GENETIC MAPPING OF MODIFIERS IN PRENATAL LETHAL *Tgfb1* KNOCKOUT MICE

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IN THE NAME OF

ALLAH

MOST GRACIOUS, MOST MERCIFUL

"SCIENCE WANTS TO KNOW THE MECHANISM OF THE UNIVERSE, RELIGION THE MEANING. THE TWO CANNOT BE SEPARATED."

CHARLES TOWNES.

DECLARATION

I certify that this thesis does not contain material previously published or written by any other person except where referred to in the text and that the results in this thesis have not been submitted for any other degree or diploma.

Morteza J. Bonyadi

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Table of Contents:

LIST OF FIGURES: PAGE

LIST OF TABLES:

PAGE

List of Publications and Presentations:

-The TGFB type II receptor, *Tgfbr2*, maps to Distal mouse chromosome 9. 1996 Mortaza Bonyadi, et al., **Genomics** 33, 328-329.

-Mapping of a major genetic modifier of embryonic lethality in TGFB1- knockout mice. 1997 Motaza Bonyadi, et al., **Nature Genetics** 15:207-211.

-Genetic mapping of loci which modify the prenatal lethal phenotype of TGFB1 knock-out mice. Mortaza Bonyadi et al., 1996 Mouse Molecular Genetic Meeting **(Cold Spring Harbor)**.August-Sept.

-Genetic mapping of loci which modify the prenatal lethal phenotype of TGFB1 knock-out mice. Mortaza Bonyadi et al., 1996 Mouse Molecular Genetics Meeting **(Institute Pastor , Paris)** October.

-Strain-Dependent phenotypic Variation in TGFB1 knock-out mice.
Sarah Rasholm, Mortaza Bonyadi, et al., 1996 Mouse Molecular Genetic Meeting **(Cold Spring Harbor)** August-Sept.

Abbreviations:

ALK: Activin receptor-like kinase.

bFGF: Basic Fibroblast Growth Factor.

BSA: Bovine Serum Albumin.

CCM: Chemical Cleavage of Mismatches.

CF: Cystic Fibrosis.

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator.

cM: centiMorgan.

DEPC: Diethylpyrocarbonate.

dpp: *decapentaplegic.*

dsDNA: Double-stranded DNA.

EDTA: Ethylenediaminetetra acitic acid.

EGFR: Epidermal Growth factor Receptor.

Eng: Endoglin.

ES : Embryonic Stem.

FGFR3: Fibroblast Growth Factor Receptor type III.

FIN13: Fibroblast Growth Factor Inducible gene 13.

FKBP12: Rapamycin Binding Protein,

GAG: Glycosaminoglycan.

GAPDH1: Glyceraldehyde-3-Phosphate Dehydrogenase.

GS: Serine / Glycine rich.

HHT: Hereditary Haemorrhagic Telangiectasia.

***Igf2r*:** Insulin Like Growth Factor Type II Receptor (gene).

IGFI-R: Insulin Like Growth Factor Type I Receptor.

IGFIIR: Insulin Like Growth Factor Type II Receptor (protein).

IL: Interleukin.

LAP: Latent-Associated Peptide.

LTBP: Latent TGFB Binding Protein.

Mad: Mothers Against dpp.

MAD: Mothers Against DPP.

MDE: Mutation Detection Enhancement.

MHC: Major Histocompatibility Complex.

Mv1Lu: Mink Lung Epithelial Cells.

NOD: Non-Obese Diabetic.

OD: Optical Density.

PBS: Phosphate Buffered Saline.

PCR: Polymerase Chain Reaction.

PFA: Paraformaldehyde.

PlanH1: Plasminogen Activator Inhibitor Type I.

RFLP: Restriction Fragment Length Polymorphism.

RI: Recombinant Inbred.

SDS: Sodium Dodecyl Sulphate.

SE: Standard Error.

SMC: Smooth Muscle Cell.

SSC: Standard Saline Citrate

SSRs: Simple Sequence Repeats.

TBRI: Transforming Growth Factor Beta Type I Receptor.

TBRII: Transforming Growth Factor Beta Type II Receptor.

TAE: Tris-acetate/EDTA Electrophoresis.

TBE: Tris-borate/EDTA Electrophoresis.

TE : Tris-ethylenediaminetertra Acitic Acid.

TEMED:N,N,N',N'-tetramethylethylenediamine.

TESPA: 3-Aminopropyltriethoxy-Silane.

TGFB: Transforming Growth Factor Beta (protein).

Tgfb: Transforming Growth Factor Beta (gene)

***Tgfbr2*:** Transforming Growth Factor Beta Type II Receptor (gene).

tPA: Tissue Plasminogen Activator.

TRIP1: Transforming Growth Factor Receptor Interacting Protein.

VNTY: Variable Number of Tandem Repeat.

Summary:

This thesis presents data obtained from genetic analysis of TGFB1 knockout neonates (Shull et al., 1992, Kulkarni et al., 1993) bred onto different genetic backgrounds. TGFB1(-/-) embryos were initially reported to develop to term normally and die by 3 weeks due to multisystemic inflammation (Shull et al., 1992; Kulkarni et al., 1993). Therefore, it was suggested that TGFB1 was not vital during embryogenesis. However, an independent group (Dickson et al., 1995) demonstrated the implication of TGFB1 in an early stage of embryogenesis. It was shown that 50% of the TGFB1(-/-) embryos died in uterus due to defects in yolk sac vasculogenesis and haematopoiesis, whereas the rest did not succumb to the yolk sac phenotype but developed to term normally and died by 3 weeks post-partum as described previously (Kulkarni et al 1993). It has also been shown that maternal TGFB1 can cross the placenta (Letterio et al., 1994). TGFB1 was detected in TGFB1(-/-) embryos born to TGFB1(+/-) females, whereas in those born to null females, TGFB1 was not detected. Thus, it has been suggested that *Tgfb1* gene knockout was not equivalent to a protein knockout and the maternally acquired protein could rescue TGFB1(-/-) foetuses and embryos.

The work accomplished in this project set out to determine the reason for the existence of at least two different phenotypes; yolk sac insufficiency and survival to birth, among TGFB1(-/-) conceptuses. During this study, the involvement of several possible genetic and/or non-genetic modifying factors in the different expressivity of the TGFB1(-/-) phenotype was examined. The phenotype of TGFB1(-/-) was studied by breeding the *Tgfb1* null allele onto two inbred strains; NIH/Ola and C57Bl/6J/Ola. Also TGFB1(-/+) heterozygous crosses between various combinations of NIH/Ola and C57Bl/6J/Ola enabled study of the possible implications of maternal factors in the different expressivity of the TGFB1(-/-) phenotype. It was estimated that one locus with a codominant pattern of inheritance was responsible for the different expressivity. Due to the codominant behaviour of the modifying gene(s), the F2(NIH/Ola x C57Bl/6J/Ola) intercross animals were considered to be the most

informative animals for genetic linkage analysis. 50 polymorphic DNA markers were utilised to initiate a genome-wide search by screening 50 TGFB1(-/-) neonates from an F1 intercross. More than 90% of the genome was screened for modifying gene(s) during this study. Four regions of the genome showed suggestive linkage ($P<0.05$) in the first screen. To confirm the linkages, 30 extra null animals were screened with the interested markers. A small region of mouse chromosome 5 harbouring a genetic modifier met the criterion of definitive linkage ($P\leq10^{-5}$).

During this project, the feasibility of mapping genetic factors involved in determining early embryonic lethality without need to access the embryos was demonstrated.

Fibroblast growth factor receptor type 3 (*Fgfr3*) and Fibroblast growth factor inducible gene 13 *(Fin13)*, which mapped in the vicinity of the modifying gene, were considered as candidate genes. The expression of these genes were assessed in 9.5 d.pc. embryos and yolk sacs bred onto either NIH/Ola or C57Bl/6J/Ola strains by application of RT-PCR. Also expression of Transforming growth factor beta I (*Tgfb1*) gene was examined in 9.5 d.pc both yolk sac and embryo bred onto either of the strains. By application of heteroduplex analysis (HA) and direct DNA sequencing, the possibility of genetic polymorphism within *Fin13* between the two mouse strains was investigated over the coding region.

