgene Approach**

The application of current inducible promoter technology to studies aimed at evaluating transgene-dependent effects upon the cell offers considerable advantages over both conventional transient or stable transfection approaches and viral transduction approaches (see sections 1.i.2 and 1.i.8).

Transient transfection efficiencies using plasmid DNA and current DNA transfer technology can vary largely between different cell types and between experiments, making accurate phenotypic comparison difficult. Transient approaches do however offer immediacy which studies of stable transgene expression do not offer. The selection of clones possessing stably integrated transgenic DNA requires at least a 10-day latency period before meaningful observations can be recorded and so often results in the analysis of transgene expression within a sub-clonal population of cells distinct from the parental non-transfected cell line.

The main advantage of an inducible transgene approach over conventional constitutively expressing transgenic approaches is that stably transfected clones can be established in the absence of transgene expression. This is particularly advantageous when the constitutive expression of a desired transgene is predicted

to confer a strong selective disadvantage upon the host cell (e.g. a tumour suppressor gene).

Following the selection of clones stably transfected with a constitutively expressing and growth limiting transgene, cells that exhibit low levels of transgene expression (as a result of position effect) will possess a selective advantage over cells that exhibit high levels of transgene expression. The likely consequence of this selective pressure will be that during the course of antibiotic selection for stably transfected clones, cells weakly expressing the transgene will become the predominant population within the culture system, as cells strongly expressing the transgene will be inhibited or removed. Such an eventuality could potentially result in the analysis of cells clonally distinct from the parental non-transfected cell, where it may not be possible to observe the full spectrum of transgene-mediated phenotypic effects as a result of weak expression.

Through the application of inducible expression technology however, stably transfected clones can be established in the absence of selective pressure conferred by transgene expression. Further, these clones possess the ability to rapidly and uniformly induce transgene expression, thus potentially enabling reproducible observation of a wider range of transgene-mediated effects within cells more likely to be representative of the original non-transfected parental cell type.

The development of recombinant adenoviral and retroviral expression vectors now permits both a rapid and highly efficient means of introducing transgene expression into cells *in vitro*. Variation of virus titre and extent of viral exposure

can result in the rapid transduction of virtually 100% of cells growing in culture. As discussed in section 1.i.2 however, the ability to use viral vectors as a means of introducing stable transgene expression into cells growing *in vivo* can be of highly variable efficiency and thus difficult to reproduce. In these respects and with regard to the aims of this project (section 1.ii.10), inducible promoter technology is superior to both conventional transfection and viral transduction technology in permitting the examination of transgene mediated effects within mammalian cells *in vivo*.

Once tumour cells have been transfected with an inducible tumour suppressor transgene and fully characterised for transgene expression levels and phenotypic effects *in vitro*, these cells can be potentially returned to the mouse to grow *in vivo* in the absence of transgene expression (see chapter 3). In this manner it may be possible to examine how fully *in vitro* characterised levels of transgene expression affect tumour cell growth *in vivo* and so permit a means of better evaluating the potential application of replacement tumour suppressor gene therapy in the treatment of cancer.

### **(2.i.1) Choice of Inducible Expression System**

In order to best achieve the experimental aims described in the opening section of this chapter, an inducible expression system was required that would permit both a low level of “leaky” transgene expression when in the non-induced state, yet also produce a high level of transgene expression when induced.

As summarised in section 1.i.8, there are presently a wide variety of inducible gene expression systems currently available. For this study however, two

inducible promoter systems were chosen to regulate the expression of a p53 transgene: the human 6-16 promoter system (interferon  $\alpha/\beta$  inducible) and the tetracycline inducible expression system.

The tetracycline inducible promoter system (described in section 1.i.8c) was selected because it affords very tight regulation of transgene expression; i.e. very low basal transgene expression in the non-induced state and a high level of transgene expression in the induced state. This system is also desirable because two forms of Tet repressor molecule exist which permit the researcher to either repress or induce transgene expression following administration of tetracycline (or tetracycline derivative). Other inducible gene expression systems do not offer such a degree of flexibility.

Further, the Tet system can efficiently induce or repress transgene expression in mammalian cells at very low concentrations ( $0.1\mu\text{g ml}^{-1}$ ) of inducer. This effective concentration of tetracycline does not exert any other gross pleiotropic effects upon mammalian cells, thus this system permits the examination of transgene mediated effect on cell phenotype without further complication arising in the form of effects mediated by transgene inducer.

The one slight disadvantage regarding the use of this system to regulate transgene expression is that a greater length of time is often required to optimise experimental conditions before it is possible to successfully achieve induction of transgene expression. Two plasmid components must be stably introduced into the host cell and sequentially optimised before transgene expression can be manipulated precisely.



Further, the cytotoxic effects associated with high levels of tetR expression often necessitates screening a greater number of clones than is otherwise required in the other 'two-component' inducible systems (listed in table 1.i.a of chapter 1) before inducible clones are identified. The advantages associated with the application of this system however far outweigh the disadvantages.

The interferon inducible expression system was also selected to achieve these experimental goals, as this system is also capable of tightly regulating transgene expression. The effective inducing concentration of interferon  $\alpha/\beta$  does however exert several pleiotropic effects upon a wide variety of mammalian cells (see section 1.i.8b).

Whilst the cellular effects elicited by interferon  $\alpha/\beta$  may preclude the choice of this conditional expression system for the routine analysis of effects conferred by transgene expression, the application of this conditional expression system to regulate the expression of tumour suppressor genes does open some interesting possibilities with regard to observing tumour cell phenotype. In addition to enabling the investigation of the experimental goals outlined at the beginning of this chapter, the application of this inducible expression system also permits the investigation of possible adjuvant anti-tumour effects resulting from the introduction of p53 gene expression simultaneously in the presence of interferon  $\alpha/\beta$ .

### **(2.i.2) Cloning Strategy of Inducible p53 Expression Constructs**

The p53 cDNA cassette used in the following experiments detailed in this chapter was derived from a previously cloned and characterised version, (from pBKOP53wt, Roger Malcomson (Malcomson, 1996), by a double EcoRI and MluI restriction digest. The resultant 1.9Kb DNA fragment, which includes full-length p53 cDNA sequence, a small intron and SV40 polyA sequence, was then blunt ended with Klenow fragment and cloned into a cut and de-phosphorylated EcoRV site within the polylinker of pBluescript to generate pBSP53.

The p53 cDNA cassette was then orientated relative to the other restriction sites within the pBS polylinker, to determine an appropriate position in which to directionally clone promoter sequence, such that the expression of p53 would be rendered inducible.

**N.B.** all plasmids referred to within sections 2.i.2, 2a and 2b are mapped and included separately within the appendix.

#### **(2.i.2a) Cloning Strategy of Interferon $\alpha/\beta$ Inducible p53 Constructs**

The human 6-16 interferon  $\alpha/\beta$  inducible promoter exists in both a long and a short format (see section 1.i.8a). These will be referred to as the 6-16<sub>L</sub> and 6-16<sub>S</sub> promoter sequences respectively for the remainder of this thesis.

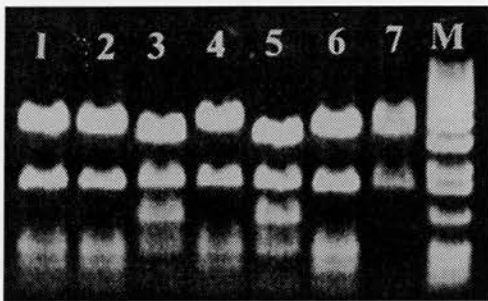
Both 6-16<sub>L</sub> and 6-16<sub>S</sub> promoter sequences were removed from p1CX and p3CX respectively (both plasmids kindly provided by Austin Smith) by a BglIII/HindIII double restriction digest. The resultant 1.1Kb and 0.6Kb fragments were then blunt ended with Klenow fragment and cloned into pBSP53 polylinker sequence

at a cut and Klenow fragment blunted HindIII site to generate p6-16<sub>L</sub>p53 and p6-16<sub>S</sub>p53 respectively.

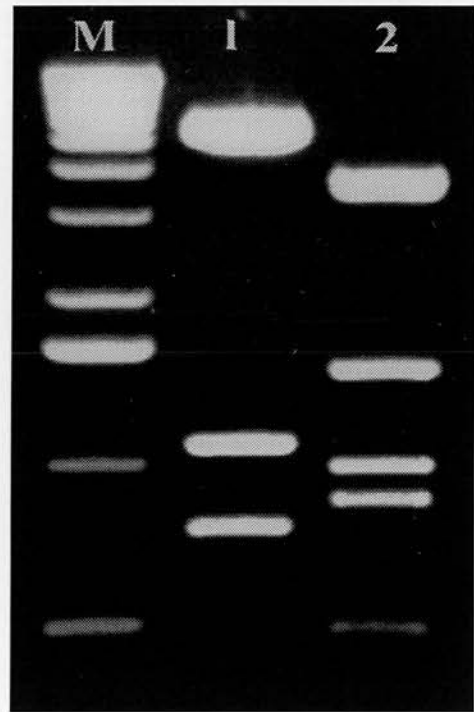
To enable the selection and derivation of stably transfected tumour and ES cell clones with these constructs, a PGK promoter driven Hygromycin resistance cassette was cloned into both p6-16<sub>L</sub>p53 and p6-16<sub>S</sub>p53 at Klenow fragment blunted XbaI and SpeI sites respectively to generate p6-16<sub>L</sub>p53-HYG and p6-16<sub>S</sub>p53-HYG. Bacterial cultures transformed by the latter construct grew very poorly and yielded very low amounts of DNA, therefore only fully constructed p6-16<sub>L</sub>p53-HYG is depicted in figure 2.1 below.

**Fig 2.1**

(i)



(ii)



**Fig2.1** Shows two gel photographs depicting both p6-16<sub>L</sub>p53 (i) and p6-16<sub>L</sub>p53-HYG (ii) constructs. Correctly constructed p6-16<sub>L</sub>p53 will produce a 3.9Kb, 1.6Kb, 1.0Kb, 0.5Kb and 0.48Kb banding pattern following PstI digestion [lanes 3 and 5 of (i)]. Correctly constructed p6-16<sub>L</sub>p53-HYG will produce a 5.9Kb, 1.2Kb and 0.75Kb banding pattern following digestion with EcoRI [lane 1 of (ii)] and a 3.9Kb, 1.6Kb, 1.0Kb, 0.8Kb and 0.48Kb banding pattern following digestion with PstI [lane 2 of (ii)].

### **(2.i.2b) Cloning Strategy of Tetracycline Inducible p53 Construct**

As detailed within the appendix, constructs were also generated to render the expression of transgenic p53 inducible to tetracycline within the cell. Although these constructs were stably transfected into p53 deficient sarcoma cell lines (see section 2.i.3) no further analyses were conducted and the remaining portion of this chapter will detail only experimental work undertaken with the interferon  $\alpha/\beta$  inducible p53 construct, p6-16<sub>L</sub>p53-HYG.

### **(2.i.3) Derivation of p53 Deficient Sarcoma Cell Lines**

Tumour cells that have always lacked p53 gene expression (i.e. tumour cells arising from targeted p53 deficient mice) will theoretically respond better to p53 replacement gene therapy than other tumour cell types that have lost p53 gene function at a later stage of tumourigenesis. In theory, little further selective advantage can be gained from the additional loss of gene function in an already inherited dysfunctional p53 dependent pathway. Thus, re-introduction of p53 gene expression in these cells will more likely restore p53 dependent pathways in comparison to other tumour cells that have lost p53 gene function at a later stage of tumourigenesis.

As such, eight sarcoma cell lines were derived from tumours arising from outbred p53  $-/-$  mice (see section MM4.2 for procedure). These cultures were incubated at 37°C in 5% CO<sub>2</sub>, and were grown up over the course of several weeks to establish multiple freezes of primary tumour cell lines.

Two of the eight primary lines, SL1 and SL5, possessed near uniform morphology and grew efficiently past passage 3. These two lines were selected for subsequent transfection with p6-16<sub>L</sub>p53-HYG.

#### **(2.i.4) Derivation of p6-16<sub>L</sub>p53-HYG Stably Transfected Clones**

Both Sarcoma Line 1 (SL1) and Sarcoma Line 5 (SL5) were plated at a density of  $5 \times 10^6$  cells/10cm dish and were transfected with construct p6-16<sub>L</sub>p53-HYG by lipofectin reagent (see section MM4.2).

Following a 12-day period of hygromycin selection (80µg/ml), 60 SL1 and 20 SL5 hyg<sup>r</sup> (hygromycin resistant) clones were picked and grown further to enable characterisation of transgene expression (again see section MM4.2). All clones were cultured continuously in the presence of hygromycin following transfection.

#### **(2.ii) CHARACTERISATION OF STABLY TRANSFECTED CLONES**

The aim of these experiments was to identify SL sub-clones, stably transfected with the p6-16<sub>L</sub>p53-HYG construct, which exhibited high levels of induced transgene expression following administration of interferon  $\alpha/\beta$  to cell culture media, but which also exhibited very low amounts of transgene expression in the absence of inducer.

##### **(2.ii.1) Optimisation of rtPCR Assay**

The transgene expression levels of all 80 stably transfected clones were initially analysed by rtPCR, as this assay is both highly sensitive and can be further refined to provide comparative quantitation of transgene expression between samples.

To detect gene expression by rtPCR, total mRNA is extracted from either cultured cells or fresh tissue and is used as template for the production of cDNA by the enzyme reverse transcriptase (see materials and methods sections MM2.1 and MM2.4).

Subsequent PCR cycling of this total cDNA, with sequence specific primers, will result in the amplification of template originating from a specific transcriptional product.

To enable the screening of p53 transgene expression levels by rtPCR, primers prP53cDNA and prPA.SEN (see table 2.1), were optimised to amplify transgene specific sequence at the 3' end of the p53 cDNA cassette (see fig 2.2).

Theoretically, correctly spliced p53 transgene transcript no longer possesses intronic sequence but retains both primer annealing sites. Application of these primers to screen transgene expression can therefore permit differentiation of PCR product origin via agarose gel electrophoresis, as the predicted size of amplified product originating from genomic template is 520bp whilst amplified product originating from correctly spliced mRNA is 350bp.

**Table 2.1**

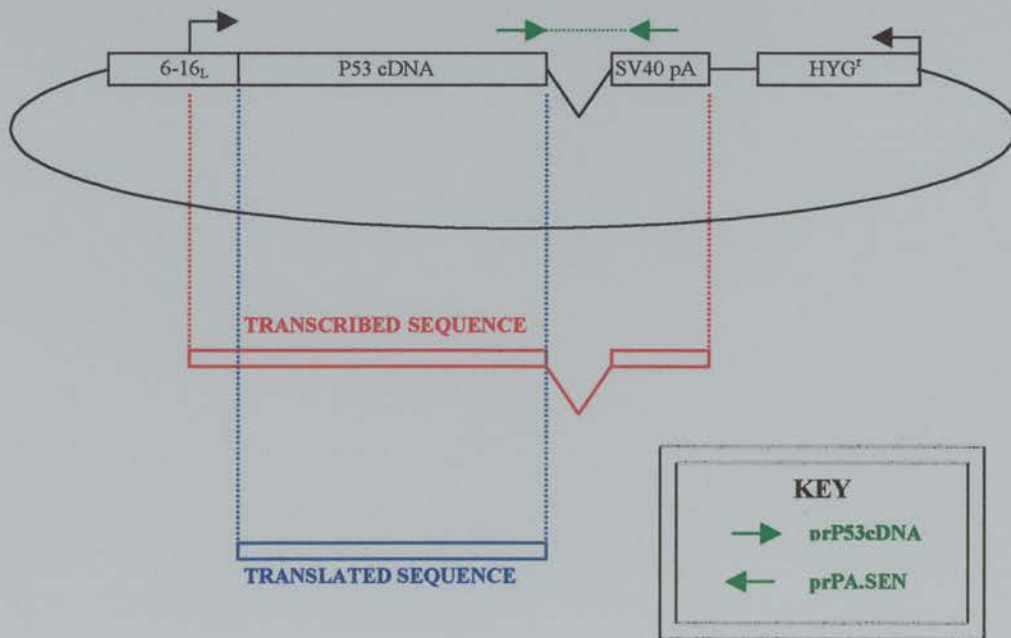
prP53cDNA	5' ATG CTA CAG AGG AGT CTG GA 3'	Located from 1172 to 1191bp on cDNA seq.
prPA.SEN	5' GAT CAT GAA CAG ACT GTG AG 3'	Located from 693 to 712bp on pBKCMV seq.
p $\beta$ -act.A	5' CAT GAA GTG TGA GGT TGA CA 3'	Located at 924bp of $\beta$ -actin cDNA sequence
p $\beta$ -act.B	5' CAG AAG CAA TGC TGT CAC CT 3'	Located at 1650bp of $\beta$ -actin cDNA sequence

To standardise quantified amplification product originating from transgenic mRNA and thus control for template loading error and variability in PCR reaction efficiency between samples (see section 2.ii.3 for discussion), primers were also designed and optimised to amplify a 740bp portion of the constitutively expressed  $\beta$ -actin gene (see table 2.1).

On the evening prior to transgene induction, stably transfected cells were seeded in triplicate, at a density of  $2 \times 10^5$  cells per  $10\text{cm}^2$  (single well of a 6 well plate). The following morning, media was aspirated from cells and replaced with medium containing 0, 500 or 1000 I.R.U (International Reference Units), of interferon  $\alpha/\beta$  (SIGMA) per ml. All clones were grown for a further 48 hours in the presence of (or absence of) interferon  $\alpha/\beta$ , at which time cells were lysed using TRIZOL and RNA extracted for rtPCR analysis (see section MM.2.1 of materials and methods).

Fig 2.2

p6-16<sub>L</sub>P53-HYG



### **(2.ii.2) p6-16<sub>1</sub>p53-HYG Transgene is Inducible, but does not Splice**

Following reverse transcription of mRNA derived from these induced clones, there are a number of possible transgene specific PCR amplification products that can theoretically arise through the application of primers prP53cDNA and prPA.SEN (see fig.2.2).

Amplification product can originate from correctly spliced transgenic cDNA, from unspliced transgenic cDNA and from contaminating genomic DNA carried over during the preparation of mRNA. It is not possible to differentiate the amplification products arising via the latter two origins by agarose gel electrophoresis.

Although reaction conditions were readily optimised to efficiently amplify the background 550bp cDNA/DNA template, conditions could not be optimised to detect the 350bp amplification product, predicted to correspond to correctly spliced transgenic transcript, in any of the stably transfected and induced samples. There are a number of possible scenarios that could explain this result. Firstly, failure to detect correctly spliced transgene cDNA template could simply be the result of poorly optimised PCR reaction conditions. This explanation is unlikely however as the amplification of the related background transgene specific template, using the same pair of primers, is possible through a wide-range of reaction conditions.

Another explanation could be that none of the stably transfected clones possess the ability to induce transgene expression in the presence of interferon  $\alpha/\beta$ . This eventuality could arise from either mutation of the p6-16<sub>1</sub>p53-HYG construct or from an intrinsic inability of the SL cell lines to respond to interferon  $\alpha/\beta$  (i.e.



through mutation or constitutive down-regulation of the cell-surface interferon  $\alpha/\beta$  receptor or other down-stream signalling molecules).

Failure of induced mRNA to correctly splice at the 3' end would also result in the amplification of cDNA template indistinguishable by agarose gel electrophoresis from contaminating genomic DNA. Genomic DNA can be easily removed from mRNA samples, and thus subsequent cDNA samples, by DNase I treatment of the sample prior to reverse transcription. Following rtPCR, this enzymatic step will only permit the production of amplification products originating from either spliced or unspliced transgenic mRNA.

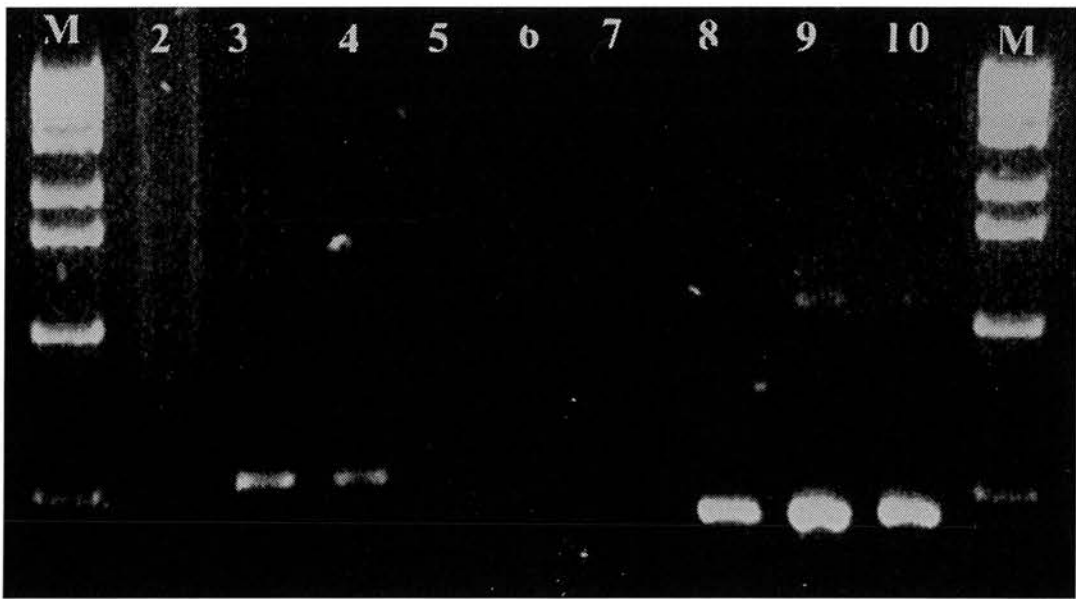
Fig2.3 shows the amplification products produced from cDNA originating from clone 49 (SL1) following DNase I treatment prior to reverse transcription.

Sample lanes (2) to (4) demonstrate that transgene expression originating from p6-16<sub>L</sub>p53-HYG is inducible to interferon  $\alpha/\beta$  and that transgene expression in the uninduced state is very low. Sample lanes (5) to (7) show that following DNase I treatment of these mRNA samples prior to reverse transcription, all traces of contaminating genomic DNA have been removed from each sample. Taken together, these results imply that the induced mRNA transcript never exists in a spliced form, thus explaining the failure to detect cDNA template corresponding to correctly spliced mRNA.

The inclusion of splice acceptor and splice donor sequences within transgene expression constructs, such that they effectively enable the splicing of transgenic mRNA, is believed to enhance translation efficiency and thus the expression of transgenes in mammalian cells (Sambrook *et al*, 1989). As shown in fig.2.2, the

failure of transcript originating from p6-16<sub>L</sub>p53-HYG to splice does not alter or affect final transgene translation product, although it must be considered that efficiency of transcript translation may be compromised. To demonstrate the usefulness of these stably transfected clones in achieving the experimental goals detailed at the start of this chapter, further analysis and characterisation of p53 induction at the protein level is required.

**Fig 2.3**



**Fig 2.3** From left to right, gel lanes (2) to (4) show amplification product originating from uninduced, 500 I.R.U. and 1000 I.R.U. interferon  $\alpha/\beta$  treated samples respectively, following DNase I treatment prior to reverse transcription. Gel lanes (5) to (7) show the amplification products produced from uninduced, 500 I.R.U. and 1000 I.R.U. interferon  $\alpha/\beta$  treated samples respectively, treated with DNase I and not reverse transcribed. Gel lanes (8) to (10) show uninduced, 500 I.R.U. and 1000 I.R.U. interferon  $\alpha/\beta$  treated samples respectively, treated with DNase I prior to reverse transcription but amplified using a set of  $\beta$ -Actin specific primers (sequence not shown).

Following DNase I treatment of all mRNA samples, rtPCR experimentation identified 9, from the total of 80 clones, exhibiting good inducible transgene expression when cultured in the presence of interferon  $\alpha/\beta$ . The remaining clones

were discarded as either no transgene expression was detectable or the basal, non-induced level of transgene expression was high.

5 of the 9 inducible SL clones lost their capacity to proliferate within several passages of identification and so could not be expanded in culture to enable further experimentation. However, of the remaining four expandable clones, three (1D, 16 and 44) were derived from SL1 and one (5A) was derived from SL5.

**N.B.** Time restraints limited much of the experimentation detailed in the remainder of this chapter to the characterisation and investigation of inducible clone 1D only.

### **(2.ii.3) Optimisation of Quantitative rtPCR Assay**

*Maria Wallace primarily undertook the practical work detailed in this section under the supervision of both Dr Alan Clarke and myself, as part requirement for her B.Sc. (Med. Sci.) Hons degree.*

Following the success of the non-quantitative rtPCR approach employed as a means to identify inducible transgene expressing clones, quantitative rtPCR analysis was the method of choice to further analyse the transcriptional kinetics of transgene induction.

Quantitative rtPCR is currently the most sensitive means of detecting levels of specific gene expression when amounts of analytical starting material are limiting. The main advantage of such an approach for these specific experiments therefore, was that it enables the analysis of inducible gene expression kinetics on a relatively small scale, thus reducing consumption of inducing reagent. In

addition, there was no further requirement to design and optimise the use of novel PCR reagents, as this approach uses reagents identical to those employed previously in the non-quantitative rtPCR assay.

To enable the quantitation of initial template copy number via this approach, total mRNA is extracted from samples, reverse transcribed and amplified as detailed in the previous section. The final concentration of transgene-specific amplification product is then quantitated and is used as a measure to determine the relative amounts of initial cDNA template (thus the relative levels of gene expression) present in each of the samples prior to amplification.

To ensure reproducibility in the final concentration of amplification product for a given sample, and to ensure that any observed differences between samples are genuine, several important considerations must be borne in mind when designing and optimising quantitative rtPCR reaction conditions.

Firstly, the amplification of DNA template by PCR does not indefinitely increase exponentially at every cycle and eventually reaches a maximum level of DNA concentration, termed the plateau phase. Both the progressive consumption and saturation of reagents with increasing cycle number and the progressive decrease in Taq DNA polymerase activity following repeat exposure to high temperature are believed to be the underlying reasons responsible for this phenomenon (Reidy *et al*, 1995).

In order for any difference between final PCR amplification product concentrations to be meaningful therefore, it is essential that the concentration of

final rtPCR product be quantitated during the exponential, or log, phase of the reaction, i.e. before the reaction reaches plateau phase.

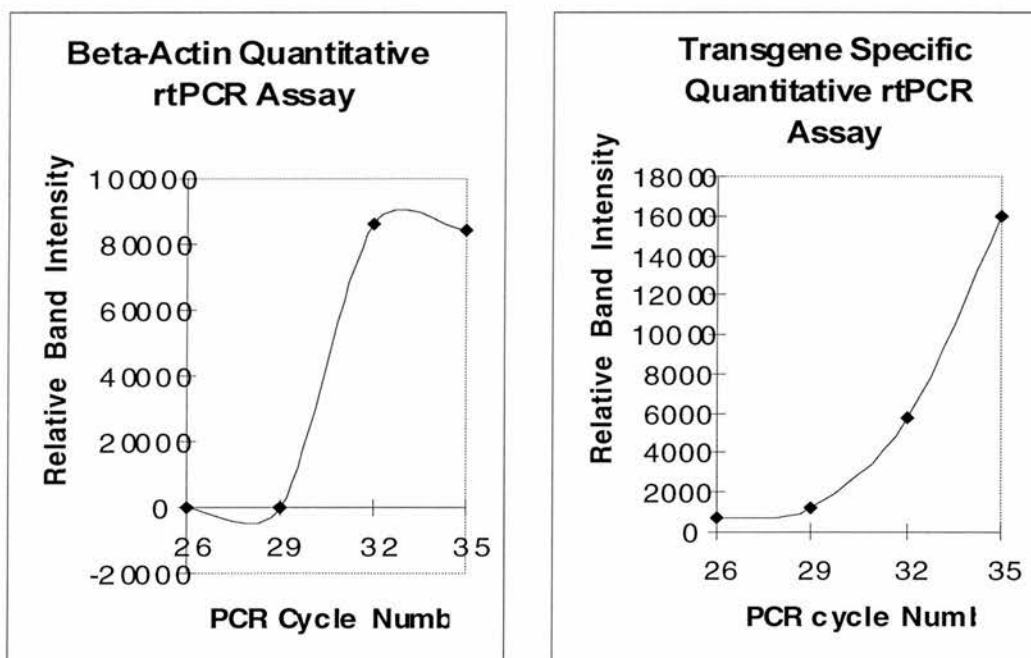
Due to the extreme sensitivity afforded by the PCR technique, it is also essential that any difference arising in final amplification product concentrations between samples are not the result of variation in the amount of extract loaded or from variation in PCR reaction efficiency between samples. The amplification of another gene, typically that of a constitutively expressed or “housekeeping” gene, in the same reaction tube as the gene of interest, can control for these variables if both the amplification reactions are terminated in their respective log phases. Constitutively expressed genes, typically  $\beta$ -actin or G3PDH, are effectively expressed at a consistent level across a diverse range of tissue and cell-types. By standardising the final concentration of amplification product resulting from the gene of interest against the concentration of amplified product originating from a housekeeping gene, it is possible to control for both template loading error and variability in PCR reaction efficiency between samples.

Finally, the presence of trace amounts of contaminating genomic DNA in the mRNA preparation will also theoretically give rise to amplification product. To facilitate the discrimination of amplification product origin from between these two possible sources, gene specific PCR primers should be designed to anneal to exonic sequence separated by a small portion of intronic sequence. In this way, amplification product originating from mRNA will be shorter than amplification product originating from genomic DNA, and hence amplification product

originating from transcriptional product can be readily discriminated from background product by agarose gel electrophoresis.

To determine the log phases for both transgene specific and  $\beta$ -actin specific amplification reactions, PCR reactions were set-up and terminated in triplicate following 26, 29, 32 and 35 completed cycles, using both primer pairs independently and cDNA template derived from clone 1D. 10% of each reaction was then run on a 0.8% agarose gel, and the specific PCR product was quantitated using the Herolab E.A.S.Y. enhanced analysis system. As shown in fig.2.4, both amplification reactions were demonstrated to be in log phase at approximately cycle number 31

Fig 2.4



#### **(2.ii.4) Further Analysis of Transgene Induction**

The aim of this experiment was to apply the quantitative rtPCR assay optimised in the previous section, to further characterise transgene induction by examining the transcriptional kinetics of transgene mRNA production following the exposure of inducible SL cells to interferon  $\alpha/\beta$ .

On the evening prior to transgene induction, inducible clone 1D was seeded in triplicate for each time-point, at a density of  $2 \times 10^5$  cells per  $10\text{cm}^2$ . The following morning, media was aspirated from all cells and replaced with media containing 1000 I.R.U of interferon  $\alpha/\beta$  per ml. All clones remained in the presence of interferon  $\alpha/\beta$  before being lysed with TRIZOL at either 0, 6, 12, 24, 36, 48 or 72 hours post-induction, at which time RNA was extracted to enable analysis by quantitative rtPCR.

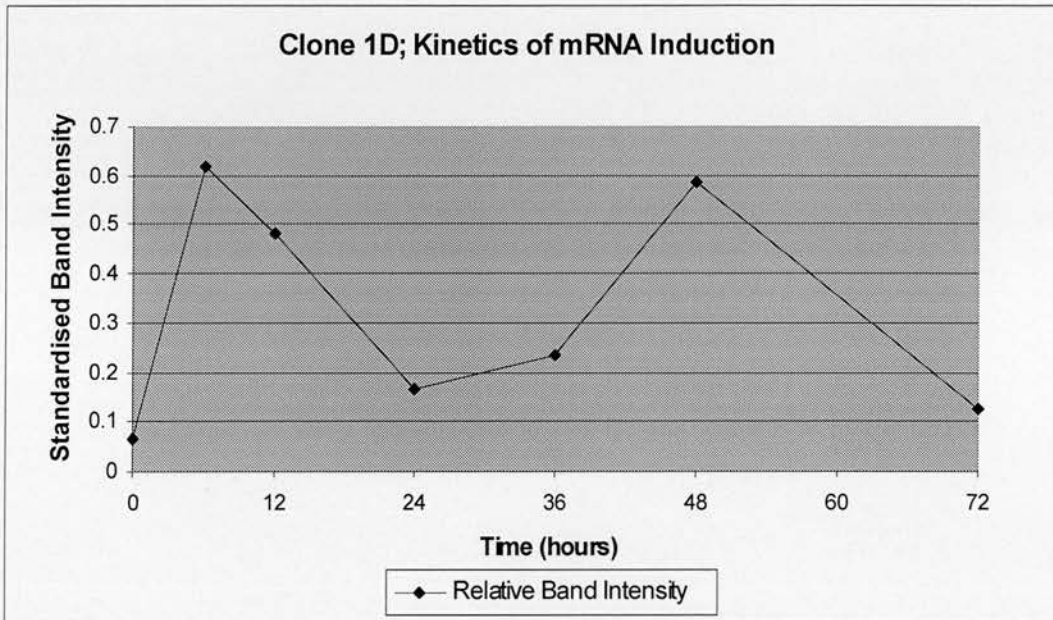
The results of this experiment are presented in the form of a graph in fig.2.5

This data demonstrates that the induction of transgenic mRNA by interferon  $\alpha/\beta$  is rapid, reaching maximal levels within the cell following 6 hours continuous exposure to inducer.

Perhaps the most interesting feature of this data however is that despite the continuous presence of inducer for the duration of the experiment, the transcriptional kinetics of transgene expression varies extensively over time. After rapidly reaching maximal levels of transgene expression at 6 hours, the relative amount of transgenic mRNA drops to near uninduced levels in the cell by 24 hours, before proceeding to rise again to near maximal levels by 48 hours. At

this time, the amount of transgene mRNA falls before reaching relatively low levels in the cell again by 72 hours.

**Fig 2.5**



**Fig2.5** The above graph shows the relative amounts of transgenic mRNA expressed over time following exposure of cells from clone 1D to 1000 I.R.U. of interferon  $\alpha/\beta$ . The final concentration of transgene specific PCR product was standardised against the final concentration of  $\beta$ -actin PCR product from each sample at every time-point.

This data would suggest that the response of SL1 cells to interferon  $\alpha/\beta$  is rapid, but following continuous stimulation of these cells, the response becomes modulated and by 24 hours SL1 cells are held in a transitory non-responsive state.

The data depicted in figure 2.5 should be considered as semi-quantitative however, as opposed to precisely quantitative, as although the general pattern of observed mRNA induction was reproducible utilising both clone 1D and 5A, the relative standardised band intensities varied considerably between identical induction experiments. As the observed pattern of transgene induction was



reproducible between clones and induction experiments, it is likely that the observed modulation of induced transgenic mRNA levels relative to constitutively expressed mRNA are not artifactual. Indeed, the results described within section 2.ii.6 demonstrate that the percentage of p53 protein expressing cells over time follows a comparatively similar pattern to the kinetics of p53 mRNA induction. This would further suggest that the observed kinetics of p53 mRNA induction is genuine.

It should also be noted however that this pattern of interferon induced transgene expression, mediated through the human 6-16 promoter, has not been previously described. Indeed, previous application of this system in ES cells reported both rapid and stable induction of reporter transgene expression following 50 hours of continuous stimulation with interferon  $\alpha/\beta$  (Whyatt *et al*, 1993).

The levels of transgenic mRNA were not investigated in the Whyatt study, so it may be possible that the nature of this observed difference in the kinetics of transgene induction is the result of cell-type specific responses to interferon  $\alpha/\beta$ . Hypothetically, a transitory induction of IRF-2 expression within SL but not ES cells may be able to influence interferon  $\alpha/\beta$  mediated gene transactivation sufficiently to produce these observed cell-type specific differences in transgene expression level (see section 1.i.8a). A further hypothesis could be that SL cells become somehow 'de-sensitised' following continuous signalling from the interferon  $\alpha/\beta$  receptor, whilst ES cells do not.

It could further be postulated that these observed differences in transgene induction kinetics are simply the result of differences in induced protein stability. It is possible that the translated reporter gene product observed in the Whyatt

study is stable in ES cells, whilst p53 protein is known to be unstable and rapidly turned-over in many unchallenged cell-types (see section 1.ii.3).

#### **(2.ii.5) Characterisation of Transgene Induction at the Protein Level**

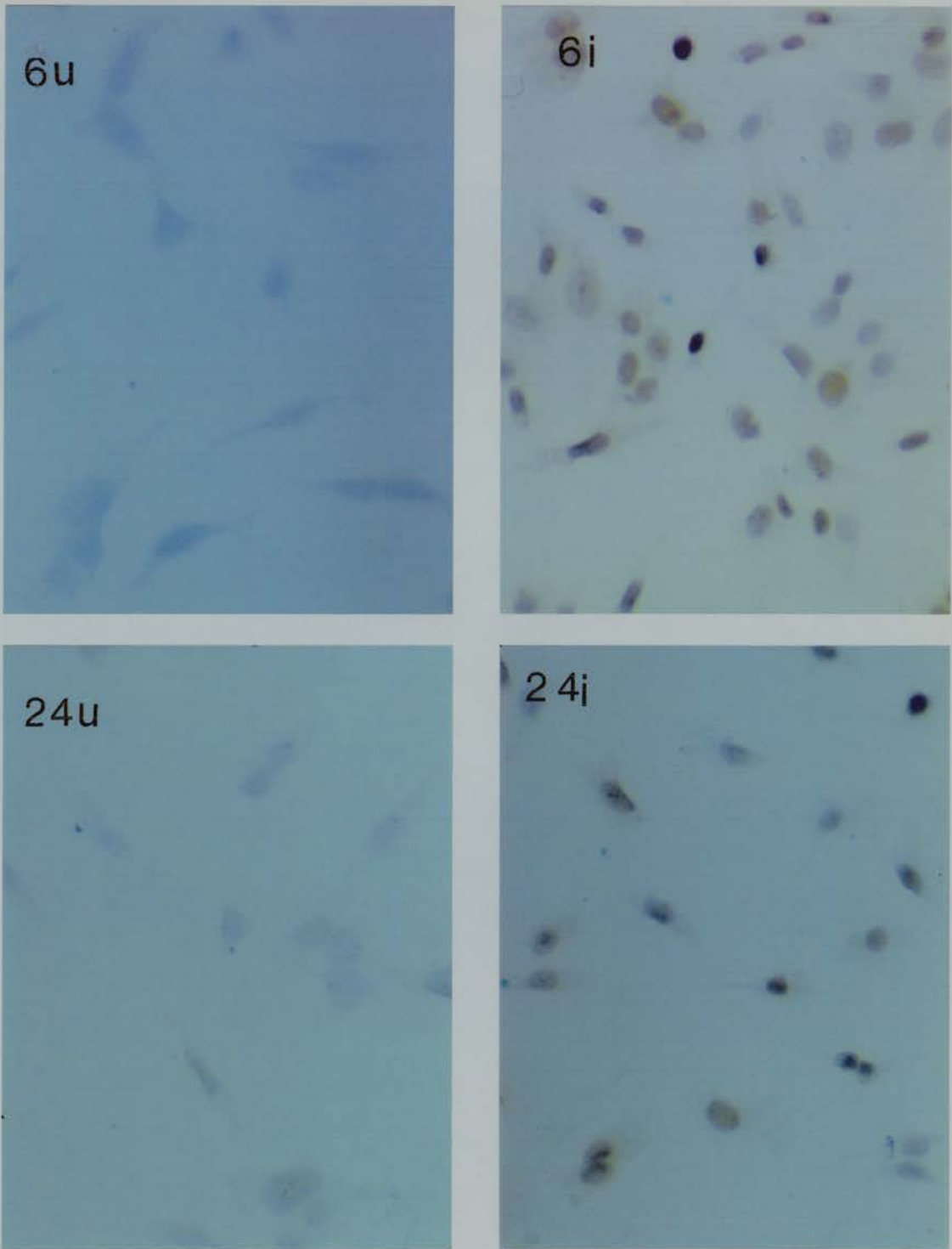
The aim of this experiment was to prove by immuno-staining of induced cells grown *in vitro*, that the translation product corresponding to previously detected transgenic mRNA was p53.

On the evening prior to transgene induction, inducible clone 1D was seeded onto chamber-slides at a density of  $3 \times 10^4$  cells per chamber well (see MM3.2). Each chamber-slide contains two chamber wells, therefore on the morning following plating, media was aspirated from both wells of every slide and replaced with media containing either 0 or 1000 I.R.U. of interferon  $\alpha/\beta$  per ml.

Wild-type p53 protein is known to stabilise following DNA damage, so all cells were exposed to  $10 \text{ J/m}^2$  UV C irradiation 6 hours following induction (time-point corresponding to maximal levels of transgenic mRNA, section 2.ii.4) in an attempt to enhance the detection of p53 protein by this approach. Following irradiation, all cells were returned to media conditions identical to those prior to genotoxic insult.

Media was aspirated at every time-point and the cells were washed once in PBS before being fixed in 50% methanol: 50% acetone for 10 minutes at room temperature. Following fixation, the cells were air-dried and stored at  $-80^\circ\text{C}$  prior to immuno-staining. The time-points analysed were 0, 6, 12, 24, 48 and 72 hours after genotoxic insult.

Fig.2.6



**Fig2.6** - shows the results of immunochemistry performed on populations of both uninduced (U; 0 I.R.U. interferon  $\alpha/\beta$ ) and induced (I; 1000 I.R.U. interferon  $\alpha/\beta$ ) 1D cells. Staining was achieved using the anti-p53 monoclonal antibody, Pab421. **N.B.** The times used to head each photograph are times following genotoxic insult. (Magnification 500X).

The immunochemistry performed on these samples used the monoclonal antibody Pab421 (see section MM.3.2 for protocol) and the results of are shown in Fig.2.6. No p53 expression can be detected within non-induced 1D cells growing *in vitro* 6 hours after exposure to DNA damage, however strong p53 expression is detected within induced cultures of 1D cells at a corresponding timepoint. At later timepoints (24 hours after DNA damage), again p53 expression can barely be detected within cultures of non-induced 1D cells, whilst p53 expression is again readily detected within induced 1D cells at a corresponding timepoint.

The results of this experiment conclude that following stable transfection of construct p6-16<sub>L</sub>p53-HYG, it is possible to induce p53 expression in response to interferon  $\alpha/\beta$ .

#### **(2.ii.6) Kinetics of Transgene Translation**

The results described in section 2.ii.5 confirm that p53 protein can be detected at all times following administration of interferon  $\alpha/\beta$ . The aim of this experiment was to measure the proportion of p53-positive cells over time, via the application of an immuno-flow cytometry method (see section MM.3.4 of materials and methods).

On the evening prior to transgene induction, inducible clone 1D was seeded at a density of  $2 \times 10^5$  cells per  $10\text{cm}^2$ . The following morning, media was aspirated from all cells and replaced with media containing 1000 I.R.U. of interferon  $\alpha/\beta$  per ml. At each time-point, cells were trypsinised and frozen at  $-20^\circ\text{C}$ . The time-points analysed were 0, 6, 12, 24, 36, 48 and 72 hours post-induction.

Following completion of the experimental time-course, all samples were defrosted and labelled with an anti-p53 1° anti-body (Ab-1)/ FITC conjugated 2°, before being run on a gated flow cytometer to determine the percentage of fluorescent cells in each sample. Untransfected SL1 (p53 deficient) cells were also labelled and included in this experiment to control for the effects of cellular auto-fluorescence within samples. Non-specific FITC background staining was controlled as specified at point seven of protocol MM3.4 within the materials and methods section.

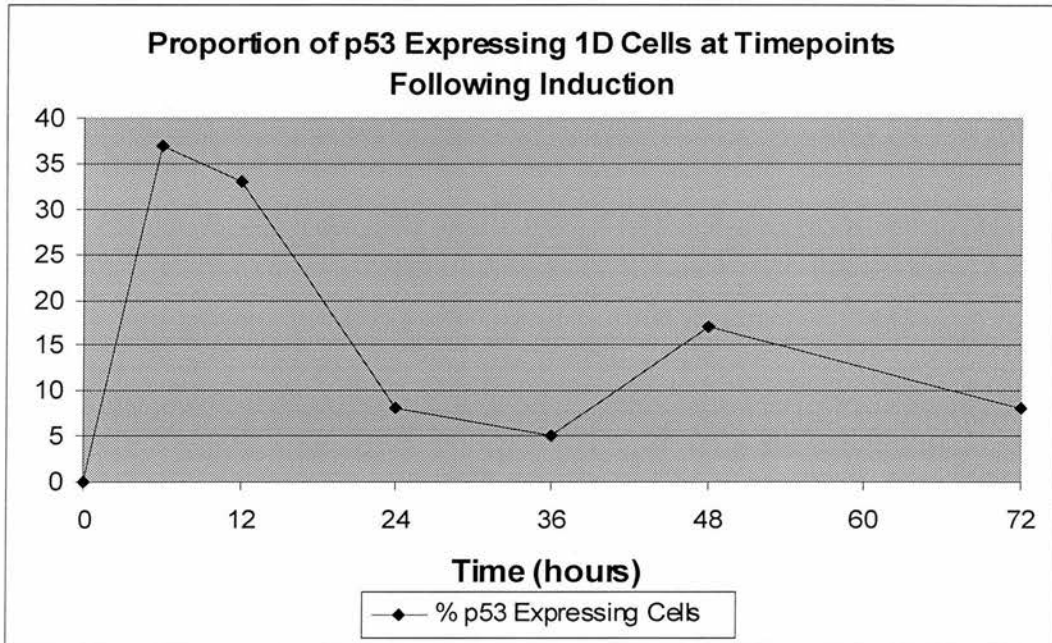
The results of this experiment are shown in fig.2.7. The percentage of cells expressing detectable levels of transgene is maximal at 6 hours post-induction (approx. 37%), however this figure falls between 6 hours to 36 hours, where only 5% of all cells express p53. Expression levels rise again between 36 and 48 hours, before falling once more at 72 hours.

The kinetics of transgene translation, as determined by this flow cytometry technique, are similar to the kinetics of transgene transcription. This would suggest that rapidly following translation of transgenic mRNA, the protein product is rapidly degraded within SL1 cells.

However, despite the success of this immuno-flow cytometry technique to detect and discriminate between both transgene expressing and non-expressing cells, the ability of this technique to exactly quantitate the percentage of transgene expressing cells appears to be inconsistent. Upon subsequent repetition of this experiment, the exact percentage value of expressing cells was variable, despite high consistency of the overall observed kinetics of transgene expression. One

must conclude therefore, that this technique is only suitable for the approximate quantitation of p53 expressing cells, thus permitting the observation of trends in transgene expression.

**Fig.2.7**



### **(2.iii) The Effect of p53 Expression upon Tumour Cell Cycle *In Vitro***

The aim of this experiment was to investigate the possible effects upon tumour cell cycle *in vitro*, resulting from the induction of p53 gene expression, in both the presence and absence of UV-C mediated DNA damage through time.

On the evening prior to transgene induction, cells from clone 1D were plated in triplicate for each time-point, at a density of  $2 \times 10^5$  cells per  $10 \text{cm}^2$ . The following morning, media was aspirated from the cells and replaced with media containing either 0 or 1000 I.R.U. of interferon  $\alpha/\beta$  per ml.

Four experimental conditions were investigated; 1D cells uninduced without damage; 1D cells induced without damage; 1D cells uninduced with damage and 1D cells induced with damage.

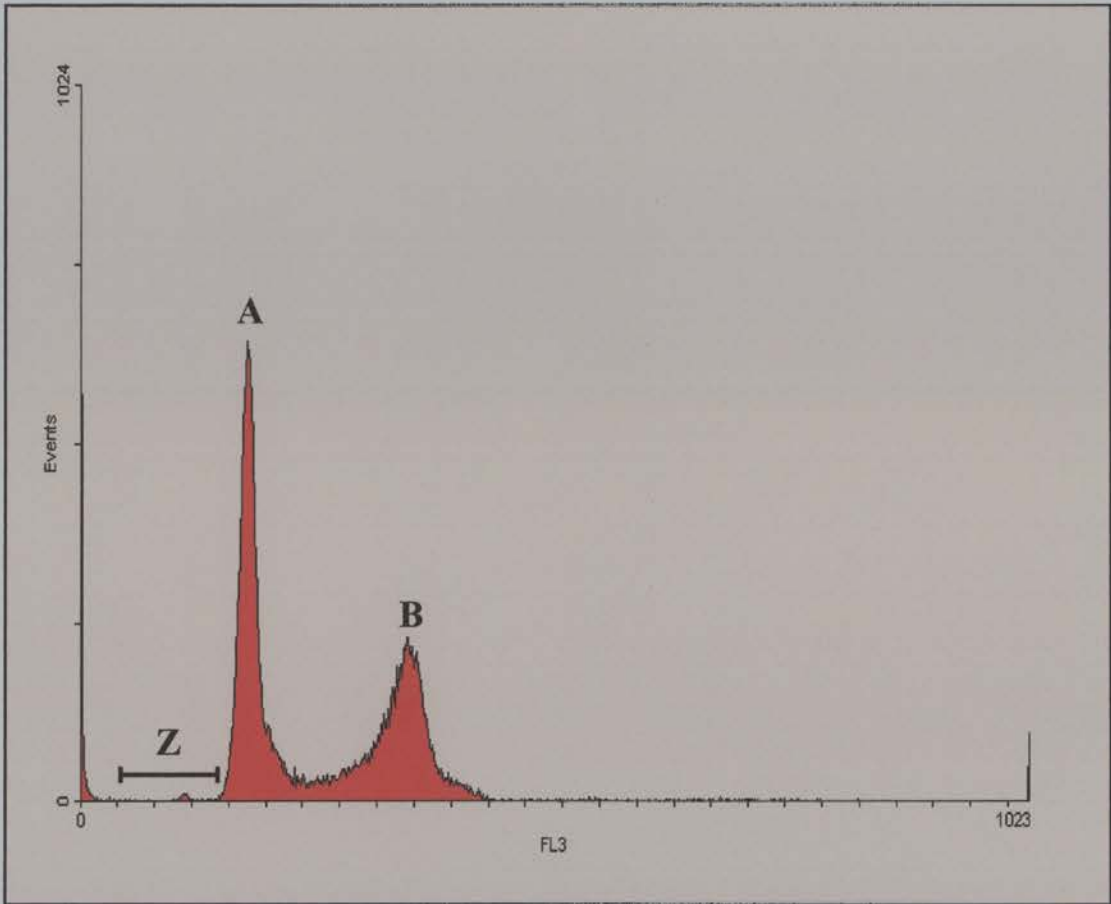
To investigate the effects of p53 induction plus DNA damage upon tumour cell cycle, the media from all populations of cells intended for damage was aspirated 6 hours following induction and exposed to 10 J/m<sup>2</sup> UV C irradiation. These cells were then returned to media conditions identical to those prior to damage. The time-points analysed were 0, 6, 12, 24, 36, 48 and 72 hours post-induction (where 6 hours post-induction is equivalent to 0 hours post-damage).

Flow cytometry and Vindalov-type analysis (see section MM4.3) was subsequently performed to determine the proportion of cells in each compartment of the cell cycle (i.e. G1/G0, S or G2/M) and thus to determine the extent of transgene mediated effect upon tumour cell cycle.

To control for any possible phenotypic effects conferred by interferon  $\alpha/\beta$  upon tumour cell cycle, it was essential that transgene<sup>-ve</sup> SL1 cells be examined under conditions identical to those as detailed for clone 1D. Only in this manner is it possible to dissect transgene-mediated effects from effects conferred by transgene inducer upon tumour cell cycle.

Flow analysis of the parental, non-transfected SL1 cells demonstrated the presence of multiple, large aneuploid populations of cells within the total cell population. These cells were therefore unsuitable to act as the control population in this experiment, as the presence of multiple aneuploid populations of cells “confuses” the cell cycle analysis software package (XL, Coulter) which in turn

**Fig.2.8**



**Fig.2.8** depicts a typical cell cycle profile of tumour cell 1D following Vindalov-type and flow cytometric analysis (see section MM4.3). The proportion of cells in G1/G0 of the cell cycle corresponds directly to the percentage of events scored at peak A. Similarly, the proportion of cells in G2 of the cell cycle corresponds to the percentage of events scored at peak B. The proportion of cells in S phase can be calculated by scoring the number of events arising between both the G1/G0 (A) and G2 (B) peaks. The proportion of cells undergoing apoptosis can also be ascertained from this histogram by scoring the number of sub-G1 events. In practical terms this can be achieved by scoring the number of events arising within zone Z.



results in aberrant and inaccurate placing of cells into the various cell cycle compartments. To facilitate meaningful analysis of the cell cycle by this flow cytometry method therefore, it is essential that the analysed population of cells possess a near uniform chromatin content.

Two p53-transgene<sup>-ve</sup> sub-clones, M25 and M48, were derived from the parental SL1 line following lipofection of a puromycin resistance cassette and the selection of puro<sup>res</sup> (1µg/ml puromycin), stably transfected clones. These two lines were shown to possess uniform chromatin content by flow cytometry and thus were suitable to act as the p53-transgene<sup>-ve</sup> control cell populations in this experiment.

### **(2.iii.1) p53 Induction and the Cell Cycle**

The effects of transgene induction upon tumour cell cycle, in the absence of DNA damage, are shown in figure 2.9 (**N.B.** – Only one control data set (M48) is shown in comparison to the experimental inducible clone 1D in Fig.2.9). The data set generated from the other transgene<sup>-ve</sup> control line, M25, supports the trends exhibited by clone M48 in all experimental conditions, therefore there was little need to additionally reproduce this control data in the following figures [fig2.9, and fig 2.10]).

At the beginning of the time course, uninduced 1D cells appear to possess a large proportion of cells in S-phase of the cell cycle, indicating very rapid cellular proliferation. At later time-points however (post-48hours) the proportion of cells in S-phase drops and begin to accumulate in G1/G0-phase. This shift in the

percentage of proliferating cells to non-proliferating cells is coincident with 1D cells reaching confluence on the tissue culture plate.

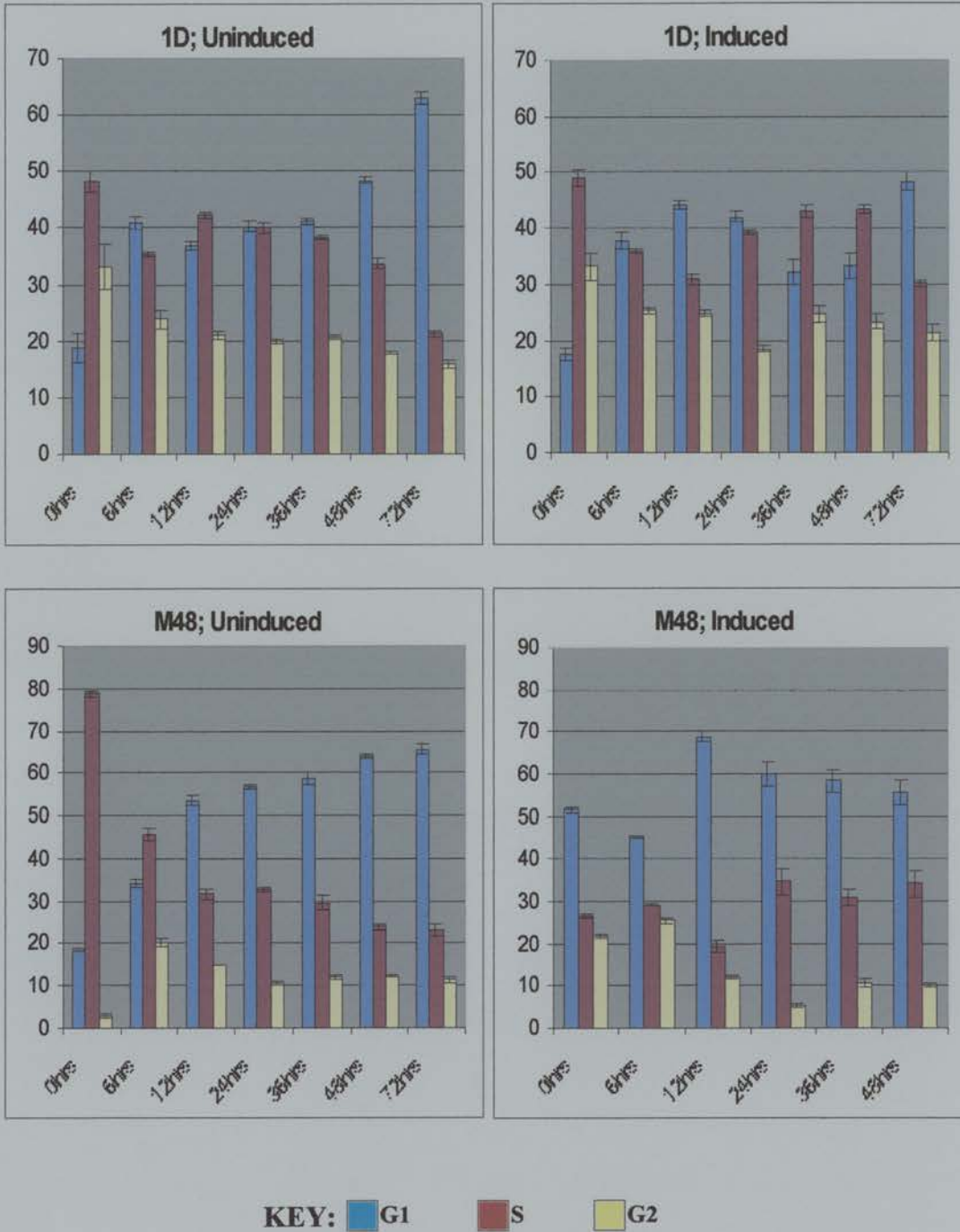
Similarly to the population of uninduced 1D cells, induced 1D cells initially possess a high proportion of cells in S-phase of the cell cycle, indicative of rapid cellular proliferation. Overall however, this data would suggest that induced 1D cells proliferate slower than uninduced 1D cells.

Within the population of induced cells, the proportion of cells in S-phase only reach levels greater than 40% 24 hours post-induction. Uninduced cells in comparison possess this proportion of cells in S-phase by 12 hours. Further, induced 1D cells only reach confluence and begin to drop out of S-phase into G1/G0 at 72 hours post-induction, thus reaching confluence a full 24 hours later than uninduced cells.

To determine whether this effect on the proliferation rate of 1D cells is transgene or inducer mediated, it is necessary to examine the cell cycle profile of M48 cells cultured in both the presence and absence of interferon  $\alpha/\beta$ .

Uninduced M48 cells initially possess a very high proportion of cells in S-phase, however between 6 and 36 hours, the proportion of cells in S-phase progressively stabilises to approximately 30% of all cells. By 48 hours, the proportion of cells in S-phase begins to fall and to accumulate in G1/G0, coincident with M48 cells reaching confluence on the tissue culture dish.

**Fig.2.9**



**Fig2.9** - shows the percentage of 1D and M48 cells in each compartment of the cell cycle when cultured in either the absence or presence of interferon  $\alpha/\beta$  (values are expressed as the mean  $\pm$  SD). The figures written on the Y-axis correspond to percentage of cells from total cell population.

Induced M48 cells however only possess greater than 30% of cells in S-phase by 24 hours, a level that is stable through to 48 hours. The 72 hour time-point is missing from this data, but it is clear that by 48 hours uninduced M48 cells drop out of S-phase into G1/G0, whilst induced M48 cells do not.

It must be concluded that this observed slowing in proliferation rate of SL1 tumour cells is interferon  $\alpha/\beta$  mediated as both transgene positive and transgene negative SL1 cells respond in essentially an identical manner.

Further, as induced experimental samples are exposed to inducer at all times of this experiment, one must also conclude that the proliferation rate of SL1 cells is only initially inhibited by interferon  $\alpha/\beta$ . This inhibition can only be temporary, as induced SL1 cells are able to resume a proliferative rate approximate to that of uninduced cells, as well as possessing the ability to reach confluence, within approximately 24 hours of non-treated cells.

#### **(2.iii.2) Effects of p53 Induction and DNA Damage upon Tumour Cell Cycle**

The effects of transgene induction upon tumour cell cycle, in the presence of DNA damage, are shown in figure 2.10

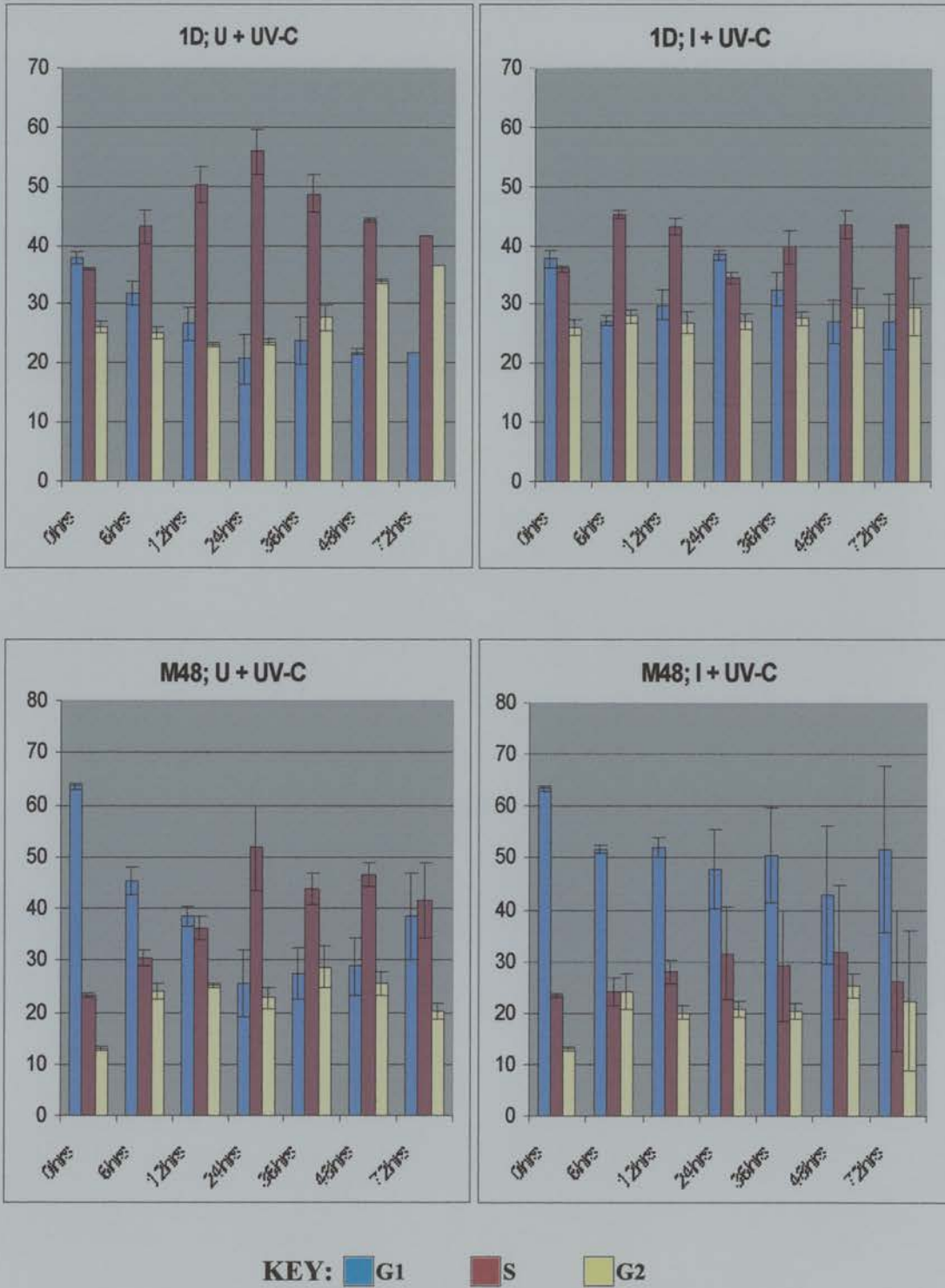
**(N.B.-** the time-scale depicted on the X-axes of the graphs displayed in fig.2.10 are not equivalent to the times depicted in fig.2.9. Zero hours, as depicted in fig.2.10 is equivalent to zero hours genotoxic insult (exposure to  $10 \text{ J/m}^2$  UV-C irradiation) or to 6 hours post-induction as depicted in fig.2.9.

At the time of genotoxic insult, uninduced 1D cells appear to predominantly reside in G1/G0 of the cell cycle. Immediately following damage however, cells exit G1/G0 and accumulate progressively into S-phase of the cell cycle reaching peak levels (approx. 55% of all cells) by 24 hours.

The number of cells in G2/M remains relatively constant between 0 and 24 hours suggesting that the accumulation of cells in S-phase is the result of an increase in the rate of cellular proliferation and not the result of a block in the cell cycle between S and G2/M phases. At later stages of the time-course (between 24 and 72 hours), the proportion of cells in G2/M rises, most likely as a consequence of the high proportion of cells in S-phase at 24 hours progressing through the cell cycle.

Induced cells also appear to reside predominantly in the G1/G0 portion of the cell cycle at the time of genotoxic insult. The main difference between the cell cycle profiles of induced and uninduced 1D cells however, is that throughout the duration of the time-course, cells treated with interferon  $\alpha/\beta$  exhibit only a slight increase in the rate of proliferation, reaching maximal levels (approx. 45% of all cells) by 6 hours. No proliferative burst is observed at 24 hours and this rate of proliferation is essentially maintained for the duration of the time-course experiment.

**Fig.2.10**



**Fig2.10** - shows the percentage of 1D and M48 cells in each compartment of the cell cycle when cultured either in the presence or absence of interferon  $\alpha/\beta$  and in the presence of UV-C induced DNA damage (values are expressed as the mean  $\pm$  SD). The figures written on the Y-axis correspond to percentage of cells from total cell population.

To determine whether this observed tumour cell phenotype is transgene or inducer mediated, it is necessary to examine the cell cycle profile of DNA damaged M48 cells, cultured in both the presence and absence of interferon  $\alpha/\beta$ .

Uninduced M48 cells exhibit a similar cell cycle profile following DNA damage to that observed for uninduced 1D cells. At the time of genotoxic insult uninduced M48 cells predominantly reside in the G1/G0 phase of the cell cycle. Immediately following DNA damage however, these cells exit G1/G0 and accumulate in S-phase, reaching peak levels (52% of all cells) by 24 hours.

Again, the number of cells in G2/M remains relatively constant throughout the course of the experiment, suggesting that the accumulated cells in S-phase are the result of an increase in proliferation rate and not of a block in the cell cycle between S and G2/M.

M48 cells treated with interferon  $\alpha/\beta$  also appear to exhibit a similar cell cycle profile following DNA damage to that observed for induced 1D cells. At the time of DNA damage, treated M48 cells reside predominantly in the G1/G0 portion of the cell cycle. This cell cycle profile is essentially maintained throughout the duration of the experiment, although there is a slight observed increase in the proportion of cells in S-phase, exiting from G1/G0, reaching maximum levels (32%) by 24 hours.

It must be concluded therefore that inhibition of SL1 cell proliferation following DNA damage is interferon  $\alpha/\beta$  mediated as both transgene positive and transgene negative SL1 cells respond in essentially an identical manner.

### **(2.iii.3) Conclusions from Section (2.iii)**

Interferon  $\alpha/\beta$  temporarily inhibits the proliferation of SL1 tumour cells in the absence of damage, and also inhibits the rapid increase of SL1 tumour cell proliferation following DNA damage.

The introduction of p53 expression appears to have no effect upon SL1 tumour cell cycle in either the presence or absence of DNA damage. The existence of any transgene mediated phenotypic effects must therefore be slight, as they are not detectable by flow cytometric analysis.

### **(2.iv) The Effect of p53 Expression upon the Rate of Apoptosis *In Vitro***

The aim of this experiment was to investigate the rate of SL1 tumour cell apoptosis following induction of p53 expression, in both the presence and absence of DNA damage.

On the evening prior to transgene induction, cells from clone 1D were plated in triplicate for each time-point, at a density of  $2 \times 10^5$  cells per  $10\text{cm}^2$ . The following morning, media was aspirated from the cells and replaced with media containing either 0 or 1000 I.R.U of interferon  $\alpha/\beta$  per ml.

Four experimental conditions were investigated; 1D cells uninduced without damage; 1D cells induced without damage; 1D cells uninduced with damage and 1D cells induced with damage.

To investigate the effects of p53 induction plus DNA damage upon the rate of tumour cell apoptosis, the media from all populations of cells intended for damage was aspirated 6 hours after induction and exposed to  $10 \text{ J/m}^2$  UV-C irradiation.



These cells were then returned to media conditions identical to those prior to damage. The time-points analysed were 0, 6, 12, 24, 36, 48 and 72 hours post-induction (where 6 hours post-induction is equivalent to 0 hours post-damage).

At each time point, the medium from each sample was aspirated and reserved prior to trypsinisation. Following trypsinisation of adherent cells, the reserved medium was subsequently used to neutralise trypsin activity for every corresponding sample. The rate of tumour cell apoptosis was determined as the percentage of cells sub-G1/G0, following Vindalov-type analysis and flow cytometry (see section MM4.3). The data generated by this method was confirmed to reflect apoptosis following staining of samples with Acridine Orange and manual counting of apoptotic bodies by fluorescence microscopy.

#### **(2.iv.1) Effects of p53 Induction upon the Rate of Tumour Cell Apoptosis**

As shown in figure 2.11, the induction of p53 expression in the absence of DNA damage had no measurable effect upon the rate of apoptosis of SL1 cells in culture. The percentages of apoptosis shown in this diagram are equivalent to the levels of apoptosis in untreated samples, therefore it is also possible to conclude that the transgene inducer, interferon  $\alpha/\beta$ , exerts no measurable effect upon the rate of apoptosis *in vitro*.

Following DNA damage of SL1 tumour cells, a transgene dependent apoptotic phenotype is observed in cell line 1D (see fig.2.12). All untreated SL1 cell lines exhibit levels of apoptosis equivalent to background until 12 hours post-damage.

Fig.2.11

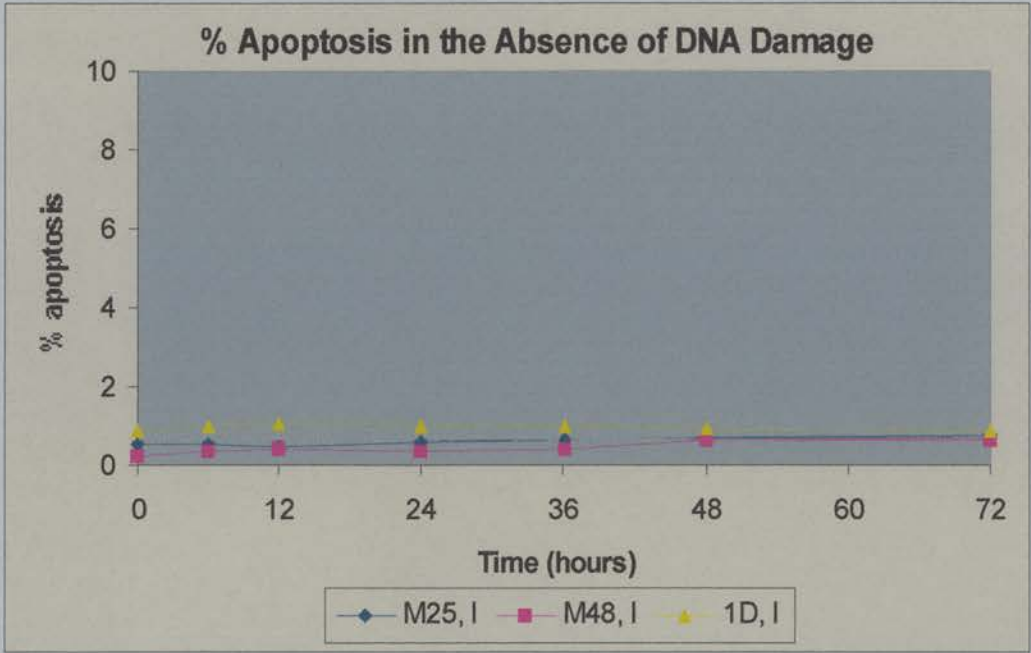


Fig.2.11 shows the percentage apoptosis of SL1 cell lines M25, M48 and 1D following administration of 1000 I.R.U. interferon  $\alpha/\beta$  at time-point 0 hours, in the absence of DNA damage. (Values are expressed as the mean  $\pm$  SD).

By 24 hours, the levels of apoptosis have risen above the level of background observed in non-DNA damaged cultures. The percentage of apoptosis in experimental line 1D is also marginally elevated above the levels of both control lines M25 and M48, however the rate of increase between all cell lines is comparable. The reason for this elevation in the apoptotic rate of 1D cells could be either the result of “leaky” transgene expression in the uninduced state, or simply because of an intrinsic difference in the properties of cell lines derived from the parental SL1 line.

By 48 and 72 hours, levels of apoptosis are maximal (approximately 4%), with no significant difference recorded between the rates of experimental line 1D and both control lines.

DNA damaged and interferon  $\alpha/\beta$  treated SL1 cells again show little difference from the level of background apoptosis observed in non-DNA damaged cultures at early time-points. By 12 hours however, the percentage of apoptosis of induced 1D cells begins to increase above the level of apoptosis detected in both control lines. By 24 hours the rate of apoptosis in induced 1D cells has reached maximal levels (approx. 8%), which is approximately 24 hours faster and at nearly double the rate of uninduced 1D cells. The level of apoptosis in the 1D samples remains high through 36 hours, from which time the level of apoptosis becomes no longer significantly different from the control lines.

The apoptosis levels of both treated control lines are essentially identical to those observed in the non-treated control lines following DNA damage. This result further confirms that interferon  $\alpha/\beta$  exerts no measurable effect upon the rate of apoptosis of SL1 cells *in vitro*.

#### **(2.iv.2) Conclusions from Section (2.iv)**

Interferon  $\alpha/\beta$  exhibits no measurable effect upon the rate of SL1 cell apoptosis in either the absence or presence of DNA damage. The introduction of p53 expression also appears to have no effect upon the rate of SL1 cell apoptosis in the absence of DNA damage.

In the presence of DNA damage however, populations of induced 1D cells reach maximal levels of apoptosis by 24 hours. This time-point is 24 hours faster than non-induced and damaged 1D cells. Further, the maximal level of apoptosis reached by induced and damaged 1D cells is approximately double that reached by non-induced and damaged 1D cells. These results clearly demonstrate the

existence of both p53 dependent and independent waves of apoptosis elicited by the cell following DNA damage.

Fig.2.12

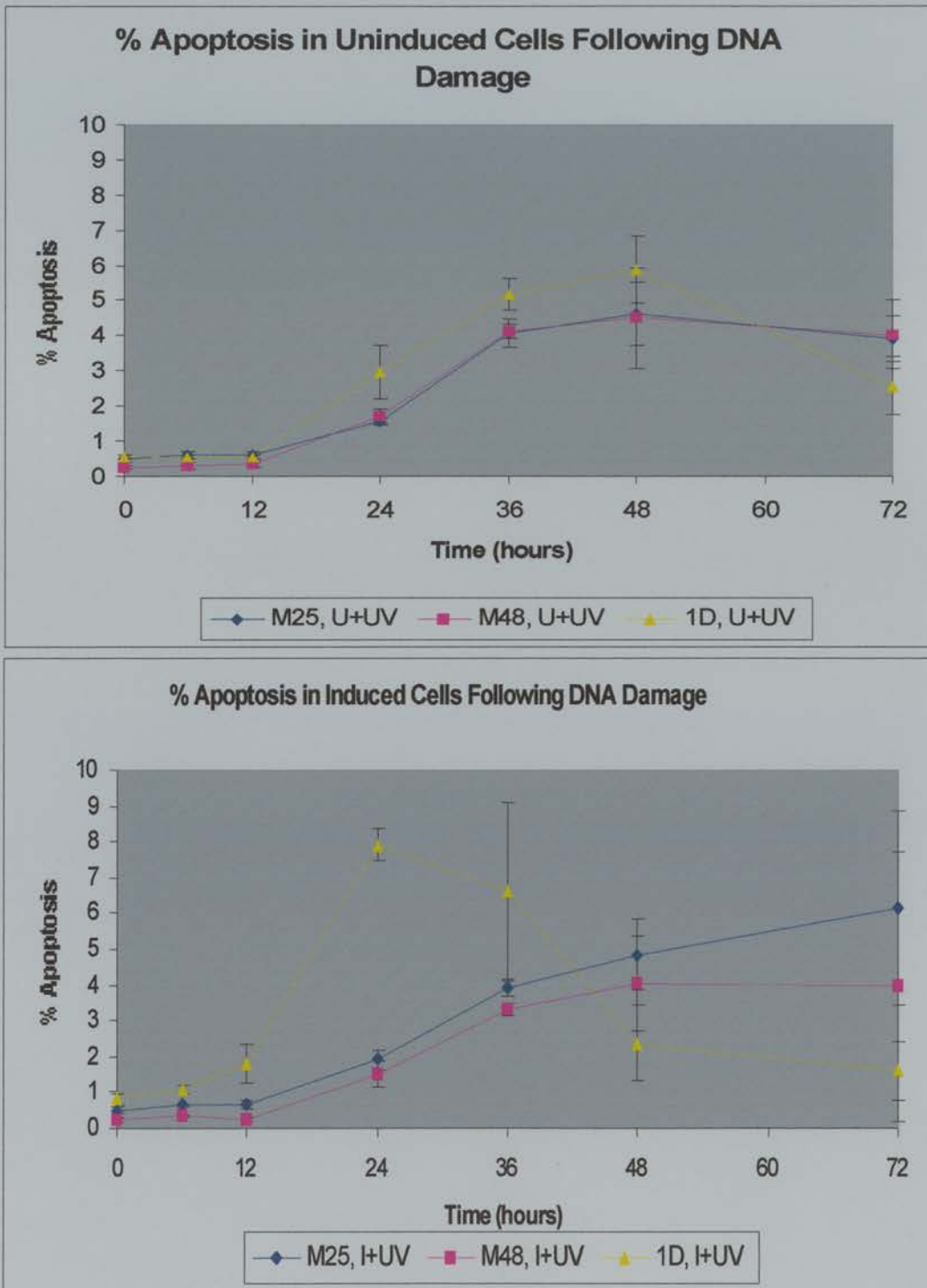


Fig.2.11 shows the percentage apoptosis of SL1 cell lines M25, M48 and 1D following administration of 1000 I.R.U. interferon  $\alpha/\beta$  at timepoint 0 hours, in the absence of DNA damage. (Values are expressed as the mean  $\pm$  SD).

## **(2.v) The Long Term Consequences of Transgene Dependent Effects *In Vitro***

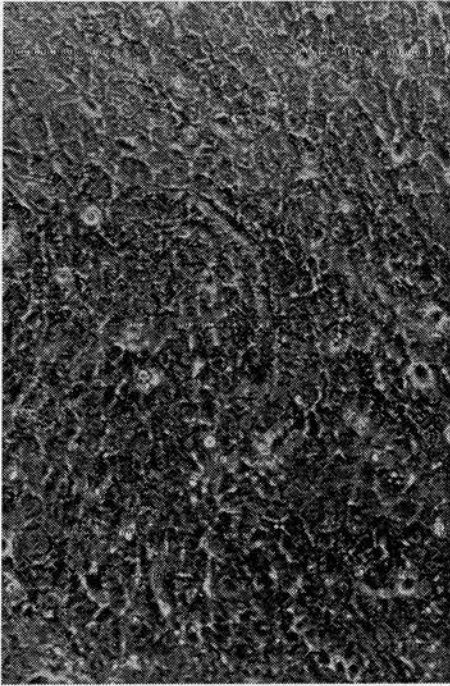
The aim of this experiment was to determine the long-term consequences of transgene mediated apoptosis upon tumour cell viability by means of a clonogenic survival assay.

The immediate consequences of transgene dependent apoptosis are illustrated in Figure 2.13. These photographs of 1D cells were taken at a time-point equivalent to 36 hours after DNA damage and clearly demonstrate that the short-term viability of 1D tumour cells, when cultured in both the presence of interferon  $\alpha/\beta$  and DNA damage, are severely compromised (Magnification 500X).

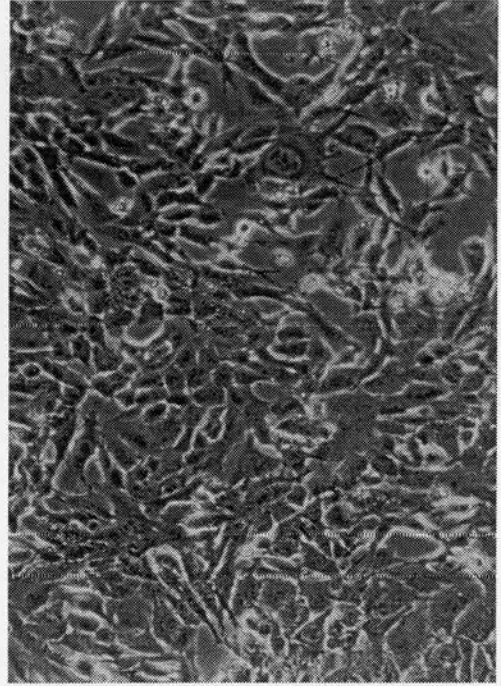
To determine the long-term consequences of transgene dependent apoptosis, both M25 and 1D cells were plated out at a density of  $5 \times 10^6$  cells per 10cm petri dish on the evening prior to transgene induction. The following morning, all cells were re-fed in media containing either 0 or 1000 I.R.U. interferon  $\alpha/\beta$ . At 6 hours post-induction, the media from all cells were again aspirated and were either exposed to  $10 \text{ J/m}^2$  UV C irradiation and re-fed in media containing 0 I.R.U. interferon  $\alpha/\beta$ , or were re-fed immediately in 0 I.R.U. interferon  $\alpha/\beta$  media in the absence of DNA damage. Two hours after DNA damage, all cells were trypsinised and plated out in triplicate at serially diluted concentrations ranging from  $1 \times 10^6$  cells to  $1 \times 10^2$  cells per 10cm petri dish. Long-term surviving clones were counted approximately 2 weeks later.

**Fig2.13**

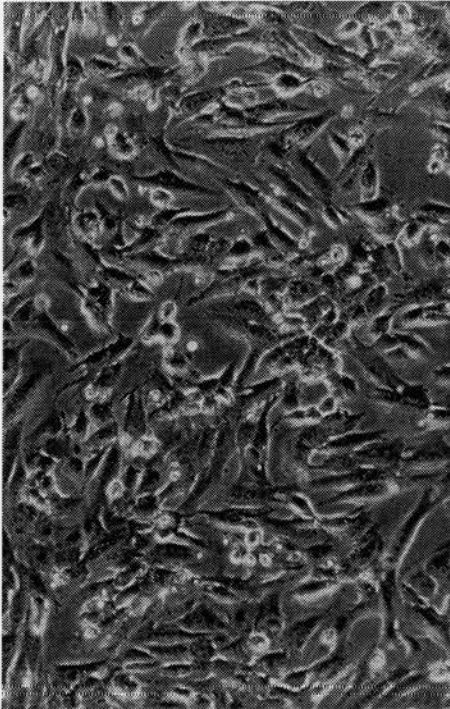
**1D, U**



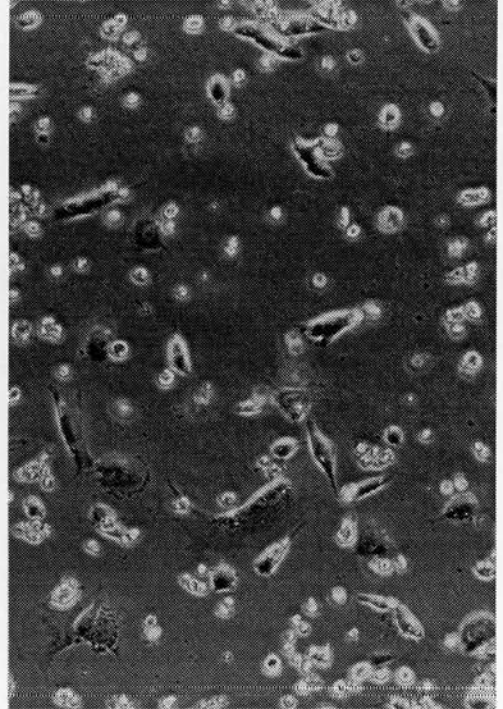
**1D,I**



**1D, U + UV-C**



**1D, I + UV-C**



**Fig. 2.13** shows the consequences of transgene induction, in both the presence and absence of UV mediated DNA damage, on 1D cells growing *in vitro*. Cells were photographed 36 hours after induction. **U** = uninduced; **I** = induced (1000 I.R.U. interferon  $\alpha/\beta$  ml $^{-1}$ ); **UV-C** indicates exposure to 10 J/m $^2$  UV- C irradiation. (Magnification 500X).

**Table 2.2****M25; U**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	-	-	-
$10^3$	-	-	-
$10^2$	31	20	16

**1D; U**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	-	-	-
$10^3$	-	-	-
$10^2$	25	22	17

**M25; I**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	-	-	-
$10^3$	-	-	-
$10^2$	28	19	21

**1D; I**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	-	-	-
$10^3$	-	-	-
$10^2$	26	16	14

**M25; U + UV-C**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	32	66	61
$10^3$	3	3	2
$10^2$	0	0	0

**1D; U + UV-C**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	11	10	13
$10^3$	0	0	0
$10^2$	0	0	0

**M25; I + UV-C**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	52	50	58
$10^3$	8	7	6
$10^2$	0	0	1

**1D; I + UV-C**

PLATING DENSITY	I	II	III
$10^6$	-	-	-
$10^5$	-	-	-
$10^4$	10	24	18
$10^3$	1	1	0
$10^2$	0	1	0

**Table 2.2** on the previous page shows the number of surviving M25 (transgene<sup>-ve</sup>) and 1D clones following treatment with both 0 and 1000 I.R.U. interferon  $\alpha/\beta$ , in both the presence and absence of DNA damage and serial dilution. Plates grown to confluence or with very large numbers of clones are denoted as a dash sign (-).

The results of this assay demonstrate that there is no gross difference in the long-term clonogenic survival of transgene positive or negative cells following treatment with interferon  $\alpha/\beta$  in either the presence or absence of DNA damage.

#### **(2.vi) Further Research**

The observation that growth arrest of tumour cell cycle did not accompany the induction of p53 gene expression in the presence of DNA damage was not predicted as this is a well characterised p53-dependent phenotypic response in many cell types, including a wide variety of tumour cell lines transfected with wild-type p53 transgenic DNA.

As discussed in section 1.ii.5, functional p53 can induce growth arrest or apoptosis in response to DNA damage, but the means through which these biological end-points are achieved are almost certainly different. Indeed, much experimental evidence has been recorded to demonstrate that lesions affecting either the central core domain or the N- terminal domain of p53 can prevent the initiation of p53-dependent growth arrest in response to cellular challenge. In addition to mutation of the p53 transgene, the genetic background of SL tumour cells could also result in this observed deficiency of cell cycle arrest. As p53 requires the specific downstream activation of gene expression to induce growth



arrest, it is possible that the inability of SL cells to initiate a p53-dependent arrest could be the result of lesions affecting the wild-type expression of these genes.

The p53 cDNA cassette used in the experiments detailed within chapter 2 had previously been sequenced and determined to encode wild-type p53 protein prior to the construction of p6-16<sub>L</sub>p53-HYG (Malcomson, 1996). Therefore, it is most likely that any mutational event arising within the p53 coding region of this construct would have had to have occurred during the *in vitro* clonal expansion of stably transfected and non-induced 1D cells (see section 6.ii for discussion). To examine whether the p53 protein encoded by p6-16<sub>L</sub>p53-HYG was wild type, both a functional reporter assay and Western analyses were attempted.

#### **(2.iv.1) RGC-LacZ Reporter Assay**

A functional reporter assay (RGC-LacZ reporter assay (Frebourg *et al*, 1992), (Bargonetti *et al*, 1992)) was attempted to determine whether the induced p53 protein encoded from p6-16<sub>L</sub>p53-HYG possessed the ability to transactivate the expression of downstream genes.

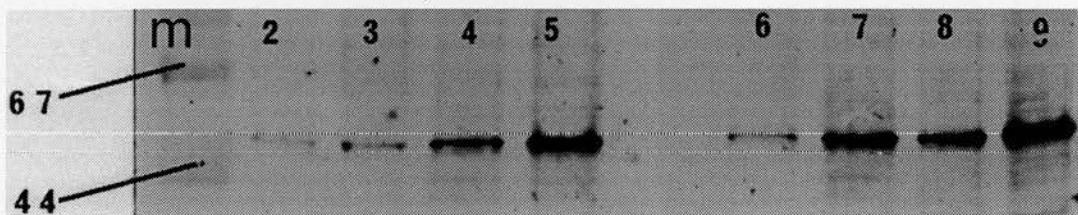
Induced 1D cells staining positive for LacZ following the transient transfection of the RGC-LacZ reporter construct would indicate that the p53 protein encoded by p6-16<sub>L</sub>p53-HYG was indeed capable of transactivating the expression of downstream genes. Suitable transient transfection conditions for SL1 cells could not be optimised within the time left available to me however, so unfortunately no conclusions could be drawn from this experiment.