The latter part of the thesis presents data about the genetical mapping of Transforming Growth Factor Beta type II receptor (*Tgfbr2*) and Plasminogen activator inhibitor type I (*PlanH1*) on the mouse genome. Following the mapping *Tgfbr2* on the mouse genome, two uncloned mouse mutants which mapped in the vicinity of the *Tgfbr2* location were examined, by Southern blotting, for the possibility of a large deletion in *Tgfbr2* gene in either of these mutants.

1: Introduction

Part I:

I: 1.1 Transforming Growth Factor B (TGFB):

TGFB is emerging as the prototype of a superfamily of polypeptides involved in control of development of various cells and tissues. The complexity of the superfamily is large (Table 1.1), and may even be greater than now known due to the existence of additional members of more distantly related molecules and by the potential formation of heterodimers between *Tgfb* gene products co-expressed in the same cell. The existence of the TGFB1/TGFB2 heterodimer has been confirmed in porcine platelets (Cheifetz et al., 1987). Family members, found in a wide variety of species from *Drosophila* to man, are related through an approximately 112-amino acid carboxyl terminal domain with varying degrees of sequence identity (Hoffmann, 1991). Each member has a set of seven cysteine residues in common.

The study of the mechanism of action of activated growth and differentiation factors has frequently been complicated by the presence of a high level of complexity at three levels: the factors themselves (isoforms), the receptors, and the cellular responses. Thus, the factors may be found to be members of a large family of structurally and functionally homologous polypeptides, to bind to multiple types of cell surface receptors and to elicit an array of apparently unrelated cellular responses. In TGFB1 complexity is found in all three levels.
In the first part of the introduction the biochemistry and biology of TGFB1 will be discussed with particular emphasis on other members/genes which might potentially modify the activity of TGFB1 *in vivo*.

I: 1.2 Transforming Growth Factor beta I (TGFB1):

Type I TGFB is an important cellular regulatory molecular which is secreted from producer cells as small and large latent high molecular weight

	Individual genes/ligands	Location in Mouse Chromosome (cM)	Refrences (et al.,)
Mammalian	**TGF-B1**	**7** (6.5)	Fujji 1986
Mammalian	**TGF-B2**	**1** (101)	Barton 1988
Mammalian	**TGF-B3**	**12** (41)	Barton 1988
Xenopus	**TGF-B5**		
Mammalian	**Activin-βA**		
Mammalian	**Activin-βB**		
Mammalian	**Activin-βC**		
Drosophila	**Dpp**		
Mammalian	**BMP2**	**2** (75)	Dickinson 1990
Mammalian	**BMP4**	**14** (14)	Dickinson 1990
Drosophila	**60A**		
Mammalian	**BMP5**	**9** (42)	Green 1961
Mammalian	**BMP6 (Vgr-1)**	**13** (18)	Dickinson 1990
Mammalian	**BMP7 (Op-1)**	**2** (≅100)	Marker 1995
Mammalian	**BMP8 (Op-2)**	**4** (59)	Marker 1995
Xenopus	**Vg-1**		
Mammalian	**GDF-1**	UN	
Mammalian	**GDF-3 (Vgr-2)**	**6** (58)	Jones 1992
Mammalian	**Nodal**	**10** (29)	Zhou 1993
Xenopus	**Xnr1**		
Xenopus	**Xnr2**		
Xenopus	**Xnr3**		
Mammalian	**BMP3 (Osteogenin)**	**5** (55)	Dickinson 1990
Mammalian	**GDF-10**		
Mammalian	**GDF-5 (CDMP-1)**	**2** (90)	Storm 1994
Mammalian	**GDF-6 (CDMP-2)**	**4** (90)	Storm 1994
Mammalian	**GDF-7**	**12** (Proximal)	Storm 1994
Mammalian	**MIS**		
Mammalian	**Inhibin-α**	**1** (41.6)	Male 1991
Mammalian	**GDF-9**	UN	
Mammalian	**GDNF**		
Chicken	**Dorsalin**		
Drosophila	**Screw**		

Table 1.1: The Transforming Growth Factor Beta (TGF-B) Superfamily (Massague and Weis-Garcia., 1996).

Type I TGFB is an important cellular regulatory molecular which is secreted from producer cells as small and large latent high molecular weight complexes (Miyazono et al., 1993). Although receptors for TGFB are found in almost all cell types (Gentry et al., 1987), these receptors do not recognise latent TGFB1. An understanding of structure, processing , and activation of latent TGFB1 in greater detail should provide clues to its mechanism of action and function. Large latent TGFB1 complex purified from human platelets (Miyazono et al., 1988; Wakefield et al., 1988; Okada et al., 1989) is composed of at least three components, identified (figure 1.1) as TGFB1 (small bioactive peptide), the NH_2-terminal part of the TGFB1 precursor known as latent-associated peptide (LAP), and another gene product, latent TGFB binding protein (LTBP). Latent TGFB which is composed of only the mature TGFB and BLAP, is denoted small latent TGFB complex , whereas that associated with LTBP is denoted large latent TGFB complex.

I: 1.2.1 TGFB latency and LTBPs:

LTBP is linked to LAP by a disulphide bond and creates a high molecular weight form of LTGFB with a molecular mass of 210,000 kD (Daniel et al., 1993). LTBP is not directly needed for the latency of TGFB, but it appears to have several important functions. It plays roles in the assembly and secretion of LTGFB complex from certain cell types (Miyazono et al., 1991). In human fibroblasts and fibrosarcoma cells, LTBP1 binds to extracellular matrix after secretion and is released from the matrix by proteolytic digestion (Taipale et al., 1994, 1995). In addition, LTBP1 is important for the activation of LTGFB in co-culture of endothelial cells and smooth muscle cells (Flaumenhaft et al., 1993). LTBP has a variable size depending upon the cell type. The difference in the sizes may be, in part, due to proteolytic processing of the protein (Miyazono et al., 1991). In addition, two different transcript sizes of LTBP1 mRNA have been observed, which suggests use of different polyadenylation sites, different promoters or the presence of alternative splice variants (Kanzaki et al., 1990; Tsuji et al., 1990). So far three LTBPs (LTBP1-3) have been identified. The overall structure of LTBP2 is very similar to that of LTBP1.