### (2.vi.2) Western Analysis of p6-16<sub>L</sub>p53-HYG Translation Product

Induced p53 mRNA encoded from p6-16<sub>L</sub>p53-HYG was shown not to splice appropriately at its 3' UTR region (see section 2.ii.2). Theoretically, the failure of the p53 transgene to splice should not alter the final translated transgene product, however if the unspliced sequence were translated, the presence of an additional C- terminal peptide 'tag' could hypothetically compromise the ability of transgenic p53 protein to tetramerise. A likely consequence of this would be less efficient sequence-specific DNA binding and correspondingly, lessened p53-dependent influence on the expression of downstream genes. Western analysis of induced p53 protein from p6-16<sub>L</sub>p53-HYG in 1D cells permits the comparison of transgene translation product size relative to that of wild-type p53 protein.

To optimise the detection of p53 protein on a western blot, total protein was extracted from UV-C irradiated wild-type ES cells and p53 protein was detected using the anti-p53 sheep polyclonal antibody BMG-1 (see section MM3.1). BMG-1 can recognise multiple epitopes of both wild-type and mutant p53, at both N- and C- terminal domains (Boehringer Mannheim). The results of this optimisation experiment are shown in figure 2.14.

**Fig 2.14**



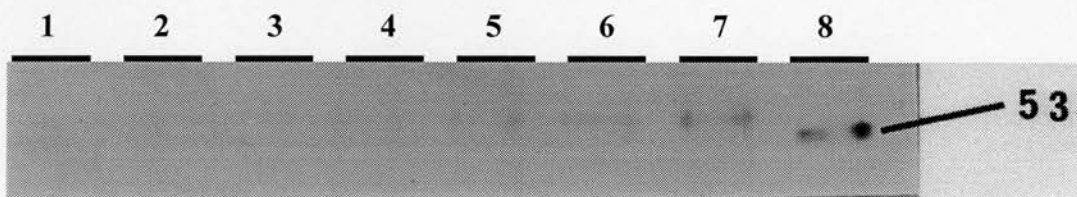
**Fig 2.14** shows the result of a western blot containing total protein extract derived from UV-C irradiated (10 J/m<sup>2</sup>) WT ES cells and the sheep poly-clonal antibody BMG-1. Reading from left to right, lane one contains protein markers (both the 44Kd and 67Kd bands are visible). Lanes 2 to 5 were loaded with 5µg, 10µg, 15µg and 20µg of total ES cell protein extract respectively and shows detected p53 protein using BMG-1 at a concentration of 1µg/ml. Lanes 6 to 9 were loaded with 5µg, 10µg, 15µg and 20µg of total ES cell protein extract respectively and shows detected p53 protein using BMG-1 at a concentration of 2µg/ml.

The result of this experiment would suggest that it is possible to specifically detect stabilised p53 protein on a western blot using only small amounts of total cell extract and low concentrations of the anti-p53 sheep polyclonal antibody BMG-1.

For the main experiment, cells from clone 1D were plated at a density of  $2 \times 10^5$  cells per  $10\text{cm}^2$  on the evening prior to transgene induction. The following morning, media was aspirated from the cells and replaced with media containing either 0 or 1000 I.R.U of interferon  $\alpha/\beta$  per ml. To stabilise p53 protein, all induced samples were exposed to  $10 \text{ J/m}^2$  of UV-C irradiation 6 hours after induction. Cells were lysed at 0, 6, 12, 24, 36, 48 and 72 hours following DNA damage and protein samples were stored at  $-20^\circ\text{C}$  prior to western analysis (see section MM3.1).

Fig 2.15 shows the western analysis results of induced and irradiated 1D cell extracts run beside the WT ES cell extract, used in the previous experiment to optimise p53 detection conditions.

**Fig. 2.15**



**Fig 2.15** shows the result of a western blot containing total protein extract derived from both uninduced and induced + DNA damaged 1D cell extracts run beside the WT ES cell extract used previously in figure 2.14. P53 protein was detected using the sheep polyclonal antibody BMG-1, at a concentration of  $1\mu\text{g/ml}$ . Reading from left to right, lane 1 contains  $20\mu\text{g}$  of total cell extract derived from uninduced 1D cells. Lanes 2 to 7 contain  $20\mu\text{g}$  of total cell extract derived from induced and DNA damaged 1D cells lysed at 6, 12, 24, 36, 48, 72 hours respectively, following damage. Lane 8 contains  $10\mu\text{g}$  of DNA damaged WT ES cell lysate, as used previously to derive figure 2.14.

The result of this western analysis shows little specific recognition p53 protein in 1D cell lysate samples until approximately 36 hours after DNA damage, at which time the signal intensity increases, reaching maximum intensity at 72 hours. There is however an apparent discrepancy in the sizes of p53 protein recognised in 1D cell lysate samples compared to that recognised in wild-type ES cell lysate. Recognised p53 protein in the former sample set appears to be at least several Kd larger than the specific product recognised in the latter.

It is currently not possible to conclude, in the absence of further experimental data, whether the protein detected in induced 1D cell lysates corresponds to p6-16<sub>L</sub>p53-HYG translation product or to non-specific background.

Although the protein recognised by BMG-1 appears to be specific in 1D cell lysate samples, previous analysis of within this chapter would suggest that maximal levels of p53 transgene expression are achieved rapidly and become diminished by 72 hours.

If however this recognised protein was shown to correspond to the translation product of p6-16<sub>L</sub>p53-HYG, then although this protein must possess p53 epitope(s) to be recognised by BMG-1, it is not the same size as wild-type p53 protein. These results would then implicate the existence of peptide 'tag' at either the N- or C- terminal domains of the transgenic p53 protein, thus perhaps explaining the inability of this protein to induce cell cycle arrest in response to DNA damage.

In the absence of time constraints, it would be ideal to repeat this blot and use cell lysate derived from the transgene<sup>-ve</sup> cell line M25 as a negative control. Absence of BMG-1 recognition within this sample and reproducible detection of protein

product within 1D cell lysates (as shown in figure 2.14) would strongly suggest that the translation product originating from p6-16<sub>L</sub>p53-HYG is larger than wild-type p53 protein.

## CHAPTER 3

The aim of this chapter was to use the sarcoma derived p53 inducible cell line 1D, as a means of evaluating possible therapeutic benefit arising from the introduction of p53 gene expression in otherwise p53 deficient tumour cells *in vivo*.

### **(3.i) The *In Vivo* Experimental System**

Retention of SL1 cell tumorigenic potential, following their establishment as a cell line *in vitro*, was determined by injecting an aliquot of untransfected SL1 cells sub-cutaneously into the flanks of SCID (Severe Combined Immuno-Deficient) mice and observing the time required before tumours arose at the site of injection. Host immuno-deficient animals were essential in this experiment as SL1 cells originate from an outbred genetic background and so would likely be rejected if injected sub-cutaneously into an inbred immune competent animal.

6 SCID mice were initially injected with  $1 \times 10^7$  untransfected SL1 cells into each flank and tumours became visible at all sites of injection approximately 2 weeks after injection (see section MM5.1). Within the following 2 weeks, all mice had to be sacrificed as a result of rapid tumour growth.

This same experiment was subsequently repeated using 1D cells, following their preliminary characterisation. These cells were derived as a tumour sub-clone from SL1 cells and were shown to induce the expression of a p53 transgene when cultured in the presence of interferon  $\alpha/\beta$  (see chapter 2). Sub-cutaneous injection of  $1 \times 10^7$  1D cells into the flanks of SCID mice also resulted in the appearance of

rapidly growing tumours at the site of injection within approximately 2 weeks. The demonstration that injected 1D cells give rise to tumours as rapidly as untransfected SL1 cells in the absence of interferon  $\alpha/\beta$ , would suggest that in the absence of transgene expression, 1D cells possess an essentially equivalent tumourigenic potential as p53 deficient SL1 cells.

It is possible to stimulate endogenous levels of interferon  $\alpha/\beta$  in the mouse by injecting micro-gram amounts of a synthetic double stranded RNA, polycytidylic: polyinosinic acid (pI:pC), into the peritoneal cavity (see sections 1.i.8b and MM5.2). Indeed, a previously published report has detailed the induction of cre protein *in vivo*, encoded by a transgene regulated by the murine and interferon  $\alpha/\beta$  responsive Mx1 promoter, via exactly such an approach (Kuhn *et al*, 1995).

As p53 transgene expression can be induced *in vitro* by culturing 1D cells in the presence of interferon  $\alpha/\beta$ , it was reasoned that it may be possible to stimulate the expression of p53 within 1D cells growing *in vivo* by following a similar pI:pC dose regime as detailed by Kuhn *et al*. In this way, it may be possible to better evaluate the potential therapeutic benefits conferred by the introduction of p53 gene expression in the treatment of p53 deficient tumours *in vivo*.

Perhaps the most serious potential drawback concerning the application of an interferon  $\alpha/\beta$  inducible expression system *in vivo*, is that sufficient amounts of endogenous interferon  $\alpha/\beta$  can be produced to induce the gene expression of loci containing ISRE promoter elements following viral or microbial infection (see chapter 1, section 1.i.8b). It is therefore highly likely that mice held in standard,

non-barrier mouse house conditions will be exposed a number of pathogens sufficient to induce transgene expression at inappropriate and undesirable times.

The necessitive use of SCID animals in the prevention of SL cell rejection for these experiments however, may possibly be advantageous with respect to reducing the constitutive background levels of systemic interferon  $\alpha/\beta$ . SCID animals must be maintained in barrier conditions to minimise both microbial and viral infection and thus to retain viability. Barrier conditions should therefore provide an environment for the mouse in which the normal physiological inducers of interferon  $\alpha/\beta$  are absent and so consequently should minimise the likelihood of uncontrolled transgene induction.

The relevance of background systemic levels of interferon  $\alpha/\beta$  with respect to induction of transgene expression does appear to be questionable however. The results published by Kuhn *et al* showed that non-induced levels of cre-mediated recombination between loxP sites was very low (~1%) in essentially all organs except the spleen of non-barrier maintained mice possessing an Mx1-cre transgene. These mice were immune-competent and so it was not essential to keep these animals under barrier conditions to retain viability.

Upon administration of three 250 $\mu$ g doses of pI:pC over a six-day period, the percentage of cre-mediated recombination in certain tissues rose to nearly 100%, perhaps suggesting that supra-physiological levels of endogenous interferon  $\alpha/\beta$  are required in the host animal before the expression of Mx1-regulated transgenes are induced efficiently.



### **(3.ii) Optimisation of *In Vivo* Transgene Induction**

The initial experiments of this chapter were intended to define a pI:pC treatment regime that would efficiently induce p53 transgene expression within 1D cells growing *in vivo*.

Accordingly, a cohort of 12 SCID mice were injected sub-cutaneously in both flanks with an aliquot of 1D cells as prepared previously. Due to the observed efficiency and rapidity of tumour appearance following the injection of 1D cells in section 3.i, only half the previous amount of 1D cells (i.e.  $5 \times 10^6$  1D cells per flank) were injected into these animals. Tumours arose at all sites of injection approximately 3-4 weeks after injection and so thus enabled a reduction in the already considerable consumption of cell culture resources required to set up experiments with these numbers of animals.

In keeping with the protocol described by Kuhn *et al*, it was intended that once tumours became visible, 4 animals would be injected intra-peritoneally (IP) with one 250 $\mu$ g dose of pI:pC, 4 animals would be injected IP with two separate 250 $\mu$ g doses of pI:pC two days apart, and 4 animals would be injected with three separate 250 $\mu$ g doses of pI:pC each two days apart. Tumours would then be collected the day following the last administered dose of pI:pC and analysed by light-microscopy for any gross phenotypic effects and for p53 transgene expression by immuno-histochemical analysis on micro-sections and by western analysis.

All SCID animals were closely monitored following the first IP administered dose of pI:pC for any signs of adverse effect. Although no animals appeared to become

significantly distressed by pI:pC treatment, it was noted that all animals showed altered coat condition within approximately 6 to 12 hours of injection.

The majority of injected SCID mice appeared to have fully recovered their coat condition within 24 hours of injection however surprisingly several animals were found dead in their cages.

This pattern of rapid sickness and recovery or death of the SCID animals following IP injection of 250 $\mu$ g pI:pC continued at all subsequent stages of this experiment. Indeed, over the course of all attempts at inducing p53 transgene expression *in vivo*, this SCID response to pI:pC accounted for the deaths of approximately 20% of all injected animals. The nature of this phenomenon is discussed in greater detail in chapter 4.

Subsequent histological examination of all tumours recovered from surviving SCID animals treated with pI:pC, from all of the induction regimes detailed above, did not reveal any gross phenotypic effect of transgene or inducer upon either the number of mitotic figures or on the rate of apoptosis relative to non-treated 1D tumours (see fig.3.1). It should be noted that it is very difficult to detect subtle histological changes of this kind within tumour samples, as the rate of both mitosis and apoptosis are already very high.

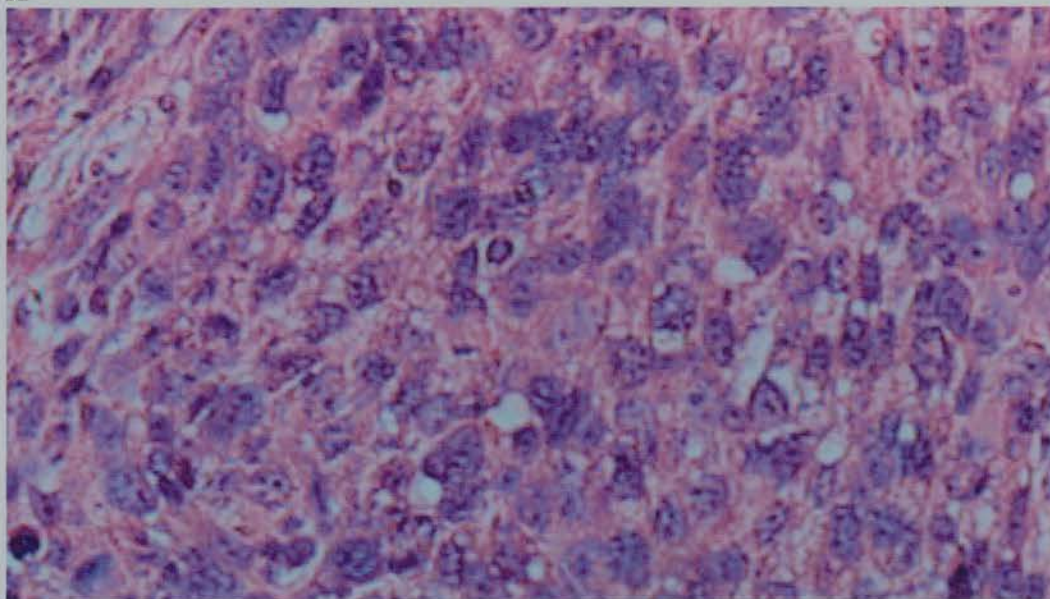
Further however, immuno-histochemical analysis of pI:pC treated tumour samples did not detect any p53 transgene expression (see fig.3.2). Western analysis of p53 expression within these tumour samples was also attempted, but no p53 protein could be detected using the partially optimised assay conditions described in section 2.vi.2 of chapter 2 and so these results are not presented.

**Fig.3.1** on the next page shows two H+E stained and fixed sections of tumours resulting from the sub-cutaneous injection of 1D cells. Photo A shows the histology of a non-pI:pC treated tumour, whilst the photo B shows the histology of a pI:pC treated tumour. No grossly apparent difference in the number of mitotic or apoptotic bodies exists between tumour sections (magnification 500X).

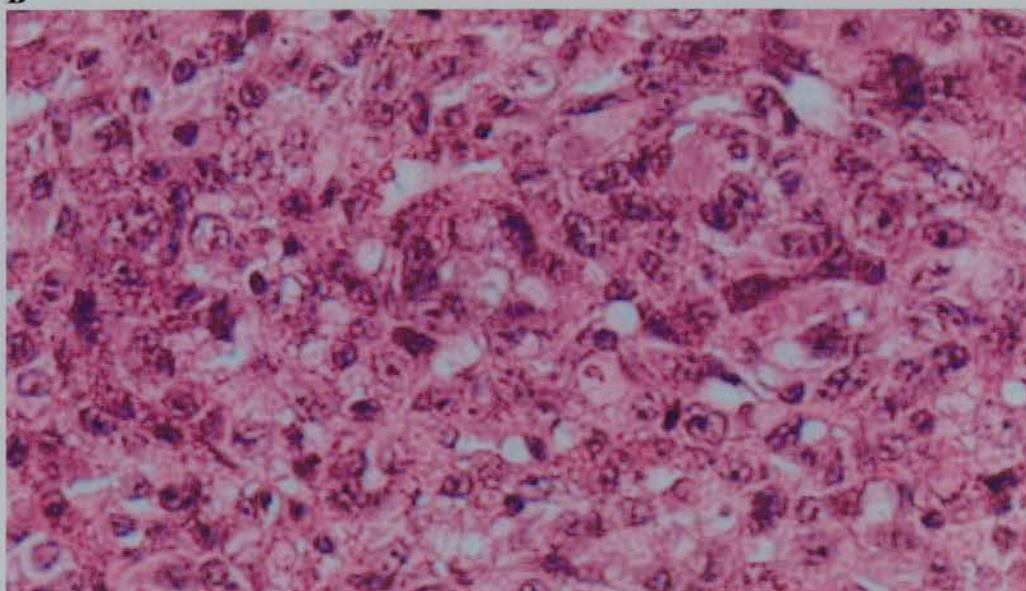
**Fig.3.2** on the page following next shows the results of immuno-staining fixed and paraffin embedded tissue sections with anti p53 CM5 antibody as detailed in section MM3.3. Photo A shows positive staining for p53 in the nuclei of wild-type epithelia cells derived from the intestinal crypt following exposure to 5 Grays whole body  $\gamma$ -irradiation. Photo B shows that 1D tumour cells grown *in vivo* do not induce p53 expression following treatment with pI:pC (magnification 400X).

Fig.3.1

A

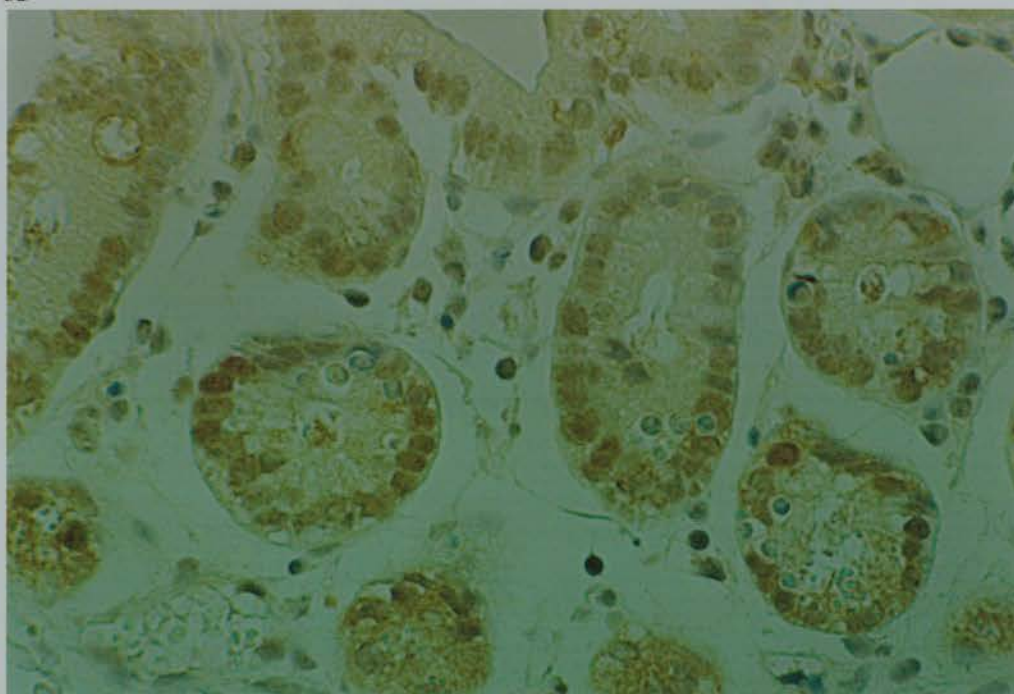


B

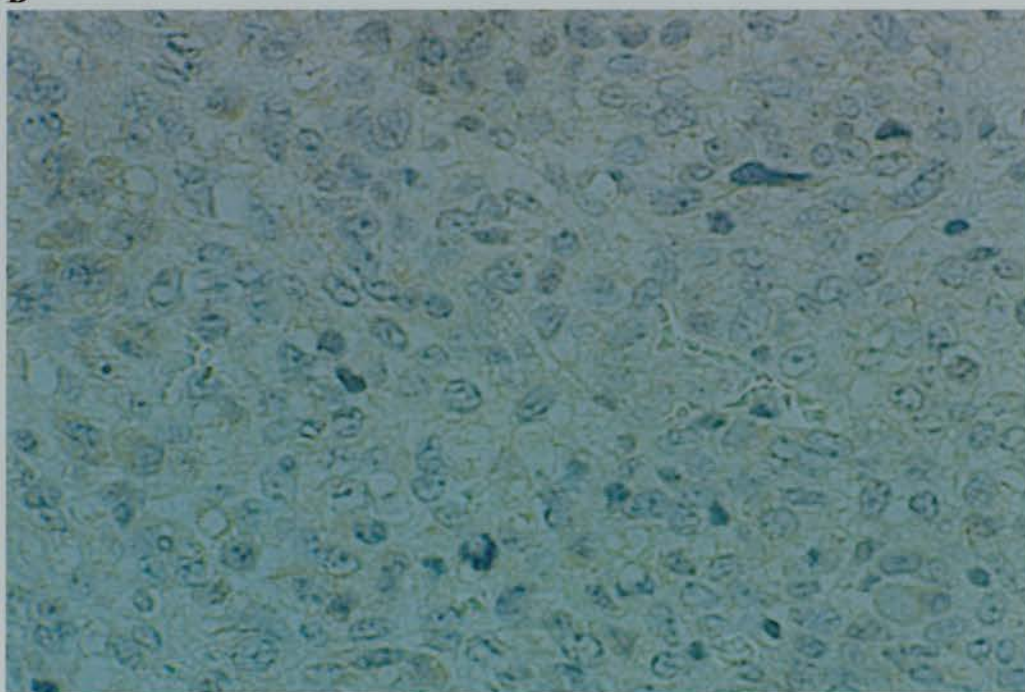


**Fig.3.2**

**A**



**B**



The fact that no obvious transgene mediated effects were detected upon the rate of tumour cell proliferation and cell death following the stimulation of endogenous interferon  $\alpha/\beta$  was not entirely surprising. The *in vitro* data presented in chapter 2 also showed no observable transgene mediated effects upon either of these two biological processes following transgene induction in the absence of DNA damage.

The fact that IP injection of pI:pC, the most potent inducer of endogenous murine interferon  $\alpha/\beta$ , did not result in the induction of transgene expression *in vivo* was not predicted however. In the study published by Kuhn *et al*, a single IP injection of 250 $\mu$ g pI:pC was sufficient to induce cre-mediated recombination between loxP sites within 97% of liver cells. The observed failure of this regime to induce 6-16 regulated transgene expression must either be the result of inefficient induction of transgenic p53 protein, which rapidly becomes degraded *in vivo* in the absence of relevant cellular challenge, or the result of a transcriptional 'silencing' of p53 transgene expression within 1D cells growing *in vivo*. To test both of these hypotheses, a further cohort of SCID animals were injected subcutaneously with 1D cells to generate additional tumours.

In an attempt to enhance the immuno-histochemical detection of possibly weak *in vivo* p53 transgene induction, it was reasoned that damaging the DNA of 1D cells growing *in vivo*, in addition to the pI:pC dose regime detailed above, may stabilise any low levels of induced transgene expression sufficiently to facilitate detection. Further, as a transgene-dependent apoptotic phenotype was detected in 1D cells growing *in vitro* (see chapter 2, section 2.iv), it was reasoned that the addition of

DNA damage to pI:pC treatment of 1D tumours may result in a transgene-dependent histological phenotype discernible from non-treated and treated but non-DNA damaged 1D tumours.

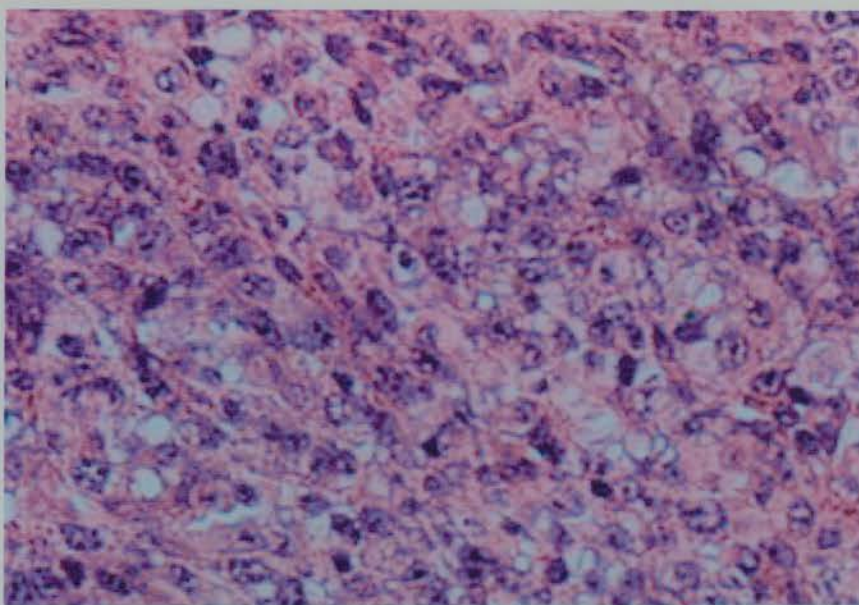
Accordingly, 12 SCID mice were injected sub-cutaneously with  $5 \times 10^6$  1D cells into each flank and upon the appearance of tumours, in addition to the previously applied pI:pC dose regime, these animals were also exposed to 5 Grays whole body  $\gamma$ -irradiation on the day following the last administered dose of pI:pC. All tumours were removed for analysis 48 hours after  $\gamma$ -irradiation.

**Fig. 3.3** on the next page shows two micro-sections of a 1D-cell tumour at 500X magnification following treatment with pI:pC and 5 Grays whole body  $\gamma$ -irradiation. Photo A shows an H+E stained tissue section and demonstrates that no gross difference in the numbers of mitotic or apoptotic figures was observed within tumours treated with both pI:pC and  $\gamma$ -irradiation relative to non-pI:pC treated or pI:pC treated and non-DNA damaged tumours (see figure 3.1).

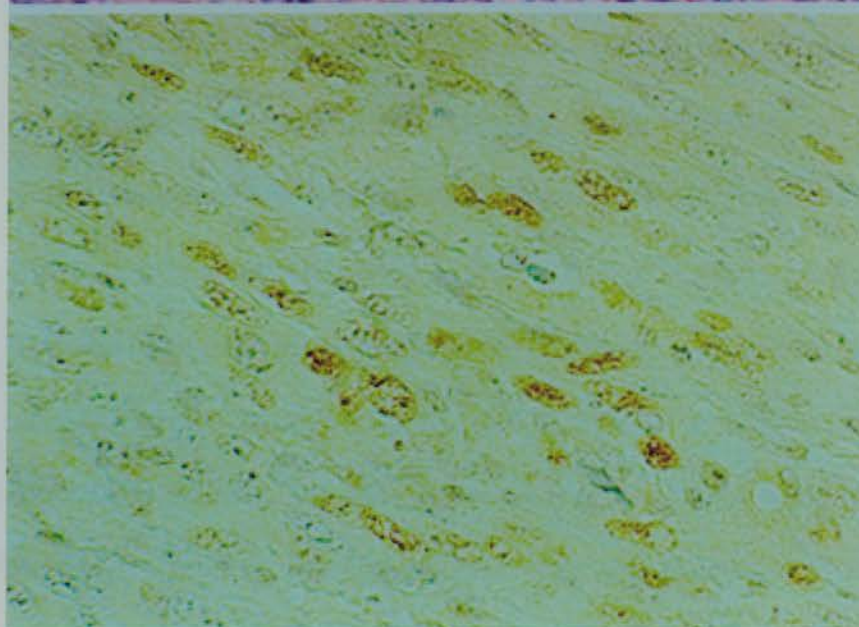
Photo B shows a tissue section derived from the same tumour following immunohistochemistry with anti p53 CM5. Whilst the vast proportion of this tumour did not appear to express p53 protein, small pockets of 1D cells grown *in vivo* appeared to express p53 protein within their nuclei.

**Fig.3.3**

**A**



**B**





These results would suggest that 1D cells modulate or lose their capacity to induce p53 transgene expression when grown *in vivo*. To further investigate the underlying causes of such a scenario, 1D-cell tumours were removed from two animals and re-established in culture as cell lines 1D.EX.1 and 1D.EX.2 (as detailed within section MM4.2).

These cell lines were initially demonstrated to have retained resistance to hygromycin in culture. This would suggest that the lack of observed *in vivo* transgene induction was not directly the result of p53 transgene loss through either gross chromosomal deletion or loss, as both hygromycin resistance cassette and 6-16 regulated p53 transgene sequences are tightly linked.

To enable a comparison of the ability of 1D.EX clonal lines to induce transgene expression relative to the non-injected and *in vitro* characterised 1D cells (see chapter 2), both explanted cell lines were further expanded in culture.

Cells from 1D.EX.1 and EX.2 were plated on chamber-slides at a density of  $3 \times 10^4$  cells per well on the evening prior to transgene induction (see section MM3.2). Each chamber-slide contains two chamber wells, therefore on the morning following plating, medium was aspirated from both wells of every slide and replaced with medium containing either 0 or 1000 I.R.U of interferon  $\alpha/\beta$  per ml. To enhance the detection of p53 transgene expression, all media was aspirated and all cells were exposed to  $10 \text{ J/m}^2$  UV-C irradiation 6 hours after induction. Following irradiation, all cells were returned to media conditions identical to those prior to genotoxic insult. 1D cells were also plated and treated as described to act as a positive control for transgene induction in this experiment.

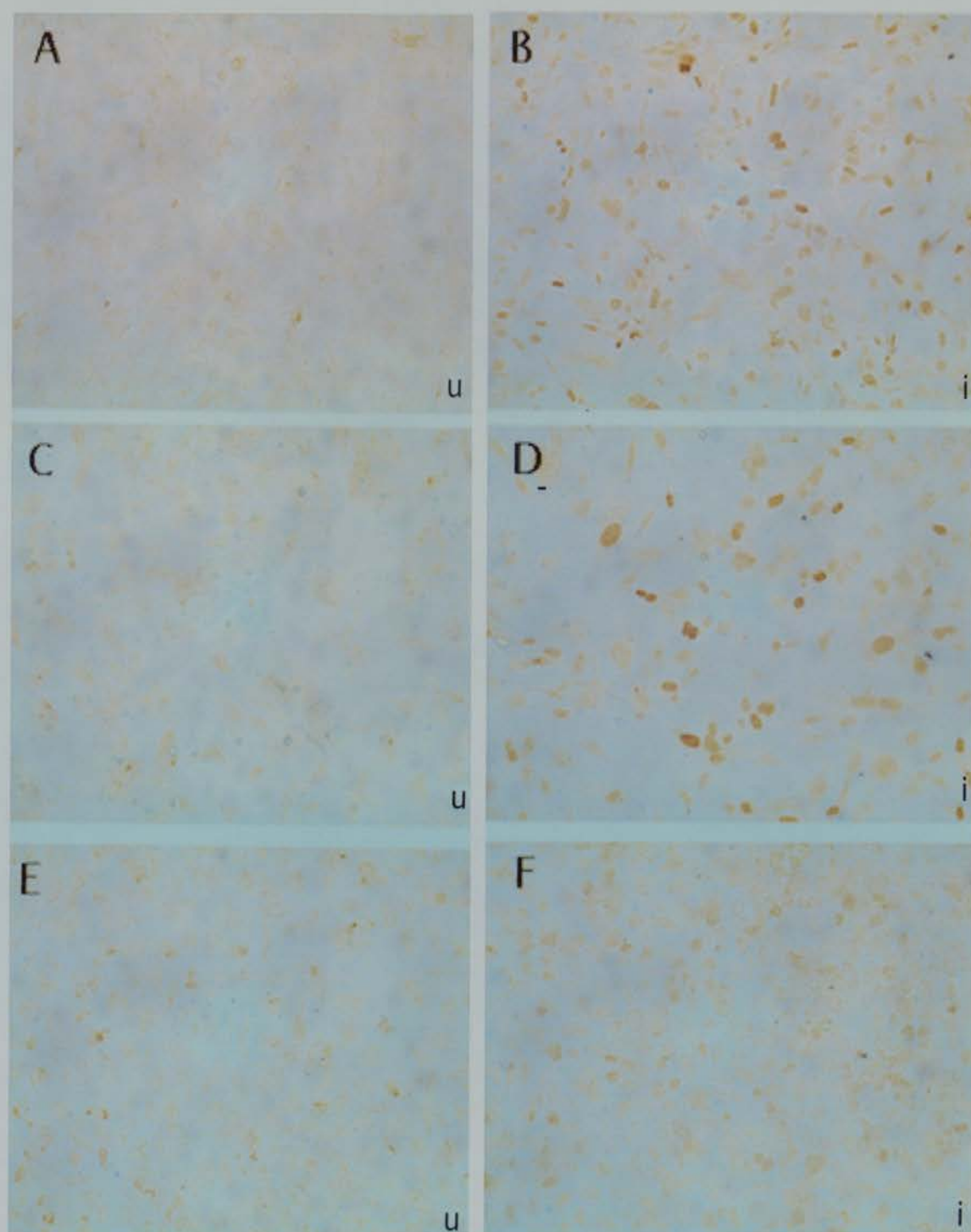
Medium was aspirated at each time-point from all appropriate cells, which were then washed once in PBS prior to fixation and storage at  $-70^{\circ}\text{C}$ . The time-points analysed were 0, 6, 12, 24, 48 and 72 hours after genotoxic insult.

Immunocytochemistry was performed on these samples using the monoclonal antibody Pab421 and the results of this experiment are shown at 6 hours after DNA damage on the following page as Fig.3.4 (see section MM.3.2 for protocol).

Photographs A and B of figure 3.4 show that in the absence of interferon  $\alpha/\beta$ , virtually no p53 expression can be detected in the nuclei of non-injected 1D cells (A), whilst p53 expression can be readily observed within the nuclei of the same cells following treatment with interferon  $\alpha/\beta$  (B). Photographs C and D also show an equivalent level of p53 induction (D) following the treatment of 1D.EX.2 cells with interferon  $\alpha/\beta$ , whilst photographs E and F demonstrate that 1D.EX.1 cells have lost the ability to induce p53 transgene expression (F).

(N.B. all photographs comprising fig.3.4 were taken at 400X magnification).

Fig.3.4



The results of this immuno-assay would appear to suggest that 1D cells grown *in vivo* can rapidly evolve and lose their ability to induce p53 transgene expression in response to interferon  $\alpha/\beta$ . It is not possible to determine the overall frequency of presenting 1D-cell tumours that lose transgene inducibility from the data presented as only two tumours were re-established as cell lines and analysed *in vitro*. However, upon the basis of the immuno-histochemistry performed upon pI:pC treated and DNA-damaged tumours, it could be predicted that loss of transgene expression would be frequently observed.

Non-induced levels of transgene expression *in vivo* must therefore confer considerable selective growth advantage, as 1D cells can lose inducible transgene expression within three to four weeks of injection.

This observation could be considered surprising as non-induced amounts of expressed p53 transgene appear to be very low in 1D cells grown *in vitro* as determined by rtPCR and immuno-analysis (see chapter 2). Further, no transgene-mediated effects were observed upon either the rates of tumour cell proliferation or apoptosis following the induction of transgene expression in the absence of additional DNA damage.

As mentioned in section 3.i, the presence of endogenous amounts of interferon  $\alpha/\beta$  within host SCID animals may account for higher levels of non-induced transgene expression *in vivo* than that previously characterised *in vitro*. Even upon that basis, the data contained within chapter 2 would suggest that 1D cells exhibiting background “leaky” expression of transgene expression should possess no short-term differences in their ability to proliferate, or in their disposition to commit to apoptosis, relative to non-transgene expressing 1D cells.

As detailed within chapter 1 however, the expression of wild-type p53 can be stabilised and activated by many more stimuli other than DNA damage. It is therefore highly likely that the selective pressures exerted on 1D cells growing *in vivo* are different from the pressures on cells growing *in vitro*. Thus, the data presented within this chapter would suggest that the amounts of non-induced p53 transgene are sufficient to confer a selective disadvantage to 1D cells growing *in vivo*.

The implications of these *in vivo* observations with regard to p53 replacement gene therapy are discussed in greater depth in chapter 6.

## CHAPTER 4

As discussed in chapter 3, attempts to stimulate the production of interferon  $\alpha/\beta$  in SCID animals possessing tumours comprised of 1D cells were made by injecting 250 $\mu$ g of synthetic double stranded RNA (pI:pC) intra-peritoneally (IP).

Within 6 to 12 hours after IP injection of pI:pC however, all SCID mice appeared to show altered coat condition. The majority of injected SCID mice made a full recovery 24 hours after injection, however approximately 20% of all SCID animals injected with pI:pC died within an equivalent timeframe. This phenomenon has not been previously reported and so the aim of this chapter was to investigate the underlying biology responsible.

### **(4.i) SCID Phenotype following Injection of pI:pC**

The main reasons for using pI:pC to induce transgene expression *in vivo* were that this reagent is much more affordable than purified preparations of interferon  $\alpha/\beta$  and a previously published report detailed a complete protocol for the use of this reagent in achieving the induction of transgene expression, regulated by an interferon  $\alpha/\beta$  responsive promoter, *in vivo* (Kuhn *et al*, 1995).

The results of Kuhn *et al* did not report any phenotypic side-effects resulting from the administration of 250 $\mu$ g pI:pC IP and so this was the dose we initially chose to inject into SCID mice whilst attempting to induce the expression of p53 from p6-16<sub>L</sub>p53-HYG within 1D cells growing *in vivo*. The observed response of SCID mice to this administered dose of pI:pC, as detailed within the opening paragraphs

of this chapter and within section 3.ii of chapter 3, was therefore highly surprising.

Initially it was suspected that the concentration of supplied pI:pC was higher than that stated by the manufacturer, therefore we injected a total of 8 further SCID mice with a range of lower doses of pI:pC to observe whether these animals were equally affected as those injected with 250µg IP. Two animals each were injected with 50µg, 100µg, 150µg and 200µg amounts of pI:pC IP.

All animals rapidly lost coat condition following the injection of pI:pC as described previously and again none of the animals appeared to be suffering unduly. The following day however, one of the animals injected with 50µg, one of the animals injected with 150µg and both of the animals injected with 200µg of pI:pC were found dead in their cages. The surviving animals appeared to have regained coat condition and appeared normal, thus the observed SCID response to pI:pC administered IP does not appear to be dependent upon the amounts of pI:pC injected within this range.

The next concern was that the SCID response to pI:pC was the result of impurities present within the manufacturers preparation of this reagent as no potentially toxic chemicals are added to prepare pI:pC prior to injection. Poly(I):poly(C) reagent is supplied as a sterile, lyophilised potassium salt, which is resuspended in PBS, heated to 50°C to aid solubilization and gradually cooled to room temperature to ensure efficient annealing of complementary RNA strands, as specified by the manufacturer, prior to use.

To test the hypothesis of pI:pC batch contamination, small quantities of pI:pC were ordered from other leading manufacturers, prepared as specified and then injected IP as a single 250µg dose as previously described into duplicate SCID animals. The pI:pC supplied by ICN, SIGMA and Pharmacia (as used in the original IP injections) all resulted in the observed SCID response.

The transgenic mice used by Kuhn *et al*, that showed no apparent pI:pC-mediated effects following IP injection, were all on a C57BL/6 x CBA genetic background. To test the hypothesis that the observed SCID response was mouse-strain specific, 3 SCID and 3 Balb/C mice were injected with 250µg IP. All animals were sacrificed 12 hours after injection and kidneys, liver, heart, lungs and pancreas were removed from all animals and fixed in formalin. The small intestine was removed and fixed (as detailed in section MM5.3) from one SCID and one Balb/C animal only.

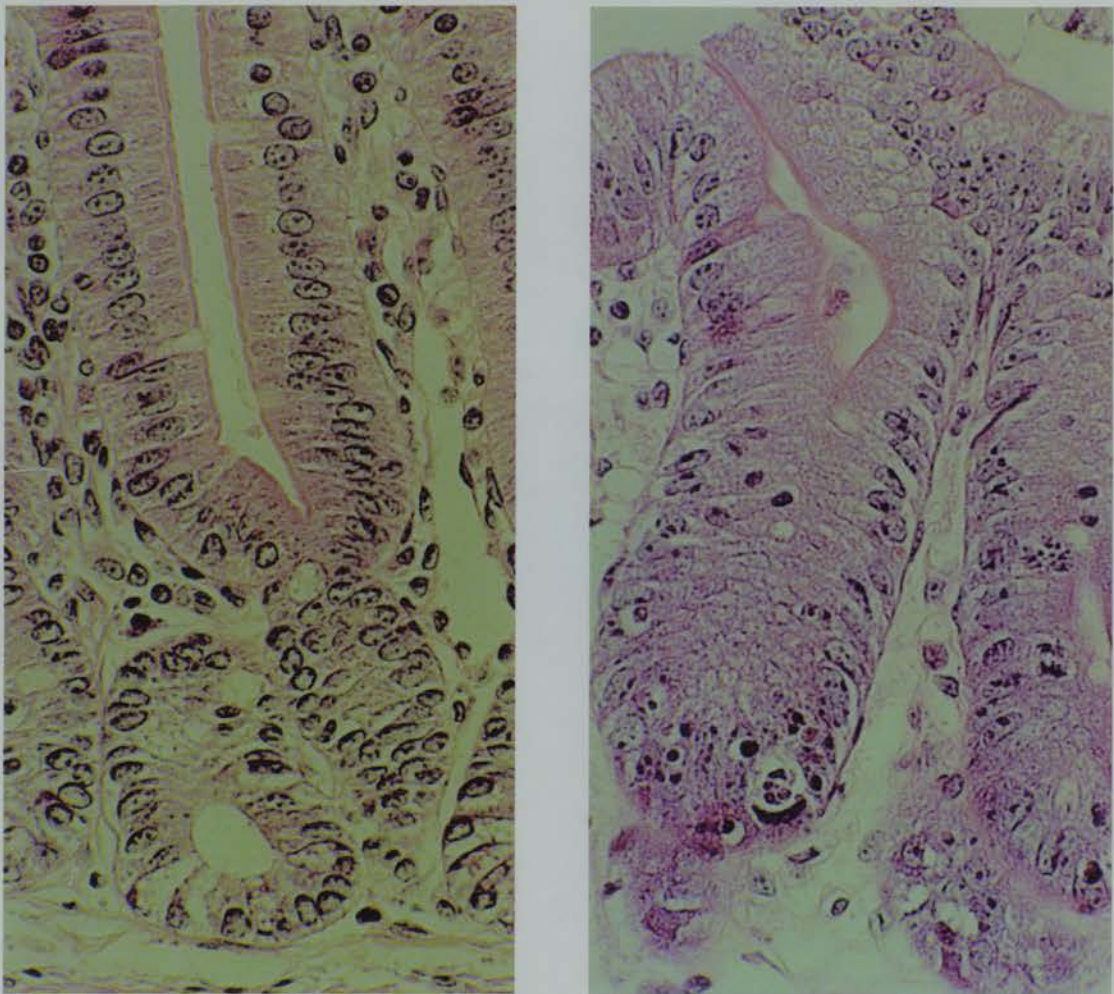
Subsequent microscopic analysis of these organs did not reveal any abnormal histology apart from the SCID small intestine, which exhibited an increased number of apoptotic bodies within the crypt compartments of the intestinal epithelium (see figure 4.1).