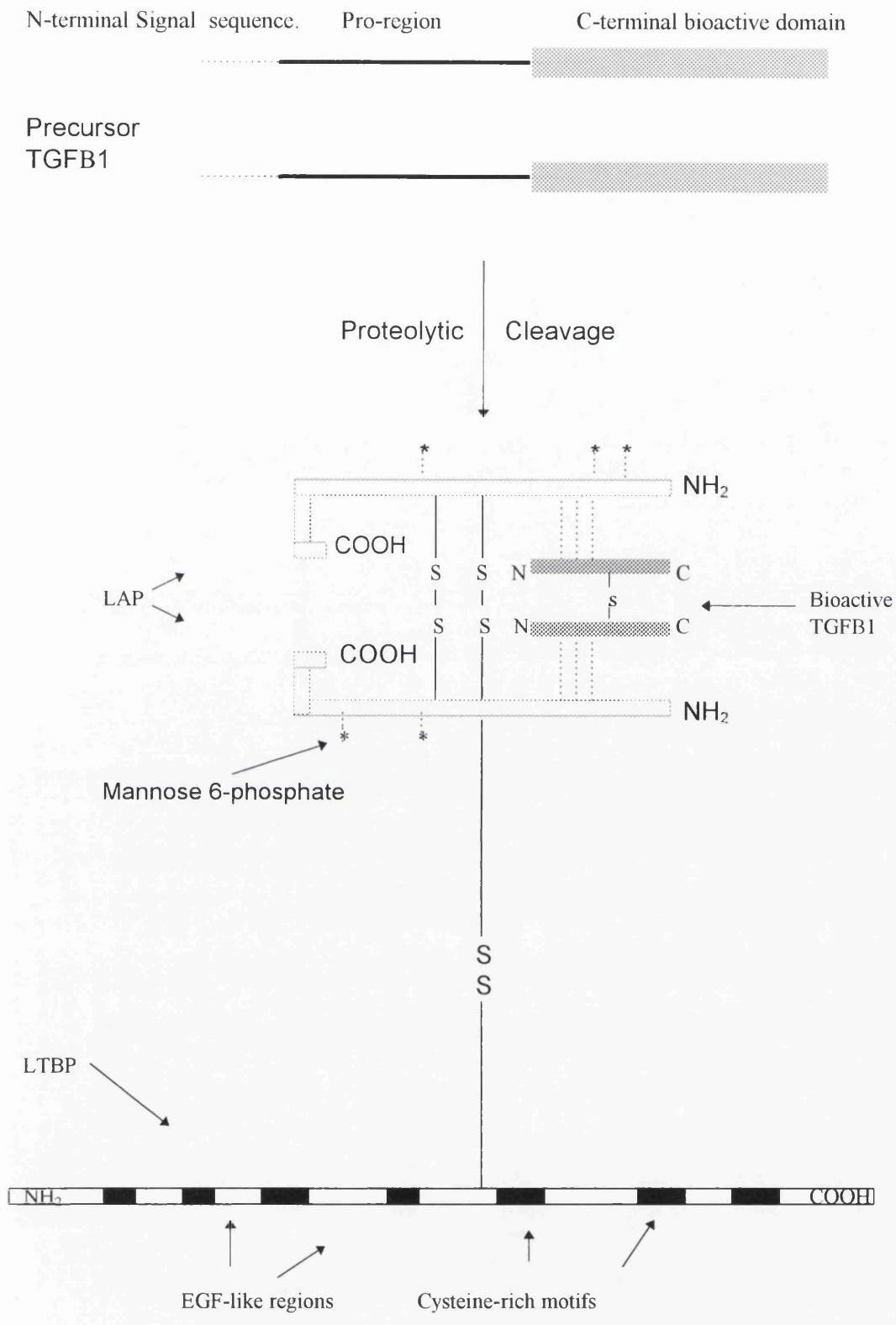


Figure 1:1 The structure of Precursor and Latent Transforming Growth factor-B (LTGFB).
LAP: Latent Associated Protein., LTBP: Latent TGF-B Binding protein.
EGF-like: Epidermal Growth Factor-like.

Similar to LTBP1, LTBP2 has been shown to form complex with the TGFB1 precursor (Moren et al., 1994). The third member in the LTBP family, LTBP3, cloned from the mouse, is expressed widely in cells during murine development and can form a complex with the TGFB1 precursor in MC 3T3-E1 osteoblasts (Yin et al., 1995).

A single LTBP1 locus appears to exist in the human genome that has been mapped to chromosome 2, region p12-q22 (Stenman et al,. 1994). LTBP2 has been localised to human chromosome band 11q12 and mouse chromosome band 19B (Li et al., 1995).

I: 1.3 Biological activation of LTGFB:

Biological activation of TGFB occurs by dissociation of the mature TGFB from the rest of the complex, which can be achieved *in vitro* by physical or chemical treatment such as heating or exposure to low pH (Brown, et al, 1990). The activation of LTGFB normally seen in co-cultures of endothelial cells and either smooth muscle cells or percytes (Sato et al., 1989; Antonelli-Olridge et al., 1989), has been suggested to require cell-cell contact and involves plasmin and urokinase.

Analysis of the carbohydrate chains of the TGFB precursor revealed that two of the three carbohydrate chains contain mannose 6-phosphate (Man-6-P) which is not seen in mature TGFB (Purchio et al., 1988). Enzymatic removal of the carbohydrate-containing LAP produces active TGFB from LTGFB complex (Miyazono and Heldin, 1989). LTGFB isolated from platelets binds to the plasma membrane form of the cation-independent Man-6-P/insulin like growth factor receptor type II (IGFII R) (Purchio et al., 1988; Kovacina et al., 1989) indicating the possible involvement of IGFII-R in activation of LTGFB.

I: 1.3.1 IGFII R/ Mannose-6-Phosphate:

IGFII-R is involved in targeting Man-6-P containing proteins to lysosomes. It also plays a role in the binding and internalisation of insulin-like growth factor II (IGFII) at the cell surface which leads to degradation of the growth factor by the lysosomes (Cohick & Clemmons, 1993). IGFII is a potent growth stimulant (Lau et al,. 1994; Oka et al., 1985) which binds to both IGFI-R and IGFII-R, but with higher affinity to IGFI-R. Binding of IGFII by IGFI-R promotes cellular proliferation and suppresses apoptosis (Baserga, 1994; 1995; Harrington et al., 1994). In contrast, binding of IGFII to IGFII-R results in internalisation and subsequent degradation of the ligand, making it unable to activate IGFI-R. Thus, IGFII-R, by antagonising the growth stimulatory effect of IGFII, effectively operates as a growth suppresser gene. Furthermore, recent studies in human breast cancer cells (MCF-7) by showing that cellular proliferation and receptor tyrosine kinase-dependent is reduced in the presence of IGFII-R, support the role of IGFII-R as a tumour suppresser (Ellis et al., 1996).

It has been demonstrated that induction of cell surface IGFII-R caused an increased binding of LTGFB to adipocytes. Moreover, activation of LTGFB is inhibited by mannose 6-phosphate or anti-IGF2/mannose 6-phosphate receptor antibodies. Therefore, IGFII-R indirectly exerts growth suppressive effects by activation of TGFB which is a potent growth suppressor protein. (Kovacina et al., 1989; Prchio et al., 1987; Kojima et al., 1993; Dennis and Rifkin, 1991; Kornfeld 1992).
High levels of IGFII and IGFII-R are seen in the developing rat embryo but both decline postnatally suggesting that may have a role in embryonic growth and development (Kornfeld., 1992).

The *IGF2R* gene has been localised to the long arm of human chromosome 6, region 6q25→ 6q27 and mouse chromosome 17, region A-C (Laureys et al., 1988). *Igf2r* has been shown to be paternally-imprinted in the mouse (Barlow et al., 1991).

I: 1.3.2 Plasminogen activator inhibitor type I (PAI1):

PAI1 is a member of the serine protease inhibitor, or serpin, superfamily of proteins (Huber& Carrell., 1989). It is a key regulator of plasmin-mediated proteolytic cascades, which contributes to both fibrinolysis and matrix degradation.

Proteolytic activation of latent TGFB by plasmin has been demonstrated (Lyons et al., 1990). Plasmin is produced proteolytically from plasminogen by tissue plasminogen activator (tPA) at the cell surface (Kirschenlohr et al., 1993). PAI1 blocks the activation of latent TGFB by competitively inhibiting tPA.

PAI1 has been implicated in a number of vascular diseases. During atherosclerosis, it has been proposed that PAI1 indirectly enhances human vascular smooth muscle cell (SMC) proliferation by inhibiting plasmin-mediated activation of TGFB, a potent inhibitor of SMC proliferation (Grainger et al., 1993 and 1994). Elevated levels of PAI1 in transgenic mice contribute to the development of venous, but not arterial occlusions (Erickson et al., 1990). Conversely, complete deficiency of PAI1 in human was associated with abnormal bleeding (Fay et al., 1992) and mice deficient in PAI1, display an enhanced hyperfibrinolytic state (Carmeliet et al., 1993).

I: 1.4 TGFB binding components:

TGFB and other members of the TGFB family initiate a signalling pathway through binding to transmembrane receptors with serine / threonine kinase activity (Massague et al., 1994; 1992; Attissano et al., 1994; Lin and Lodish, 1993; Miyasono et al., 1993). The TGFB receptors, which were identified through affinity-labelling assays, include the so called type I, type II, and type III receptors; proteins of ~ 53 kDa, 70-85 kDa, and 200-400 kDa, respectively (Massague, 1987).

Some mutant mink lung epithelial cells (Mv1Lu), selected through loss of TGFB responsiveness, no longer express type I receptor; others, similarly selected,

lose expression of both the type I and II receptors. However, all of these variants continue to express the type III receptor (Boyd et al., 1989, Laiho et al., 1990; 1991). This has led to the proposal the type III receptor may not be involved directly in signal transduction but mediates ligand access to the signalling receptors (Massague et al., 1990).