This experiment was repeated with additional animals to determine the time-points after IP injection of pI:pC that SCID mice exhibit elevated levels of apoptosis within this compartment of the small intestine relative to Balb/C mice. 3 SCID mice and 3 Balb/C mice were sacrificed at each of the 3, 6, 12, 24, 48 and 72 hour time-points following the IP injection of pI:pC. At every timepoint, the



small intestine was removed from each animal and prepared for histological analysis as detailed in section MM5.3. The results of this experiment are detailed in figure 4.2, which shows an immediate and SCID-specific apoptotic response in the crypt compartments of the intestinal epithelium. Further however, both animals appear to show elevated levels of apoptosis within this region of the intestine at later time-points

**Fig.4.1**



**Fig.4.1** shows fixed and H + E stained small intestine samples derived from both Balb/C and SCID mice (from left to right respectively) 12 hours after IP injection of 250 $\mu$ g pI:pC. Note the increased numbers of apoptotic bodies present within the crypt compartments of the small intestine derived from the SCID mouse.

Fig.4.2

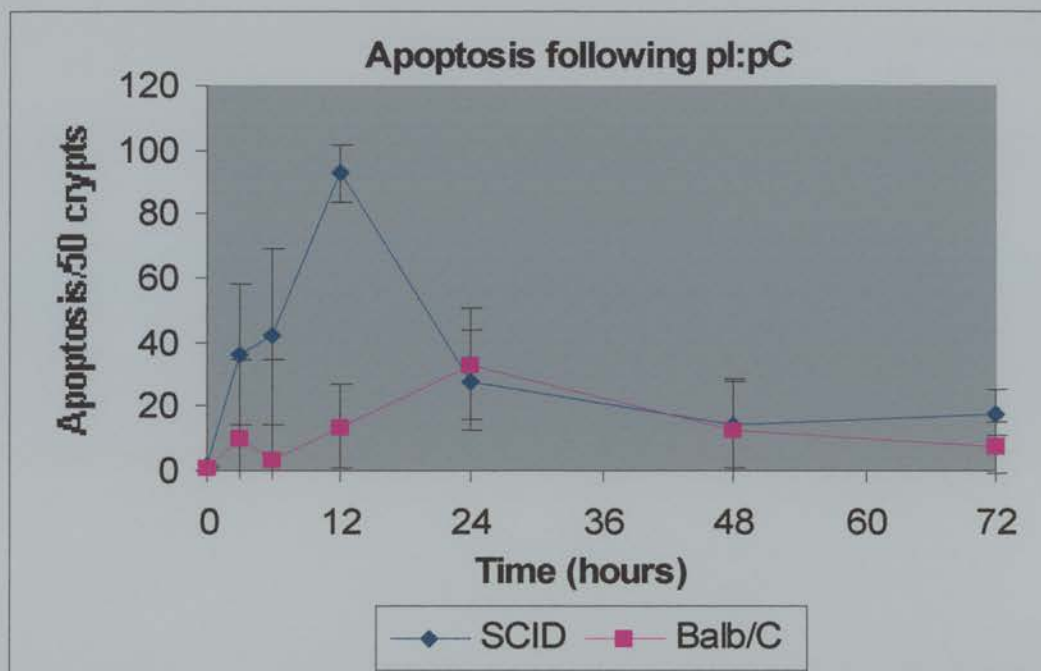


Fig.4.2 shows the number of apoptotic bodies present per 50 intestinal crypts of pI:pC treated SCID and Balb/C mice. The intestines from three animals were analysed per data point and the data is expressed as the average  $\pm$  SD.

#### (4.ii) Interferon $\alpha/\beta$ and Morphology of the SCID Intestine

The observed SCID response to pI:pC has not been previously described although a paper published by Garside *et al* examined the effects of interferon  $\alpha/\beta$  upon the induction of intestinal pathology in CBA x Balb/C mice (Garside *et al*, 1991). The results of this study suggested that either the administration of 100 $\mu$ g of pI:pC or purified interferon  $\alpha/\beta$  resulted in significant villus atrophy and crypt hypertrophy by 24 and up to 72 hours after their respective administrations. Further, these effects were abolished following NK cell depletion with anti-asialo GM1 antibody prior to the induction or administration of interferon  $\alpha/\beta$ .

The data presented by Garside *et al* did not examine levels of apoptosis within the intestine and our data would suggest that the increased degree of intestinal disruption observed in SCID animals relative to Balb/C animals following

induction of interferon  $\alpha/\beta$  is predominantly the result of a rapidly induced wave of apoptosis affecting intestinal crypt epithelial cells. Indeed, it could be hypothesised that the villus atrophy and crypt hypertrophy recorded by Garside *et al* at later timepoints could result from increased levels of crypt cell apoptosis at earlier timepoints, immediately following administration of interferon  $\alpha/\beta$ .

Interferon  $\alpha/\beta$  has previously been described as being a potent stimulator of NK cell activity (Perussia, 1991) and in addition to heightened cytotoxic activity, activated NK cells produce large amounts of the inflammatory cytokine TNF $\alpha$  (tumour necrosis factor  $\alpha$ ; Degliantoni *et al*, 1985). Elevated levels of TNF $\alpha$  have been implicated as being partly responsible for mediating an enteropathic side effect that occurs during the course of graft versus host disorder (GVHD; Garside and Mowat, 1993). The expression of many cytokines are implicated in mediating the side effects of GVHD however, which is a complex, common and serious side effect of allogenic bone transplantation (Ferrara *et al*, 1997). Our data would suggest however that mice deficient in both T and B cells are more susceptible to the enteropathic side effects conferred by elevated levels of interferon  $\alpha/\beta$  relative to immune competent strains of mice.

A speculative hypothesis that would explain this data could be that cytokines produced by either T or B cells can partially inhibit NK cell activation or modify stimulated NK cell activity. Indeed, the secretion of IL-10 from CD4<sup>+</sup> T cells has been shown to strongly inhibit the ability of IL-2 activated NK cells to secrete interferon  $\gamma$  (Hsu *et al*, 1992). SCID mice are essentially deficient in both T and B cells and therefore would perhaps be more susceptible to the effects elicited by NK cell activity relative to immune competent mice.

Preliminary experiments have accordingly been attempted to deplete NK cells within SCID animals prior to pI:pC treatment to investigate whether the heightened levels of pI:pC-mediated apoptosis observed within the intestine of SCID mice is NK cell dependent. Initial observations would suggest this is indeed the case, however more extensive experimentation is required before these observations can be considered definitive.

## CHAPTER 5

The aim of this chapter was to render the expression of p53 inducible in the mouse by replacing the host p53 promoter sequence of murine ES cells with that of an inducible promoter sequence via double replacement targeting.

### **(5.i) Targeting Approach**

Double replacement targeting involves the sequential use of two independent targeting vectors. Despite the increased length of time and effort involved in the selection of two homologous recombination events, the application of gene targeting technology in rendering the expression of a gene inducible confers a number of advantages and refinements over the inducible transgenic approach detailed in chapters two and three.

As described in section 1.i.7c (chapter 1), the first targeting vector is used to delete and replace host cell promoter sequence with a positive/negative selection marker (*e.g.* for the introduction an HPRT selectable marker cassette into otherwise HPRT deficient ES cells; see fig 5.a). Multiple second round vectors can subsequently be generated, utilising the same regions of homologous sequence as the first vector, to retarget this original locus and replace marker sequence with any desired DNA sequence.

This is of strong advantage regarding the aim of this chapter, as within recent years rapid and significant improvements have been made within the field of inducible promoter technology. This strategy therefore provides a high degree of flexibility and enables the generation of multiple, targeted ES cell lines with

inducible gene expression influenced by a variety of different promoter sequences. This could be viewed as useful in the comparison of inducible promoter systems, as well as providing an opportunity to keep pace with all technological inducible promoter advances.

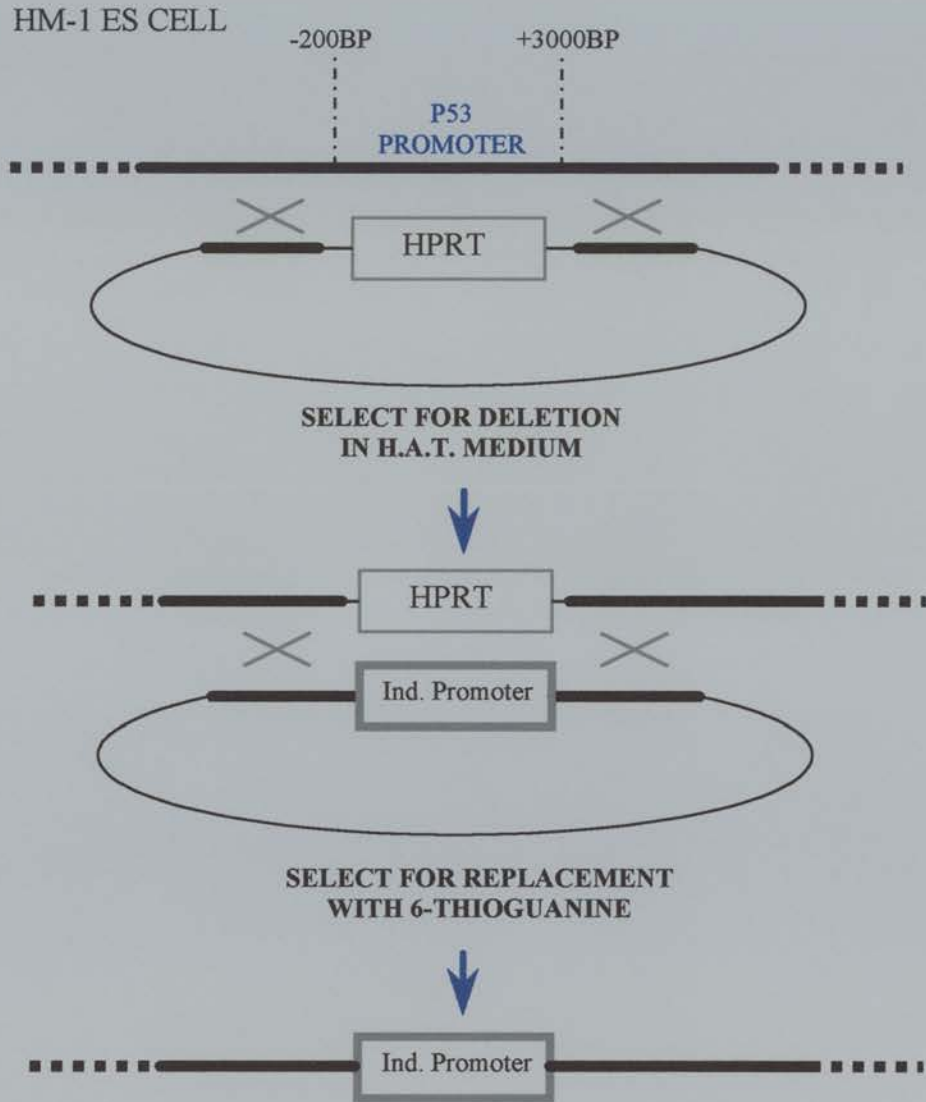
A further advantage arising from this application of gene targeting is that the expression of a gene can be rendered inducible whilst leaving downstream gene organisation and structure virtually unaltered. All exon/intron boundaries can be left intact, which should result in the normal post-transcriptional processing of induced mRNA and the optimal translation of gene product. Further, as the genetically modified locus is known and highly specific, the expression of all other loci in the cell will remain unaffected following HR and the stable integration of foreign DNA.

The level of inducible gene expression between targeted clones should also be highly consistent. Inducible cells will no longer be prone to the transgene associated problems of position effect or with variance of construct copy number. For those reasons, this type of approach would be useful in the comparison of transcriptional activity resulting from different constitutive or inducible promoter systems.

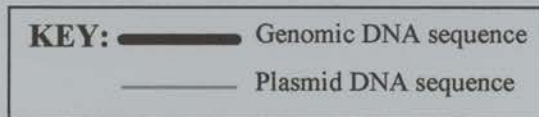
The region spanning from approximately 250 base pairs upstream of the published transcriptional initiation site of p53, plus the entire length of non-coding exon 1 contain all the transcriptional regulatory sequence elements previously demonstrated to influence p53 expression (see chapter 1, section

1.ii.4a). This region will be referred to as the promoter region of p53 for the remainder of this chapter.

**Fig.5a** Double Replacement Targeting Strategy at p53 Locus



**P53 EXPRESSION RENDERED INDUCIBLE**

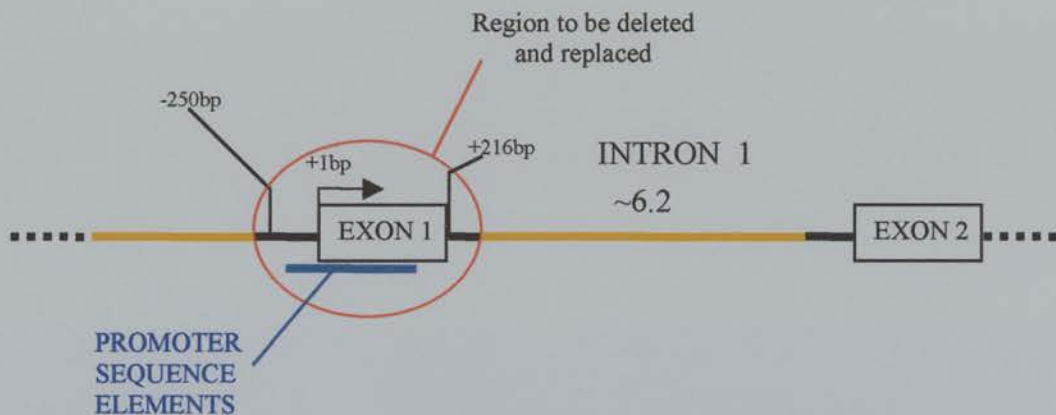


## (5.ii) Targeting Vector Construction

### (5.ii.1) Vector Homology Requirements

The construction of a targeting vector that will knock-out and replace the promoter region of p53 without altering p53 coding sequence requires the derivation of homologous sequence, flanking both immediately upstream and downstream relative to the region of p53 promoter (see fig. 5b below).

Fig.5b



Schematic diagram illustrating gene organisation at the 5' region of p53 locus in mouse. Orange sequence corresponds to candidate sequence that could be cloned as regions of homology in final targeting constructs.

The p53 locus in mouse has been previously characterised with respect to gene organisation and all exons and introns have been fully sequenced. Briefly, the first exon of p53 is wholly composed of non-coding sequence and is separated from the coding sequence of exon 2 by a 6.2kb intron.

The presence of such a large region of non-coding DNA immediately downstream of the region to be replaced is a useful feature of p53 gene



organisation and provides obvious candidate sequence to comprise the downstream “arm” of homology in the final targeting vector. Once cloned as such, all homologous recombination between host and vector sequence will occur within non-coding DNA thus reducing the risk of mutation to, or rearrangement of, p53 coding sequence during the process of HR. As the full sequence of intron 1 is known, it was possible to devise a conventional long PCR strategy to facilitate the rapid cloning of this region (see section 5.ii.3).

The region of DNA located immediately upstream of p53’s first exon was previously uncharacterised with the exception of a small region of approximately 250bp which had been cloned, sequenced and analysed for its ability to influence the expression of reporter gene constructs. In order to clone a portion of DNA rapidly from this region, an inverse PCR strategy was employed (see section 5.ii.2). **N.B.** The restriction maps relating to all plasmids constructed within this chapter are included within the appendix.

### **(5.ii.2) Derivation of the Upstream Arm of Homology**

It is not possible to amplify and clone novel sequence via conventional PCR approaches because prior sequence knowledge of the DNA template is essential to design both upstream and downstream primers. However, if one sequence specific primer can be designed, inverse PCR strategies have been developed that enable the amplification of novel DNA sequences.

The vectorette tag system is such a PCR based approach, which employs the use of small synthetic DNA tags composed of double stranded DNA with an internal

mismatch region (see fig. 5c). These tags are ligated onto the free ends of total genomic DNA fragments resulting from the treatment of the DNA with various six-cutter restriction enzymes (see MM1.7).

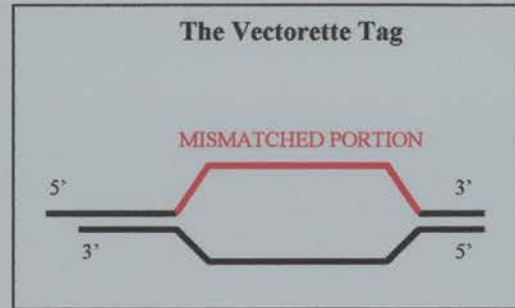
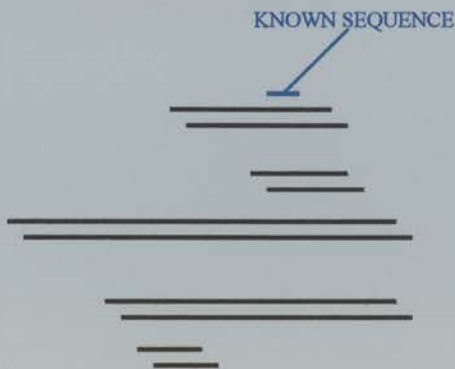
Using the mix of “tagged” genomic DNA as PCR template, the PCR reaction proceeds in the presence of the sequence specific primer and a synthetic primer. The synthetic primer is a 25mer comprising of sequence identical to that of the leading strand at the mismatch region of the vectorette tag, thus initially, this primer has no complementary sequence to anneal to.

Strand extension can only occur from the sequence specific primer during the first round of PCR amplification. The complementary strand to the leading strand at the mismatch region is synthesised at this stage, thus producing complementary sequence for the synthetic primer to anneal to in subsequent amplification rounds. The PCR reaction proceeds normally from the second amplification round onwards, exponentially synthesising increasing copy numbers of the DNA sequence located between the sequence specific and synthetic primer.

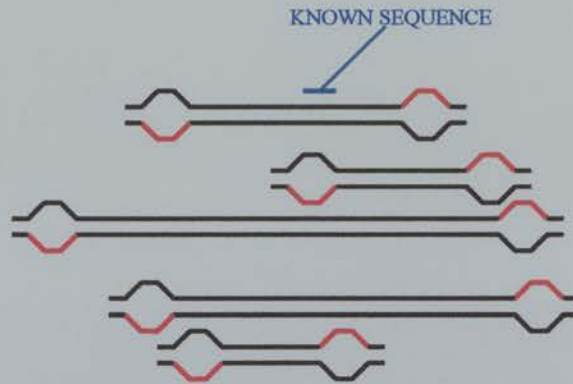
Using this Vectorette tag approach, I constructed tagged genomic libraries from EcoRI, HindIII, BamHI, BglII, BclI and XhoII digests. I then designed two nested primers (see table 5.1), complementary to the leading strand and orientated to extend away from the p53 locus into the previously uncharacterised upstream sequence. The location of the primer, expressed as ‘bp’ in the third column of table 5.1, corresponds to the location of the primer relative to the number of nucleotides upstream of the main transcriptional initiation site of p53 (+1bp).

**Fig. 5c – Schematic Illustrating Vectorette ‘Tag’ Strategy**

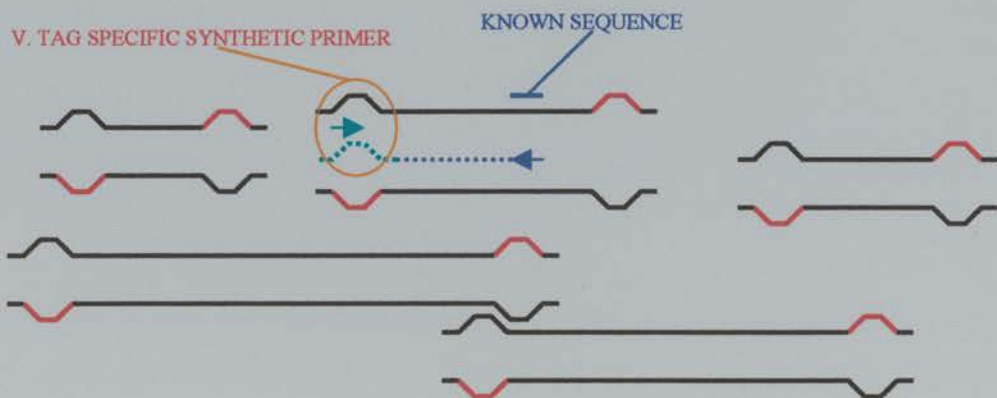
1. Digest Genomic DNA with vectorette tag compatible six-cutter restriction enzyme.



2. Ligate Vectorette tag to fragments of genomic DNA.



3. At the first round of PCR amplification, strand extension can only proceed from the sequence specific primer. The first round extension product is now tagged at the 3' end with sequence complementary to that of the synthetic vectorette primer, enabling exponential amplification of novel template with subsequent thermocycling.



**Table 5.1**

Primer 1 (P1)	5' GCT CTG TGC TCT TTC CTA TCC AG 3'	Located -261 to -239bp
Primer 2 (P2)	5' GAC TCC TGC CTC TGT GTC AAG TA 3'	Located -185 to -152bp

Initial amplification attempts from the tagged libraries were cycled at the conditions listed below in table 5.2, but resulted in the amplification of a smear of non-specific background products (see section MM1.8 for PCR reaction volumes and manufacturers).

**Table 5.2**

94°C	1 minute
60°C	1 minute
72°C	2 minutes

Cycled for 40 cycles

The problems of high background with this approach are reportedly not uncommon, and are predominantly the result of unligated vectorette tags and genomic fragments present in the PCR template mix of tagged genomic fragments. This problem, termed end-repair priming, can lead to significant amounts of non-specific background PCR amplification products.

Recently, vectorette technology has been superseded by splinkerette technology, which employs the use of a highly stable internal stem loop structure instead of the region of internal mismatch. The modified splinkerette tag is reportedly free from the problems of end-repair priming (Devon *et al*, 1995).

Following modification of PCR programme cycle and reaction volumes, I produced a band from the XhoII tagged library using primer P1 and the conditions listed below in table 5.3.

**Table 5.3**

94°C	1 1/2 minutes
68°C	1 minute
72°C	2 1/2 minutes

Cycled for 40 cycles

**Fig. 5d**

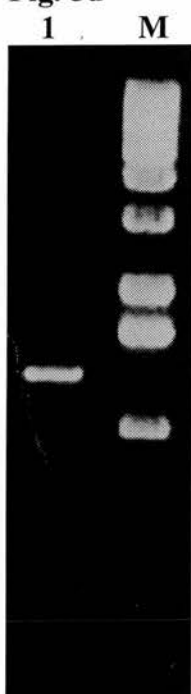


Fig 5.d shows specific 1.3Kb PCR product in lane 1, resulting from the amplification of the XhoII tagged vectorette library with primer P2.

This band sized at approximately 1.3Kb (see fig 5.d) was the predominant PCR amplification product over a small amount of non-specific background and was cloned using a TA cloning vector (Invitrogen; see MM1.6). This fragment will be referred to as FX for the remainder of the chapter (Fragment from XhoII library).

To rapidly prove that FX was the desired sequence immediately upstream of the p53 promoter and not non-specific artefact, I developed a diagnostic PCR assay. This screen used primer P2 and another existing primer, designed previously to anneal at the very limit of previously characterised sequence, termed Sou 1 (see table 5.4). The screen was optimised (see table 5.5) to amplify a 117bp fragment from genomic DNA template. If genuine, the 1.3Kb band FX should contain both primer annealing sites to enable amplification of this product.

**Table 5.4**

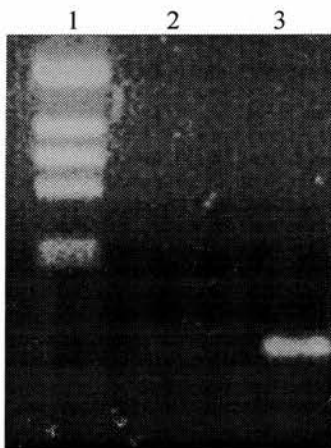
Sou 1	5' GAT GGC TAT GAC TAT CTA GCT GG 3'	Located -280 to -258bp relative to p53 +1bp
-------	--------------------------------------	---------------------------------------------

**Table 5.5**

94°C	1 minute
60°C	1 minute
72°C	2 minutes

Cycled for 32 cycles

**Fig. 5e**



**Fig 5e** shows results of diagnostic PCR using primers P2 and Sou 1. From left to right, lane 1 is 1Kb DNA ladder, lane 2 is +ve control (wild-type ES cell DNA as template), lane 3 is amplification product from FX.

As shown in fig. 5e, FX was positive for the 117bp diagnostic band.

Subsequent sequencing proved conclusively that the PCR derived sequence of FX was immediately upstream of the p53 promoter (see section MM1.9). This limited amount of sequence data also permitted the design of another primer, PIN 1 (see table 5.6) to work in conjunction with primer P1, and enabled the re-derivation of FX using high-fidelity Taq DNA polymerase and genomic DNA as template (see table 5.7). The re-derived, hi-fidelity version of FX was cloned into pCRII (Invitrogen) as pCRII.FX, then sub-cloned and fully sequenced (see fig. 5f and 5g).

**Table 5.6**

PIN 1	5' GAG GCA CCG GTT CAA AGT CTG TAT 3'	Located from 59bp to 89bp of FX sequence (see fig. 5g)
-------	------------------------------------------	--------------------------------------------------------

**Table 5.7**

94°C	2 minutes
------	-----------

For one cycle

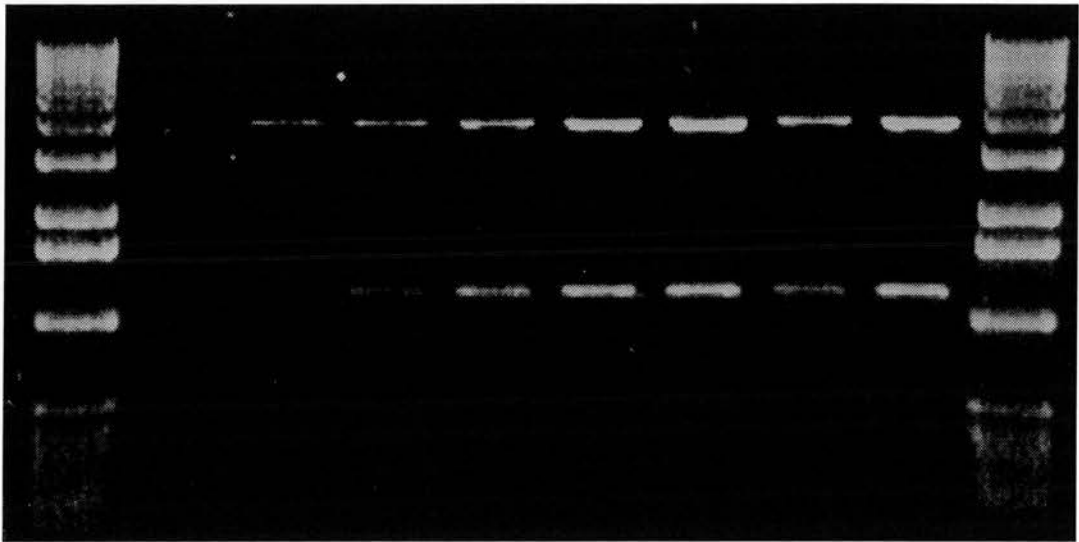
94°C	45 seconds
64°C	1 minute
72°C	2 minutes

For 10 cycles

94°C	45 seconds
64°C	1 minute
72°C	2 minutes + 20 second increments per cycle

For 20 cycles

**Fig. 5f**



**Fig 5f** shows the successful cloning of Hi-fidelity amplified FX (at 1.3Kb) into pCRII plasmid backbone (4Kb) in all lanes to generate pCRII.FX. DNA marker is 1Kb ladder.



Fig. 5g

FX - COMPLETE SEQUENCE

1

TCGCTGTCCT CTCCTTCGAT CTGTGGCTAG CTGGGGTTGG TCATCACCAC

51

CGCATGGCGA GGCACCGGT CAAAGTCTGT ATTTTCCTC CGCCTGGGGA

Pin AI            PIN 1 →

101

ACCTTGGGGT ACCGGAGCTG GGGCCAGGTC AGGAGGGAGG CTATCCGGAG

KpnI

151

CTAAGAGTCG CTCCTCCGAC GTCTTCATTC TGTAGAGTAA GCCCCCGGAA

201

GGCAGAGGTC GGGCAAGTCT CGCTGAGCCG GCTACCAGCT GCCGAGGCTA

PvuII

251

GAGTGCATTA CCGTTCCCAG GGATGCTCAG AGACCGGAGT CCGCTTTCCT

301

CTTCCGGAAA ATGTAAGCCG AACCTAAAGC AATCACCAGG GAACGAGTGT

351

CCAAAGCCAA GCGCCTAGGG TCGCTAGGCG CCGCCAGGGC TTCTTGCTCT

401

CGCGGGAGTC GGGCCACCTT CCGATAGGCT CTCCGCATCC TCCTCCGATT

451

CCGAGCGGGA AGGCGGGAAG GAGCGACTTT GCCTACACCT CAAGCGCTGG

501

**AGAATTCCTA** GAGGTTTCTG GAAGTTGTAG TCTGAACTCT GGCCTTGCG

Eco RI

551

AAACTACAC GAGCGCCCC TACCGTCCCC TGGGGGTAAT TCTTAAAGCG

601

CCTATCCTCC CTGGCCTGCA GAGGGCGCAT AATTTCTACA GTTTTTGCCC

651

CTCTTGACTA TCTTGTTTTG AATCCCGTAA CCTCAGGTTT CCTTTCTCCC

701

CATCTCTCCC CCCTTCTTGT TCCTCTCTTT CCCTTTCTCC CCCGCCCTCC

751

CTTCATTCAT TCGACATTTA TTGTCAAGTT CTTACTGCCT AACCCAGGAC

801

TATACAAGGC ATTGGGAAAA AAATAGCAAT GTTTTCTAGT TCTTAATCTC

851

CATAAAGTTT TCGTTGCTGT GCAATTAAAG GCTGTGAAAA CAGTCTTTAC

901

AGAGAGTGAT AAGGACTGTA CAGGAAATTA AACACGGTGG TCGGATACCA

951

AGTATCTCGG AGAACACGTT AGATTGAGAT ACTATGAAAA GCCTTTCTAA

1001

AGTGACATTT TAGCTAATGA GGGGAAAAAG AACTTAGGGG CCCGTGTTGG

1051

TTCATCCCTG TACTTGGGAA GCCTAAAGCA GGAAGACGGC CGCGAATTCC

Eco RI

1101

AGGCCAGCCT TGGCTACAAA GACTCTATCT TAAAAATCCA AAAAGATGGC

1151

TATGACTATC TAGCTGGATA GGAAAGAGCA CAGAGCTCAG AACAGTGGCG

← P1

1201

GTCCACTTAC GATAAAAAC TAAATCTTTC CACTCTTTAT ACTTGACACA

1251

GAGGCAGGAG T

## KEY.

Red text = Primer Sequences

Green text = Restriction Sites used in mapping fragment

Blue text = Previously characterised sequence

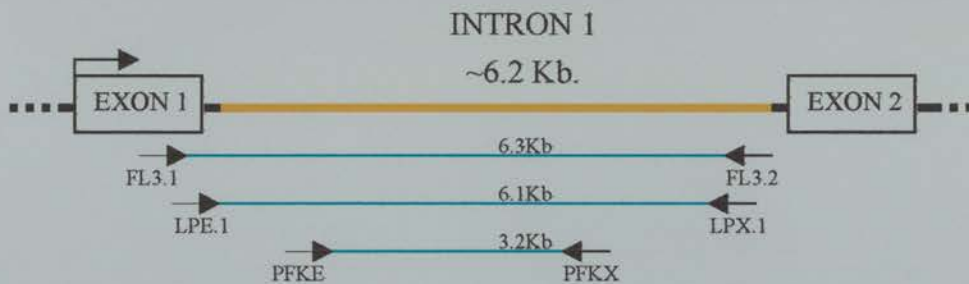
### **(5.ii.3) Derivation of the Downstream Vector Arm**

The successful amplification of DNA template by PCR is generally limited to product sizes of no greater than 5kb using conventional Taq DNA polymerase. This size limitation is thought to result from the inability of Taq DNA polymerase to proof-read and correct nucleotide misincorporations and this consequently affects the ability of Taq to continue primer extension (Barnes, 1994). Different formulations of DNA polymerase are now commercially available that combine both the thermostable properties of Taq DNA polymerase with DNA polymerases originating from other species of micro-organism, which possess proof-reading activity (e.g. *Pwo* DNA polymerase). Using such a mix of DNA polymerase, amplification of DNA templates up to 40Kb in length have been reported, however, it should be noted that high quality template DNA is essential when attempting to amplify fragments larger than 12Kb in size. All PCR reactions subsequently listed in section 5.ii.3 have been conducted using the Expand Long Template PCR system from Boehringer Mannheim (see section MM1.8 and accompanying literature provided by supplier with kit).

The complete 6.2Kb sequence of p53's intron 1 has previously been published (GENBANK; accession U10088) and primer pairs were designed to anneal and amplify DNA template from this region. The initial primer pair FL3.1 and FL3.2 (see fig 5h and table 5.8), were designed to anneal to sequence within exon 1 and the 3' end of intron 1. Theoretically this should result in the amplification of the entire region immediately downstream of the p53 promoter. Subsequent restriction enzyme modification and cloning of this amplification product would

entire region immediately downstream of the p53 promoter. Subsequent restriction enzyme modification and cloning of this amplification product would enable its use as the downstream arm of homologous sequence in the final replacement targeting vector.

**Fig. 5h**



**Fig. 5h** illustrates approximate primer annealing sites and resultant amplification product size relative to both exons 1 and 2 at the 5' end of the p53 locus. See table 5.8 for primer data.

**Table 5.8**

FL3.1	5' CTG TAG GTA GCG ACT ACA GTT AGG 3'	Located +125 to +149bp within exon 1
FL3.2	5' TGT AGA GAG AAG AGA TTG TGT ACT G 3'	Located 6156bp to 6180bp at 3' end of intron 1
LPE.1	5' CTC CGA ATT CTG GGT AAG TAA TTG ATG AGC GTG 3'	Located +204 to 20bp at 3' end of exon 1
LPX.1	5' AGT CTA GAA TAA AAA GAA GGT GGG ACA TCC AGT 3'	Located 6059bp to 6091bp at 3' end of intron 1
P3KE	5' GCA CAG TTC TCT TGC AAT CAG CTA GA 3'	Located 4746bp to 4771bp of intron 1
P3KX	5' TCA TCT AGA GCT TAC ATA TGA CCC TG 3'	Located 1605bp to 1630bp of intron 1

Following extensive modification of PCR reaction component concentrations and PCR cycling conditions, primers FL3.1 and FL3.2 could not be optimised to produce the desired 6.3Kb product.

A further primer pair, LPE.1 and LPX.1, was designed to anneal to sequence at both the 5' and 3' ends of intron 1. These primers were designed to amplify a 6.1Kb template which, with respect to its moderately shorter length, would perhaps be amplified more efficiently than the previously desired 6.3Kb product. Again, following extensive attempts at reaction optimisation, this primer pair also proved to be unsuccessful at producing the specific amplification product. Further reaction optimisation attempts were made using alternative primer pair combinations from the 2 primer pair sets, but this approach was also unsuccessful.

Further decreasing the length of DNA template, a third primer set, P3KE and P3KX was designed to amplify a 3.2Kb fragment. This primer pair combination was optimised to amplify the desired template (see fig. 5i) using the cycle conditions detailed in table 5.9.

This 3.2Kb amplification product, termed F3K for the remainder of this chapter (**F**ragment of **3 K**b), was subsequently cloned into pCRII (Invitrogen), to form pCRII.F3K (see figures 5.ia and 5.ib). This plasmid was then extensively restriction mapped and approximately 200bp, at both the 5' and 3' ends of F3K, was sequenced to prove the authenticity of this PCR amplification product.

**Table 5.9**

94°C	2 minutes
------	-----------

For one cycle

94°C	1 minute
55°C	45 seconds
72°C	2 minutes

For 10 cycles

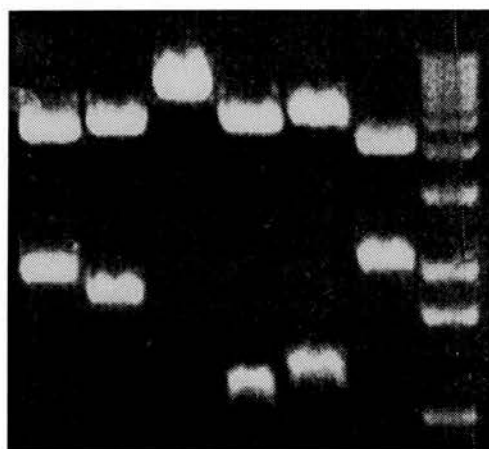
94°C	1 minute
55°C	45 seconds
72°C	2 minutes + 20 second increments per cycle

For 20 cycles

**Fig. 5i.a**



**Fig. 5i.b**



**Fig 5i .a** shows the successful amplification of F3K.

**Fig.5.i.b** shows pCRII.F3K, following digestion with BamHI, HindIII, uncut, SacI, KpnI and XhoI respectively (looking at gel lanes from left to right). Resultant fragment sizes are as predicted (see pCRII.F3K plasmid map in appendix).

Despite the possibility that F3K may have been cloned with occasional point mutations arising from the process of PCR amplification, F3K was directly cloned into the final targeting construct without prior sequencing. The reasons for this are essentially two-fold.

Firstly, F3K is wholly comprised of non-coding sequence, therefore following homologous recombination, the introduction of any point mutation into this region of the host ES cell will not affect resultant wild type gene expression.

Further, it is not essential for homologous targeting vector sequence to be entirely composed of isogenic DNA. Although isogenic targeting constructs reportedly possess enhanced homologous recombination frequencies, the presence of occasional Taq DNA polymerase mediated nucleotide misincorporations within F3K does not preclude against its use as an arm of homology in the final replacement targeting vector. It should be noted however that F3K was amplified from isogenic 129/OLA genomic DNA template with high fidelity Taq DNA polymerase in an attempt to derive sequence with the highest possible degree of homology to that of the host cell locus. DNA template amplified by both Taq and *Pwo* DNA polymerase, reportedly exhibit a nucleotide misincorporation rate approximately 10 fold lower than template amplified with Taq DNA polymerase alone (ref. technical data sheet, Boehringer Mannheim).

#### **(5.ii.4) Selection Marker Strategy and Construction of Targeting Vector**

A successful double replacement targeting strategy must enable both the positive selection of the deletion event and the negative selection of the marker replacement event at both the first and second rounds of HR respectively (see fig



5a). This can be achieved by either replacing host cell sequence with both positive and negative selectable marker sequences (e.g. *neo* and *tk*), or by replacing with a single selectable marker cassette that will permit both positive and negative selection (e.g. HPRT selectable marker cassette in otherwise HPRT deficient cells). There is no significant advantage to be gained from either approach and so the HPRT approach was chosen as it simplified subsequent targeting vector construction.

The basis of positive and negative HPRT selection in mammalian cells arises from the ability of normal cells to utilise both HPRT mediated salvage and *de novo* purine synthesis pathways to synthesise inosinic acid; a prerequisite for nucleic acid synthesis.

In HPRT deficient mammalian cells, only the *de novo* purine synthesis pathway exists and gain of HPRT function can be positively selected when these cells are cultured in the presence of HAT (hypoxanthine, aminopterin and thymidine) containing media (see section MM4.1). Aminopterin blocks *de novo* purine synthesis, thus HPRT deficient cells become completely dependent upon the gain of HPRT function for their survival when cultured in the presence of HAT. Media containing Aminopterin must also be supplemented with hypoxanthine and thymidine to enable the positive selection of HPRT expression however, as the former is a required substrate of HPRT and the latter because aminopterin also inhibits the synthesis of this nucleoside (Szybalski, 1992).

It is also possible to select for cells that lack HPRT gene expression by culturing cells in the presence of 6-Thioguanine (6-TG). 6-TG is metabolised by HPRT to

a toxic nucleotide, therefore HPRT deficiency confers cellular resistance to this molecule (Stutts and Brockman, 1963).

Both F3K and a modified version of FX were positionally cloned to flank an HPRT mini-gene (kindly provided by David Melton).

F3K was removed from pCRII.F3K following EcoRI digestion and was ligated into the EcoRI site of the HPRT mini-gene containing plasmid, pPolyIII/PGK-HPRT(RV) V.17. HindIII digestion was subsequently performed on recombinant clones to orientate F3K relative to the HPRT mini-gene. This new construct was termed pHPRT-F3K.

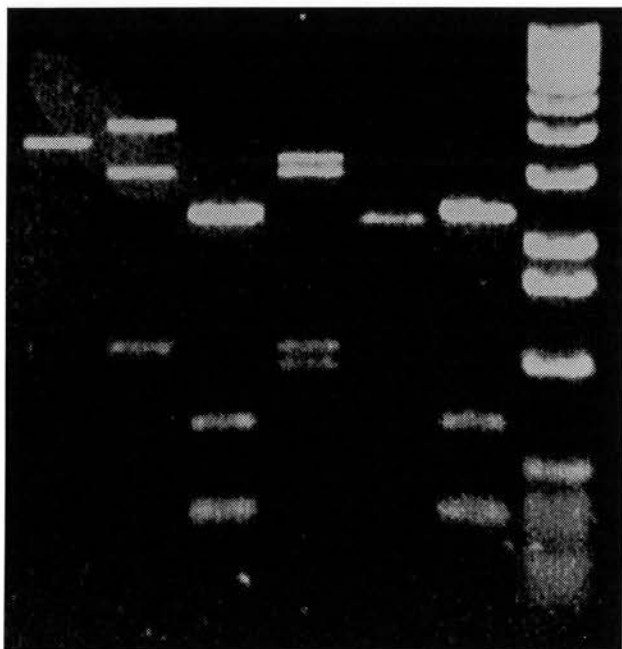
To enable subsequent PCR screening of HAT resistant ES cells (see section 5.iii.1) the plasmid pCRII.FX was digested with KpnI to remove ~80bp from the 5' end of FX prior to targeting vector construction. Re-ligation of KpnI digested pCRII.FX generated the plasmid pCRII.FX $\Delta$ K.

An essential requirement for the application of a PCR screen to identify targeted clones is that one of the primers used must anneal within a region of sequence external to sequences involved in the targeting event. As the sequence upstream of FX is not known, this enzymatic step enabled the previously designed primer PIN1 (see table 5.6) to be used in conjunction with a primer specific to targeting vector sequence for the screening of targeted ES cell clones.

FX $\Delta$ K was removed from pCRII.FX $\Delta$ K following digestion with XbaI and HindIII and was cloned into a Klenow fragment blunted XbaI site of pHPRT-F3K

following blunt-ending with Klenow fragment. FXΔK was then orientated with respect to HPRT and F3K sequences by PvuII restriction digest (see Fig. 5.j).

**Fig. 5.j**



**Fig.5.j** illustrates the completed construction of the 1<sup>st</sup> round targeting vector pFXΔK-HPRT-F3K. Lane 2 shows the correct orientation of FXΔK sequence relative to HPRT-F3K sequences by producing a 4.2Kb, 3.1KB, 1.1Kb and 0.4Kb band following digestion with PvuII. Lane 4 shows FXΔK sequence in the other orientation relative to HPRT-F3K, producing 3.2Kb, 3.1KB, 1.1Kb, 1.0Kb and 0.4Kb bands following PvuII digestion.

The replacement of host cell sequence located between FXΔK and F3K will remove sequences previously demonstrated to influence both the transcriptional and post-transcriptional regulation of p53 gene expression. Following HR, all known transcription factor binding sites will be removed from one allele of p53. The deletion of this region also disrupts the putative region of dyad symmetry located at the 5' end of the p53 locus. This DNA sequence has been implicated in the post-transcriptional regulation of p53 expression by coding to form a stable stem loop structure in resultant mRNA (see chapter 1, section 1.ii.4b). In theory

therefore, the replacement of this region with inducible promoter sequence will produce cells with the ability to conditionally express p53.

**(5.iii) Targeting Event Screening Strategies.**

Both PCR and southern strategies were devised to screen HAT resistant ES cell clones to demonstrate the successful replacement of the p53 promoter region with HPRT mini-gene sequence via HR.

**(5.iii.1) Development of PCR Screen to Detect HR at 5' Vector Arm**

A PCR based screening approach can confirm HR between vector and host locus sequences providing that the amplification product is of reasonable size (see comments made in section 5.ii.3), that one primer anneals to sequence external of recombining sequences and that the other primer is vector specific. The application of such an approach can greatly facilitate the rapid analysis of potentially targeted ES cells.

Primer PIN1 (see table 5.6) was used in conjunction with primer Pr.T.V., designed to anneal to the 3' end of the HPRT mini-gene (see table 5.10), to confirm HR between the upstream vector arm FXΔK and host locus sequence. Only targeted cells possess both primer annealing sites and therefore only these cells can amplify the diagnostic 1.3Kb product from genomic template (see fig 5m).

**Table 5.10**

Pr.T.V	5' CTG AAG CTC TCG ATT TCC TAT CAG 3'	Located at 1054bp to 1077bp of HPRT sequence (Melton '85).
--------	------------------------------------------	------------------------------------------------------------

As no PCR product can be amplified from wild-type cells, a plasmid, pFX.HPRT, was constructed to mimic the correctly targeted locus and enable optimisation of PCR reaction conditions. Full-length FX sequence was cloned adjacent to HPRT mini-gene sequence, thus positioning both primer annealing sites adjacent relative to one another and enabling amplification of the appropriate 1.3Kb template.

PCR reaction conditions were optimised in the presence of wild-type ES cell DNA to ensure that the amplification of plasmid template occurred within a complex DNA template background. For this approach to be successful when applied to the screening of targeted ES cell DNA, the PCR reaction must be sufficiently sensitive to detect and amplify one single gene copy per copy of the genome. Upon the assumption that there are approximately  $3 \times 10^9$  bp per murine genome and that there are approximately  $7 \times 10^3$  bp per copy of pFX.HPRT, the number of plasmid copies per 0.7pg of DNA is equal to the number of genome copies per of 300ng DNA.

To determine the absolute boundaries of reaction sensitivity using primers PIN1 and Pr.T.V and the cycling conditions detailed in table 5.11, 1µl from each of a series of  $1/10$  serially diluted plasmid DNA samples was used as PCR template in reactions containing a consistent background amount of 300ng wild-type genomic DNA.

The initial experiment demonstrated that the PCR reaction was highly sensitive, as the 1.3Kb amplification product was readily visible following  $10^{-7}$  dilution of 1µg plasmid DNA, approximately equivalent to one single gene copy per copy of the genome. The experiment was repeated with two further serial dilutions and still the 1.3Kb amplification product was detectable following  $10^{-9}$  dilution (see

fig. 5.k). The amplification of the desired product following dilution to  $10^{-9}$ , approximates to the detection of 1fg plasmid DNA per 300ng of genomic DNA, or to one single gene copy per hundred genome copies. These results would suggest that primers PIN1 and Pr.T.V are sufficiently sensitive to detect HR between FX $\Delta$ K and the host locus sequences.

**Table 5.11**

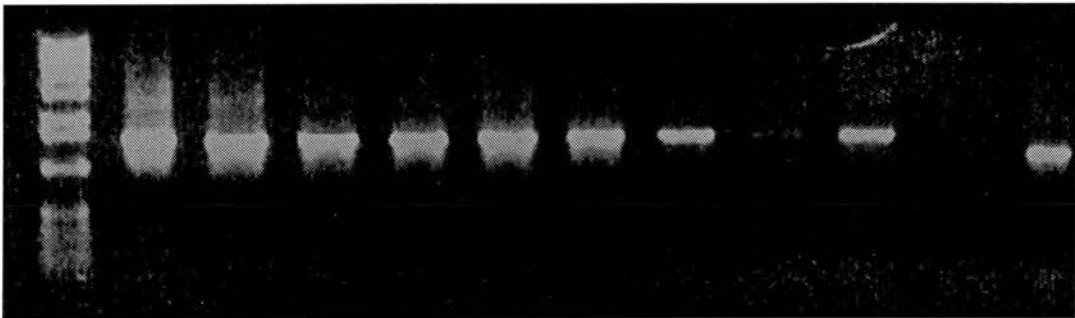
94°C	2 minutes
------	-----------

For one cycle

94°C	1 minute
58°C	1 minute
72°C	1 1/2 minutes

For 35 cycles

**Fig. 5.k**



**Fig 5.k** is a gel photograph demonstrating PCR results following the amplification of serially diluted concentrations of pFX-HPRT DNA plus 300ng genomic DNA. The lanes read from left to right as, 1Kb DNA marker,  $10^{-1}$  dilution,  $10^{-2}$  dilution,  $10^{-3}$  dilution,  $10^{-4}$  dilution,  $10^{-5}$  dilution,  $10^{-6}$  dilution,  $10^{-7}$  dilution,  $10^{-9}$  dilution,  $10^{-8}$  dilution, negative control and positive control consisting of primers PIN1 and P1, with wild-type genomic DNA template alone. This positive control demonstrates that the quality of genomic DNA used as complex background in all other samples is of sufficient quality to enable PCR amplification. 10 $\mu$ l from a total of 100 $\mu$ l final PCR reaction was loaded per lane.

Despite producing convincing data to demonstrate that the PCR screening assay is sufficiently sensitive to detect a single targeted locus from within the genome, failure to amplify the 1.3Kb targeted locus specific PCR product can result for three reasons.

Firstly, if the p53 locus is not targeted, PIN1 and Pr.T.V primer annealing sites never become adjacently positioned relative to one another and therefore are unable to amplify the diagnostic amplification product from genomic template.

An additional reason is that the genomic DNA preparation may be of insufficient quality to enable template amplification. This eventuality can be roughly controlled by using primers PIN1 and P1 to amplify 1.2Kb of wild-type and overlapping genomic DNA template, in addition to using primers PIN1 and Pr.T.V to screen samples.

Finally, it should be noted that the PCR assay was optimised from simple template within a complex genomic background and not from targeted genomic and therefore complex DNA template. In theory, this should make little difference to the optimisation of successful PCR reaction conditions, as the presence of any problematic secondary structure within the complex genomic template should be removed at every PCR cycle following heat denaturation at 94°C. However, any difference in optimal reaction conditions between these two template types could potentially result in the inability to detect HR events in correctly targeted cells via this approach.

Although PCR based screening approaches can greatly facilitate the rapid analysis of potentially targeted ES cells, rather than risk discarding correctly targeted ES

cell clones solely upon the basis of the absence of a diagnostic amplification product, all clones were further screened by Southern analysis.

**(5.iii.2) Development of Screen to Detect HR at 3' Targeting Arm**

To enable the detection of HR between F3K and host locus sequences, a probe was generated by PCR to anneal to sequence external of the targeting event and immediately downstream from F3K. The probe was produced using two primers, Pro3A and Pro3B (see table 5.12) and was amplified from wild-type ES cell DNA using cycle conditions detailed in table 5.13. The resultant 224bp probe was fully sequenced and was shown to be 99.8% identical relative to the sequence of intron 1 stored in Genbank.

**Table 5.12**

Pro 3A	5' AGC CTG TGA CGC ACT AGA TT 3'	Located from 4777bp to 4796bp of intron 1
Pro 3B	5' CAG CAA AGG CAA GCA TTT C 3'	Located from 4983bp to 5001bp of intron 1

**N.B.** Primer Pro 3B forms an internal stable secondary structure and so must be heat denatured for 10 minutes at 94°C immediately prior to being added hot to PCR reaction.

**Table 5.13**

94°C	2 minutes
------	-----------

For one cycle

94°C	1 minute
60°C	45 seconds
72°C	1 minute

For 30 cycles



Following the successful replacement of p53 sequence with HPRT mini-gene sequence, it is predicted that both a BclII restriction site will be introduced and a BstXI site removed from the host locus (see fig 5m). Southern analysis of BclII and BstXI digested wild-type ES cell DNA, with the 3' probe described above, results in the hybridisation and detection of 13Kb and 4.5Kb bands respectively (see fig.5m). Although it is not possible to predict the exact size and mobility shift of hybridised bands following the digestion of a successfully targeted locus, it can be predicted that the size of targeted hybridisation products will be readily discernible from wild-type hybridisation products. Following successful HR, the additionally hybridised band resulting from BclII digestion must be between approximately 10Kb and 4Kb in length, whilst the additionally hybridised band resulting from BstXI digestion must be greater than 7.5Kb.

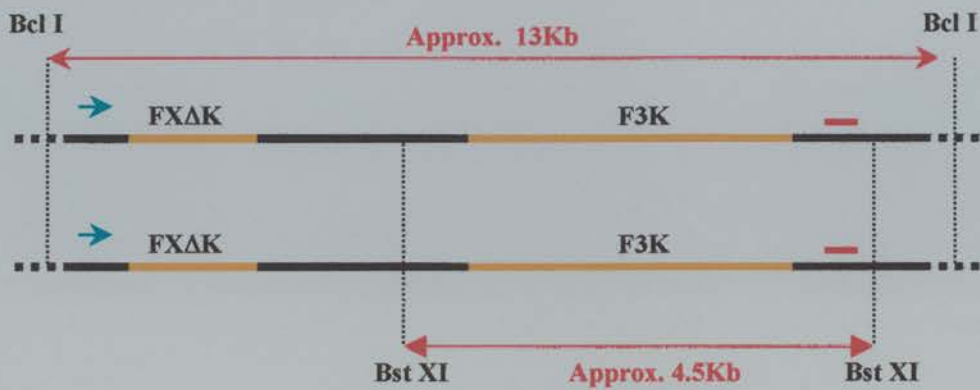
**Fig 5L**



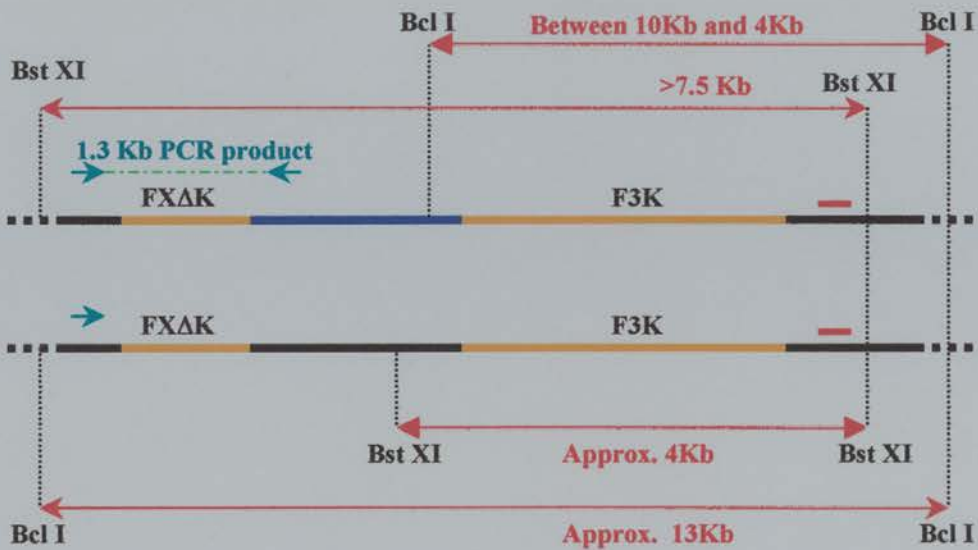
**Fig 5L.** shows an autoradiograph of BclII digested wild-type ES cell DNA, hybridised to P<sup>32</sup> labelled 3' probe. The detected band runs at approximately 13Kb.

Fig 5m - Summary of Targeting Event Screening Strategies

Wild-type p53 locus



P53 locus following 1st round of targeting



KEY

- |                                      |                                         |
|--------------------------------------|-----------------------------------------|
| Genomic Sequence                     | External Probe Sequence                 |
| Homologous Targeting Vector Sequence | PIN1 PCR primer Sequence                |
| HPRT mini-gene Sequence              | HPRT mini-gene Specific Primer Sequence |

#### **(5.iv) Targeting Results using pFX $\Delta$ K-HPRT-F3K**

Initial attempts to target the p53 locus with pFX $\Delta$ K-HPRT-F3K were problematic as large numbers of false-positive ES cell clones proved resistant to, and survived HAT selection following electroporation and plating at a density of  $8 \times 10^5$  cells per  $10\text{cm}^2$  dish. The underlying cause of this phenomenon is well understood, in that cells that stably integrate and express the HPRT mini-gene can rescue non-HPRT expressing clones within close proximity during HAT selection (Hooper and Subak-Sharpe, 1981). Repeat electroporation and plating at a reduced density of  $5 \times 10^5$  cells per  $10\text{cm}^2$  dish produced far fewer, but true HAT resistant ES cell clones (on average 5 HAT resistant clones per dish).

Southern analysis on 128 of these true HAT resistant clones concluded that none had been successfully targeted. All clones produced only the wild-type 13Kb band following BclI digestion and hybridisation with the 3' screening probe. Subsequent PCR analysis on these clones also concluded that no clones had been targeted as all clones failed to amplify the diagnostic 1.3Kb product. To ensure that this was genuinely the result of a zero percentage targeting efficiency and not the result of poor quality genomic DNA preparation, primers PIN1 and P1 were used to amplify 1.2Kb of overlapping genomic template. 121/128 of clones amplified this 1.2Kb template.

Southern analysis of BstXI digested DNA with the 3' probe was not performed on these samples.

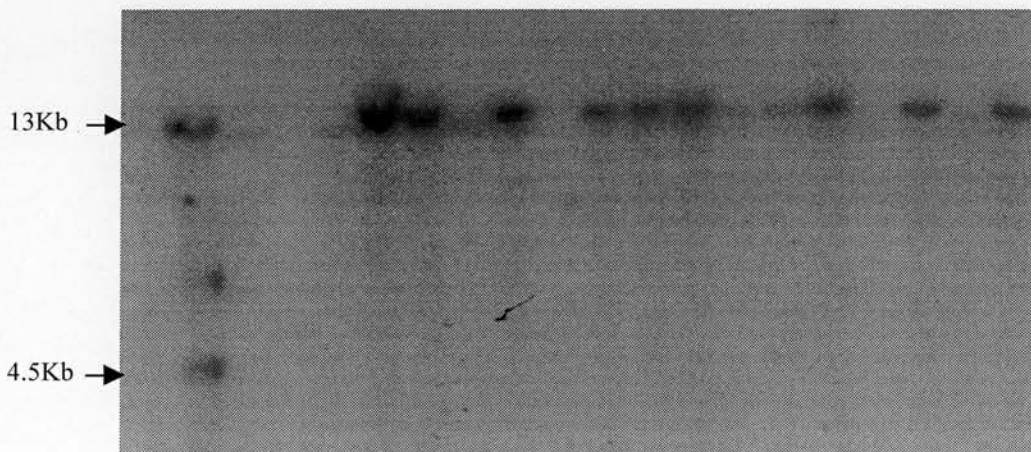
It is well known that the frequency of successful HR can vary extensively during the course of targeting experimentation at different gene loci, and that reported

targeting efficiencies of less than 1/200 are not uncommon. Thus, despite the fact that 0/128 clones proved to be successfully targeted via this approach, it would be wrong to abandon this targeting strategy before more HAT resistant ES cell clones were analysed.

Accordingly a further 420 HAT resistant clones were grown in 24 well plates following electroporation with pFXΔK-HPRT-F3K. 380/420 clones grew sufficiently to enable analysis.

Southern analysis of BclI digested DNA demonstrated that 1/380 clones possessed a second detectable band following hybridisation with P<sup>32</sup> labelled 3' probe (see MM1.4 and MM1.5). Sample 272, as shown in fig.5n, possesses both the wild-type band at 13Kb as well as an additional hybridising band at approximately 4.5Kb. This mobility shift is within the size range predicted to result following the successful replacement of p53 sequence with HPRT mini-gene sequence.

**Fig.5n**



**Fig.5n** shows the results of a southern analysis conducted on the genomic DNA derived from multiple ES cell clones following targeting with pFXΔK-HPRT-F3K and HAT selection. Note that the sample furthest on the left (clone 272) possesses two hybridising bands at approximately 13KB and 4.5Kb.

To confirm that the recombinant p53 allele of clone 272 is the desired targeting event and not the product of illegitimate recombination, attempts were made to analyse this DNA further by PCR screen to confirm HR between FXΔK and host locus sequences.

Using the reaction conditions optimised in section 5.iii.2, it was not possible to amplify the diagnostic 1.3Kb product from clone 272. However, the amplification of this diagnostic template also failed in all positive control samples. *i.e.* amplification failed in the serially diluted pFX-HPRT template plus wild-type genomic DNA background. Further alteration of PCR reaction conditions was not permitted in the time left available, but all individual PCR reagents were renewed in an attempt to re-establish this screen. Unfortunately, technical difficulties persisted with this assay and as a result it was not possible to confirm the successful HR between FXΔK and host locus sequences in clone 272.

Attempts, by Southern type analysis, to additionally confirm that clone 272 had undergone the desired HR event between F3K and host locus sequences, were made by hybridisation of P<sup>32</sup> labelled 3' probe to BstXI digested genomic DNA. In similar circumstances to the PCR targeting screen mentioned above, time was the limiting factor with this experiment, and again technical problems prevented the direct confirmation or otherwise, regarding the nature of the re-arrangement present at the p53 locus of clone 272.

#### **(5.v) Conclusions**

The results clearly show that these attempts to target and delete the 5' promoter region of p53 are highly inefficient with this construct. Despite the possibility

that this approach may have been successful in producing one clone from five hundred and eight analysed, time constraints do not permit the complete characterisation of this ES cell clone, neither do they permit the second round of targeting to render expression from the p53 locus inducible.

Given more time, there are several modifications that could be made to this targeting strategy that would possibly enhance the frequency of successful homologous recombinant clones.

The most obvious would be to increase the overall length of targeting vector homology by increasing the length of the downstream vector arm F3K. Potentially this could be achieved rapidly by further attempting to optimise amplification conditions for new PCR primer sets. Alternatively, considering the difficulties already faced whilst attempting to amplify DNA template from this region of the genome, the existing arms of vector homology could be radio-labelled and used as probes to screen a  $\lambda$  phage genomic library. If successful, this approach would likely produce clones spanning between 10 to 16kb in length, encompassing the p53 locus.

Increasing the length of FX $\Delta$ K, the upstream targeting vector arm, would also be a means of increasing the overall length of targeting vector homology, but this would require further inverse PCR or  $\lambda$  phage cloning and characterisation of novel sequence. Significant extension in the length of FX $\Delta$ K would also make it increasingly difficult to develop a sufficiently robust PCR assay to reliably screen for the successful HR of the upstream targeting arm in ES cell clones surviving selection. Failure to develop a suitable PCR assay of this type would necessitate further cloning and characterisation of novel sequence upstream of FX $\Delta$ K, to

enable the generation of probes and the derivation of a southern assay to confirm the successful HR of this vector arm into the target locus.

Cloning a negative selection marker (e.g. a *tk* cassette) outwith the two arms of vector homology is another approach that could be employed to increase the ratio of correctly targeted to randomly integrated surviving ES cell clones (see chapter 1, section 1.i.7b for discussion).

Following HR between both targeting vector arms and the desired host locus, *tk* expression will be lost. Non targeted clones resistant to HAT by virtue of the random integration of the HPRT mini-gene, will also likely retain the expression of *tk*. Therefore, culturing HAT resistant ES cell clones in media containing gancyclovir will theoretically enrich for the desired targeting event.

Although it is not possible to simultaneously select for loss of *tk* expression and gain of HPRT expression (the thymidine in HAT protects against the kill by gancyclovir), it would be possible to select for and against the expression of these markers sequentially.

A further means of improving targeting efficiency would be to alter electroporation conditions. A recent publication by Templeton *et al* describes an alternative ES cell transfection procedure utilising a modified electroporation and plating procedure which considerably reduces the amount of cell death associated with other well established electroporation procedures. Templeton claims that these protocol modifications are sufficient to raise the absolute efficiency of gene targeting from  $10^{-5}$  to approximately  $10^{-1}$  cells. This targeting frequency is

sufficiently high to enable the isolation of targeted ES cell clones in the absence of selection.

An alternative vector design and targeting strategy, utilising cre/loxP technology, is proposed in fig.5o, which enables both the replacement of p53 promoter sequence with inducible promoter and splice donor sequence within only one round of homologous recombination.

Transient expression of cre recombinase in successfully targeted cells will cause recombination to occur specifically between loxP sequences, resulting in the deletion of both selectable marker cassettes.

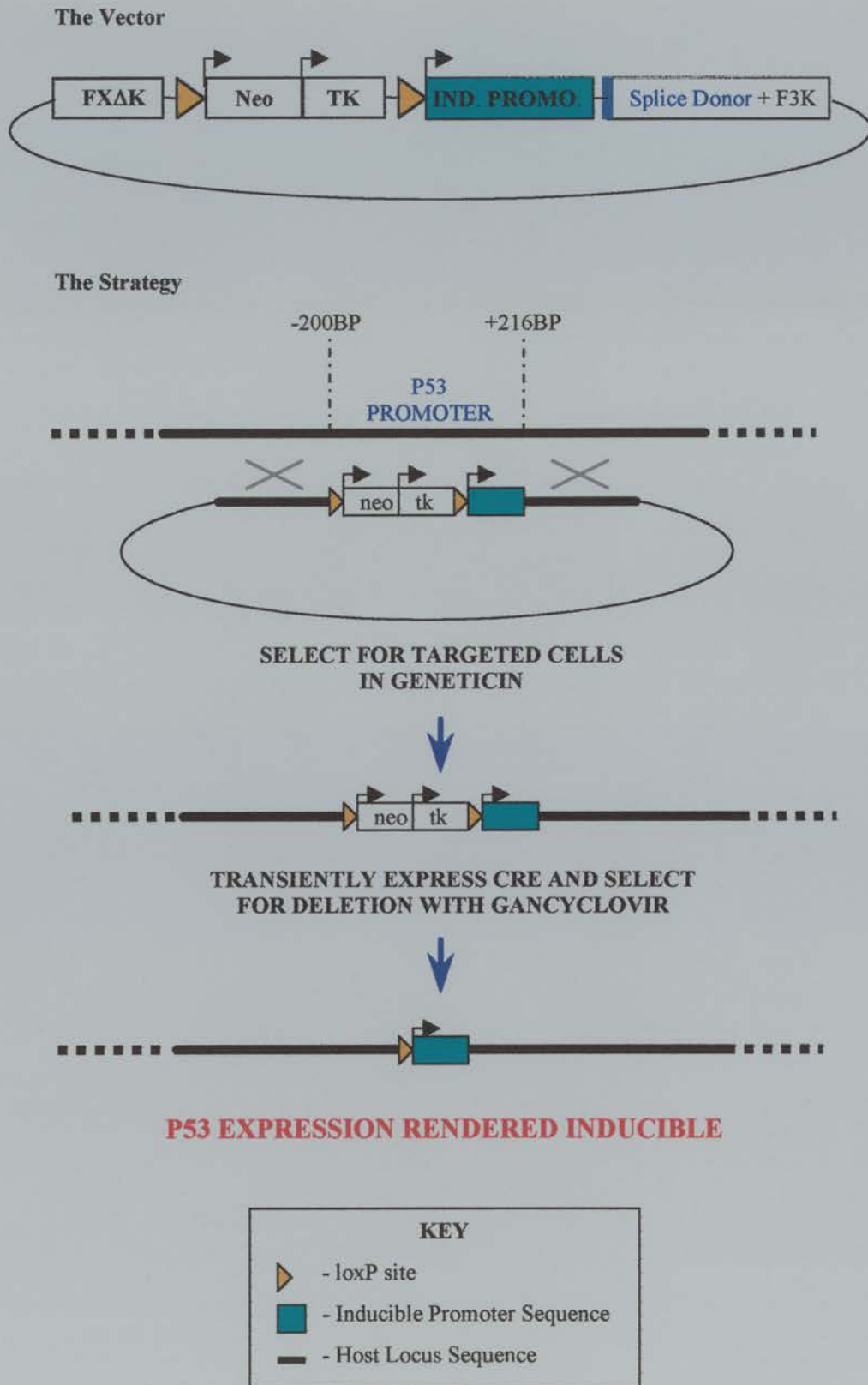
Thus, following one round of HR, targeted cells that successfully undergo cre-mediated recombination will be able to conditionally express p53, in the absence of potential transcriptional interference resulting from the presence of residual selectable marker cassettes at the host locus.

The implications for further research concerning clone 272 and these alternative targeting approaches are discussed in chapter 6.



Fig.5o

### 1-Step Replacement Targeting Vector and Strategy



## CHAPTER 6

### DISCUSSION

#### **(6.i) A Brief Summary of Aims and Experimental Approaches**

The main aim of the experimental work described within this thesis was to render the expression of p53 inducible in murine cells and thus generate animal models with which to better evaluate the therapeutic benefits conferred by p53 replacement gene therapy in the treatment of cancer.

As discussed within chapter 1, multiple human gene therapy trials are currently being undertaken whereby tumours are treated with recombinant viral vectors that constitutively express the tumour suppressor gene p53. These trials were initiated primarily upon the basis of observations made from extensive *in vitro* experimentation suggesting that the re-introduction of p53 expression, within an otherwise p53 deficient tumour cell background, can induce the growth arrest or apoptosis of tumour cells. Indeed, preliminary observations from these gene therapy trials would appear to suggest that in certain instances tumour size can be stabilised or even regression induced via such a therapeutic approach (Roth *et al*, 1996).

The major limitation of all human gene therapy trials is the current inability of vectors to efficiently deliver and maintain stable gene expression within cells growing *in vivo* (Blau, 1997). It is therefore highly unlikely that these current p53 replacement gene therapy trials will form an effective and permanent anti-tumour treatment.

To formally test the hypothesis that the introduction of p53 gene expression into tumour cells growing *in vivo* can form the basis of an effective anti-tumour treatment, animal models are required whereby the expression of wild-type p53 can be precisely manipulated. Many p53 deficient cell types are known to be predisposed to tumourigenesis, therefore the ability to efficiently upregulate p53 expression within these cells growing *in vivo* would essentially mimic highly efficient vector-mediated gene delivery and thus would permit an opportunity to better evaluate subsequent therapeutic effects.

To achieve these aims both an inducible transgene approach (chapters 2 and 3) and a double replacement targeting approach (chapter 5) were attempted to effectively render the expression of all p53 protein within the cell inducible. As described within the opening section of these respective chapters, both approaches have associated advantages and disadvantages relative to one another. In brief, the inducible transgene approach is rapid to establish compared with the targeting approach, however the targeting approach is not affected by ‘position effect’ and so potentially offers greater reproducibility in modelling the effects of highly efficient vector gene delivery between different tumour types.

Clearly the efficacy of both approaches in achieving the experimental aims stated is also dependent upon maintaining the transcriptional silence of desired gene expression in the absence of inducer. Significant non-induced levels of transgene expression may be sufficient to confer phenotypic effects upon the cell, therefore to truly model and evaluate the consequences of replacement gene therapy by

these approaches, the expression of the desired gene must be silent or at least at very low levels within the cell in the absence of inducer.

With regard to these considerations, the inducible expression systems initially intended to be applied to these studies were the tetracycline inducible and the human 6-16 (interferon  $\alpha/\beta$ ) inducible promoter systems. The plasmid constructs required to render the expression of a p53 transgene inducible to both these stimuli were constructed as detailed within section 2.i.2, however lack of time precluded further experimental work utilising the tetracycline system.

To render the expression of p53 from the genomic locus inducible to various stimuli, a replacement targeting vector was constructed to delete endogenous p53 promoter sequence and enable the subsequent introduction of inducible promoter sequence (chapter 5). The resultant targeting efficiency was very low, but one clone appeared to possess a re-arrangement at the 3' end of targeting homology. The results and implications from both studies are discussed in the following section.

#### **(6.ii) Discussion of Experimental Data**

The experimental data described within chapter 2 was generated following the stable transfection of an interferon  $\alpha/\beta$  inducible p53 transgene (p6-16<sub>L</sub>p53-HYG) into a novel sarcoma derived cell line termed SL1 (Sarcoma-Line 1). SL1 tumour cell sub-clones were subsequently derived, which demonstrated very low levels of p53 transgene expression when in the non-induced state, but the ability to induce p53 transgene expression when cultured in the presence of interferon  $\alpha/\beta$ . Transgene dependent effects upon tumour cell cycle and the rate of apoptosis

were measured in both the presence and absence of UV-C induced DNA damage by flow cytometric analysis.

In brief, proliferation of SL1 tumour cells was shown to be rapidly, but temporarily reduced when cultured in the presence of interferon  $\alpha/\beta$ , irrespective of additional genotoxic insult. No further significant cell cycle effect was observed within SL1 cell cultures following the induction of p53 transgene expression.

Conversely, interferon  $\alpha/\beta$  appeared to exert no effect upon the induction of apoptosis in transgene<sup>-ve</sup> SL1 cells in either the presence or absence of DNA damage, whilst a clear 'early' p53-dependent wave of apoptosis was detected within cultures of transgene<sup>+ve</sup> SL1 cells following treatment with interferon  $\alpha/\beta$  and exposure to UV-C irradiation.

Transgene<sup>-ve</sup> cells retained the ability to enter apoptosis in response to an equivalent dose of DNA damage, but this p53-independent apoptotic response was initiated and reached maximal levels approximately 12 and 24 hours respectively after the p53-dependent apoptosis observed in transgene<sup>+ve</sup> cells. The initiation of p53-dependent apoptosis did not however appear to significantly affect the long-term clonogenic survival of transgene<sup>+ve</sup> SL1 cells relative to transgene<sup>-ve</sup> cells.

As described within section 1.i.8b, interferon  $\alpha/\beta$  has been used as an anti-tumour treatment for several human malignancies, therefore the observation that this reagent can temporarily inhibit SL1 tumour cell cycle should not be considered surprising. Indeed, in addition to tightly regulating transgene expression, this

inducible expression system potentially enables observation of synergistic or adjuvant anti-tumour effects elicited by the introduction of p53 gene expression in the presence of interferon  $\alpha/\beta$ .

The lack of observed p53-dependent growth arrest, especially in the presence of DNA damage was not predicted. The ability of p53 to cause cell cycle arrest at multiple checkpoints is well characterised in a wide variety of wild-type and transformed cells possessing either damaged DNA template or elevated levels of oncogene expression.

Perhaps the most obvious hypothesis to explain this observation is that the p53 protein expressed from p6-16<sub>L</sub>p53-HYG is not wild-type p53 protein. P53-mediated growth arrest appears to be dependent upon the ability of p53 to transactivate or repress the expression of specific downstream loci. As discussed in section 1.ii.2a and 2b, mutant p53 proteins have previously been identified that are unable to transactivate gene expression as a result of point-mutations affecting the coding of amino acid residues within their N- terminal and core domains. Mutations affecting the N- terminal domain of p53 appear to prevent appropriate interaction between p53 and other protein components of the basal transcriptional machinery, whereas mutations within the core domain of p53 can affect wild-type protein conformation or the ability of p53 to bind to its cognate sequence.

The RGC-reporter assay, mentioned within section 2.vi, was intended to directly test the ability of transgenic p53 protein to transactivate the expression of downstream genes. Time constraints however limited the opportunity to define optimal and reproducible transient transfection conditions and thus the results of this experiment could not be concluded.

A further scenario that could explain the observed lack of transgene-mediated cell cycle arrest of 1D cells is that additional sequence may be translated from p6-16<sub>L</sub>p53-HYG and 'tagged' to the C- terminal domain of transgenic p53. Messenger RNA transcribed from p6-16<sub>L</sub>p53-HYG was shown not splice appropriately at the 3' end (see section 2.ii.2), although theoretically this should not alter the final p53 translation product.

As p53 tetramerizes within this protein domain however, it may be possible that the presence of an additional C- terminal peptide 'tag' could inhibit or de-stabilise the multimerisation of transgenic p53 protein and thus alter the ability of p53 to modify the expression level of specific downstream loci.

To examine and compare the size of transgenic p53 protein relative to wild-type p53, whole cell extracts from interferon  $\alpha/\beta$  treated 1D cells were subjected to western analysis using the anti p53 polyclonal antibody BMG-1 (section 2.vi).

Specific p53 epitope(s) appeared to be most abundant at 72 hours after DNA damage on a protein several kilodaltons larger than wild-type p53. Although this recognised protein would appear to be specific, all previous data examining transgene expression levels at times following transgene induction and DNA damage suggest that transgene expression is maximal within 6 hours of exposure to interferon  $\alpha/\beta$ . No specific protein was recognised on the western blot at this time, therefore it is not possible to conclude in the absence of further experimentation whether the protein recognised on this blot is genuinely expressed from p6-16<sub>L</sub>p53-HYG or is non-specific background.

The p53 cDNA cassette used in these studies was fully sequenced prior to vector construction and was shown to encode wild-type p53 protein accordingly (Malcomson, 1996). Presumably therefore, the underlying nature of both of these hypotheses must be a mutational event affecting the coding region of transgenic p53 that occurred after the transfection of p6-16<sub>L</sub>p53-HYG into SL1 cells.

Non-induced levels of transgenic p53 protein appear to be very low in 1D cells, but this level of protein may still have been sufficient to confer a selective disadvantage to these cells growing *in vitro*. Indeed, an observation that was not at first deemed of relevance was that from the total of 9 clones initially shown to exhibit good-fold induction of p53 expression, 5 lost their proliferative capacity within a few passages in culture. It is therefore feasible that the 4 remaining clones either expressed non-physiologically relevant amounts of non-induced wild-type p53 protein or had gained selective advantage through the mutation of p53 coding sequence enabling escape from this 'crisis' in culture. Further circumstantial evidence to support the latter notion is that following the induction of p53 transgene expression, no transgene-dependent effects were observed upon either the cell cycle or rate of apoptosis of 1D cells when cultured in normal conditions (i.e. in the absence of additional DNA damage).

To formally prove such a theory, both the comparison of p53 transgene-mediated effects and direct scrutiny of the sequence comprising the coding region of stably integrated transgenic DNA would be required between multiple SL1 derived cell lines.



Interferon  $\alpha/\beta$  and p53 appear to inhibit tumour progression through different molecular pathways, therefore one could predict that the appropriate use of both reagents in conjunction would at least confer an additive anti-tumour effect. A further speculative hypothesis to explain the lack of observed p53 transgene-dependent growth arrest however, may be that interferon  $\alpha/\beta$  is indirectly responsible for editing p53 mRNA.

Interferon  $\alpha/\beta$  is known to stimulate the expression of the enzyme dsRAD in response to double stranded RNA (see section 1.i.8b). The expression of dsRAD has also been shown to be physiologically relevant regarding the generation of isoform diversity amongst glutamate and serotonin 2C receptors within the brain (Seeburg, 1996 and Burns *et al*, 1997).

The expression of dsRAD can effectively mutagenise coding sequences residing within dsRNA as this enzyme binds to dsRNA and catalyses the conversion of adenosine to inosine. tRNA recognises rITP as rGTP, thus dsRAD-mediated editing of dsRNA is equivalent to an adenosine to guanosine conversion.

The 5' portion of p53 mRNA encodes for the N- Terminal (transactivation) domain of p53 protein and possesses extensive dyad symmetry that potentially forms a stable dsRNA stem loop structure (see section 1.ii.4b).

In addition to interferon  $\alpha/\beta$  inducing the expression of p53 from p6-16<sub>L</sub>p53-HYG therefore, interferon  $\alpha/\beta$  mediated induction of dsRAD may be able to alter the coding sequence of transgenic p53 mRNA molecules sufficiently to impair p53-dependent transactivation (and consequently growth arrest) within the cell.

Although all of the above theories concerning the lack of p53-dependent growth arrest relate to mutation or modification of transgenic p53 coding sequence, it

could also be reasoned that this observation might result from lesions affecting the expression of p53-dependent genes.

An examination of the expression levels of known downstream components of the p53-dependent growth arrest pathway (*e.g.* p21, GADD45 *etc.*) may enable the investigation of such a scenario. However, as not all the components or requirements regarding the initiation and maintenance of this pathway are yet known, the detection of p53-dependent p21 gene expression would not necessarily confirm that all the molecules through which p53-dependent growth arrest can be achieved are present within the cell and are functional.

Transient transfection studies utilising the original p6-16<sub>L</sub>P53-HYG construct and SL1 cells cultured in the presence of interferon  $\alpha/\beta$  may be able to demonstrate the ability of transgene expression to arrest cell growth. Indeed, such experiments could provide further insight regarding whether the lack of observed transgene dependent cell cycle arrest in response to DNA damage is cell line or transgene dependent.

One of the major limitations of current human gene therapy trials is the inability to introduce and maintain the expression of genes in cells growing *in vivo*. The results detailing attempts to induce transgene expression *in vivo*, following sub-cutaneous injection of 1D cells (see chapter 3), also appear to highlight this problem as a potentially major limitation of utilising genes that restrain cell growth as candidates for replacement gene therapy.

Following sub-cutaneous injection of 1D cells, tumours appeared rapidly at the site of injection (within 3 weeks), yet transgene expression could barely be

detected following stimulation of endogenous interferon  $\alpha/\beta$  and whole body  $\gamma$ -irradiation.

Following the explantation of two 1D-cell tumours back into culture, the cells from one tumour retained the ability to induce similar levels of p53 transgene expression as cultured 1D cells, whilst the cells from the other tumour cell line had lost the ability to induce p53 expression. This suggests that during the establishment of tumours derived from 1D cells *in vivo*, selective advantage could be gained from loss of non-induced transgene expression. Although the *in vitro* characterisation of 1D cells demonstrated low levels of non-induced p53 protein, it is possible that levels of non-induced transgene expression are significantly higher (and physiologically relevant) within 1D cells growing *in vivo* as a result of exposure to systemic levels of interferon  $\alpha/\beta$  within the mouse.

The observed loss of inducible transgene expression could have occurred through multiple mechanisms including chromosomal loss or gross deletion, point- or frame-shift mutation of transgene coding sequence or through modulation of the host cell interferon  $\alpha/\beta$  signalling pathway.

Both explanted cell lines retained resistance to a concentration of hygromycin comparable to that of non-injected 1D cells suggesting that transgene expression was not lost as a result of chromosomal loss or by large chromosomal deletion.

An experiment that could potentially discriminate between loss of transgene expression by mutation of p53 transgene coding sequence or modulation of host cell interferon  $\alpha/\beta$  signalling involves the sequential stable transfection of both p6-16<sub>L</sub>p53-HYG and a 6-16 regulated luciferase reporter construct into SL1 cells. These cells could be injected sub-cutaneously into SCID animals as detailed

previously and following the appearance of tumours, would be explanted back into culture prior to treatment with interferon  $\alpha/\beta$ .

Low luciferase expression levels from the cells passaged *in vivo* relative to the cells maintained *in vitro* would suggest that host cell interferon  $\alpha/\beta$  signalling had been modified. Such a situation could account for the inability of these cells to induce p53 transgene expression.

An approximately equivalent level of luciferase expression within the cells passaged *in vivo* and cells maintained *in vitro* would suggest that any observed loss of inducible transgene expression had resulted from genetic lesions directly affecting p53 coding sequence.

Theoretically a single mutational event conferring less efficient interferon  $\alpha/\beta$  signalling within 1D cells would modify or even ablate the anti-tumour effects conferred by both interferon  $\alpha/\beta$  and transgenic p53. A single mutation arising within p53 transgene coding sequence however, would impart less selective advantage as 1D cells would still be susceptible to the anti-proliferative effects conferred through exposure to interferon  $\alpha/\beta$ . It would therefore be interesting to conduct the experiment as detailed above and to observe whether the frequency of functional inactivation of interferon  $\alpha/\beta$  signalling pathway relative to p53 transgene product reflects these predictions.

The inability to efficiently induce transgene expression *in vivo* effectively precluded any evaluation of therapeutic transgene-mediated effects upon tumours comprised of 1D cells. An experiment that could potentially examine the ability of injected 1D cells to establish tumours *in vivo* would be to sequentially treat 1D

cells growing *in vitro* with interferon  $\alpha/\beta$  and DNA damage immediately prior to sub-cutaneous injection. This experiment represents an *ex vivo* equivalent to the long-term clonogenic survival assay described within section 2.v. Recording the mean time taken for tumours to present, following all combinations of transgene induction and exposure to DNA damage, would potentially enable some insight regarding the transgene-mediated effects within 1D cells growing *in vivo* before transgene inducibility is selectively modified or lost.

One of the major limitations of the inducible transgene study described within chapters 2 and 3 is that the effects of p53 expression cannot be analysed in p53 deficient tumours arising *de novo*. Time constraints precluded against the generation of p53 inducible mice, although the construct p6-16<sub>L</sub>p53-HYG was transfected into ES cells and stable recombinant clones were selected.

The intrinsic polygenic nature of cancer means that any conclusions drawn upon the basis of observations within 1D cell culture or tumours, regarding the potential efficacy of p53 replacement gene therapy in the treatment of malignant disease, would have to be qualified. The genetic background of 1D cells is unlikely to be generally representative of all tumour cell types, therefore the ability (or otherwise) to treat 1D-cell tumours by this approach cannot directly be extrapolated as being of relevance to the treatment of malignant disease in general.

The double replacement targeting approach described within chapter 5 would theoretically permit an examination of induced wild-type p53 gene expression

within tumours arising *de novo*. Indeed, the effects of p53 replacement gene therapy could also potentially be modelled within tumours originating from an outbred genetic background following successful replacement targeting and a conventional breeding programme. Such animals would provide the most accurate means of assessing the ability of p53 to treat a wide spectrum of malignancies.

If ES cell clone 272 proves to be correctly targeted, then it will be possible to re-target and replace the HPRT marker sequence of these cells with that of an inducible promoter by using the same regions of homology as the first targeting vector. Multiple second round targeting vectors could potentially be constructed rendering the expression of the p53 locus inducible to a wide variety of stimuli, however following the experience gained through experimentation with the 6-16 promoter, the targeted introduction of Tet-O sequences would be the favoured option.

Animals targeted with 6-16 promoter sequence would not possess the SCID mutation and so would likely not be negatively affected by attempts to stimulate endogenous levels of interferon  $\alpha/\beta$  following administration of pI:pC. Use of the tetracycline inducible system however, would have the advantage of being effective at concentrations that do not elicit pleiotropic effects *in vivo*, and so would permit a clearer examination of transgene mediated effects upon the treatment of tumours.

An alternative 1-step targeting strategy utilising Cre/loxP technology has also been proposed within section 5.v of chapter 5, which would rapidly render the expression of p53 inducible from its genomic locus.

### **(6.iii) Future Perspectives and Concluding Remarks**

Current DNA vector technology is unable to efficiently deliver stable gene expression to cells growing *in vivo*, thus it is presently not possible to accurately evaluate the therapeutic benefits conferred by replacement gene therapy in the treatment of genetic disease.

The value of modeling replacement gene therapy within animals utilising inducible promoter technology is therefore likely to remain high until significant improvements are made in the field of DNA vector technology, sufficient as to enable a direct measure of therapeutic effects conferred by replacement gene therapy treatment regimes *in vivo*.

Inducible promoter technology is both relatively new and improving, but is also widely available and so will undoubtedly prove to be of great utility in efforts to understand the basis of genetic disease. It is now known that certain inducible expression systems are better than others for precisely regulating gene expression and enabling clear examination of transgene-mediated effects within the cell. Thus it is likely that increasing amounts of experimental data will be generated in the short-term utilising both the choice tetracycline and ecdysone based inducible expression systems. Indeed, the results of such a research effort will certainly prove highly informative regarding both the evaluation of prospective replacement gene therapy treatment regimes, as well as providing further insight into the underlying molecular basis of genetic disease.

Recent technical improvements have been reported within the field of inducible promoter technology that should further enable these experimental goals to be

reached rapidly. In an attempt to reduce background levels of transgene expression within non-induced cells, tet repressor molecules have been engineered to possess altered dimerisation domains (Rossi *et al*, 1998). The system, designated Retro-Tet-ART, possesses repressor molecules that can bind to tet-O sequences and inhibit transgene expression in the absence of tetracycline, as well as activator molecules that bind to tet-O sequences in the presence of tetracycline and transactivate transgene expression. In this manner, background levels of transgene expression are reportedly further reduced, thus this system would appear to be the current inducible expression system of choice for analysing the effects of potentially toxic or growth-limiting transgene expression within the cell and for modelling replacement gene therapy.

Even in the light of improved inducible expression systems and correspondingly in the ability to generate improved and accurate animal models, the ultimate goal to effectively cure human genetic disease remains.

In this respect, a recently developed recombinant lentiviral (HIV-derived) vector has been described as being able to deliver stable reporter gene expression to cells within the brain, muscle and liver at higher efficiency relative to existing adeno- and retroviral vectors (Naldini *et al*, 1996 and Kafri *et al*, 1997). Lentiviral vectors are reportedly superior to both adenoviral and retroviral vectors as they can efficiently transduce non-dividing cells and because they are pseudotyped with VSVG (Vesicular Stomatitis Virus G) glycoprotein, they can potentially transduce a wide variety of cell types *in vivo* with a high degree of efficiency.