I: 1.4.1 Transforming Growth factor beta type I (TBRI) and type II receptors (TBRII):

The transforming growth factor B (TGFB) type II receptor (TBRII) is a constitutively phosphorylated serine-threonine kinase receptor for the ligands TGFB1, TGFB2, and TGFB3 (Wrana et al., 1994). This ligand/receptor system is important in negative growth regulation and has profound effects on cellular phenotype and extracellular matrix deposition (Cui et al., 1996). TBRII is active as an oligoheteromeric complex with the TGFB type I receptor (TBRI). Binding of TGFB to the constitutively phosphorylated TBRII results in recruitment of TBRI (figure 1.2), which itself is unable to bind TGFB. After this type I-type II receptor complex has been formed, TBRII phosphorylates TBRI in its GS (serine / glycine rich) domain. The GS domain preceding the kinase domain is the distinguishable feature of TBRI (Wrana et al., 1994; 1996). The kinase activity of TBRI, as well as its phosphorylation by TBRII, are required for all the downstream responses induced by TGFB. Although the role of this receptor trans-phosphorylation is not currently understood, phosphorylation of the type I receptor could activate its own kinase activity or the phosphorylated residues could serve as substrate binding sites.

TBRII is essential in mediating the negative growth response of cells to TGFB (Laiho et al., 1990), and its level of expression dictates the magnitude of this biological response *in vivo* (Cui et al., 1995). Somatic cell mutations in TBRII have been found in human colon carcinomas (Markowitz et al., 1995). TBRII deficiency embryos die by 13.5 dpc. due to yolk sac hematopoiesis and vasculogenesis deficiency (Oshima et al., 1996). The histological features of the Tgfbr2(-/-) yolk sac were almost identical with those in the TGFB1(-/-)

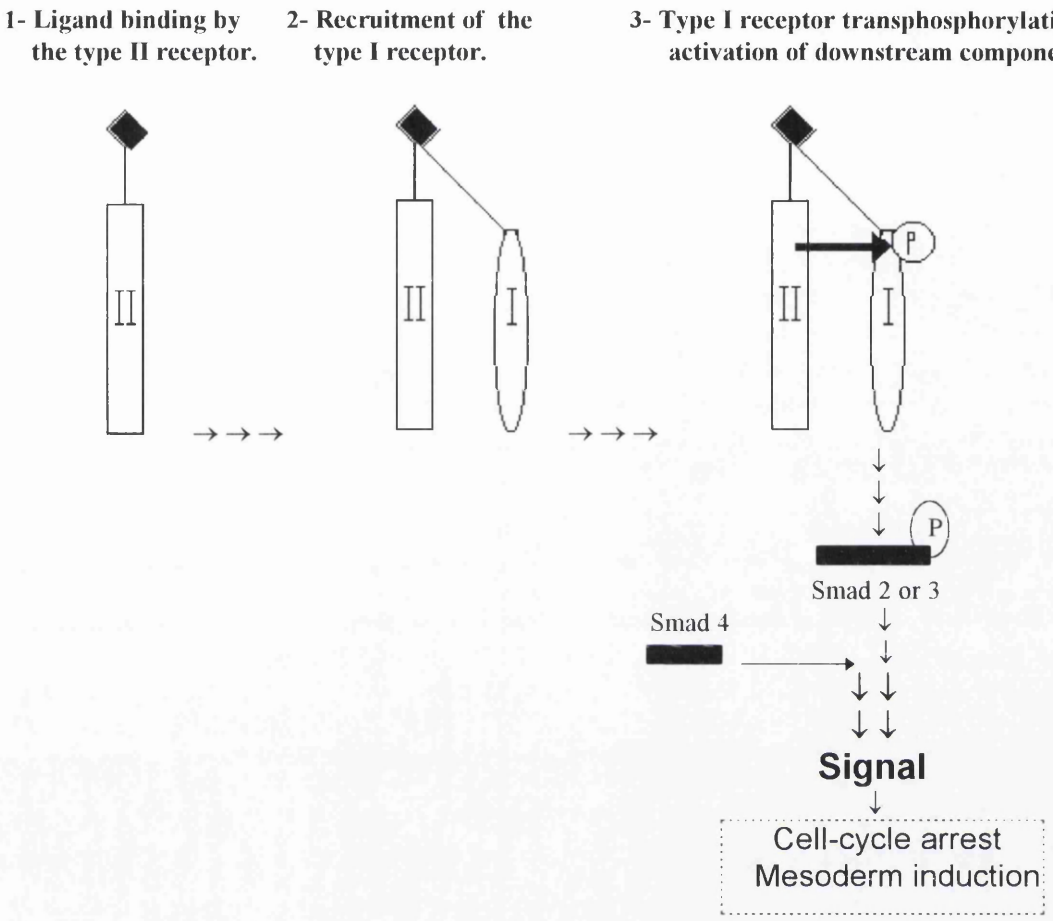


Figure 1.2 Mechanism of activation of the TGFB receptors. The TGFB type II receptor is the primary ligand (TGFB-indicated with black diamond) binding receptors, while the TGFB type I receptor, once recruited to the ligand-bound type II receptor, serve as the first substrate for the type II and initiates down stream responses.

embryos dying around 10.5 dpc. (Dickson et al., 1995) indicating that TGFB1 and TBRII are essential for hematopoeisis *in vivo.*

The search for TGFB receptor substrates has been approached using the yeast two hybrid system and complementary DNA (cDNA) from neonatal rat heart. The rapamycin binding protein, FKBP12, interacted with TBRI used as a "bait". However, no interaction between FKBP12 and the cytoplasmic domain of TBRII or activin type II receptor was observed (Wang et al., 1994). The binding of FKBP12 to the TBRI is potentially interesting because of its involvement in another growth inhibitory pathway induced by rapamycin (Chung et al., 1992; Jayaraman et al., 1993). The FKBP12-rapamycin complex, similarly to TGFB, inhibits G1 phase progression in various mammalian cell types as well as yeast (Schreiber 1992). It could be hypothesised that certain components of the TGFB and rapamycin signalling pathways might be the same or similar proteins.

The first major milestone in identifying the downstream components that turn TGFB receptor signals into specific effects on gene expression has been reached with the identification of the Smad proteins as TGFB signal transducers.

The discovery of Smad proteins in vertebrates as downstream components of TGFB signalling pathway stems from identifying of *Mad* (mothers against *dpp*) gene in *Drosophila*. *dpp* (*decapentaplegic*) encodes a fly homologue of BMP2, and BMP4 from vertebrates (Sekelsky et al., 1995) and its function is required for the embryo development and for the development of eyes and wings (Newfeld et al., 1996). It was found that mutation in *Mad* gene exacerbate the phenotype of weak *dpp* allele (Sekelsky et al., 1995). After this finding, several MAD homologues, now referred to as Smad proteins, were reported from vertebrates (Hoodles et al., 1996; Liu et al., 1996; Baker & Harland, 1996). It was noticed that mutations in *Mad* block the signalling ability of a constitutively active form of the DPP type I receptor encoded by *tkv* (*thick vein*) gene. This indicated that MAD functions downstream of the receptor (Hoodles et al., 1996). Further evidence to support that Smads function downstream of TGFB

receptors has been obtained by observing that introduction of Smads from human and mouse into frog early embryo mimics the effects of activated TGFB-family receptors on mesoderm development (Graff et al., 1996; Liu et al., 1996). Moreover it was found that Smads are phosphorylated (Hoodles et al., 1996; Baker & Harland, 1996) and accumulate in the nucleus (Hoodles et al., 1996; Liu et al., 1996) in response to TGFB-family agonists. These findings argue that Smad proteins are mediators of TGFB receptors' signals. Upon phosphorylation by activated receptors (Macias-Silva et al., 1996), Smads form complexes (Zhang et al., 1996; Wiersdorff et al., 1996) (figure 1.2), move into the nucleus, associate with DNA-binding proteins (Chen et al., 1996) and activate gene transcription.
The human, mouse and frog *Smads* 1 through 6 have been cloned. *Smads 4* and *2* are located at 18q21, and they are the target of mutations in cancer (Riggins et al 1996; Yingling et al., 1996; Eppert et al., 1996).

Although both type I and type II TGFB receptors are required for TGFB-induced growth inhibition and extracellular matrix protein synthesis in Mv1Lu cells (Massague et al., 1990), there could be divergent signalling pathways for the two sets of responses and association of TBRII functions with TGFB's anti-proliferation activity (Derynck et al., 1994; Chen et al., 1993). Using the cytoplasmic domain of TBRII as a "bait", the protein called transforming growth factor receptor interacting protein (TRIP1) was isolated. No interaction between this protein and activin type II or type I receptors was observed. Phosphorylation of TRIP1 on serine and threonine by the receptor kinase, and its co-expression with TBRII receptor during development, suggest a signalling role for TRIP1 (Chen et al., 1995).

Type I receptors so far identified include TSR-R (ALK1), ActRI (ALK2), BMPR-IA (ALK3), ActR-IB (ALK4), TBRI (ALK5), and BMPR-IB (ALK6) (Attisano et al., 1993; ten Dijick et al., 1994; Frolik et al., 1984; Carcamo et al., 1994). The activin receptor-like kinase 1 (ALK1) which is a type I cell surface receptor for the TGFB ligands is highly expressed in endothelial cells and other

highly vascularized tissues (such as lung and placenta) (Attisano et al., 1993). The ALK1 protein can associate with the TGFB or activin type II receptors *in vitro*, with the complex binding TGFB and activin respectively (Attisano et al., 1993; ten Dijke et al., 1994(b); Miyazono et al., 1994). However, its ligand *in vivo* remains to be revealed (ten Dijke et al., 1994(a)).

Type II receptors include ActRII, ActRIIB, TBRII, and BMPRII (Mathews & Vale, 1991; lin et al., 1992; Attisano et al., 1992; Estevez et al., 1993).

Using somatic cell hybrids and fluorescence *in situ* hybridisation, *TGFBRII* has been mapped to human chromosome 3p22 (Mathew et al., 1994). Human ALK1, ALK2, ALK3, ALK4, ALK5, and ALK6 have been assigned to chromosomes 12q11-q14, 2q22-q37, 6, 12q11-q14, 9, and 4 respectively (Johnson et al., 1995; Roijer et al., 1997).

I: 1.4.2 TGFB type III receptor (betaglycan):

The type III receptor for TGFB, known as betaglycan has a biological function distinct from that of the type I and II receptors. This receptor is heterogeneous in nature and typically runs on SDS-polyacrylamide gels as a broad band with an average mass of 280 to 330 kDa (Massague 1985). One of the most remarkable characteristics of betaglycan is that it is an integral membrane proteoglycan consisting of approximately 200 kDa of glycosaminoglycan (GAG) chain, in which the relative proportion GAG varies in different cell types. The GAGs are not required for functional expression of the receptor on the cell surface (Cheifetz & Massague 1989) nor for binding to TGFB (Cheifetz & Massague 1986; Cheifetz & Massague 1988).

As discussed before (1.3.1), TBRIII may not be involved directly in signal transduction but serve some other functions such as concentrating ligand before presentation to bona fide signal-transducing receptors (Massague et al., 1990). The type III receptor shows comparable affinities for all TGFB isotopes. It is also the most abundant cell surface TGFB receptor in many cell lines

(Massague 1995, Massague & Like., 1985; Cheifetz et al., 1986.; Fanger et al 1986). Johnson et al (1995) have assigned human betaglycan to 1p32-p33.

I: 1.4.3 Endoglin (Eng):

Endoglin is a homodimeric integral membrane glycoprotein composed of disulphide-linked subunits of 95 kDa (Gougos & Letarte, 1988, 1990). The transmembrane domain and the relatively short (43 amino acids) cytoplasmic tail of this protein (Lopez-Casillas et al., 1991; Wang et al., 1991) were remarkably similar to the corresponding regions in betaglycan (71% amino acid sequence similarity with 63% identity) (Gougos & Letarte., 1990). The extracellular regions of these two proteins show limited amino acid sequence homology (Gougos & Letarte., 1990; Lopez-Casillas et al., 1991; Bork et al., 1992). Endoglin has been shown to bind TGFB1 and TGFB3 with high affinity, while betaglycan binds all three isoforms (Cheifetz et al., 1992). Moreover, TGFB2 was shown to be less potent than TGFB1 and TGFB3 on certain cell types, including hematopoietic cells and endothelial cells (Ohata et al., 1987; Jennings et al., 1988; Cheifetz et al., 1990; Hiai & kaji., 1992) which could be due to absence (or less) of affinity between TGFB2 and endoglin.

On human vascular endothelial cells of capillaries, arterioles and venules in all tissues examined (Gougos et al., 1990) endoglin is the most abundant transforming growth factor B binding protein (Cheifetz et al., 1992). In the presence of TGFB ligand, endoglin can associate with the signalling receptors TBRI and TBRII, and is thought to potentiate response to the growth factor (Yamashit et al., 1994). The human autosomal dominant condition hereditary haemorrhagic telangiectasia type I which shares many cellular and histological similarities to the yolk sac phenotype of TGFB1(-/-) knockout mice, is caused by mutations in endoglin (McAllister et al., 1994).

ENG has been assigned to human chromosome 9 using a human x hamster somatic cell hybrid mapping panel, and regional localisation to 9q34→qter was determined by fluorescence *in situ* hybridisation to metaphase

chromosomes (Fernandez-Ruiz et al., 1993). The murine *Eng* maps to chromosome 2 to a region syntenic with human chromosome 9q34→qter (Qureshi et al., 1995)

I: 1.5 TGFB isoforms and their functions:

Three TGFB isoforms (TGFB1-3) have been identified in mammals. The biologically active TGFB isoforms are homodimers, and each monomer of each isoform has nine conserved cysteine residues. The bioactive peptide of TGFB among these isoforms is also highly conserved (approximately 70% amino acid content)(Roberts & Sporn, 1990). In the mouse, TGFB1 is encoded by the gene *Tgfb1*, TGFB2 by *Tgfb2,* and TGFB3 by *Tgfb3.* Conservation is also evident at the genomic level. The *Tgfb1* gene has seven exons and this structure is largely conserved in other *Tgfb* genes. Murine *Tgfb* type I, 2, and 3 have been assigned to chromosomes 7 (6.5cM) (Fujji et al., 1986), 1 (101cM), and 12 (41cM) respectively (Baton et al., 1988).

I: 1.5.1 TGFB and Reproduction:

I: 1.5.1.1 Testicular function:

TGFB1 is one of the chemotrophic factors involved in the migration of primordial germ cells to the genital ridge and the initiation of gonad development (Godin and Wylie, 1991). Expression of all TGFB isoforms has been observed in embryonic and/or adult testis (Derynck et al., 1988; Miller et al., 1989 a, b; Watrin et al., 1991). Two TGFB1 transcripts; 2.4 and 1.8 kb TGFB1 mRNAs, are found. Both TGFB1 mRNAs have been observed in testis and most tissues. However, a 1.8 kb TGFB1 transcript is reported to predominate in the testis and it appears to be germ cell-specific. It has been observed in all germ cell populations examined, including meiotic prophase spermatocytes, early spermatids, and residual bodies (Wartin et al., 1991). Also, all three TGFB isoforms are produced by Sertoli and peritubular cells, and appear among proteins secreted by them (Skinner and Moses, 1989). TGFBs are involved in autocrine and paracrine regulation of testis function and

germ cell development and differentiation. TGFB1 promotes production of proteins and stimulates clustering and colony formation by peritubular cells *in vitro* (Skinner and Moses, 1989). TGFB1 from Sertoli cells acts in a paracrine fashion to modulate steroidogenesis in Leydig cells (Avallet et al., 1987; Lin et al., 1987; Fauser and Hsueh, 1988; Morera et al., 1988). All these results imply the possible involvement of TGFBs in the process of spermatogenesis. TGFB1 has been localised on human spermatozoa at neutral pH (by immunocytochemical studies) which suggests that TGFB1 could have some roles in human gametes (i.e. transmission or viability of spermatozoa). The TGFB1 immunostaining pattern at an acidic pH was similar to that at neutral pH, but at a higher intensity. Thus, it was suggested that an *in vivo* activation of latent TGFB1 in seminal plasma may take place in the acidic environment of the vagina (Chui et al., 1996).