The application of recombinant lentivirus as means of introducing stable gene expression *in vivo* has yet to be extensively tested and fully optimised however, therefore their true potential as a means of efficiently transducing cells growing *in vivo*, sufficient to enable direct evaluation of gene replacement therapeutic effect, cannot presently be estimated.

Disruption of p53-mediated tumour suppression pathways appears to be a near unifying feature of human tumourigenesis and therefore is an obvious candidate gene to base the development of future anti-cancer treatments. Before replacement gene therapy can be considered a viable alternative to conventional chemotherapeutic and drug-based cancer treatments however, significant technical improvement in the ability to deliver stable therapeutic gene expression to target cells growing *in vivo* currently appears to be an absolute requirement. Considering the inherent genetic instability of tumour cells, the task of attempting to improve the means through which gene expression is introduced and maintained, such that replacement gene therapy can become an effective means of treating malignant disease, would appear to be all the more difficult.

## CHAPTER 7

### MATERIALS AND METHODS

#### Section 1 - DNA Techniques

##### MM1.1

##### **Heat-Shock Transformation of CaCl<sub>2</sub> Competent *E. Coli* with Plasmid DNA**

1. Add either 50ng of supercoiled plasmid DNA, or 10% volume of a plasmid DNA ligation mixture to a freshly thawed 100µl aliquot of CaCl<sub>2</sub> Competent XL-1 blue *E. Coli* (Stratagene) on ice.
2. Gently mix samples by stirring with a p200 pipette tip and keep on ice for a further 30 minutes.
3. Heat-shock sample tubes at 42°C for 45 seconds, before replacing back on ice for 2 minutes.
4. Add 250µl of LB broth to each sample tube and incubate at 37°C with shaking (approx. 225 rpm) for 1 hour.
5. For routine transformation of supercoiled plasmid DNA, plate 1/5<sup>th</sup> sample volume, and for DNA ligation experiments, plate entire sample volume, onto appropriate antibiotic containing LB-agar plate and incubate inverted at 37°C overnight.

##### MM1.2

##### **Preparation of Plasmid DNA from *E. Coli***

##### Mini-Prep

The preparation of plasmid DNA by this method should be sufficient to produce between 20-50µg DNA. This method was routinely used to provide sample DNA for the analysis of cloning steps involved during the construction of all vectors detailed within this thesis.

1. Using a white-capped universal tube (Griener), inoculate 5mls of LB media (supplemented with appropriate antibiotic) with a single bacterial colony previously grown on a LB-agar plate (see section MM1.1).
2. Incubate sample at 37°C overnight with vigorous shaking (~225rpm).

**N.B.** – To promote efficient growth of bacterial culture, leave lid of universal sample tube slightly loose to enable good oxygenation of sample.

### **The Following Day**

1. The overnight culture should now appear turbid. Remove 1.5ml from each sample and spin at 4,000rpm for 5 minutes on a desktop microfuge (Sanyo).
2. Carefully remove and discard all LB supernatant and add 100µl of ice cold Resuspension Buffer to remaining pellet. Vortex vigorously to ensure complete resuspension of pellet.
3. Add 200µl of freshly prepared Lysis Buffer and gently invert sample tube several times to mix. The bacterial suspension should clear.
4. Add 150µl of Neutralisation Buffer and again gently invert sample tube several times to mix. A cloudy white precipitate should form.

**N.B.** – **Do not** vortex sample tubes at stages **3** and **4**, as this will shear bacterial genomic DNA which could cause contamination of final plasmid DNA prep.

5. Incubate samples from stage **4** on ice for 5 minutes before spinning at full speed (13,000 rpm) on a desktop microfuge for 20 minutes.
6. Carefully remove and transfer supernatant to a clean 1.5ml eppendorf tube.
7. To remove protein from plasmid DNA sample, add 300µl of Tris-buffered phenol and 300µl of chloroform: iso-amyl alcohol mix (24 parts: 1 part respectively). Vortex each sample thoroughly, before spinning at full speed (13,000 rpm) on a desktop microfuge for 5 minutes.
8. Carefully remove aqueous (upper) phase to a clean 1.5ml eppendorf tube, ensuring that no organic phase is carried over.

9. Add 150µl of 3M Sodium Acetate (CH<sub>3</sub>COONa), pH 5.5 and 1ml of absolute ethanol to precipitate plasmid DNA. Vortex thoroughly and place at -80°C for 1 hour.
10. Remove samples from -80°C freezer and spin at full speed (13,000 rpm) on a desktop microfuge at 4°C for 20 minutes. A blue/white DNA pellet should now be visible at the base of the eppendorf tube. Carefully remove and discard supernatant without disturbing pellet.
11. To remove excess salt from plasmid DNA prep add 1ml of 70% ethanol, gently invert tube several times and spin at full speed (13,000 rpm) on a desktop microfuge for 5 minutes. Again, carefully remove and discard supernatant without disturbing DNA pellet
12. All traces of ethanol must be removed from each sample prior to resuspension of DNA pellet. Either, open and invert sample tubes on the bench to air-dry for several hours, or place open sample tubes in a vacuum dryer for 5-10 minutes. To ensure resuspension of high-quality plasmid DNA, care should be taken not to over-dry pellets.
13. The dried plasmid DNA pellets are finally resuspended in a 50µl volume of either dH<sub>2</sub>O or TE buffer. Plasmid DNA prepared in this manner will be stable long term at -20°C.

## **SOLUTIONS**

### LB Media (per litre)

10g – NaCl

10g - Bacto-Tryptone

5g - Yeast Extract

### Resuspension Solution

50mM Glucose

25mM Tris.HCl (pH=8.0)

10mM EDTA (pH=8.0)

Autoclave and store at 4°C

### Lysis Solution

(To make 5mls)

4.55mls dH<sub>2</sub>O

0.25mls 20% SDS

0.20mls 5M NaOH

### Neutralisation Solution

(To make 100mls)

60mls 5M Potassium Acetate  
(C<sub>2</sub>H<sub>3</sub>KO<sub>2</sub>)

11.5mls Glacial Acetic Acid

28.5mls dH<sub>2</sub>O

TE Buffer (pH 7.5)

10mM Tris.Cl (pH=7.5)

1mM EDTA (pH=8.0)

**N.B.** – Final pH of solution is dependent upon the initial pH of Tris.Cl

### **Maxi-Prep**

The preparation of plasmid DNA by this method should be sufficient to produce between 200-500µg DNA. This method employs the use of QIAGEN columns to produce very high quality plasmid DNA in large quantities, suitable for the purposes of mammalian cell transfection and DNA sequencing.

1. Using a sterile 500ml conical flask, inoculate 100mls of LB media (supplemented with appropriate antibiotic) with a single bacterial colony previously grown on a LB-agar plate (see section MM1.1).
2. Loosely cover the top of the conical flask with tinfoil and incubate sample at 37°C overnight with vigorous shaking (~225rpm).

### **The Following Day**

All subsequent protocol steps and buffer compositions are detailed in the “QIAGEN Plasmid Purification Handbook, January 1995”, which is included with every batch of QIAGEN plasmid purification kits.

### **MM1.3**

#### **Restriction Digestion of DNA**

Examples of typical restriction digest reaction volumes are detailed below. These include typical volumes for the analysis of plasmid DNA, for the cloning of plasmid DNA and for the analysis of genomic DNA. Although total reaction volumes and incubation times differ for each application of this technique, the relative volume of restriction enzyme(s) should be consistent and never exceed 10% of the total reaction volume.

### Analysis of Plasmid DNA

1µl appropriate 10X React buffer (Gibco-BRL)

1µl of plasmid DNA (at an approximate concentration of 1µg/µl)

0.5µl of RNase A

6.5µl of dH<sub>2</sub>O

1µl (or 10 units) of appropriate restriction enzyme(s) (Gibco-BRL)

\*10µl total reaction volume

**{Incubate at appropriate temperature for 1 hour}**

### Cloning of Plasmid DNA

3µl appropriate 10X React buffer (Gibco-BRL)

5µl of plasmid DNA (at concentration of 1µg/µl)

0.5µl of RNase A

18.5µl of dH<sub>2</sub>O

3µl (or 30 units) of appropriate restriction enzyme(s) (Gibco-BRL)

\*30µl total reaction volume

**{Incubate at appropriate temperature for 3 hours}**

### Analysis of Genomic DNA

6µl appropriate 10X React buffer (Gibco-BRL)

10µg of genomic DNA

0.5µl of RNase A

Sufficient dH<sub>2</sub>O to increase total reaction volume to 57µl

3µl (or 30 units) of appropriate restriction enzyme (Gibco-BRL)

\*60µl total reaction volume

**{Incubate at appropriate temperature over-night}**

## **MM1.4**

### **Southern Analysis of Genomic DNA**

1. Digest 10µg genomic DNA overnight as detailed in section MM1.3

2. Run out genomic digest on a 0.8% agarose gel until 500bp DNA marker reaches the foot of the gel. Photograph gel and DNA markers beside a ruler to enable accurate sizing of bands which subsequently hybridise to P<sup>32</sup> radiolabelled probe.
3. (Optional) If fragment of interest is greater than 10Kb, depurination of DNA within the gel **prior** to denaturation reportedly increases the efficiency of DNA transfer to nylon filter.  
Transfer gel to a tray containing several volumes of Depurination Solution. Soak the gel, with gentle agitation, for 10 minutes. Rinse briefly with dH<sub>2</sub>O.
4. Transfer gel to a tray containing several volumes of Denaturation Solution. Soak the gel, with gentle agitation, for 45 minutes. Rinse briefly with dH<sub>2</sub>O.
5. Transfer gel to a tray containing several volumes of Neutralisation solution. Soak the gel, with gentle agitation, for 30 minutes. Rinse briefly with dH<sub>2</sub>O.

## SOLUTIONS

<u>Depurination Solution</u>	<u>Denaturation Solution</u>	<u>Neutralisation Solution</u>
0.2N HCl	1.5M NaCl	1.5M NaCl
	0.5M NaOH	1M Tris-HCl, pH 7

### Capillary Transfer of DNA from Gel to Nylon Filter

1. Fill a tray with 10x SSC.
2. Rest a piece of plastic on the sides of the tray to form a platform over the 10 x SSC solution. Wrap the platform with a piece of Whatmann 3MM filter paper soaked in 2 x SSC, ensuring that all trapped air bubbles that arise between paper and platform are removed and that the edges of the 3MM paper rest within the 10 x SSC solution in the tray.
3. Place the neutralised gel on top of the 2 x SSC soaked 3MM paper. Cut pieces of para-film to cover all wet areas of the 2 x SSC soaked 3MM paper not already covered by the gel.

4. Cut a piece Hybond N+ (Amersham) nylon filter to size, wet in dH<sub>2</sub>O and place on top of gel. Ensure the complete removal of all air bubbles that may arise between gel and Hybond N+ filter.

**N.B.** - Marking one side of the nylon filter with pencil facilitates subsequent orientation of the autoradiogram to the filter.

5. Cut two pieces of Whatmann 3MM paper to the size of the gel, wet in 2 x SSC and place on top of Hybond N+ filter. Ensure the complete removal of all air bubbles that may arise between 3MM paper and Hybond N+.
6. Place a stack of dry paper towels on top of the wet 3MM paper. Place a glass plate over the top of the paper towel stack and weigh down with a large book (or equivalent!). Cover the edges of the tray with cling-film to minimise the evaporation of 10 x SSC and leave to transfer overnight.

This procedure should set up a flow of liquid from the reservoir in the tray, through the gel and into the paper towels, transferring the digested DNA fragments from within the gel onto the nylon membrane.

7. The following day, dismantle stack and dry Hybond N+ filter between two sheets of 3MM Whatmann filter paper.

**N.B.** – the filter must dry completely to ensure fixation of the DNA to the nylon membrane.

#### Hybridisation of Radio-Labelled Probe to DNA Immobilised on Nylon Membrane

1. Wet the dry DNA bound nylon filter membrane in 2 x SSC solution and place on top of a piece of nylon gauze, wetted in dH<sub>2</sub>O. The layer of gauze ensures even distribution of probe over the entire surface of the DNA bound nylon membrane
2. Roll the two pieces of nylon together to form a tube and place inside of a Hybaid hybridisation tube.
3. Add 25mls of pre-hybridisation solution (with 500µl boiled Salmon Sperm DNA (Sigma; 10mg/ml)) to the hybridisation tube and incubate membrane at 65°C in a rolling incubator for 3 hours.
4. Boil P<sup>32</sup> radiolabelled probe (see section MM1.5) for 10 minutes and pipette into blue-capped Falcon tube. Immediately decant pre-hybridisation solution



into Falcon tube containing probe, invert to mix, then quickly pour back into Hybaid tube containing membrane. Incubate the probe with the membrane at 65°C, in a rolling incubator, overnight.

## SOLUTIONS

### Dextran Sulphate Stock Solution

50g Dextran Sulphate dissolved in  
300mls of dH<sub>2</sub>O

### Pre-Hybridisation Solution

15mls 20x SSC  
5mls 10% SDS  
30mls Dextran Sulphate Stock Solution

5. The following morning, decant probe/hybridisation solution from Hybaid tube and rinse twice with 2 x SSC.
6. Add 100mls Wash Solution I (pre-warmed to 65°C) to hybaid tube and incubate at 65°C in the rolling incubator for 20 minutes before decanting.
7. Repeat this step a further four times by sequentially adding 100mls pre-warmed Wash Solution II through to V. In theory, this step removes non-hybridised and non-specifically hybridised probe from the nylon filter membrane.

Wash Solution I	2x SSC + 1% SDS in dH <sub>2</sub> O	Lowest Stringency
Wash Solution II	1x SSC + 1% SDS in dH <sub>2</sub> O	↓
Wash Solution III	0.5x SSC + 1% SDS in dH <sub>2</sub> O	↓
Wash Solution IV	0.25x SSC + 0.1% SDS in dH <sub>2</sub> O	↓
Wash Solution V	0.1x SSC + 0.1% SDS in dH <sub>2</sub> O	Highest Stringency

8. Following the last wash, remove filter membrane from Hybaid tube and gently blot dry with a piece of Whatmann 3MM paper.
9. Seal filter into polythene bag and tape securely into auto-rad cassette (with enhancer screens).
10. Overlay a piece of KODAK Biomax MS film (Amersham) over hybridised filter membrane and place at -70°C for between 1- 4 days prior to developing.

## **MM1.5**

### **Radiolabelling Probe with P<sup>32</sup> by Random Priming**

All probes were labelled with Stratagene's "Prime-it RmT Random Prier Labelling Kit".

1. Add 50ng of probe DNA and dH<sub>2</sub>O to a volume of 42µl in a single reaction tube.
2. Boil sample tube for 5 minutes before briefly centrifuging to collect condensation.
3. Add 3µl of Magenta DNA polymerase and 5µl of P<sup>32</sup> labelled dCTP (ICN). Mix thoroughly and place reaction tube at 37°C for a minimum of 1 hour.
4. At end of labelling reaction add 2µl of stop reaction buffer.

### Determination of Radio-Nucleotide Incorporation

1. Empty the contents of a Sephadex G50 column (Amersham) and wash resin by allowing a minimum of 2mls TE buffer to pass through column.
2. Add 20µl of Salmon Sperm DNA to stopped probe labelling reaction tube, mix thoroughly and add total volume directly onto Sephadex resin (take care not to splash any volume of probe labelling mixture on column walls).
3. Add 400µl TE directly onto resin and collect flow through. This volume of TE should not be significantly radioactive.
4. Add a further 400µl of TE directly onto resin and collect flow through. This volume of TE should contain probe.
5. Compare residual counts in Sephadex column with those present in the fraction of TE containing labelled probe. 50% label incorporation is achieved if counts from both sources are approximately equal. This is the minimum radio-nucleotide incorporation efficiency that should be used for subsequent experimentation.

## MM1.6

### Ligation of DNA Fragments

The following section details my lab protocols used to clone fragments of DNA into plasmid vector backbones.

#### “Sticky” End Cloning

This technique is applicable when the restriction enzyme(s) used in the preparation of DNA vector and insert are either identical and thus generate identical 5' or 3' overhangs, or are different but generate compatible 5' or 3' overhangs. (e.g. BglII and BamHI recognise and cut at different sites, but generate identical 5' overhangs).

1. Cut both insert and vector with the appropriate restriction enzyme(s) as detailed in section MM1.3.
2. Following digestion, supplement the restriction digest of intended **vector sequence only** with:

- 1µl appropriate 10x reaction buffer
- 8µl dH<sub>2</sub>O
- 1µl Shrimp Alkaline Phosphate (S.A.P.)

Vortex sample tube to mix thoroughly and place reaction at 37°C for a further hour.

**N.B.** - This step is optional but reduces the frequency of background cloning products considerably by preventing the recircularisation of vector sequences in the absence of insert. Enzymatic treatment with S.A.P. removes the terminal-end phosphate groups from linearised vector sequence and thus prevents the ligation of free vector ends in the absence of insert sequence.

3. Stop restriction digest and de-phosphorylation by adding 10% volume of Loading Buffer.

4. Run DNA on a 0.8% agarose gel (or appropriate) to separate desired DNA fragments from all non-required or undigested sequences present in the sample.
5. Excise the appropriate digested vector and insert bands from the gel with a scalpel blade and purify using QIAquick Gel extraction kit (Qiagen). See manual provided with kit for protocol.
6. Run 10% of the gel-purified vector and insert fragments on a 0.8% agarose gel beside known DNA standards. This step enables evaluation of both the integrity and concentration of the purified DNA fragments.  
**N.B.** – **do not** attempt to clone DNA fragments that appear as a slightly diffuse or “smear” band on an agarose gel. Anything other than a tight band with little background at this stage of the cloning procedure indicates poor integrity and/or degradation of the desired DNA fragment.
7. Set up ligation reaction using 50ng vector DNA and an approximate 3:1 molar ratio (insert: vector) of DNA fragments.

#### Ligation reaction

- 2 $\mu$ l 10x Ligase buffer
- x $\mu$ l DNA vector (equivalent to 50ng)
- x $\mu$ l DNA insert (to generate 3:1 molar ratio of insert to vector)
- x $\mu$ l dH<sub>2</sub>O to adjust total reaction volume

\*Total reaction volume = 20 $\mu$ l.

8. Incubate ligation reaction at 16°C overnight.
9. The following day, transform CaCl<sub>2</sub> competent bacteria with 10% of ligation reaction as detailed in section MM1.1.

#### “Blunt” End Cloning

This technique is applicable when the restriction enzyme(s) used in the preparation of DNA vector and insert sequences either generate 5' or 3' overhangs that are not compatible or cut to generate blunt ended DNA.

1. Cut both insert and vector with the appropriate restriction enzyme(s) as detailed in section MM1.3.
2. (a) If enzymes cut to generate non-compatible 5' overhangs, proceed to step 3.  
(b) If enzymes cut to generate non-compatible 3' overhangs, proceed to step 4.  
(c) If enzymes cut to generate a blunt ended DNA fragment, proceed to step 5.

3. Blunt ending DNA Fragments with 5' overhangs using Klenow Fragment

Supplement restriction digestion with the following components:

- 1µl appropriate 10x reaction buffer
- 4µl 10mM dNTP's (final reaction concentration = 1mM)
- 4µl dH<sub>2</sub>O
- 1µl Klenow fragment (Large)

Vortex to mix thoroughly and incubate sample for a further hour at 37°C.

**(Proceed to step 5).**

4. Blunt ending DNA Fragments with 3' overhangs using T4 DNA Polymerase

Supplement restriction digestion with the following components:

- 1µl appropriate 10x reaction buffer
- 4µl 10mM dNTP's (final reaction concentration = 1mM)
- 4µl dH<sub>2</sub>O
- 1µl T4 DNA Polymerase

Vortex to mix thoroughly and incubate sample for a further hour at 37°C.

**(Proceed to step 5).**

5. Supplement the restriction digest of intended **vector sequence only** with:

- 1µl appropriate 10x reaction buffer
- 8µl dH<sub>2</sub>O
- 1µl Shrimp Alkaline Phosphate (S.A.P.)

Vortex sample tube to mix thoroughly and place reaction at 37°C for a further hour.

6. Exactly follow protocol steps 4, 5 and 6 detailed in the previous protocol for the cloning of “Sticky” ended fragments of DNA.
7. Set up ligation reaction using 50ng vector DNA and an approximate 1:1 molar ratio (insert: vector) of DNA fragments.

#### Ligation reaction

- 2µl 10x Ligase buffer
- xµl DNA vector (equivalent to 50ng)
- xµl DNA insert (to generate 1:1 molar ratio of insert to vector)
- xµl dH<sub>2</sub>O to adjust total reaction volume

\*Total reaction volume = 20µl.

8. Incubate ligation reaction at 16°C overnight.
9. The following day, transform CaCl<sub>2</sub> competent bacteria with 10% of ligation reaction as detailed in section MM1.1.

### **MM1.7**

#### **Construction of Vectorette Genomic DNA Libraries**

The Vectorette genomic DNA libraries were constructed from 129/OLA ES cell DNA (extracted as detailed in section MM4.1) by digesting total genomic DNA with BamHI, ClaI, EcoRI, HindIII, EcoRV, XhoII restriction enzymes and ligating the appropriate compatible vectorette tag.

1. Digest 1µg of 129/OLA ES cell genomic DNA with 20 units of restriction enzyme for 1 hour at 37°C (as detailed in section MM1.3), in a total reaction volume of 50 µl.

2. Add the following to restriction digest:
  - 5µl Vectorette units (3pmol)
  - 1µl 100mM ATP
  - 1µl 100mM DTT
  - 1 unit T4 DNA ligase
3. Incubate ligation reaction at 20°C for 1 hour, then 37°C (or appropriate) for 30 minutes. **Repeat 3 times.**

**N.B.** this repeated thermocycling enables optimal ligation efficiency of synthetic tags to genomic DNA by enabling the digestion of tag: tag ligation products and not tag: Genomic DNA products at 37°C.

If using a compatible 4-cutter enzyme, **do not** thermocycle and incubate sample tube at a constant 20°C for 5 hours.
4. Following ligation, add 200µl dH<sub>2</sub>O and divide total reaction volume into 10µl aliquots. Store at -20°C.
5. Use 1µl of Vectorette library as template per PCR amplification reaction, in conjunction with sequence specific and synthetic DNA tag specific primers, as detailed in section MM1.8

Synthetic Vectorette Primer Sequence:

5' CGA ATC GTA ACC GTT CGT ACG AGA A 3'

## MM1.8

### PCR amplification of DNA

The following reaction mix was standard for all PCR amplifications detailed within this thesis. Only Mg<sup>2+</sup> concentration and cycle conditions were varied to enable optimisation of specific template amplification for each individual primer pair (detailed at each relevant section throughout thesis).

The following reagents were prepared in a template free area:

10µl 10X PCR Reaction buffer IV (Advanced Biotechnologies)

2mM dNTP's

40µM Primer A

40µM Primer B

1 to 3mM MgCl<sub>2</sub>

dH<sub>2</sub>O to 98µl

Once prepared, individual reaction tubes were transferred to a dedicated template loading area where 1µl of template was added to each sample, excepting negative control (approx. 300ng genomic DNA or 1µl of a 10<sup>-6</sup> plasmid dilution). 60µl of paraffin oil was then overlaid to reduce sample evaporation during thermocycling.

All PCR reactions were "hot-started" to reduce the potential amplification of background templates. Samples were pre-heated to 94°C for 1 minute prior to 1<sup>st</sup> cycle and 1µl Taq DNA polymerase (Advanced Biotechnologies) was added to each sample tube below level of paraffin oil.

## SOLUTIONS

### 10X Reaction Buffer IV

200mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

750mM Tris-HCl, pH 9.0

0.1% Tween 20

## **MM1.9**

### **Dideoxy-Sequencing of Plasmid DNA with Sequenase**

For best sequencing results, use plasmid DNA of the highest quality, preferably prepared through a QIAGEN DNA column.

1. Take 10µg of plasmid DNA and add dH<sub>2</sub>O to a final volume of 45µl
2. Denature DNA by adding 5µl of 2M NaOH, 2mM EDTA. Mix thoroughly by inversion and keep at room temperature for 5 minutes.



3. Neutralise this strong alkali by adding 5µl of 2M ammonium acetate, pH 4.6 and vortex.
4. To precipitate DNA, add 185µl of ice-cold absolute ethanol, vortex and place at -70°C for 30 minutes.
5. Spin sample tubes at 13,000 rpm for 10 minutes at 4°C and decant supernatant. Wash pellet once with 200µl ice-cold ethanol.
6. Dry DNA under vacuum for approximately 15 minutes then resuspend pellet in 6µl of dH<sub>2</sub>O.
7. Anneal sequencing primer by adding 2µl 5X Annealing Buffer (Amersham) and a 2µl volume (0.5pmol) of primer to resuspended DNA. Heat this mixture to 65°C for 2 minutes, then allow temperature to drop slowly (approx. 30 minutes) to room temperature. Chill samples on ice.
8. Add 2.5µl of each dideoxy termination mix (Amersham) to 4 eppendorfs and pre-warm to 37°C.
9. To the annealed reaction tube add:
  - 1µl of 0.1M DTT
  - 2µl Labelling Nucleotide Mix (Amersham)
  - 0.5µl or 5µCi radio-labelled dATP ( $\alpha$ -S<sup>35</sup>)
  - 2µl of a 1:8 diluted stock of Sequenase Enzyme
  - (Addition of 1µl 0.1M MnCl<sub>2</sub>, 0.15M Sodium Isocitrate (optional) to the labelling reaction will enable preferential sequencing of DNA close to primer).
10. Mix total volume (approx. 15µl) and incubate at room temperature for between 2 to 5 minutes, depending on requirement to read sequence either close or increasingly distant from the primer.
11. Transfer 3.5µl of labelling reaction to each of the four pre-warmed dideoxy termination mixes.
12. Incubate termination reactions at 37°C for between 2-5 minutes before adding 4µl of Stop Solution (Amersham). Mix and either keep on ice prior to running on sequencing gel, or store at -20°C.

### Preparation of a Polyacrylamide Sequencing Gel

1. Take two large sequencing plates and wash once with water and detergent, then wash once with acetone. Coat the inner side of one plate with “Gel-slick” (FMC BioProducts, USA) to facilitate the separation of both plates following electrophoresis of samples. Ensure the inner sides of each plate are completely clean, as any dust or residues remaining from previous gels will trap air when pouring gel.
2. Mix the following in a 500ml beaker:  
80mls 6%Acrylamide, 6M Urea, 1X TBE  
80µl 25% Ammonium Persulphate  
80µl TEMED

Immediately following the addition of TEMED pour gel-mix between glass plates, place inverted sharks-tooth comb at the top of gel (so as to form a single large well) and leave to polymerise at room temperature for between 45 to 60 minutes.

3. Following removal of comb, immediately transfer gel to electrophoresis rig, ensuring that the smaller of the two glass plates is closest to rig apparatus. Fill buffer tanks with 1X TBE. Using a 50ml syringe, rinse out the single well with buffer, ensuring that all non-polymerised gel is washed out.
4. Replace sharks tooth comb in the other orientation and apply pressure until the tips of the “teeth” penetrate about 2-3mm into the gel. This will form individual wells in which to load samples.

### Denaturing PAGE of Sequencing Reactions

1. Immediately prior to the loading of the sequencing reactions, pre-run the sequencing gel at 40W, or 1.7kV for 20 minutes or until the temperature of the outer glass plate reaches approximately 50°C.
2. Heat samples to 80°C for 10 minutes, then place immediately on ice.
3. Load 2µl of each reaction into one of the slots formed by the replaced sharks-tooth comb. Try to ensure that the loading order of reactions is consistent

(e.g. G, A, T, C from left to right) to enable subsequent ease and certainty in the interpretation of sequencing results.

4. Run samples at 40W or 1.7kV until the bromophenol blue dye front from the samples reaches the foot of the gel. The availability of free wells permitting, stop the current and reload samples in spare wells. Re-apply electrical current and run gel until either the bromophenol blue dye front of the newly loaded samples, or the cyanol blue of the original samples, reaches the foot of the gel.
5. Dismantle electrophoresis rig, taking great care as the bottom TBE buffer reservoir now contains  $S^{35}$  nucleotide. Separate the glass plates with a knife and remove one to leave gel exposed.
6. Ensuring that the base plate upon which the gel rests is completely horizontal, pour Gel Fix Solution over the entire surface area of the gel. Leave solution in contact with the gel for 10 minutes (bromophenol blue dye turns yellow), then drain by carefully tipping plate, ensuring that the gel does not become mobile on plate surface.
7. To remove gel from the base plate, place a piece of Whatmann 3MM paper on top of gel and press down firmly until the entire gel surface has come into contact. Immediately pull the Whatmann 3MM paper away from the glass plate in one rapid and constant movement. The gel should stick to the 3MM paper and easily peel off from the surface of the plate.
8. Cover gel surface in cling film and dry in a Vacuum Dryer at 80°C for 2 hours, or until the gel is completely dry.
9. Place dried gel in an autoradiography cassette and overlay a piece of Bio-Max MR film (Amersham). Expose film to gel at room temperature overnight and develop the following day.

## SOLUTIONS

### Gel Fix Solutions

10% Methanol

10% Acetic Acid

in dH<sub>2</sub>O

## Section 2 - RNA Techniques

### MM2.0

Precautions that should be taken **at All Times** to avoid RNase contamination of RNA samples

1. **Gloves must be worn at all times.**
2. **Normal pipettes may be used ONLY in conjunction with RNase-free filter tips.**
3. **All eppendorf or sample tubes must be removed from new bag with gloved hands. Autoclave and dry tubes prior to use.**
4. **Dispense all solutions from dedicated “RNA only” stocks with a sterile strippette into previously sealed 50ml blue-capped Falcon tubes.**
5. **Make up all solutions requiring dH<sub>2</sub>O with DEPC treated dH<sub>2</sub>O.**

### MM2.1

#### **Extraction of mRNA from Cultured Cells**

1. Aspirate media from cells and wash 1x in PBS.
2. Aspirate PBS from cells and add 1ml of TRIZOL Reagent (Gibco-BRL) per 10cm<sup>2</sup> of culture dish.  
**N.B.** TRIZOL is highly hazardous substance therefore read accompanying health and safety information carefully prior to use and exercise great caution.
3. Leave at room temperature for approximately 2 minutes, and pass resultant whole cell lysate several times through a blue (p1000) pipette tip.
4. Transfer cell lysate to a RNase-free eppendorf tube and add 200µl of chloroform per 1ml TRIZOL.
5. Shake tubes vigorously for 15 seconds, then centrifuge samples at 13,000 rpm for 15 minutes at 4°C. Remove the upper (colourless) aqueous phase and transfer to a clean eppendorf.

6. To precipitate RNA, add 500µl isopropyl alcohol per 1ml of TRIZOL reagent used in the initial cell lysis. Mix thoroughly and incubate at room temperature for 10 minutes. Centrifuge samples at 13,000 rpm for 10 minutes at 4°C, at which time, RNA precipitate should be visible as a pellet at the base of the sample tube.
7. To wash the RNA, remove supernatant from pellet and add 1ml of 70% ethanol per 1ml of TRIZOL reagent used in the initial cell lysis. Vortex sample thoroughly and centrifuge at 10,000 rpm for 5 minutes at 4°C. Discard ethanol supernatant and briefly air-dry RNA pellet for between 5 to 10 minutes.
8. Resuspend pellet in an appropriate volume of RNase-free water by passing the solution several times through a yellow (p200) tip and incubating at 55°C for 10 minutes.

## **MM2.2**

### **Extraction of RNA from Fresh Tissue**

1. For this technique it is essential that all samples of fresh tissue are immediately frozen in liquid nitrogen following removal from host animal, then rapidly transferred to -80°C storage prior to use.
2. Add 1ml of TRIZOL per 100mg fresh tissue and homogenise thoroughly using a “drill-bit” homogeniser.

For all subsequent instructions, follow steps **3** to **8** of section MM2.1.

## **MM2.3**

### **Agarose Gel Analysis to Determine Integrity of Prepared RNA**

1. To prepare agarose gel, melt the appropriate amount of agarose in DEPC treated dH<sub>2</sub>O. Cool to approx. 60°C then add 5X formaldehyde gel-running buffer and formaldehyde to generate a mix of:

- 3.5 parts molten agarose/dH<sub>2</sub>O
- 1 part 12.3M formaldehyde stock solution
- 1.1 parts 5X formaldehyde gel-running buffer

**Gel should be cast in a fume cabinet**

2. To prepare RNA sample mix the following:
  - 4.5µl RNA sample (up to 30µg)
  - 2.0µl 5X formaldehyde gel-running buffer
  - 3.5µl Formaldehyde
  - 10.0µl Formamide
  - (Optional: add 1µl of Ethidium Bromide at 1mg/ml in DEPC treated dH<sub>2</sub>O to enable visualisation of RNA under UV light)
3. Incubate sample tube at 65°C for 15 minutes, then chill immediately on ice. (briefly centrifuge to collect tube contents)
4. Add 2µl of Formaldehyde gel-loading buffer to each sample.
5. Run gel in 1X formaldehyde gel-running buffer until dye-front reaches the foot of the gel
6. The presence of well resolved and strong 28S and 18S rRNA bands infers high quality of RNA preparation.

## SOLUTIONS

### DEPC Treated dH<sub>2</sub>O

0.1% DEPC + dH<sub>2</sub>O

Leave to stir over-night at room temperature, with bottle cap slightly open to equalise gas pressure.

Autoclave solution prior to use

### 5X Formaldehyde Gel-Running Buffer

0.1M MOPS, pH 7.0 (pH adjusted with

2M NaOH)

40mM Sodium Acetate

5mM EDTA, pH 8.0

Filter sterilise (0.2µm) prior to use

### Formaldehyde Gel Loading Buffer

50% Glycerol

1mM EDTA, pH 8.0

0.25% Bromophenol Blue

0.25% Xylene Cyanol FF

### **MM2.4**

#### **Generation of cDNA from RNA Template with Reverse Transcriptase**

##### First Strand cDNA Synthesis

1. To a PCR tube add the following:

1 $\mu$ l Oligo (dT)<sub>12-18</sub> at 500 $\mu$ g/ml

1-5 $\mu$ g total RNA

Make up volume to 12 $\mu$ l with DEPC treated dH<sub>2</sub>O

2. Heat mixture to 70°C for 10 minutes, then quickly chill on ice. Briefly centrifuge to collect contents of tube.

3. Now add:

4 $\mu$ l 5X First Strand Buffer (Gibco BRL; supplied with enzyme)

2 $\mu$ l 0.1M DTT

1 $\mu$ l 10mM dNTP mix

4. Gently mix tube contents and incubate at 42°C for 2 minutes.

5. Add 1 $\mu$ l (200 units) of Superscript II (Gibco BRL) and gently mix. Incubate samples at 42°C for 50 minutes.

6. Following inactivation of reaction by incubating at 70°C for 15 minutes, sample is ready to be used as template in conventional PCR reaction (see section MM1.8).

## Section 3 - Protein and Immunological Techniques

### MM3.1

#### Western Analysis of Protein

##### Extraction of Protein from Cultured Cells

It should be possible to extract 100µg protein/per  $10^6$  cells via this method.

1. Trypsinise cells as detailed in section MM4.1 and wash cells once in ice cold PBS.
2. Spin cells at 1,200 rpm for 5 minutes, discard supernatant and slightly loosen cell pellet by flicking sample tube several times. Resuspend cell pellet in an appropriate aliquot of ice-cold NP-40 Lysis Buffer (use approximately 5X volume buffer: pellet). Invert tube several times and pipette up and down several times with a blue (p1000) tip to ensure total cell lysis. Keep samples on ice at all times.
3. To reduce viscosity of protein preparation, pass sample several times through a 25-gauge syringe needle to shear chromosomal DNA.
4. Samples may be stored at -20°C prior to use.

##### Extraction of Protein from Fresh Tissue

1. For this technique it is essential that all samples of fresh tissue are immediately frozen in liquid nitrogen following removal from host animal, then rapidly transferred to -80°C storage prior to use.
2. Add the equivalent of 5X volume of NP-40 lysis buffer to each sample of fresh tissue (i.e. 500µl lysis buffer per 100mg fresh tissue) and homogenise thoroughly using a “drill-bit” homogeniser.
3. Samples may be stored at -20°C prior to use.



## SOLUTIONS

### NP-40 Lysis Buffer

50mM Tris/HCl pH 8.0

150mM NaCl

1% NP-40

1:1000 protease inhibitor cocktail stock (add immediately prior to use)

1:1000 PMSF stock (add immediately prior to use)

### 1000X Protease Inhibitor Cocktail

{Leupeptin; Pepstatin; Antipain Dihydrochloride; Chymostatin} at 1mg/ml in DMSO.

### 1000X PMSF (Phenylmethyl-sulfonyl Fluoride)

100mM PMSF in Ethanol

### Quantitation of Sample Protein Concentration

Quantitation of sample protein concentration was achieved by Bradford Assay using the “Bio-Rad Protein Assay” kit (Bio-Rad).

1. Dilute an appropriate volume of 5X assay reagent to 1X with dH<sub>2</sub>O, sufficient to provide 1ml reagent per protein standard /protein sample.
2. Measure and record the absorbance of known protein standards (ie 0µg, 5µg, 10µg, 15µg, 20µg, 25µg and 50µg B.S.A.) at 595nm on a spectrophotometer.
3. Add 5µl from each sample to each 1ml aliquot of 1X assay reagent, mix by inversion and record absorbance at 595nm on a spectrophotometer.
4. Calculate the protein concentration of each sample by comparing recorded absorbance with the recorded absorbencies of the known protein standards.

### Preparation of SDS-Polyacrylamide Gels

All SDS-PAGE of protein samples was performed using Bio-rad mini-PROTEAN II equipment and gel multi-caster. The solution volumes described below are sufficient to pour ten 12.5% mini-gels (0.75mm thickness) using gel multi-caster apparatus.

1. To pour the separating phase (or the Running Gel) of the SDS-PAGE gel, mix the following reagents in a 100ml conical flask:

25mls Monomer Solution  
15mls 4X Running Gel buffer  
0.6mls 10% SDS  
19.1mls dH<sub>2</sub>O  
300µl 10% Ammonium Persulphate

Finally add 20µl TEMED, mix thoroughly and pour into the multi-gel caster until level of fluid reaches approximately    of the way from the top of the plates.

2. Immediately overlay the un-polymerised gel mix with water-saturated butanol and leave gel to polymerise at room temperature for between 45-60 minutes.
3. Pour off water saturated butanol and wash polymerised gel once with dH<sub>2</sub>O.
4. To pour the stacking phase of the SDS-PAGE gel, mix the following reagents in a 100ml conical flask:

2.66mls Monomer Solution  
5mls 4X Stacking Gel Buffer  
0.2mls 10% SDS  
12mls dH<sub>2</sub>O  
100µl 10% Ammonium Persulphate

Finally add 10µl TEMED, mix thoroughly and pour into the multi-gel caster until level of fluid reaches the top of the glass plates.

5. Immediately insert 0.75mm combs into the un-polymerised stacking gel mix, ensuring that no air becomes trapped and leave to polymerise at room temperature for between 45-60 minutes.
6. Gels can be stored at 4°C for up to 1 month, providing they are kept moist with TBS and sealed in clingfilm.

## SOLUTIONS

### Monomer Solution

40% acrylamide: bis-acrylamide mix  
(19:1); [Instagel]

### 4X Running Gel Buffer

1.5M Tris.Cl, pH 8.8

### 4X Stacking Gel Buffer

0.5M Tris.Cl, pH 6.8

### Water Saturated Butanol

50mls n-butanol

5mls dH<sub>2</sub>O

(use upper phase only)

## Preparation of Protein Samples Prior to SDS-PAGE

1. Choose to run amount of sample equivalent to either 10 or 20µg protein per lane.
2. Add equivalent volume of 2X Sample Buffer to each protein sample.
3. Boil samples for 2 minutes, briefly centrifuge to collect tube contents and place immediately on ice prior to loading onto gel.

## SOLUTIONS

### 2X Sample Buffer

0.125M Tris.Cl, pH 6.8

4% SDS

20% v/v Glycerol

0.2M DTT

0.02% Bromophenol Blue

### Running Protein Samples by SDS-PAGE

1. Rinse out the wells of the SDS-PA gel several times with dH<sub>2</sub>O and fix gel in PROTEAN II electrophoresis tank, filled to approximately 1/3 capacity with 1X Running Buffer.
2. Fill the top compartment of gel tank with 1X Running Buffer and carefully load prepared protein samples, attempting to place entire sample as near to the base of the well as possible.
3. Run at 30mA per gel for 50 minutes, or until bromophenol blue dye front reaches base of the gel (PROTEAN II rig can accommodate a maximum of 2 gels).

### Semi-Dry Transfer of Protein from SDS-PA to Nitrocellulose Membrane

1. Remove gel from between glass plates and place in 1X Transfer Buffer for 5 to 10 minutes.
2. Assemble a stack of Whatmann 3MM filter paper (pre-wet in transfer buffer), nitrocellulose membrane [Hybond ECL; Amersham] (pre-wet in dH<sub>2</sub>O), "soaked" gel and Whatmann 3MM filter paper (pre-wet in transfer buffer) on top of the base plate of a semi-dry electrophoretic transfer unit (Fastblot B34, Biometra).
3. If transferring one gel, run unit at 220mA for 25 minutes; transferring 2 gels, run unit at 400mA for 30 minutes.
4. Stain nitrocellulose with Ponceau's Reagent for 5 minutes and rinse several times in dH<sub>2</sub>O to determine the success of both PAGE and protein transfer.

### SOLUTIONS

#### 1X Running Buffer

0.025M Tris.Cl, pH 8.3

0.192M Glycine

0.1% SDS

#### 1X Towbin Transfer Buffer

0.025M Tris

0.192M Glycine

20% Methanol

0.1% SDS

(final pH should be between 8.2 and 8.4)

### Immuno-Detection of p53 Fixed on Nitrocellulose

1. Pre-block nitrocellulose filter with 5% powdered milk (Marvel) in 1X TTBS, for 30 minutes.
2. Add 1° antibody (BMG-1; anti-p53 sheep polyclonal [Boehringer Mannheim]) at a concentration of 1mg/ml in 5% powdered milk/ 1X TTBS for 1 hour.
3. Wash filter three times in 1X TTBS, for 10 minutes each wash, then add 2° antibody (Rabbit anti-sheep, conjugated to peroxidase [DAKO]) diluted 1/1000 in 5% powdered milk/ 1X TTBS. Leave 2° on for 30 minutes.
4. Wash filter three times in 1X TTBS, for 10 minutes each wash. Leave last wash on (for no longer than 15 minutes) until ready to perform ECL detection of 1°.

### SOLUTIONS

#### 1X TTBS

100mM Tris.Cl, pH 7.5

0.9% NaCl

0.1% Tween-20

#### 1X TTBS; 5% Powdered Milk

5g Marvel

100mls TTBS

### ECL Detection of 1° Antibody

Follow protocol as detailed in the literature accompanying ECL detection kit (Amersham). Once detected, place film optimised for ECL detection over filter [Hyperfilm ECL; Amersham] and seal in an autoradiography cassette for between 5 minutes to 1 hour (film laid down initially for 5 minutes to determine strength of signal).

### **MM3.2**

#### **P53 Immuno-analysis of Cultured Cells on Chamber Slides**

1. Grow cells on Nunc 2-well (4.2cm<sup>2</sup> per well), permanox coated, chamber slides (Gibco BRL).

2. At the appropriate time, aspirate media from cells, separate the microscope slide (with cells adherent) from the culture wells and immerse slide in a solution of 50% Acetone: 50% Methanol for 5 minutes at room temperature to fix cells.
3. Remove cells from fix, air-dry and store at  $-70^{\circ}\text{C}$  prior to immunostaining.
4. Following storage at  $-70^{\circ}\text{C}$ , leave slides at room temperature for 30 minutes to defrost. **All subsequent steps are carried out at room temperature unless specified.**
5. Cover fixed cells with PBS for 10 minutes.
6. Shake off PBS and cover cells with a solution of 1%  $\text{H}_2\text{O}_2$  for 10 minutes to block endogenous peroxidase activity.
7. Shake off  $\text{H}_2\text{O}_2$  solution and cover cells with PBS for 5 minutes. Shake off PBS from cells and repeat a further two times.
8. Cover cells with solution containing 20% Normal Rabbit Serum (NRS) and 0.5% Tween 20 in PBS for 10 minutes.
9. Shake off NRS/PBS/Tween and cover cells in PBS for 5 minutes
10. Shake off PBS and cover cells with Avidine D solution (from Biotin blocking kit, SP 2001, Vector Laboratories) for 15 minutes to block endogenous biotin.
11. Shake off Avidine D and cover cells in PBS for 5 minutes. Shake off PBS and repeat one further time.
12. Cover cells with Biotin solution (from Biotin blocking kit, SP 2001, Vector Laboratories) for 15 minutes.
13. Shake off Biotin solution and cover cells with solution containing the primary anti-p53 monoclonal antibody Pab421 (p53 (Ab-1) from Calbiochem, ref. OP03) at a 1/1000 dilution in 5% NRS/ 0.5% Tween 20 in PBS. Leave cells covered in this solution, in a humid chamber at  $4^{\circ}\text{C}$  overnight.
14. Shake off 1<sup>o</sup> antibody and cover cells in PBS for 5 minutes at room temperature. Shake off PBS and repeat a further two times.
15. Cover cells with solution containing 2<sup>o</sup> antibody (Rabbit anti mouse biotinylated  $\text{F}(\text{ab}')_2$  fragment, Rambio) at a 1/400 dilution in 5% NRS/ 0.5% Tween 20 in PBS. Leave cells covered in this solution for 30 minutes at room temperature.