I: 1.5.1.2 Ovarian function:

TGFB1 and TGFB2 are produced in ovarian tissue. TGFB1 appears in all tissues (Derynck et al., 1988; Thompson et al., 1989; Akhurst et al., 1990a; Ksander et al., 1990; Mulheron and Schomberg, 1990; Roy et al., 1992), but TGFB2 is lacking in oocytes (Chegini and Flanders, 1992; Roy et al., 1992; Teerds and Dorrington, 1992). TGFB may act in an autocrine/paracrine manner to regulate ovarian functions. Folliculogenesis, oocyte maturation, and differentiation of granulosa, thecal, and luteal cells are processes dependent on a complex interaction of gonadotropins, steroid hormones, and growth factors, the latter including TGFB (Chegini and Flanders, 1992). TGFB is also involved in modulating follicle stimulating hormone secretion from the pituitary (Knecht et al., 1986; 1987; Ying et al., 1986b).

I: 1.5.2 TGFB and uterine function:

Localisation studies suggest that TGFB1 is involved in uterine function, including implantation, decidualization, and placentation (Altman et al., 1990; Tamada et al., 1990; Dungy et al., 1991; Das et al., 1992; Lea et al., 1992;

Manova et al., 1992). TGFB1 appears in luminal and glandular epithelium before implantation (Tamada et al., 1990), and in the deciduum after implantation (Akhurst et al., 1990b; Tamada et al., 1990; Manova et al., 1992). TGFB2 also appears in luminal and glandular epithelium, and in myometrium and vascular smooth muscle, before implantation. Postimplantation, it is present in myometrium, epithelium, and deciduum (Das et al., 1992). TGFB3 is expressed only in the myometrium and vascular smooth muscle throughout the pre-implantation period (Das et al., 1992). An immunosuppressive factor closely related to TGFB2 is released from decidual tissue after implantation, and helps prevent maternal rejection of the foetus (Altman et al., 1990). These studies suggest the possible involvement of maternal sources of TGFBs in embryonic development during pre- and post-implantation stages.

I: 1.5.3 TGFB1 and embryo development:

I: 1.5.3.1 Preimplantation stage:

Both gene product localisation and functional studies indicate an important role for TGFB1 in preimplantation embryos. Present at the single cell stage, it may almost disappear at the two-cell stage, but reappears in four-cell and later embryos. It may be involved in regulation of differentiation during preimplantation development, in transformation of the morula to the blastocyst, or blastocyst maturation. Production of TGFB1 continues into postimplantation development, the different isoforms showing both overlapping and distinct expression patterns throughout (Paria et al., 1992; Slager et al., 1991).

In addition to expression studies, functional studies also support a role for TGFB1 in preimplantation development. *In vitro* culture of individual two-cell embryos results in impaired development to the blastocyst stage, with fewer numbers of cells per blastocysts and fewer numbers of embryos developing into blastocysts relative to that observed when embryos are cultured in groups. Addition of TGFB1 to the culture medium significantly enhances the percentage of embryos that develop into blastocysts, although it does not increase the number of cells per blastocyst (Paria and Dey 1990) suggesting the important

function of TGFB1 in transformation of the morula to the blastocyst, and in blastocyst maturation.

I: 1.5.3.2 Postimplantation stage:

TGFBs are also implicated in postimplantation development. The three TGFB isoforms exhibit both overlapping and distinct patterns of expression throughout development. Expression has been observed mainly in areas undergoing morphogenesis.

Expression of TGFB1 mRNA in the early murine embryo has been associated with both vasculogenesis and angiogenesis. High level mRNA expression of TGFB1 is seen in the forming yolk-sac blood islands, in the cardiac mesoderm prior to the differentiation of endothelial cells, and in endothelial cells per se (Akhurst et al., 1990b). It has been suggested that an important function of TGFBs in the early events of vasculogenesis and angiogenesis could be the augmentation of bFGF activity, either by increasing synthesis of this growth factor (Plouet and Gospodarowicz, 1989), or by the induction of glycosaminoglycan (GAGs) which are essential for the binding of bFGF to its receptor (Nugent and Edelman., 1992). Expression of TGFB transcripts and proteins are seen during cardiac and skeletal myogenesis (Pelton et al., 1991; Dickson et al., 1993) though they appear to be localized to nonmyogenic cells (Akhurst et al., 1990b; Millan et al., 1991; Dickson et al., 1993). TGFBs are implicated in inductive tissue interactions that result in the formation of mesenchymal cardiac cushion tissue, which contributes to valve and septum formation (Akhurst et al., 1990).

During formation of both bone and cartilage, TGFBs are expressed in different subsets of cells (Pelton et al., 1990; Millan et al., 1991). The positive effects of TGFBs on proliferation and differentiation of cells from the chondrocyte and osteocyte lineages could be compatible with their function as inducers of bone and cartilage formation (Akhurst, 1994). Interleukin-10 suppresses osteogenic differentiation of mouse bone marrow by suppressing

synthesis of TGFB1, which is essential for commitment of bone marrow cells to osteogenesis (van Vlasselaer et al., 1994). Exogenous TGFB added to long term mouse bone marrow cultures inhibits cell growth, and neutralisation of the added factor accelerates growth (Waegell et al., 1994).

The three mammalian TGFB isoforms are also expressed in palatal epithelium and mesenchyme during formation of the secondary palate (Fitzpatrick et al., 1990), in the lung during branching morphogenesis (Heine et al., 1990), in the tooth epithelium and mesenchyme during odontogenesis; (Vaahtokari et al., 1991), in the cells and tissues of the developing central and peripheral nervous system (Flanderz et al., 1991), and in the mammary gland epithelium during ductal development(Robinson et al., 1991). Observing the expression of all three TGFB isoforms during postimplantation as well as preimplantation development emphasises the importance of their functions throughout the embryogenesis.

Part II:

II: 1.1: Genetic analysis of complex traits:

II: 1.1.1 Simple traits and Complex traits:

The term "simple trait" refers to any phenotype which exhibits a pattern of classical Mendelian recessive or dominant inheritance, attributable to a single gene locus, which overrides other genetic or non-genetic factors. That is , there is a simple correspondence between genotype and phenotype. This perfect co-segregation of a trait with the gene, makes linkage analysis and positional cloning very straightforward. In contrast to simple Mendelian traits, complex traits do not override the effects of other genetic and non-genetic factors. Due to factors other than the gene itself, typically other loci and often the environment, correspondence between phenotype and genotype breaks down. This problem can be exacerbated by non-additive (epistatic) interactions between factors. In human such complex traits include susceptibilities to neural tube defects (Laurence 1990), autoimmune disease (Vyse & Todd, 1996), diabetes (Deng et al., 1995), and cancers (Ponder, 1990).

Some human conditions were initially classified as simple traits. However, these conditions show markedly different clinical manifestations among individuals carrying the identical causative locus. For example, sickle cell anaemia has been classified as a recessive, simple trait, however, individuals carrying identical alleles at the beta-globin locus show different clinical manifestations ranging from early childhood mortality to a virtually unrecognised condition even at age 50 (Huisman, 1979; Steinberg and Hebbel, 1983). Therefore, there must be other modifying factors, genetic or non-genetic, that influence on the phenotypic expressivity of sickle cell anaemia.

The indication of a complex trait can be categorised as follows (Lander & Schork, 1994):

1- Polygenic traits, 2- Genetic heterogeneity, and 3- Incomplete penetrance.

II: 1.1.2 Polygenic traits:

The term "polygenic trait" refers to any phenotype influenced by different genetic loci. Polygenic traits can be classified as: quantitative traits (e.g. height of an individual), or qualitative traits (e.g. individuals grouped into discrete traits such as death from myocardial infarction).
In polygenic traits, the presence of epistatic (non-additive) effects among the involved loci complicates the process of genetic mapping especially in outbred populations such as humans.

II: 1.1.3 Genetic heterogeneity:

The term "genetic heterogeneity" refers to cases in which mutations in any one of several genes results in the same or similar phenotype. Genetic heterogeneity can occur when several genes are required for a common biochemical pathway or cellular structure.

It must be born in mind that "genetic heterogeneity" differs from "allelic heterogeneity" in which different mutations at a single locus result in different manifestations of the disease. Allelic heterogeneity does not usually hamper the genetic mapping process. Cystic Fibrosis (CF) is an example of "allelic heterogeneity". CF is a common autosomal recessive disorder diagnosed by obstructive lung disease, elevated sweat electrolyte level, and exocrine pancreatic insufficiency (Welsh et al., 1995). The clinical manifestation of CF is heterogeneous which could be partly due to the diverse spectrum of mutations in the CF transmembrane conductance regulator (*CFTR*) gene (Rommens et al., 1989; Riordan et al., 1989).

Genetic heterogeneity poses problems in approaching genetic mapping because the inheritance pattern of chromosomal regions harbouring the disease genes in affected families may differ. Hereditary non-polyposis colon cancer, for instance, appears to be due to defects in DNA mismatch repair genes (Strand et al., 1993; Fishel et al., 1993; Leach et al., 1993; Parsons et

al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994). These genes have been highly conserved throughout evolution; five human genes homologous to those responsible for mismatch repair in unicellular organisms have been discovered (Strand et al., 1993; Fishel et al., 1993; Leach et al., 1993; Parsons et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994, Fujii & Shimada 1989; Palombo et al., 1994). This disease is caused by mutations in any of the genes involved in DNA mismatch repair (Liu et al., 1994).

II: 1.1.4 Incomplete penetrance and phenotype:

The penetrance of some loci is not complete, and as a consequence, some carriers manifest the phenotypes whereas others may not. This incomplete penetrance could be due to interference of some factors such as age, sex, environment, infection, and other genes with the penetrance of the locus (or loci).

In autoimmune type I diabetes mellitus, which is an organ-specific autoimmune disease, the identical twins of affected individuals have only 36% risk of developing the disease (Olmoss et al., 1988), demonstrating the importance of the environmental factors in developing the disease. The risk to siblings of type I diabetic individuals is about 6% (Thomson et al., 1988) whereas that of European descent population as a whole is 0.2-0.3%. Although, penetrance of the disease genes is determined by unknown environmental factors, genetic factors are essential (Cordell & Todd, 1995).

II: 1.2 Animal models for human genetic disorders:

Animal models such as mouse and rat can be utilised for mapping genes of relevance to human genetic disease due to the fact that experimental crosses override many factors which hamper genetic mapping in human families. In nearly all cases of linkage analysis in animal models, for example in the mouse, the parental combinations of alleles, the so-called *phase of linkage* -will be known with absolute certainty. However, in the analysis of human

pedigrees the *phase of linkage* is often not known with certainty. As a consequence, human geneticists are forced to employ more sophisticated statistical tools that evaluate results in the light of the probabilities associated with each possible phase relationship for each parent in a pedigree (Elston & Stewart 1971). The experimental crosses, with providing the opportunity to study hundreds of meioses from a single set of parents, override the problem of genetic heterogeneity. Also far more complex genetic interactions such as susceptibility to cancer (Nagase et al., 1995), drug sensitivity, resistance to infections, and aggressive behaviour (Festing, 1979) can be probed in experimental crosses than is possible for human families.

The mouse has been chosen as an animal model for genetic analysis mainly due to its short gestation period and relatively large litter size, the ability to perform controlled matings, and most importantly due to the availability of inbred strains showing a wide range of natural strain variations (Copeland et al., 1993). Moreover as cloning and mapping of both the mouse and human genomes began, two important evolutionary facts became clear. First, nearly all human genes have homologies in the mouse and vice versa. Second, not only are the genes themselves conserved, but so is their order- to a certain extent- along the chromosome. In 1984, Nadeau and Taylor used linkage data obtained from 83 loci that had been mapped in both species to estimate the average length of conserved autosomal segments as 8.2 cM, in the mouse (Nadeau, 1984). The practical implication of conserved chromosomal segments is that the mapping of a gene in one species can provide a clue to the location of the equivalent gene in other species by interpreting synteny information. There are many examples of smaller genomic segments that have popped out or into larger syntenic regions. Thus, even if a human gene maps between two human loci with demonstrated synteny in the mouse, there is still a small chance that it will have moved to another location in the mouse genome. Nevertheless, over 80% of the autosomal genomes of mice and humans have now been matched up at the subchromosomal level (Copeland et al., 1993). Thus, with map information for a gene in humans, it will often be possible to predict a corresponding mouse chromosomal segment of ~10 cM in length as a likely location to test first for linkage with nearby DNA markers.

The mouse models such as the non-obese diabetic (*NOD*) mouse for autoimmune type I diabetes mellitus in humans (Makino et al., 1980), the Splotch (*Sp*) mouse for Waardenburg syndrome type I in humans (Baldwin et al., 1992; Tassabehji et al., 1992), the Extra-toes (*Xt*) mouse for Greg's cephalopolysyndactyly, a rare multi-system syndrome in humans (Hui and Joyner, 1993), the curly-tail *(ct)* mutant mouse for neural tube defects in humans (Embury et al, 1979) have been reported. These natural mutant animals were selected as models for human genetic disease on the basis of similarities in pathology, associated anomalies, inheritance and the influence of potential teratogens. Moreover, in laboratory animal models such as mouse, some complex traits are induced by chemicals and diets (e.g. Nagase et al., 1995). The different manifestation of the complex traits in different strains is due to influence of natural strain variation. Applying a genetic mapping approach enables genetic determination of these strain variations. The method of genetic mapping, by which one compares the inheritance pattern of a trait (or disease) with the inheritance patterns of chromosomal regions, allows one to locate a gene without knowledge about the function of the gene. Genetic mapping of trait-causing genes to chromosomal locations dates back to the work of Haldane in 1915. Haldane found evidence for coupling between mutations of the albino (*c*) and pink-eyed dilution (*p*) loci, which we now know to lie 15 cM apart on chromosome 7.

Because disease-causing mutations may occur at many steps in a biochemical pathway, animal models may not point to those genes most frequently mutated in human disease. However, animal studies should identify key genes acting in the same biochemical pathway or physiological system. Moreover, the genetic model underlying the disease, in terms of number of genes involved and interaction effects between loci, may present similarities between the two species.

Typical experimental crosses consist of crossing one inbred strain, which has a high risk of developing a disease (or trait), with another inbred strain, which has very low or no risk. The F1 generation is typically unaffected

(e.g. Nagase et al., 1995), which could be interpreted for involvement of one recessive locus or several loci with recessive outcome. However, studying a large number of F1 animals could show small percentage of affected animals.

II: 1.2.1 F1 backcross or F1 intercross animals:

Upon beginning a new linkage study there is a choice whether to screen F1 backcross or F1 intercross animals. Both the F1 backcross and intercross approaches have advantages and disadvantages. However, it is important to bear in mind that males from F1 interspecific hybrids in some mouse strains are infertile ruling out the intercross approach. The sterility of males in these strains follow Haldane's (1922) rule which states, when in the F1 offspring one sex is absent, rare or sterile, that sex is the heterozygous sex. Therefore, in these crosses the only practical approach for genetic mapping purposes is to create a backcross generation by backcrossing F1 females to the male from a high-risk parental strain.

The primary advantages of the backcross approach are all based on the fact that each offspring from the backcross can be viewed as representing an isolated meiotic event. Therefore, the analysis of backcross data is very straightforward. However, in practice this approach can not be used to map loci defined only by recessive phenotypes that interfere with viability or absolute fecundity in both males and females, which could be mapped by applying an F1 intercross approach. Moreover, the F1 intercross approach as a consequence of the fact that informative meiotic events will occur in both parents will lead to essentially twice as much recombination information on a per animal basis compared to the backcross approach. Therefore, it leads to obtain high-resolution mapping of the locus of interest relative to closely linked markers which is the first requirement for the process of positional cloning.

Besides conventional crosses, i.e. backcross and intercross, there is another very powerful mouse cross known as "recombinant inbred strains" utilised for genetic mapping purposes.