16. Shake off 2° antibody and cover cells in PBS for 5 minutes. Shake off and repeat a further two times.
17. Incubate samples in ABCComplex/HRP (DAKO) for 30 minutes at room temperature
18. Shake off HRP-conjugated AB and cover cells in PBS for 5 minutes. Shake off PBS and repeat one further time.
19. Visualise antibody binding by covering cells with DAB (Diaminobenzidine) for approximately 5 minutes. **Do not** exceed 5 minutes to prevent non-specific background staining.
20. Wash samples immediately under running water to stop DAB staining.
21. Counter stain lightly with haematoxyline and light green. Dehydrate samples in ascending grades of alcohol and mount cover slip.

## SOLUTIONS

### 1% H<sub>2</sub>O<sub>2</sub>

3mls of 100vols stock H<sub>2</sub>O<sub>2</sub>

97mls of dH<sub>2</sub>O

### DAB Substrate Solution

0.2M Tris

0.1N HCl

0.01M Imidazole

(Adjust pH to 7.6)

## **MM3.3**

### **P53 Immuno-histochemistry of Fixed Tissue on Microscope Slides**

1. De-wax paraffin fixed samples in xylene for 1 minute, then re-hydrate samples in descending grades of alcohol (i.e. from absolute ethanol through 74op to 64op; 1 minute in each). Wash samples in tap water after last ethanol incubation.
2. Cover all samples in citrate buffer and microwave in a 700W oven at full power for 3 x 5 minutes. Ensure that samples remain covered by buffer for the entire duration of this treatment.
3. Cool samples to room temperature then wash in running tap water.

4. Block endogenous peroxidase by covering samples in 0.5% H<sub>2</sub>O<sub>2</sub> in methanol for 15 minutes **only**. (This step appears to be critical).
5. Wash samples in tap water, then cover in TTBS buffer (see solutions section MM3.1) for 5 minutes at room temperature twice.
6. Cover all samples in 20% Normal Swine Serum (NSS) in TTBS for 20 minutes at room temperature.
7. Incubate samples in 1° antibody (CM5; Rabbit polyclonal) at a dilution of 1:750 in 20% NSS in TTBS for either 1 hour at room temperature or at 4°C overnight in a humid chamber.
8. Wash samples 5 times in TTBS for 2 minutes per wash.
9. Incubate samples in 2° antibody (swine anti-rabbit IgG Biotinylated; DAKO) at a concentration of 1:400 in 20% NSS in TTBS for 30 minutes at room temperature.
10. Wash samples 5 times in TTBS for 2 minutes per wash.
11. Incubate samples in ABCComplex/HRP (DAKO) for 30 minutes at room temperature.
12. Wash samples 5 times in TTBS for 2 minutes per wash.
13. Visualise antibody binding by covering cells in DAB for approximately 5 minutes. To reduce non-specific background staining **do not** exceed 5 minutes.
14. Wash samples immediately under running water to stop DAB staining.
15. Counter stain lightly with haematoxyline. Dehydrate samples in ascending grades of alcohol and mount cover slip.

## SOLUTIONS

### Citrate Buffer

300mM Trisodium Citrate, pH 6.0

### 0.5% H<sub>2</sub>O<sub>2</sub> in Methanol

1.5mls of 100vols stock H<sub>2</sub>O<sub>2</sub>

98.5mls of high-grade methanol



### MM3.4

#### Immuno-Flow Detection of p53

1. Trypsinize a minimum of  $1 \times 10^5$  cells and centrifuge at 1,200 rpm for 5 minutes. Wash the pellet once in PBS then repeat centrifugation step.
2. Aspirate PBS and add 1ml of 0.4% formaldehyde directly to the pellet. Incubate the pellet with formaldehyde for 15 minutes on ice.
3. Pour off formaldehyde and resuspend pellet in 500 $\mu$ l of PBS. Centrifuge cells at 3000 rpm for 5 minutes.
4. Pour off PBS and add 500 $\mu$ l of Permeabilisation Buffer to the pellet. Incubate the pellet at 37°C for 15 minutes.
5. Pour off Permeabilisation Buffer and resuspend pellet in 500 $\mu$ l of PBS. Centrifuge cells at 3000 rpm for 5 minutes.
6. Pour off PBS and add 100 $\mu$ l of normal sheep serum to pellet. Incubate pellet for 5 minutes on ice with normal serum. Resuspend pellet in 500 $\mu$ l of PBS. Centrifuge cells at 3000 rpm for 5 minutes.
7. Pour off PBS and resuspend pellet in 200 $\mu$ l of PBS. Split this total volume between 2 wells of a 96 well plate. One well acts as a control for non-specific FITC staining background. One well acts as the experimental sample.
8. Add 15 $\mu$ l of a 1/100 dilution (in Flow Buffer) of 1° anti-p53 (Ab-1) antibody **only** to experimental sample. Incubate samples on ice for 1 hour.
9. Add 250 $\mu$ l of PBS and centrifuge on a plate-centrifuge at 300 G for 5 minutes.
10. Shake off PBS and add 15 $\mu$ l of a 1/50 dilution of 2° antibody (anti-mouse IgG FITC conjugate) to **both** samples. Incubate on ice for 40 minutes.
11. Add 250 $\mu$ l of PBS and centrifuge as detailed in step 9.
12. Shake off PBS and resuspend cells in 200 $\mu$ l of Flow Buffer. Run samples on gated flow cytometer.

#### SOLUTIONS

##### Permeabilisation Buffer

0.05% Tween 20 in PBS

##### Flow Buffer

1% BSA  
0.1% Sodium Azide  
in PBS

## Section 4 - Cell Culture Techniques

### MM4.1

#### ES cell culture

ES cells were routinely cultured at 37°C in CM media supplemented with 5% (v/v) fetal calf serum, 5% (v/v) newborn calf serum (selected batches),  $\beta$ -mercaptoethanol and LIF (Leukaemia Inhibiting Factor) upon gelatinised tissue culture plates.

#### Gelatinisation of Tissue Culture Plates

Gelatin (Sigma) is made up to 1% in dH<sub>2</sub>O, double autoclaved and stored at 4°C. This is equivalent to a 10x stock solution.

Make up 1x (0.1%) Gelatin solution and add sufficient volume to cover entire base of tissue culture plate. Leave the gelatin solution on the tissue culture plate for a minimum of 30 minutes at room temperature. Tissue plate is now gelatinised and is ready for plating with ES cells.

#### Complete Media (CM)

500mls G-MEM (BHK-21) media (Gibco-BRL)  
50mls Serum (25mls Fetal Calf Serum: 25mls Newborn Calf Serum)  
5mls 100x Non-Essential Amino Acids (Gibco-BRL)  
5mls 100x Sodium Pyruvate (Gibco-BRL)  
500 $\mu$ l LIF (prepared and titred in lab as per standard protocols)  
500 $\mu$ l 1000x  $\beta$ -mercaptoethanol solution

#### 1000x $\beta$ -mercaptoethanol solution

100 $\mu$ l pure  $\beta$ -mercaptoethanol  
14.1mls dH<sub>2</sub>O  
(Filter sterilise solution through 0.2 $\mu$ m filter. Store at 4°C)

### Trypsinisation of ES Cells

ES cells should be routinely passaged 1 in 5 upon reaching approximately 70-80% confluence.

1. Aspirate media from cells and wash once in PBS
2. Add appropriate volume of (1X) Trypsin-EDTA (Gibco-BRL) to cover entire base of tissue culture plate.
3. Incubate cells at 37°C for 5 minutes, with occasional agitation of plate. This should provide sufficient time to ensure that ES cells have detached from tissue culture plate and have formed a single cell suspension. **N.B.** – monitor ES cell detachment carefully and avoid over-trypsinisation as this can result in cell lysis and a reduction in overall ES cell viability.
4. Neutralise Trypsin by adding a minimum of 2 volumes CM. ES cells are now ready to be directly transferred to an area of gelatinised tissue culture plate or for any of the further manipulations detailed in this section.

### Freezing ES Cells

1. Following trypsinisation of ES cells, centrifuge cells at 1,200 rpm for 5 minutes.
2. Aspirate media and resuspend pellet in an appropriate volume of Freezing Media (FM).

Plate area	Volume of FM	To Fill No. of Cryovials	Area to Re-thaw Into
24-well	800µl	1	24-well
6-well	800µl	1	6-well
25cm <sup>2</sup>	1.6mls	2	6-well
75cm <sup>2</sup>	6.5mls	8	6-well
175cm <sup>2</sup>	13mls	16	6-well

3. Aliquot 800µl of FM ES cell resuspension into appropriate number of cryovials as detailed in table MM.4.1.

4. Wrap rack of cryovials in paper towel and place at  $-80^{\circ}\text{C}$  freezer overnight.

The following day, store cryovials in liquid nitrogen.

#### Freeze Media (FM)

80% CM

10% Serum (50% FCS: 50% NCS)

10% DMSO (Di-methyl Sulphoxide)

#### Thawing Frozen Stocks of ES Cells

1. Remove cryovial-containing cells from liquid nitrogen or  $-80^{\circ}\text{C}$  storage and thaw rapidly (with agitation) in  $37^{\circ}\text{C}$  waterbath.
2. Dilute contents of cryovial in 10x volume of CM media and spin immediately at 1,200 rpm for 5 minutes.
3. Aspirate pellet and resuspend in volume of CM media appropriate to the area of tissue culture plate to be used (1ml=24-well plate; 3mls=6-well plate; 5mls= $25\text{cm}^2$  flask)

#### Electroporation of ES Cells

1. Following trypsinisation of ES cells, centrifuge volume equivalent to  $10^8$  cells at 1,200 rpm for 5 minutes.
2. Resuspend pellet in 0.7mls of PBS and transfer to electroporation cuvette (Bio-Rad) containing 150 $\mu\text{g}$  of linearised vector DNA (suspended in 100 $\mu\text{l}$  PBS).
3. Pipette volume of cells up and down in electroporation cuvette once, to ensure good mix of cells with DNA. Place cap on cuvette and pulse with a charge of 0.8kV, 3 $\mu\text{F}$  capacitance, resulting in a time constant of 0.1 seconds (Bio-Rad Gene-Pulser).
4. Allow cells to recover for 20 minutes before adding 20mls of CM.
5. Plate out cells on 10cm gelatinised plates in non-selective media at an appropriate density to permit selection the following day ( $1 \times 10^5$  per plate for HAT selection;  $1 \times 10^6$  per plate for all other antibiotic selections)

### HAT Selection and Picking of ES Cell Clones

1. 24 hours after transfection, replace non-selective CM with selective CM containing 1 x HAT; 0.1mM hypoxanthine, 0.4 $\mu$ M aminopterin, 16 $\mu$ M thymidine (50x stock available from Boehringer Mannheim).
2. Refresh selective media on cells every 3 or 4 days until colonies of HAT resistant clones become visible to the eye (usually within 12-13 days post-transfection).
3. Prepare a 96 well plate containing 100 $\mu$ l of trypsin-EDTA in each well. Aspirate selective media from 10cm plate, wash 1x in PBS and partially aspirate leaving residual volume PBS sufficient to cover base of tissue culture plate. This measure prevents dehydration of clones.
4. Place a p200 (yellow) tip over the HAT resistant ES cell clone and apply gentle suction, thus taking the clone up into the pipette.
5. Pipette clone into a trypsin-EDTA containing well of the 96-well plate and leave for 5 minutes at room temperature.
6. Pipette contents of well gently up and down once before transferring to a single gelatinised well of a 24-well plate, containing 1ml of non-selective CM.
7. Refresh media the following day and replace with CM containing HAT (optional).

### Extraction of ES Cell DNA to Enable Subsequent Genetic Analysis

This protocol is essentially identical to that published by Laird *et al*, Nucleic Acids Research, Vol. 19, no.15, page 4293, 1991.

1. Following trypsinisation of ES cells, centrifuge cells at 1,200 rpm for 5 minutes. Wash cells once in PBS and re-centrifuge at 1,200 rpm for a further 5 minutes.
2. Partially aspirate PBS, leaving sufficient residual PBS to cover the pellet. Flick the side of the tube until pellet has resuspended in this volume.
3. Add 600 $\mu$ l of Lysis Buffer and either incubate at 55°C for 3 hours, or at 37°C overnight.

4. Add 500µl of isopropanol and shake on an orbital shaker for 15 minutes. The DNA precipitate should now become visible.
5. Carefully remove DNA precipitate using a fine glass hook made from a Pasteur pipette, and wash once in 70% ethanol.
6. Transfer DNA to an eppendorf tube containing 50µl TE buffer and resuspend. Incubation at 60°C for 30 minutes should aid resuspension in addition to evaporating any trace residual ethanol.

#### Lysis Buffer

100mM Tris-HCl, pH 8.5

5mM EDTA

0.2% SDS

200mM NaCl

100µg Proteinase K per ml

### **MM4.2**

#### **Sarcoma Cell Line (SL) Culture**

##### Media Requirements

##### Media used for first 3 passages

500ml bottle of D-MEM-12 (Gibco BRL)

50mls FCS

500µl Fungizone (50 mg/ml)

500µl Gentamycin (50mg/ml)

##### Media used for subsequent passages

500ml bottle of BHK-21 (Gibco BRL)

50mls FCS

500µl Gentamycin (50mg/ml)

Following transfection of p6-16<sub>L</sub>p53-HYG add:-

800µl Hygromycin B (50mg/ml)

Using the above media, SL cells were passaged, trypsinised, frozen and thawed as detailed for ES cells in section MM.4.1.

**N.B.** Gelatin treatment of tissue culture plates was not required prior to the plating of SL cells.

### Derivation of SL Cell Lines from p53 Deficient Mice

1. Carefully remove sarcomas from p53 <sup>-/-</sup> mice and immerse each one in a solution of 70% ethanol for 5 seconds to minimise risk of microbial infection.
2. Finely dissect sarcomas with a pair of sterile scalpel blades in a 10cm petri dish. Drizzle resultant tumour pieces with a small volume (approximately 4mls) of culture medium to prevent dehydration.
3. Remove this volume of media from the mechanically sheared tumour pieces and filter through a fine wire mesh sieve to produce a mix of single and small aggregates of cells.
4. Plate 50% of these cells directly onto a 10cm petri dish.
5. To the remaining 50%, add several millilitres of medium containing collagenase (concentration 100U/ml; Gibco BRL). Place cells in a sealed white capped universal and incubate at 37°C with gentle agitation for approximately 30 minutes.
6. Centrifuge cells at 1,200rpm for 5 minutes twice, aspirating and replacing media both times, before plating as before at point 4.
7. Further dissect the remaining tumour pieces in the initial petri dish with sterile scalpel blades as before. Drizzle tumour pieces with several millilitres of medium containing 100U/ml collagenase.
8. Remove this volume of media from the mechanically sheared and enzymatically treated tumour pieces and filter through a fine wire mesh sieve to produce a mix of single and small aggregates of cells. Centrifuge cells at 1,200rpm for 5 minutes twice, aspirating and replacing media both times, before plating as before at point 4.
9. Place the remaining pieces of tumour into a white capped universal tube and several millilitres of medium containing collagenase at a concentration of 100U/ml. Incubate these cells at 37°C for 30 minutes with gentle agitation.
10. Centrifuge cells at 1,200rpm for 5 minutes twice, aspirating and replacing media both times, before plating as before at point 4.

### Transfection of SL Cells via Liposomes

1. Plate  $6 \times 10^4$  cells per  $2\text{cm}^2$  (single well of a 24 well plate) on the evening prior to transfection.
2. The following day, dilute DNA in normal serum containing media (approximately  $1\mu\text{g}$  plasmid DNA in  $250\mu\text{l}$  medium per  $2\text{cm}^2$ ). **N.B.** It is possible to transfect cells in both serum containing and serum free media conditions. Determine optimal transfection conditions empirically
3. Vigorously vortex stock solution of Tfx-50 (Promega) for 30 seconds, then add an appropriate volume to diluted DNA to generate optimal charge ratio of lipid: DNA (see section 2.vi.1).
4. Vortex Tfx/DNA/medium mix thoroughly and leave at room temperature for a minimum of 10-15 minutes. **Do not** leave longer than 30 minutes.
5. Immediately prior to transfection, aspirate medium from cells. Vortex Tfx/DNA/medium mix again and add  $250\mu\text{l}$  of mix to each 24 well.
6. Leave Tfx/DNA/medium mix on cells for 2 hours at  $37^\circ\text{C}$ .
7. Following this 2 hour exposure, add 1ml of normal serum containing medium to each well and return plates to  $37^\circ\text{C}$  incubator.
8. Collect cell lysates 48 hours after transfection and store at  $-20^\circ\text{C}$  prior to reporter gene analysis. **N.B.** Reporter cell lysis buffer comes included with both the  $\beta$ -Galactosidase Enzyme Assay System (Promega, Cat. # E2000) and the Luciferase Assay System (Promega, Cat. #E4030). The protocols describing both  $\beta$ -Gal and Luciferase reporter assays can be found at [<http://www.promega.com>]. The extent of  $\beta$ -Gal expression was quantitated using an ELISA plate reader (Dynatech MR5000) and Biolinx v2.20 software. The extent of luciferase expression was quantitated by standard luminometer detection.

### Selection and Picking of SL Clones

1. The day following transfection with p6-16<sub>L</sub>p53-HYG, SL cells were cultured in media supplemented with  $80\mu\text{g/ml}$  Hygromycin B (Boehringer Mannheim).
2. Individual clones were visible by the 12<sup>th</sup> day of selection. Clones grew as monolayers and therefore could not be picked by the p200 pipette tip method



as described for the picking of ES cell clones. All hygromycin resistant clones were picked using a cloning “ring” method.

3. To prepare cloning rings, slice off and collect the wide ends of p1000 (blue) pipette tips using a red-hot scalpel blade. Autoclave rings prior to use.
4. Aspirate culture dish containing clones and wash once with PBS. Using fine forceps, dip each cloning ring in sterile paraffin wax and place over each individual clone to form a series of small wells.
5. Add 150 $\mu$ l of (1X) Trypsin-EDTA (Gibco BRL) to each cloning well and incubate at 37°C for 5 minutes.
6. Add 500 $\mu$ l culture medium to each well to neutralise trypsin and pipette up and down several times to ensure single cell suspension.
7. Transfer total volume to a single well of a 24 well plate and culture normally.

#### Induction of Transgene Expression

1. Plate  $2 \times 10^5$  cells per 10cm<sup>2</sup> on the day preceding induction.
2. The following day, cells should be approaching 70% confluence. Aspirate medium from cells and refresh in media containing either 500 or 1000 I.R.U. (International Reference Units) of interferon  $\alpha/\beta$  (SIGMA, cat no. I1258).

### **MM4.3**

#### **Flow Cytometric DNA Analysis of Cultured SL Cells**

1. Following trypsinisation of cells as detailed in section MM4.1, centrifuge a minimum of  $1 \times 10^5$  cells at 1,200 rpm for 5 minutes, wash once in PBS and centrifuge again as before.
2. Aspirate PBS until a small volume remains covering the pellet of cells. Flick sample tube wall to resuspend pellet in this small volume.
3. Add 225 $\mu$ l of Solution A to cells. Leave at room temperature for 15 minutes.
4. Add 163.5 $\mu$ l of Solution B to cells. Leave at room temperature for 15 minutes. At this stage, samples can be stored at 4°C overnight.

5. Immediately prior to analysing samples on flow cytometer, add 125µl of Solution C. Briefly vortex sample to mix and leave at room temperature for 5 minutes before running on flow cytometer.

**N.B.** Samples must be run rapidly following addition of Solution C as they may be ruined following exposure times of greater than 30 minutes.

## SOLUTIONS

### Stock Solution

1g Trisodium Citrate

60.5g Tris

522mg Spermine

1ml NP-40

Make up to 1 litre in dH<sub>2</sub>O and pH to 7.6

<u>Solution A</u>	<u>Solution B</u>	<u>Solution C</u>
15mg Trypsin (SIGMA) in 500ml Stock Solution	250mg Trypsin Inhibitor (SIGMA) 50mg RNase A in 500ml Stock Solution	208mg Propidium Iodide 500mg Spermidine Tetra- hydrochloride in 500ml Stock Solution

**N.B.** All the above solutions should be made up in the volumes specified and stored as 10ml aliquots at -20°C prior to use.

## **MM4.4**

### **Visualisation of Apoptosis by Acridine Orange Staining**

1. Trypsinise cells as detailed in section MM4.1, spin at 1,200 rpm for 5 minutes and wash pellet 1x in PBS.
2. Aspirate PBS until a small volume remains covering the pellet of cells. Flick sample tube wall to resuspend pellet in this volume. Add 100µl of Fix

Solution (90% ethanol: 10% formalin) and leave at room temperature for 5 minutes.

3. Take up 20 $\mu$ l of the fixed single cell suspension and add 20 $\mu$ l of Acridine Orange solution (10 $\mu$ g/ml in PBS). Visualise cells by fluorescence microscopy and score the numbers of apoptotic cells in relation to total cell number.

## Section 5 - Whole Animal Techniques

### MM5.1

#### Subcutaneous Injection of SL Cells

1. Trypsinise cells as detailed in section MM4.1 and count using haemocytometer.
2. Centrifuge cells at 1,200 rpm for 5 minutes and resuspend in sufficient volume of PBS to provide  $1 \times 10^7$  cells per ml.
3. Inject 500 $\mu$ l of cells sub-cutaneously into each flank of a SCID animal.

### MM5.2

#### *In vivo* Induction of Type I Interferons with pI:pC

1. Resuspend lyophilised pI:pC (Pharmacia, cat.# 27-4729) in PBS to a final concentration of 1mg/ml.
2. Inject between 50 $\mu$ g to 250 $\mu$ g pI:pC intra-peritoneally per SCID mouse.

**N.B. – Both the sub-cutaneous injections of SL cells and the IP injections of pI:pC were undertaken by the animal handling staff (John Verth, Will Mungall and Linda Clark) of the University of Edinburgh Medical School.**

### MM5.3

#### Apoptotic Analysis of Whole Length Small Intestine

1. Sacrifice animal and remove an approximate 10cm length of intact small intestine.
2. Using a 50ml Syringe with a yellow (p200) tip attached, flush out contents of intestine with dH<sub>2</sub>O.
3. Place intestine on a piece of dry Whatmann 3MM paper. Once stretched out, drizzle with dH<sub>2</sub>O to prevent dehydration.
4. Using a fine pair of dissection scissors, carefully cut the entire length of intestine longitudinally to form a single flat strip of exposed lumen

5. To fix section, transfer intestine on Whatmann 3MM paper to a glass tray containing Methocarn Solution. Leave intestine in methocarn for a minimum of 1 hour at room temperature.
6. Using two pairs of watchmaker's forceps, gently lift intestine from Whatmann 3MM paper and roll to form a moderately packed spiral of tissue. Hold structure together by piercing through with a hypodermic syringe needle and store in 70% ethanol prior to sectioning and mounting on microscope slide.

## SOLUTIONS

### Methocarn Solution

4 parts Methanol

2 parts Chloroform

1 part Concentrated Acetic Acid

## BIBLIOGRAPHY

- Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci U S A* 1995 Aug 29;92(18):8493-7
- An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature* 1998 Mar 26;392(6674):405-8
- Arnheiter H, Skuntz S, Noteborn M, Chang S, Meier E. Transgenic mice with intracellular immunity to influenza virus. *Cell* 1990 Jul 13;62(1):51-61
- Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 1991 Oct;6(10):1915-22
- Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990 Aug 24;249(4971):912-5
- Bargonetti J, Reynisdottir I, Friedman PN, Prives C. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. *Genes Dev* 1992 Oct;6(10):1886-98
- Barnes WM. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc Natl Acad Sci U S A* 1994 Mar 15;91(6):2216-20.
- Basham T, Smith W, Lanier L, Morhenn V, Merigan T. Regulation of expression of class II major histocompatibility antigens on human peripheral blood monocytes and Langerhans cells by interferon. *Hum Immunol* 1984 Jun;10(2):83-93
- Basham TY, Smith WK, Merigan TC. Interferon enhances antibody-dependent cellular cytotoxicity when suboptimal concentrations of antibody are used. *Cell Immunol* 1984 Oct 15;88(2):393-400
- Bedell MA, Jenkins NA, Copeland NG. Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. *Genes Dev* 1997 Jan 1;11(1):1-10

- Bedell MA, Largaespada DA, Jenkins NA, Copeland NG. Mouse models of human disease. Part II: recent progress and future directions. *Genes Dev* 1997 Jan 1;11(1):11-43
- Bellamy CO. p53 and apoptosis. *Br Med Bull* 1997;53(3):522-38
- Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* 1998 Oct 9;282(5387):290-3
- Bienz B, Zakut-Houri R, Givol D, Oren M. Analysis of the gene coding for the murine cellular tumour antigen p53. *EMBO J* 1984 Sep;3(9):2179-83
- Bienz-Tadmor B, Zakut-Houri R, Libresco S, Givol D, Oren M. The 5' region of the p53 gene: evolutionary conservation and evidence for a negative regulatory element. *EMBO J* 1985 Dec 1;4(12):3209-13
- Blau H, Khavari P. Gene therapy: progress, problems, prospects. *Nat Med* 1997 Jun;3(6):612-3
- Blyth K, Terry A, O'Hara M, Baxter EW, Campbell M, Stewart M, Donehower LA, Onions DE, Neil JC, Cameron ER. Synergy between a human c-myc transgene and p53 null genotype in murine thymic lymphomas: contrasting effects of homozygous and heterozygous p53 loss. *Oncogene* 1995 May 4;10(9):1717-23
- Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996 Jun 14;85(6):803-15
- Bollag RJ, Waldman AS, Liskay RM. Homologous recombination in mammalian cells. *Annu Rev Genet* 1989;23:199-225
- Bouck N. P53 and angiogenesis. *Biochim Biophys Acta* 1996 May 16;1287(1):63-6
- Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 1984 May 17-23;309(5965):255-6

- Brandon EP, Idzerda RL, McKnight GS. Targeting the mouse genome: a compendium of knockouts. *Curr Biol* 1995 Jul 1;5(7):758-65. Published erratum appears in *Curr Biol* 1995 Sep 1;5(9):1073
- Brown JP, Pagano M. Mechanism of p53 degradation. *Biochim Biophys Acta* 1997 Apr 18;1332(2):O1-6
- Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR, Kley N. Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* 1995 Oct 19;377(6550):646-9
- Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E, Emeson RB. Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature* 1997 May 15;387(6630):303-8
- Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature* 1994 Jul 21;370(6486):220-3
- Canman CE, Gilmer TM, Coutts SB, Kastan MB. Growth factor modulation of p53-mediated growth arrest versus apoptosis. *Genes Dev* 1995 Mar 1;9(5):600-11
- Chada K, Magram J, Raphael K, Radice G, Lacy E, Costantini F. Specific expression of a foreign beta-globin gene in erythroid cells of transgenic mice. *Nature* 1985 Mar 28-Apr 3;314(6009):377-80
- Chen J, Wu X, Lin J, Levine AJ. mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. *Mol Cell Biol* 1996 May;16(5):2445-52
- Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev* 1996 Oct 1;10(19):2438-51
- Chene P. In vitro analysis of the dominant negative effect of p53 mutants. *J Mol Biol* 1998 Aug 14;281(2):205-9
- Chiou SK, Rao L, White E. Bcl-2 blocks p53-dependent apoptosis. *Mol Cell Biol* 1994 Apr;14(4):2556-63. Published erratum appears in *Mol Cell Biol* 1994 Jun;14(6):4333



- Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* 1994 Jul 15;265(5170):346-55
- Clarke AR, Cummings MC, Harrison DJ. Interaction between murine germline mutations in p53 and APC predisposes to pancreatic neoplasia but not to increased intestinal malignancy. *Oncogene* 1995 Nov 2;11(9):1913-20
- Clarke AR, Gledhill S, Hooper ML, Bird CC, Wyllie AH. p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. *Oncogene* 1994 Jun;9(6):1767-73
- Clarke AR, Gledhill S, Hooper ML, Bird CC, Wyllie AH. p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. *Oncogene* 1994 Jun;9(6):1767-73
- Clarke AR, Howard LA, Harrison DJ, Winton DJ. p53, mutation frequency and apoptosis in the murine small intestine. *Oncogene* 1997 May 1;14(17):2015-8
- Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 1993 Apr 29;362(6423):849-52
- Clayman GL, el-Naggar AK, Lippman SM, Henderson YC, Frederick M, Merritt JA, Zumstein LA, Timmons TM, Liu TJ, Ginsberg L, Roth JA, Hong WK, Bruso P, Goepfert H. Adenovirus-mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J Clin Oncol* 1998 Jun;16(6):2221-32
- Clontech; Tet-off and Tet-on Expression Systems and Cell Lines  
<http://www.clontech.com>
- Clurman B, Groudine M. Tumour-suppressor genes. Killer in search of a motive? *Nature* 1997 Sep 11;389(6647):122-3
- Coffin JM, Goff S, Hughes SH. *Retroviruses*. Cold Spring Harbor Laboratory; ISBN: 0879694971
- Copp AJ. Death before birth: clues from gene knockouts and mutations. *Trends Genet* 1995 Mar;11(3):87-93

- Crabbe JC, Belknap JK, Mitchell SR, Crawshaw LI. Quantitative trait loci mapping of genes that influence the sensitivity and tolerance to ethanol-induced hypothermia in BXD recombinant inbred mice. *J Pharmacol Exp Ther* 1994 Apr;269(1):184-92
- Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994 Sep 9;265(5178):1582-4
- Debbas M, White E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* 1993 Apr;7(4):546-54
- Deffie A, Wu H, Reinke V, Lozano G. The tumor suppressor p53 regulates its own transcription. *Mol Cell Biol* 1993 Jun;13(6):3415-23
- Degliantoni G, Murphy M, Kobayashi M, Francis MK, Perussia B, Trinchieri G. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. *J Exp Med* 1985 Nov 1;162(5):1512-30
- Demant P, Hart AA. Recombinant congenic strains--a new tool for analyzing genetic traits determined by more than one gene. *Immunogenetics* 1986;24(6):416-22
- Deng C, Capecchi MR. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol Cell Biol* 1992 Aug;12(8):3365-71
- Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 1995 Aug 25;82(4):675-84
- Devon RS, Porteous DJ, Brookes AJ. Splinkerettes--improved vectorettes for greater efficiency in PCR walking. *Nucleic Acids Res* 1995 May 11;23(9):1644-5
- Di Leonardo A, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev* 1994 Nov 1;8(21):2540-51

- Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S, Smithies O. Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* 1987 Dec 10-16;330(6148):576-8
- Donehower LA, Bradley A. The tumor suppressor p53. *Biochim Biophys Acta* 1993 Aug 23;1155(2):181-205
- Donehower LA, Godley LA, Aldaz CM, Pyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D, Varmus HE. Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. *Genes Dev* 1995 Apr 1;9(7):882-95
- Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992 Mar 19;356(6366):215-21
- Doolittle DP, Davisson MT, Guidi JN, Green MC. (1996). Catalogue of mutant genes and polymorphic loci. In *Genetic variants and strains of the laboratory mouse*, 3<sup>rd</sup> ed. (eds. Lyons MF, Rastan S, Brown SDM.), pages 17-854. Oxford University Press, Oxford, UK.
- Dutta A, Ruppert JM, Aster JC, Winchester E. Inhibition of DNA replication factor RPA by p53. *Nature* 1993 Sep 2;365(6441):79-82
- Eilers M, Picard D, Yamamoto KR, Bishop JM. Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* 1989 Jul 6;340(6228):66-8
- Eizenberg O, Faber-Elman A, Gottlieb E, Oren M, Rotter V, Schwartz M. Direct involvement of p53 in programmed cell death of oligodendrocytes. *EMBO J* 1995 Mar 15;14(6):1136-44
- Ekert PG, Vaux DL. Apoptosis and the immune system. *Br Med Bull* 1997;53(3):591-603
- el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. *Nat Genet* 1992 Apr;1(1):45-9

- el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993 Nov 19;75(4):817-25
- Eliyahu D, Michalovitz D, Oren M. Overproduction of p53 antigen makes established cells highly tumorigenic. *Nature* 1985 Jul 11-17;316(6024):158-60
- Elson A, Deng C, Campos-Torres J, Donehower LA, Leder P. The MMTV/c-myc transgene and p53 null alleles collaborate to induce T-cell lymphomas, but not mammary carcinomas in transgenic mice. *Oncogene* 1995 Jul 6;11(1):181-90
- Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992 Apr 3;69(1):119-28
- Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981, 292:154-156
- Farrell PJ, Balkow K, Hunt T, Jackson RJ, Trachsel H. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. *Cell* 1977 May;11(1):187-200
- Ferrara JLM, Deeg HJ, Burakoff SJ (eds). *Graft-vs-Host Disease*. M Dekker: New York, 1997.
- Festing MFW. Inbred strains of mice and rats  
<http://www.informatics.jax.org/bin/strains/search>
- Field AK, Tytell AA, Lampson GP, Hilleman MR. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc Natl Acad Sci U S A* 1967 Sep;58(3):1004-10
- Fijneman RJ, Oomen LC, Snoek M, Demant P. A susceptibility gene for alveolar lung tumors in the mouse maps between Hsp70.3 and G7 within the H2 complex. *Immunogenetics* 1995;41(2-3):106-9
- Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. *Cell* 1989 Jun 30;57(7):1083-93

- Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, O'Donnell M, Hurwitz J. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc Natl Acad Sci U S A* 1994 Aug 30;91(18):8655-9
- Folger KR, Thomas K, Capecchi MR. Nonreciprocal exchanges of information between DNA duplexes coinjected into mammalian cell nuclei. *Mol Cell Biol* 1985 Jan;5(1):59-69
- Frankel WN. Taking stock of complex trait genetics in mice. *Trends Genet* 1995 Dec;11(12):471-7
- Frankel WN, Taylor BA, Noebels JL, Lutz CM. Genetic epilepsy model derived from common inbred mouse strains. *Genetics* 1994 Oct;138(2):481-9
- Frebourg T, Barbier N, Kassel J, Ng YS, Romero P, Friend SH. A functional screen for germ line p53 mutations based on transcriptional activation. *Cancer Res* 1992 Dec 15;52(24):6976-8
- Fu L, Benchimol S. Participation of the human p53 3'UTR in translational repression and activation following gamma-irradiation. *EMBO J* 1997 Jul 1;16(13):4117-25
- Fu XY, Kessler DS, Veals SA, Levy DE, Darnell JE Jr. ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. *Proc Natl Acad Sci U S A* 1990 Nov;87(21):8555-9
- Fu XY, Schindler C, Improta T, Aebersold R, Darnell JE Jr. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci U S A* 1992 Aug 15;89(16):7840-3
- Garkavtsev I, Grigorian IA, Ossovskaya VS, Chernov MV, Chumakov PM, Gudkov AV. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. *Nature* 1998 Jan 15;391(6664):295-8
- Garside P, Felstein MV, Green EA, Mowat AM. The role of interferon alpha/beta in the induction of intestinal pathology in mice. *Immunology* 1991 Oct;74(2):279-83
- Garside P, Mowat AM. Natural killer cells and tumour necrosis factor-alpha-mediated enteropathy in mice. *Immunology* 1993 Feb;78(2):335-7

- Gearing DP, Bruce AG. Oncostatin M binds the high-affinity leukemia inhibitory factor receptor. *New Biol* 1992 Jan;4(1):61-5
- Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* 1998 Oct 1;12(19):2973-83
- Ginsberg D, Oren M, Yaniv M, Piette J. Protein-binding elements in the promoter region of the mouse p53 gene. *Oncogene* 1990 Sep;5(9):1285-90
- Godley LA, Kopp JB, Eckhaus M, Paglino JJ, Owens J, Varmus HE. Wild-type p53 transgenic mice exhibit altered differentiation of the ureteric bud and possess small kidneys. *Genes Dev* 1996 Apr 1;10(7):836-50
- Gopalkrishnan RV, Lam EWF, Kedinger C. The p53 tumor suppressor inhibits transcription of the TATA-less mouse DP1 promoter. *J Biol Chem* 1998 May 1;273(18):10972-8
- Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci U S A* 1980 Dec;77(12):7380-4
- Goring DR, Rossant J, Clapoff S, Breitman ML, Tsui LC. In situ detection of beta-galactosidase in lenses of transgenic mice with a gamma-crystallin/lacZ gene. *Science* 1987 Jan 23;235(4787):456-8
- Gossen M, Bonin AL, Bujard H. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem Sci* 1993 Dec;18(12):471-5
- Gossen M, Bonin AL, Bujard H. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem Sci* 1993 Dec;18(12):471-5
- Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992 Jun 15;89(12):5547-51
- Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995 Jun 23;268(5218):1766-9

- Gottlieb E, Haffner R, King A, Asher G, Gruss P, Lonai P, Oren M. Transgenic mouse model for studying the transcriptional activity of the p53 protein: age- and tissue-dependent changes in radiation-induced activation during embryogenesis. *EMBO J* 1997 Mar 17;16(6):1381-90
- Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996 Jan 4;379(6560):88-91
- Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ Jr, Giaccia AJ. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol Cell Biol* 1994 Sep;14(9):6264-77
- Greenhalgh DA, Wang XJ, Donehower LA, Roop DR. Paradoxical tumor inhibitory effect of p53 loss in transgenic mice expressing epidermal-targeted v-rasHa, v-fos, or human transforming growth factor alpha. *Cancer Res* 1996 Oct 1;56(19):4413-23
- Griffiths SD, Clarke AR, Healy LE, Ross G, Ford AM, Hooper ML, Wyllie AH, Greaves M. Absence of p53 permits propagation of mutant cells following genotoxic damage. *Oncogene* 1997 Feb 6;14(5):523-31
- Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 1997 Aug 22;90(4):595-606
- Haines DS, Strauss KI, Gillespie DH. Cellular response to double-stranded RNA. *J Cell Biochem* 1991 May;46(1):9-20
- Hale TK, Braithwaite AW. Identification of an upstream region of the mouse p53 promoter critical for transcriptional expression. *Nucleic Acids Res* 1995 Feb 25;23(4):663-9
- Hall PA, Kearsley JM, Coates PJ, Norman DG, Warbrick E, Cox LS. Characterisation of the interaction between PCNA and Gadd45. *Oncogene* 1995 Jun 15;10(12):2427-33
- Hall PA, McKee PH, Menage HD, Dover R, Lane DP. High levels of p53 protein in UV-irradiated normal human skin. *Oncogene* 1993 Jan;8(1):203-7

- Hall SR, Campbell LE, Meek DW. Phosphorylation of p53 at the casein kinase II site selectively regulates p53-dependent transcriptional repression but not transactivation. *Nucleic Acids Res* 1996 Mar 15;24(6):1119-26
- Hammer RE, Krumlauf R, Camper SA, Brinster RL, Tilghman SM. Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. *Science* 1987 Jan 2;235(4784):53-8
- Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993 Nov 19;75(4):805-16
- Harris AW, Pinkert CA, Crawford M, Langdon WY, Brinster RL, Adams JM. The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. *J Exp Med* 1988 Feb 1;167(2):353-71
- Harvey M, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat Genet* 1993 Nov;5(3):225-9
- Harvey M, Vogel H, Morris D, Bradley A, Bernstein A, Donehower LA. A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. *Nat Genet* 1995 Mar;9(3):305-11
- Harvey M, Vogel H, Morris D, Bradley A, Bernstein A, Donehower LA. A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. *Nat Genet* 1995 Mar;9(3):305-11
- Hasty P, Ramirez-Solis R, Krumlauf R, Bradley A. Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* 1991 Mar 21;350(6315):243-6. Published erratum appears in *Nature* 1991 Sep 5;353(6339):94 (B)
- Hasty P, Rivera-Perez J, Bradley A. The length of homology required for gene targeting in embryonic stem cells. *Mol Cell Biol* 1991 Nov;11(11):5586-91 (A)
- Haupt Y, Rowan S, Shaulian E, Vousden KH, Oren M. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev* 1995 Sep 1;9(17):2170-83
- Hermeking H, Eick D. Mediation of c-Myc-induced apoptosis by p53. *Science* 1994 Sep 30;265(5181):2091-3



- Hogan B, Beddington R, Costantini F, Lacey E. *Manipulating the Mouse Embryo: A Laboratory Manual* (2nd edition) Cold Spring Harbor Laboratory; ISBN: 0879693924
- Holley MC, Lawlor PW. Production of conditionally immortalised cell lines from a transgenic mouse. *Audiol Neurootol* 1997 Jan-Apr;2(1-2):25-35
- Holliday R. A mechanism for gene conversion in fungi. *Genetical Research* 1964, 5:282-304
- Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* 1994 Sep;22(17):3551-5
- Hooper ML. *Embryonal stem cells : introducing planned changes into the animal germline* :Chur : Harwood Academic, c1992
- Hooper ML, Subak-Sharpe JH. Metabolic cooperation between cells. *Int Rev Cytol* 1981;69:45-104
- Horikoshi N, Usheva A, Chen J, Levine AJ, Weinmann R, Shenk T. Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. *Mol Cell Biol* 1995 Jan;15(1):227-34
- Hsu DH, Moore KW, Spits H. Differential effects of IL-4 and IL-10 on IL-2-induced IFN-gamma synthesis and lymphokine-activated killer activity. *Int Immunol* 1992 May;4(5):563-9
- Hu MC, Davidson N. The inducible lac operator-repressor system is functional in mammalian cells. *Cell* 1987 Feb 27;48(4):555-66
- Hug H, Costas M, Staeheli P, Aebi M, Weissmann C. Organization of the murine Mx gene and characterization of its interferon- and virus-inducible promoter. *Mol Cell Biol* 1988 Aug;8(8):3065-79
- Hupp TR, Meek DW, Midgley CA, Lane DP. Activation of the cryptic DNA binding function of mutant forms of p53. *Nucleic Acids Res* 1993 Jul 11;21(14):3167-74

- Hupp TR, Meek DW, Midgley CA, Lane DP. Regulation of the specific DNA binding function of p53. *Cell* 1992 Nov 27;71(5):875-86
- Iotsova V, Crepieux P, Montpellier C, Laudet V, Stehelin D. TATA-less promoters of some Ets-family genes are efficiently repressed by wild-type p53. *Oncogene* 1996 Dec 5;13(11):2331-7
- Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 1994 Jan 1;4(1):1-7
- Jaenisch R. Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc Natl Acad Sci U S A* 1976 Apr;73(4):1260-4
- Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, Jaenisch R. De novo methylation and expression of retroviral genomes during mouse embryogenesis. *Nature* 1982 Aug 12;298(5875):623-8
- Jayaraman L, Murthy KG, Zhu C, Curran T, Xanthoudakis S, Prives C. Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes Dev* 1997 Mar 1;11(5):558-70
- Jeannotte L, Ruiz JC, Robertson EJ. Low level of Hox1.3 gene expression does not preclude the use of promoterless vectors to generate a targeted gene disruption. *Mol Cell Biol* 1991 Nov;11(11):5578-85
- Jeffrey PD, Gorina S, Pavletich NP. Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. *Science* 1995 Mar 10;267(5203):1498-502
- Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 1995 Nov 9;378(6553):206-8
- Jordan E, Collins FS. A march of genetic maps. *Nature* 1996 Mar 14;380(6570):111-2
- Justice MJ, Jenkins NA, Copeland NG. Recombinant inbred mouse strains: models for disease study. *Trends Biotechnol* 1992 Apr;10(4):120-6

- Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* 1997 Nov;17(3):314-7
- Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G, Sherr CJ. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997 Nov 28;91(5):649-59
- Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 1991 Dec 1;51(23 Pt 1):6304-11
- Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 1992 Nov 13;71(4):587-97
- Katsuki M, Sato M, Kimura M, Yokoyama M, Kobayashi K, Nomura T. Conversion of normal behavior to shiverer by myelin basic protein antisense cDNA in transgenic mice. *Science* 1988 Jul 29;241(4865):593-5
- Kelly JM, Gilbert CS, Stark GR, Kerr IM. Differential regulation of interferon-induced mRNAs and c-myc mRNA by alpha- and gamma-interferons. *Eur J Biochem* 1985 Dec 2;153(2):367-71
- Kelly JM, Porter AC, Chernajovsky Y, Gilbert CS, Stark GR, Kerr IM. Characterization of a human gene inducible by alpha- and beta-interferons and its expression in mouse cells. *EMBO J* 1986 Jul;5(7):1601-6
- Kemp CJ, Donehower LA, Bradley A, Balmain A. Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. *Cell* 1993 Sep 10;74(5):813-22
- Kemp CJ, Wheldon T, Balmain A. p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nat Genet* 1994 Sep;8(1):66-9
- Kerr IM, Stark GR. The control of interferon-inducible gene expression. *FEBS Lett* 1991 Jul 22;285(2):194-8

- Kleinschmidt C, Tovar K, Hillen W, Porschke D. Dynamics of repressor-operator recognition: the Tn10-encoded tetracycline resistance control. *Biochemistry* 1988 Feb 23;27(4):1094-104
- Knudson AG. Antioncogenes and human cancer. *Proc Natl Acad Sci U S A* 1993 Dec 1;90(23):10914-21
- Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971 Apr;68(4):820-3
- Ko LJ, Prives C. p53: puzzle and paradigm. *Genes Dev* 1996 May 1;10(9):1054-72
- Komarova EA, Chernov MV, Franks R, Wang K, Armin G, Zelnick CR, Chin DM, Bacus SS, Stark GR, Gudkov AV. Transgenic mice with p53-responsive lacZ: p53 activity varies dramatically during normal development and determines radiation and drug sensitivity in vivo. *EMBO J* 1997 Mar 17;16(6):1391-400
- Komarova EA, Chernov MV, Franks R, Wang K, Armin G, Zelnick CR, Chin DM, Bacus SS, Stark GR, Gudkov AV. Transgenic mice with p53-responsive lacZ: p53 activity varies dramatically during normal development and determines radiation and drug sensitivity in vivo. *EMBO J* 1997 Mar 17;16(6):1391-400
- Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol Chem* 1991 Oct 25;266(30):19867-70
- Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat Med* 1997 Jun;3(6):614-20. Published erratum appears in *Nat Med* 1997 Aug;3(8):934
- Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997 May 15;387(6630):299-303
- Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995 Sep 8;269(5229):1427-9
- Kulesz-Martin MF, Lisafeld B, Huang H, Kisiel ND, Lee L. Endogenous p53 protein generated from wild-type alternatively spliced p53 RNA in mouse epidermal cells. *Mol Cell Biol* 1994 Mar;14(3):1698-708

- Lacy E, Roberts S, Evans EP, Burtenshaw MD, Costantini FD. A foreign beta-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. *Cell* 1983 Sep;34(2):343-58
- Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. *Nature* 1979 Mar 15;278(5701):261-3
- Lang RA, Metcalf D, Cuthbertson RA, Lyons I, Stanley E, Kelso A, Kannourakis G, Williamson DJ, Klintworth GK, Gonda TJ, et al. Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. *Cell* 1987 Nov 20;51(4):675-86.
- Le Mouellie H, Lallemand Y, Brulet P. Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. *Cell* 1992 Apr 17;69(2):251-64
- Leach FS, Tokino T, Meltzer P, Burrell M, Oliner JD, Smith S, Hill DE, Sidransky D, Kinzler KW, Vogelstein B. p53 Mutation and MDM2 amplification in human soft tissue sarcomas. *Cancer Res* 1993 May 15;53(10 Suppl):2231-4
- Lee F, Hall CV, Ringold GM, Dobson DE, Luh J, Jacob PE. Functional analysis of the steroid hormone control region of mouse mammary tumor virus. *Nucleic Acids Res* 1984 May 25;12(10):4191-206
- Lee JM, Abrahamson JL, Kandel R, Donehower LA, Bernstein A. Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. *Oncogene* 1994 Dec;9(12):3731-6
- Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997 Feb 7;88(3):323-31
- Li G, Ho VC. p53-dependent DNA repair and apoptosis respond differently to high- and low-dose ultraviolet radiation. *Br J Dermatol* 1998 Jul;139(1):3-10
- Li R, Waga S, Hannon GJ, Beach D, Stillman B. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* 1994 Oct 6;371(6497):534-7
- Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. Binding and modulation of p53 by p300/CBP coactivators. *Nature* 1997 Jun 19;387(6635):823-7

- Lin FL, Sperle K, Sternberg N. Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process. *Mol Cell Biol* 1984 Jun;4(6):1020-34
- Linke SP, Clarkin KC, Di Leonardo A, Tsou A, Wahl GM. A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev* 1996 Apr 15;10(8):934-47
- Lipoldova M, Kosarova M, Zajicova A, Holan V, Hart AA, Krulova M, Demant P. Separation of multiple genes controlling the T-cell proliferative response to IL-2 and anti-CD3 using recombinant congenic strains. *Immunogenetics* 1995;41(5):301-11
- Ljungman M, Zhang F. Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis. *Oncogene* 1996 Aug 15;13(4):823-31
- Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993 Sep 24;74(6):957-67
- Mack DH, Vartikar J, Pipas JM, Laimins LA. Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature* 1993 May 20;363(6426):281-3
- Malcomson RDG. Investigation of apoptosis by conditional gene expression. PhD. Thesis, University of Edinburgh library.
- Maltzman W, Czyzyk L. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol Cell Biol* 1984 Sep;4(9):1689-94
- Mansour SL, Thomas KR, Capecchi MR. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* 1988 Nov 24;336(6197):348-52
- Marin MC, Hsu B, Meyn RE, Donehower LA, el-Naggar AK, McDonnell TJ. Evidence that p53 and bcl-2 are regulators of a common cell death pathway important for in vivo lymphomagenesis. *Oncogene* 1994 Nov;9(11):3107-12
- Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 1981 Dec;78(12):7634-8

- McCarrick JW 3d, Parnes JR, Seong RH, Solter D, Knowles BB. Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. *Transgenic Res* 1993 Jul;2(4):183-90
- McNeall J, Sanchez A, Gray PP, Chesterman CN, Sleigh MJ. Hyperinducible gene expression from a metallothionein promoter containing additional metal-responsive elements. *Gene* 1989 Mar 15;76(1):81-8
- Merlin G, Falcoff E, Aguet M. 125I-labelled human interferons alpha, beta and gamma: comparative receptor-binding data. *J Gen Virol* 1985 May;66 ( Pt 5):1149-52
- Meselson MS, Radding CM. A general model for genetic recombination. *Proc Natl Acad Sci U S A* 1975 Jan;72(1):358-61
- Midgley CA, Owens B, Briscoe CV, Thomas DB, Lane DP, Hall PA. Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo. *J Cell Sci* 1995 May;108 ( Pt 5):1843-8
- Milner J, Medcalf EA. Temperature-dependent switching between "wild-type" and "mutant" forms of p53-Val135. *J Mol Biol* 1990 Dec 5;216(3):481-4
- Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* 1994 Jun 15;54(12):3131-5
- Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995 Jan 27;80(2):293-9
- Moen CJ, Groot PC, Hart AA, Snoek M, Demant P. Fine mapping of colon tumor susceptibility (Scc) genes in the mouse, different from the genes known to be somatically mutated in colon cancer. *Proc Natl Acad Sci U S A* 1996 Feb 6;93(3):1082-6
- Mombaerts P, Clarke AR, Hooper ML, Tonegawa S. Creation of a large genomic deletion at the T-cell antigen receptor beta-subunit locus in mouse embryonic stem cells by gene targeting. *Proc Natl Acad Sci U S A* 1991 Apr 15;88(8):3084-7
- Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 1995 Nov 9;378(6553):203-6

- Mosner J, Mummenbrauer T, Bauer C, Sczakiel G, Grosse F, Deppert W. Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J* 1995 Sep 15;14(18):4442-9
- Muller-Tiemann BF, Halazonetis TD, Elting JJ. Identification of an additional negative regulatory region for p53 sequence-specific DNA binding. *Proc Natl Acad Sci U S A* 1998 May 26;95(11):6079-84
- Mummenbrauer T, Janus F, Muller B, Wiesmuller L, Deppert W, Grosse F. p53 Protein exhibits 3'-to-5' exonuclease activity. *Cell* 1996 Jun 28;85(7):1089-99
- Mummery CL, Feyen A, Freund E, Shen S. Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. *Cell Differ Dev* 1990 Jun;30(3):195-206
- Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A* 1996 Oct 15;93(21):11382-8
- Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol Cell Biol* 1994 Mar;14(3):1815-23
- Neubergh M, Buckbinder L, Seizinger B, Kley N. The p53/IGF-1 receptor axis in the regulation of programmed cell death. *Endocrine* 1997 Aug;7(1):107-9
- Nishino H, Knoll A, Buettner VL, Frisk CS, Maruta Y, Haavik J, Sommer SS. p53 wild-type and p53 nullizygous Big Blue transgenic mice have similar frequencies and patterns of observed mutation in liver, spleen and brain. *Oncogene* 1995 Jul 20;11(2):263-70
- No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc Natl Acad Sci U S A* 1996 Apr 16;93(8):3346-51
- Norimura T, Nomoto S, Katsuki M, Gondo Y, Kondo S. p53-dependent apoptosis suppresses radiation-induced teratogenesis. *Nat Med* 1996 May;2(5):577-80
- Oberosler P, Hloch P, Ramsperger U, Stahl H. p53-catalyzed annealing of complementary single-stranded nucleic acids. *EMBO J* 1993 Jun;12(6):2389-96



- O'Connell MA. RNA editing: rewriting receptors. *Curr Biol* 1997 Jul 1;7(7):R437-9
- Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* 1993 Apr 29;362(6423):857-60
- Orr-Weaver TL, Szostak JW. Fungal recombination. *Microbiol Rev* 1985 Mar;49(1):33-58
- Orr-Weaver TL, Szostak JW, Rothstein RJ. Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci U S A* 1981 Oct;78(10):6354-8
- O'Shea-Greenfield A, Smale ST. Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription. *J Biol Chem* 1992 Jan 15;267(2):1391-402
- Patterson JB, Thomis DC, Hans SL, Samuel CE. Mechanism of interferon action: double-stranded RNA-specific adenosine deaminase from human cells is inducible by alpha and gamma interferons. *Virology* 1995 Jul 10;210(2):508-11
- Perussia B. Lymphokine-activated killer cells, natural killer cells and cytokines. *Curr Opin Immunol* 1991 Feb;3(1):49-55
- Petrocelli T, Poon R, Drucker DJ, Slingerland JM, Rosen CF. UVB radiation induces p21Cip1/WAF1 and mediates G1 and S phase checkpoints. *Oncogene* 1996 Apr 4;12(7):1387-96
- Player MR, Torrence PF. The 2-5A system: modulation of viral and cellular processes through acceleration of RNA degradation. *Pharmacol Ther* 1998 May;78(2):55-113
- Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. *Nature* 1997 Sep 18;389(6648):300-5
- Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW, Cordon-Cardo C, DePinho RA. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998 Mar 20;92(6):713-23

- Porter A. Controlling your losses: conditional gene silencing in mammals. *Trends Genet* 1998 Feb;14(2):73-9
- Porter AC, Itzhaki JE. Gene targeting in human somatic cells. Complete inactivation of an interferon-inducible gene. *Eur J Biochem* 1993 Dec 1;218(2):273-81
- Potter H, Dressler D. On the mechanism of genetic recombination: electron microscopic observation of recombination intermediates. *Proc Natl Acad Sci U S A* 1976 Sep;73(9):3000-4
- Purdie CA, Harrison DJ, Peter A, Dobbie L, White S, Howie SE, Salter DM, Bird CC, Wyllie AH, Hooper ML, et al. Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene* 1994 Feb;9(2):603-9
- Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995 Dec 15;83(6):993-1000
- Ramirez-Solis R, Liu P, Bradley A. Chromosome engineering in mice. *Nature* 1995 Dec 14;378(6558):720-4
- Reid LH, Shesely EG, Kim HS, Smithies O. Cotransformation and gene targeting in mouse embryonic stem cells. *Mol Cell Biol* 1991 May;11(5):2769-77
- Reid LM, Minato N, Gresser I, Holland J, Kadish A, Bloom BR. Influence of anti-mouse interferon serum on the growth and metastasis of tumor cells persistently infected with virus and of human prostatic tumors in athymic nude mice. *Proc Natl Acad Sci U S A* 1981 Feb;78(2):1171-5
- Reihnsaus E, Kohler M, Kraiss S, Oren M, Montenarh M. Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. *Oncogene* 1990 Jan;5(1):137-45
- Reisman D, Elkind NB, Roy B, Beamon J, Rotter V. c-Myc trans-activates the p53 promoter through a required downstream CACGTG motif. *Cell Growth Differ* 1993 Feb;4(2):57-65
- Reynolds GA, Basu SK, Osborne TF, Chin DJ, Gil G, Brown MS, Goldstein JL, Luskey KL. HMG CoA reductase: a negatively regulated gene with unusual promoter and 5' untranslated regions. *Cell* 1984 Aug;38(1):275-85

- Riedy MC, Timm EA Jr, Stewart CC. Quantitative RT-PCR for measuring gene expression. *Biotechniques* 1995 Jan;18(1):70-4, 76
- Ronen D, Rotter V, Reisman D. Expression from the murine p53 promoter is mediated by factor binding to a downstream helix-loop-helix recognition motif. *Proc Natl Acad Sci U S A* 1991 May 15;88(10):4128-32
- Rossi FM, Guicherit OM, Spicher A, Kringstein AM, Fatyol K, Blakely BT, Blau HM. Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. *Nat Genet* 1998 Dec;20(4):389-93
- Roth J, Dobbstein M, Freedman DA, Shenk T, Levine AJ. Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO J* 1998 Jan 15;17(2):554-64
- Roth JA, Nguyen D, Lawrence DD, Kemp BL, Carrasco CH, Ferson DZ, Hong WK, Komaki R, Lee JJ, Nesbitt JC, Pisters KM, Putnam JB, Schea R, Shin DM, Walsh GL, Dolormente MM, Han CI, Martin FD, Yen N, Xu K, Stephens LC, McDonnell TJ, Mukhopadhyay T, Cai D. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nat Med* 1996 Sep;2(9):985-91
- Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, Donehower LA, Levine AJ. Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. *Proc Natl Acad Sci U S A* 1993 Oct 1;90(19):9075-9
- Rubnitz J, Subramani S. The minimum amount of homology required for homologous recombination in mammalian cells. *Mol Cell Biol* 1984 Nov;4(11):2253-8
- Sabbatini P, Lin J, Levine AJ, White E. Essential role for p53-mediated transcription in E1A-induced apoptosis. *Genes Dev* 1995 Sep 1;9(17):2184-92
- Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* 1995 Jun;10(2):175-80
- Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW, Appella E. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* 1998 Sep 15;12(18):2831-41

- Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning; A Laboratory Manual* (2<sup>nd</sup> edition), Cold Spring Harbour Laboratory Press, 1989.
- Sanchez Y, Elledge SJ. Stopped for repairs. *Bioessays* 1995 Jun;17(6):545-8
- Sands AT, Suraokar MB, Sanchez A, Marth JE, Donehower LA, Bradley A. p53 deficiency does not affect the accumulation of point mutations in a transgene target. *Proc Natl Acad Sci U S A* 1995 Aug 29;92(18):8517-21
- Schindler C, Shuai K, Prezioso VR, Darnell JE Jr. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 1992 Aug 7;257(5071):809-13
- Schindler C, Shuai K, Prezioso VR, Darnell JE Jr. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 1992 Aug 7;257(5071):809-13
- Seeburg PH. The role of RNA editing in controlling glutamate receptor channel properties. *J Neurochem* 1996 Jan;66(1):1-5
- Sen GC, Ransohoff RM. Interferon-induced antiviral actions and their regulation. *Adv Virus Res* 1993;42:57-102
- Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* 1997 Oct 31;91(3):325-34
- Shockett P, Difilippantonio M, Hellman N, Schatz DG. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc Natl Acad Sci U S A* 1995 Jul 3;92(14):6522-6
- Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev* 1997 Dec 15;11(24):3471-81
- Simons JP, McClenaghan M, Clark AJ. Alteration of the quality of milk by expression of sheep beta-lactoglobulin in transgenic mice. *Nature* 1987 Aug 6-12;328(6130):530-2

- Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 1988 Dec 15;336(6200):688-90
- Smith AG, Hooper ML. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev Biol* 1987 May;121(1):1-9
- Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* 1985 Sep 19-25;317(6034):230-4
- Soriano P, Cone RD, Mulligan RC, Jaenisch R. Tissue-specific and ectopic expression of genes introduced into transgenic mice by retroviruses. *Science* 1986 Dec 12;234(4782):1409-13
- Southern PJ, Berg P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet* 1982;1(4):327-41
- Spencer CA, Groudine M. Control of c-myc regulation in normal and neoplastic cells. *Adv Cancer Res* 1991;56:1-48
- Stacey A, Schnieke A, McWhir J, Cooper J, Colman A, Melton DW. Use of double-replacement gene targeting to replace the murine alpha-lactalbumin gene with its human counterpart in embryonic stem cells and mice. *Mol Cell Biol* 1994 Feb;14(2):1009-16
- Strasser A, Harris AW, Bath ML, Cory S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* 1990 Nov 22;348(6299):331-3
- Stutts P, Brockman RW. *Biochem Pharmacol.* 12, 97 (1963).
- Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. The double-strand-break repair model for recombination. *Cell* 1983 May;33(1):25-35
- Szybalski W. Use of the HPRT gene and the HAT selection technique in DNA-mediated transformation of mammalian cells: first steps toward developing hybridoma techniques and gene therapy. *Bioessays* 1992 Jul;14(7):495-500

- Takenaka I, Morin F, Seizinger BR, Kley N. Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. *J Biol Chem* 1995 Mar 10;270(10):5405-11
- te Riele H, Maandag ER, Berns A. Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc Natl Acad Sci U S A* 1992 Jun 1;89(11):5128-32
- Templeton NS, Roberts DD, Safer B. Efficient gene targeting in mouse embryonic stem cells. *Gene Ther* 1997 Jul;4(7):700-9
- Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 1987 Nov 6;51(3):503-12
- Thut CJ, Chen JL, Klemm R, Tjian R. p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science* 1995 Jan 6;267(5194):100-4
- Tishler RB, Lamppu DM, Park S, Price BD. Microtubule-active drugs taxol, vinblastine, and nocodazole increase the levels of transcriptionally active p53. *Cancer Res* 1995 Dec 15;55(24):6021-5
- Tuck SP, Crawford L. Characterization of the human p53 gene promoter. *Mol Cell Biol* 1989 May;9(5):2163-72
- Unger T, Nau MM, Segal S, Minna JD. p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J* 1992 Apr;11(4):1383-90
- Varmus HE. Form and function of retroviral proviruses. *Science* 1982 May 21;216(4548):812-20.
- Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 1994 Jun 16;369(6481):574-8
- Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. *Genes Dev* 1994 Dec 1;8(23):2817-30

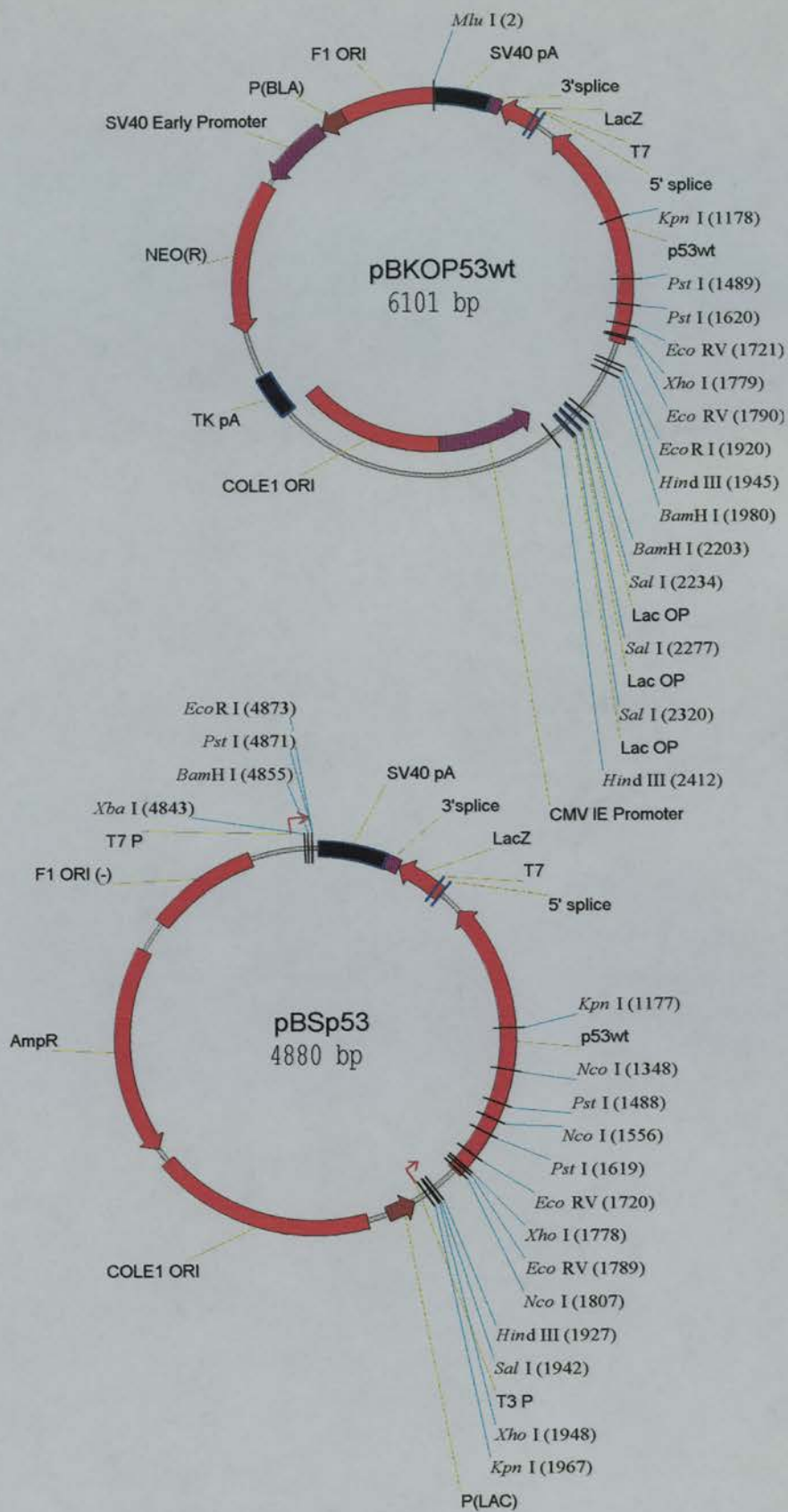
- Waldman AS. Targeted homologous recombination in mammalian cells. *Crit Rev Oncol Hematol* 1992 Jan;12(1):49-64
- Wang XW, Vermeulen W, Coursen JD, Gibson M, Lupold SE, Forrester K, Xu G, Elmore L, Yeh H, Hoeijmakers JH, Harris CC. The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. *Genes Dev* 1996 May 15;10(10):1219-32
- Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin V, Egly JM, Wang Z, Freidberg EC, Evans MK, Taffe BG, et al. p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nat Genet* 1995 Jun;10(2):188-95
- Wang Y, Reed M, Wang P, Stenger JE, Mayr G, Anderson ME, Schwedes JF, Tegtmeier P. p53 domains: identification and characterization of two autonomous DNA-binding regions. *Genes Dev* 1993 Dec;7(12B):2575-86
- Waterman MJ, Stavridi ES, Waterman JL, Halazonetis TD. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nat Genet* 1998 Jun;19(2):175-8
- Webster NJ, Green S, Jin JR, Chambon P. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* 1988 Jul 15;54(2):199-207
- Weinberg RA. The molecular basis of oncogenes and tumor suppressor genes. *Ann N Y Acad Sci* 1995 Jun 30;758:331-8
- Whyatt LM, Duwel A, Smith AG, Rathjen PD. The responsiveness of embryonic stem cells to alpha and beta interferons provides the basis of an inducible expression system for analysis of developmental control genes. *Mol Cell Biol* 1993 Dec;13(12):7971-6
- Wilkie TM, Brinster RL, Palmiter RD. Germline and somatic mosaicism in transgenic mice. *Dev Biol* 1986 Nov;118(1):9-18
- Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 1988 Dec 15;336(6200):684-7

- Witt PL, Lindner DJ, D'Cunha J, Borden EC Chapter 25; Cancer Chemotherapy and Biotherapy, 2<sup>nd</sup> Edition (ed. Chabner BA and Longo DL) Lippincott-Raven Publishers, Philadelphia 1996
- Wolf E, Kramer R, Polejaeva I, Thoenen H, Brem G. Efficient generation of chimaeric mice using embryonic stem cells after long-term culture in the presence of ciliary neurotrophic factor. *Transgenic Res* 1994 May;3(3):152-8
- Wu L, Bayle JH, Elenbaas B, Pavletich NP, Levine AJ. Alternatively spliced forms in the carboxy-terminal domain of the p53 protein regulate its ability to promote annealing of complementary single strands of nucleic acids. *Mol Cell Biol* 1995 Jan;15(1):497-504
- Wu Y, Liu Y, Lee L, Miner Z, Kulesz-Martin M. Wild-type alternatively spliced p53: binding to DNA and interaction with the major p53 protein in vitro and in cells. *EMBO J* 1994 Oct 17;13(20):4823-30
- Wubah JA, Ibrahim MM, Gao X, Nguyen D, Pisano MM, Knudsen TB. Teratogen-induced eye defects mediated by p53-dependent apoptosis. *Curr Biol* 1996 Jan 1;6(1):60-9. Published erratum appears in *Curr Biol* 1996 Jun 1;6(6):753
- Xu Y, Yang EM, Brugarolas J, Jacks T, Baltimore D. Involvement of p53 and p21 in cellular defects and tumorigenesis in *Atm*<sup>-/-</sup> mice. *Mol Cell Biol* 1998 Jul;18(7):4385-90
- Yew PR, Liu X, Berk AJ. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. *Genes Dev* 1994 Jan;8(2):190-202
- Yin C, Knudson CM, Korsmeyer SJ, Van Dyke T. Bax suppresses tumorigenesis and stimulates apoptosis in vivo. *Nature* 1997 Feb 13;385(6617):637-40
- Yonish-Rouach E, Deguin V, Zaitchouk T, Breugnot C, Mishal Z, Jenkins JR, May E. Transcriptional activation plays a role in the induction of apoptosis by transiently transfected wild-type p53. *Oncogene* 1995 Dec 7;11(11):2197-205
- Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 1991 Jul 25;352(6333):345-7

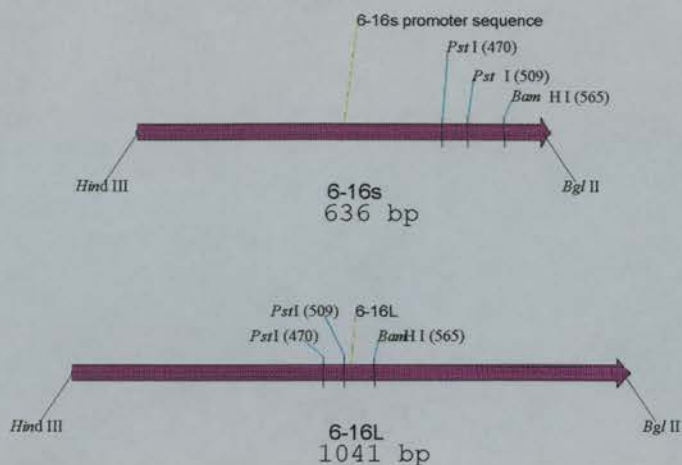


- Yuan J, Yeasky TM, Havre PA, Glazer PM. Induction of p53 in mouse cells decreases mutagenesis by UV radiation. *Carcinogenesis* 1995 Oct;16(10):2295-300
- Zakut-Houri R, Oren M, Bienz B, Lavie V, Hazum S, Givol D. A single gene and a pseudogene for the cellular tumour antigen p53. *Nature* 1983 Dec 8-14;306(5943):594-7
- Zhan Q, Carrier F, Fornace AJ Jr. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol Cell Biol* 1993 Jul;13(7):4242-50. Published erratum appears in *Mol Cell Biol* 1993 Sep;13(9):5928
- Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998 Mar 20;92(6):725-34
- Zhu J, Zhou W, Jiang J, Chen X. Identification of a novel p53 functional domain that is necessary for mediating apoptosis. *J Biol Chem* 1998 May 22;273(21):13030-6
- Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, Brash DE. Sunburn and p53 in the onset of skin cancer. *Nature* 1994 Dec 22-29;372(6508):773-6
- Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 1998 Aug 1;12(15):2424-33
- Zornig M, Hueber AO, Evan G. p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice. *Curr Biol* 1998 Apr 9;8(8):467-70

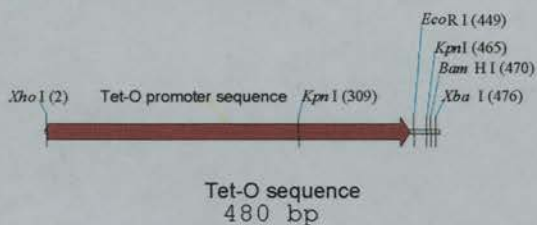
# APPENDIX - Inducible p53 Transgene Constructs



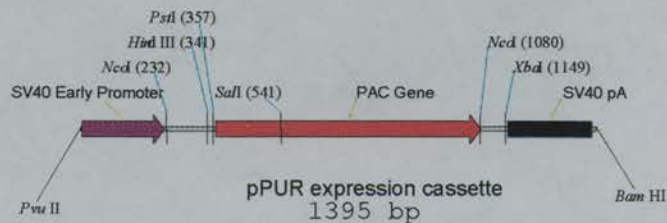
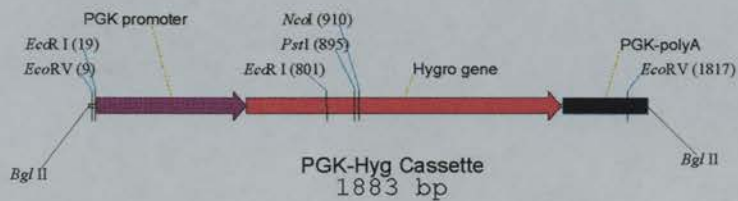
## Interferon Inducible Promoter Sequences

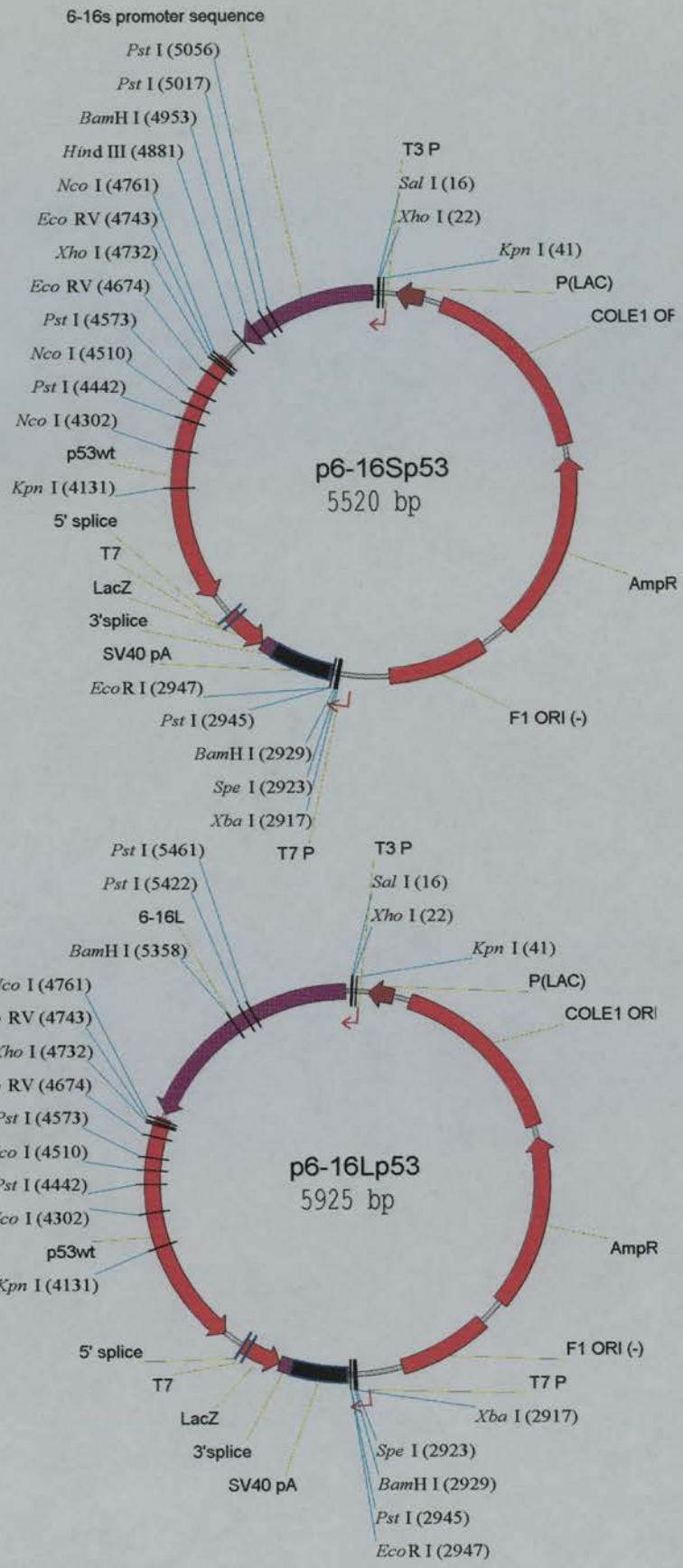


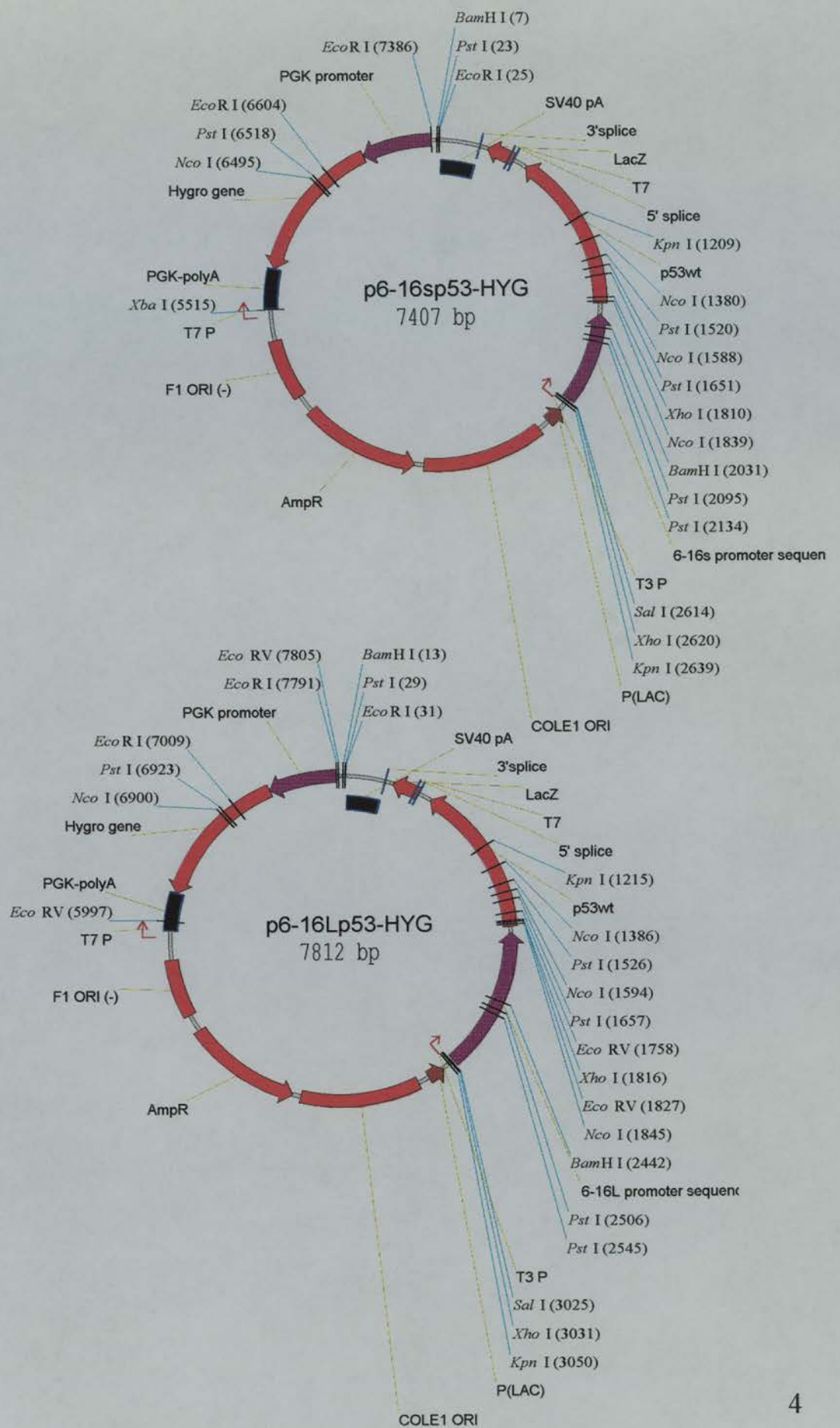
## Tet-O Promoter Sequence

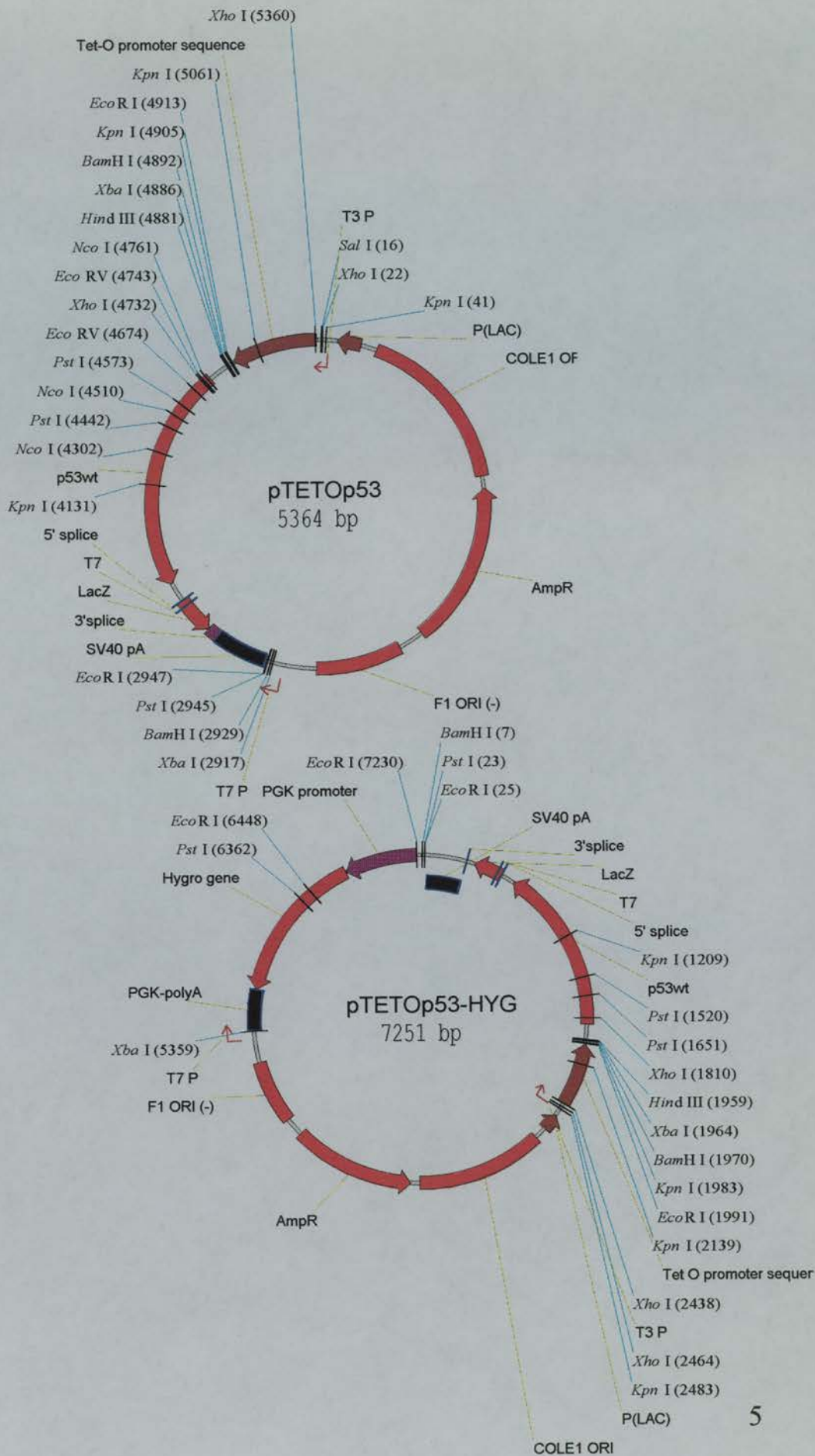


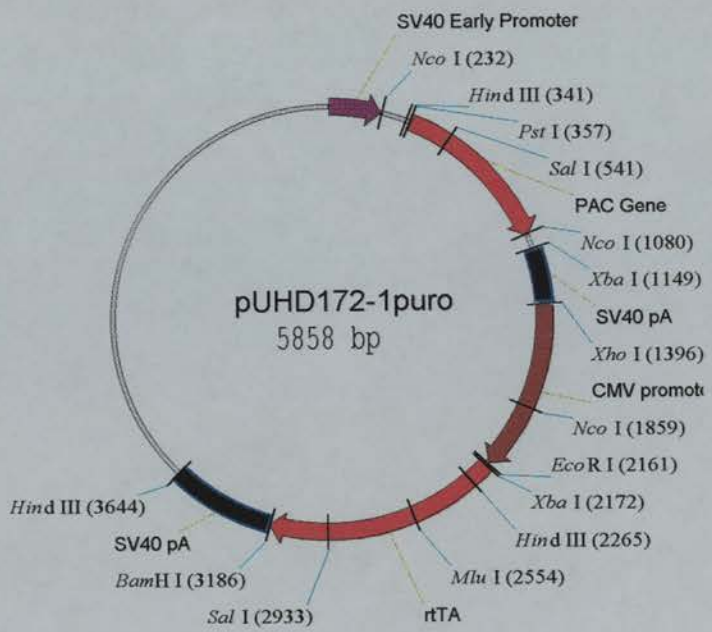
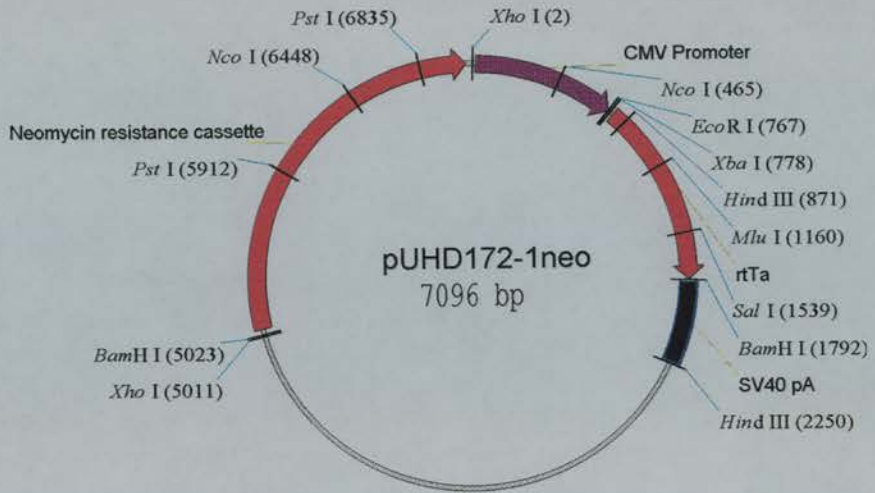
## Selection Marker Cassettes











# Targeting Vector Constructs