II: 1.2.2 Recombinant inbred (RI) strains:

Like all inbred strains, RI strains are fixed to homozygosity at essentially all loci. However, in RI strains there are two choices for the origin of allele that can be present at each locus. The construction of a set of RI strains is quite simple in theory. It begins with an outcross between two well established highly inbred strains of mice, such as B6 and DBA. These are considered the progenitor strains. The F1 progeny from this cross are all identical and thus, in genetic terms, they are all interchangeable. F1 hybrid animals are bred to each other to produce a large set of F2 animals which are not identical. At this stage, pairs of F2 animals are chosen at random to serve as the founders for new inbred strains of mice. The offspring from each F2 founder pair are maintained separately from all other offspring, and just two are chosen randomly for brother-sister mating to produce the next generation. The same process is repeated at each subsequent generation until at least 20 sequential rounds of strict brother-sister matings have been completed and a new inbred strain with special properties is established. Each of the new inbred strains produced according to this breeding scheme is called a "recombinant inbred" strain.

Establishment of recombinant inbred (RI) strains by Bailey and Taylor at the Jackson Laboratory (Bailey, 1971; 1981; Taylor., 1978) was the first important conceptual breakthrough aimed at reducing the time, effort, and mice required to map single loci. RI strains are powerful mapping tools for systemic linkage studies, offering two major advantages over conventional crosses: first, genotype information is cumulative, thus, researchers need not type many reference markers, and second, identical genotypes can be phenotyped multiple times, therefore improving the reliability of quantitative measurements. On the basis of these advantages, RI strains have launched major efforts to map complex traits including: chemically induced-lung tumorigenesis,

(Gariboldi et al., 1993), thymocyte apoptosis induced by radiation, and genetically induced-dactylaplasia (Johnson et al., 1995). However, there are two drawbacks of utilising RI strains; first, there are only two choices for the allele that can be present at each locus, second, because there is only a limited number of opportunities for recombination to occur between the two sets of progenitor chromosomes before homozygosity sets in, complete homogenisation of the genome can not take place.

II: 1.3 Markers for linkage analysis:

Linkage analysis can only be performed on loci that are polymorphic with two or more distinguishable alleles. As discussed before, the initial choice of the mouse as an experimental genetic system was due to the collection of rare genetic variants presents in hand. However, even this variation was restricted in its scope and usefulness for geneticists. This was because of the severe limitation in the number of informative markers. Although over 50 independent phenotypic markers (loci) were identified with effects on coat colour (Silver, 1979), it was impossible to follow more than a handful at any one time since mutant alleles at any one locus would act to obscure the expression of mutant alleles at other loci. An alternative approach was to utilise restriction fragment length polymorphism (RFLPs). A RFLP is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other. RFLPs are visualised by digesting DNA from different individuals with a restriction enzyme, followed by gel electrophoresis to separate fragments according to size, then blotting and hybridisation to a labelled probe that identifies the locus under investigation. In the mouse, the RFLP approach proved to be extremely powerful in interspecies crosses (Avner et al., 1988). Using interspecific crosses, which show high polymorphism, detailed genetic maps have been constructed showing the position of hundreds of genes (Kingsley et al., 1989).

Notwithstanding the great utility of RFLPs, they still have several major limitations: 1-The rate of polymorphism is considerably lower among inbred laboratory strains. 2-Typing RFLPs is time-consuming and difficult to automate.

Additionally, although interspecies crosses are quite useful, there are many circumstances in which it is preferable to use crosses between two inbred laboratory strains of the same species. Applications include mapping of mutations whose phenotypes are affected by genetic background, mapping of modifier genes and mapping of polygenic factors underlying physiological differences between strains.

An alternative source of DNA polymorphism is based on variation in the length of simple sequence repeats (SSRs). Because these variable number of tandem repeat (VNTR) polymorphisms exhibit high heterozygosity within the population, they are especially useful in linkage studies to track a specific gene as well as identifying the genes/loci responsible for genetic diseases. Variation in the number of repeats within a block of tandem repeats appears to be a universal feature of eukaryote DNA, regardless of the length of the repeat unit (Weber & May, 1989). There are two classes of variable number of tandem repeats one of which consists of larger tandem repeats called minisatellites. These show marked variability in the number of repeats and about 70% of human individuals are heterozygous for any one marker. Unfortunately these hypervariable VNTR loci tend to be biased in their distribution with a tendency to localise in telomeric bands. Minisatellites are not so useful for following specific genes in families or populations, although they have been applied in several genetic analyse, such as determining family relationships, as well as identifying the origin of tissue samples in forensic medicine. Developing the polymerase chain reaction (PCR) has provided a rapid means of detection of these VNTRs in linkage studies. This application of the PCR to type polymorphic DNA markers consumes less DNA and is faster than for routine genotyping of a block of markers, and PCR is even capable of amplifying DNA from a single template molecule (Saiki et al., 1988).

The second type of variable number of tandem repeats is the microsatellite repeat. These consist of around 10-50 copies of a motifs of 1 to 6 bp in size which occur, on average, every 6 kb. Dinucleotide repeats specially CA and GA, are particularly frequent with an estimated total of 50,000 dispersed through the genome. The availability of dense genetic linkage maps

of mammalian genomes makes feasible a wide range of studies, including positional cloning of monogenic traits, genetic dissection of polygenic traits, construction of genome-wide physical maps, rapid marker-assisted construction of congenic strains, and evolutionary comparisons (Copeland et al., 1993).

II: 1.4 Congenic mice:

In order to assess the contributions made by single locus to polygenic disease, congenic strains have been bred in which chromosomal regions from the resistant strain have been introgressed onto the backgrounds of sensitive to disease strains (Snell, 1948). This is done by successive backcrosses and selection for genotype; using markers that conservatively span the trait locus region. If the resultant strain pair retains a phenotypic difference, then crosses can be fine mapped as a simple locus.

The relative contributions of the major histocompatibility complex (MHC) and non-MHC genes to susceptibility to type I diabetes were analysed by the use of experimental crosses and congenic mouse strains (Hattori et al., 1986; Prochazka et al., 1987; Wicker et al., 1987; 1989; Livingston et al., 1991). A congenic strain NOD.B10-H-2^b (NOD.H-2^b), in which the H-2^g of the NOD was replaced with the H-2 region from the diabetes-resistant C57Bl/10SnJ (B10) strain, did not develop insulitis or diabetes demonstrating that the NOD MHC was essential for beta-cell destruction. However, the non-MHC genes in the NOD strain, in the absence of the NOD MHC, significantly contribute to the development of autoimmunity (Wicker et al., 1992).

II: 1.5 Assignment of the function of cloned genes by application of animal models:

The classic approach to identify the function of a cloned gene is to inactivate the gene and to study the biological consequence of lack-of-function of the gene. However, due to the fact that biology is full of interactions of gene products with each other and with the environment, some of the proteins could

show functional redundancy. Although, this could be misleading in assignment of the actual function of some genes, it can be employed to identify some possible hidden interactions between loci.

The function of a gene , in principle, can be inactivated at several levels. It can be approached either by manipulation of the gene by homologous recombination (Knockout approach) or by applying dominant negative mutations or antisense oligonculeotides approach.

II: 1.5.1 Knockout technology:

The general term of "knockout" relates to artificially generated null mutations of an endogenous gene. Such mouse mutants, which are devoid of a particular protein, are created by a process called homologous recombination or targeted disruption. The process involves the inactivation of an endogenous gene by insertion of cloned sequences. This takes place in embryonic stem (ES) cells which are then used to produce transgenic animals. ES cells are non-transformed totipotent cells derived from the inner cell masses of normal late blastocysts, which give rise to all organs of the growing foetus including germ line.

The knockout technique has been successful in generating loss-of-function mutations (null mutations) in transgenic mice, lacking expression of particular genes. Therefore, it has been possible to investigate the physiological consequence associated with the selective elimination of a particular protein in transgenic animals. The application of this technology led to generation of animal models for some genetic disorders in human such as cystic fibrosis (Snouwaert et al., 1992; Ratliff et al., 1992; O'Neal et al., 1993; Dorin et al., 1992) and inflammatory disorders (Kulkarni et al., 1995).

The introduction of this technology also led to the discovery of a number of null mutations which result in an embryo lethal phenotype (Gridley et al., 1987). Even though the mouse embryo is relatively accessible for study, it has often proven difficult to draw definite conclusions about the cause of death in

particular mutants. These mutants have been divided into three groups in terms of the stage of lethality (Copp, 1995) as follows:

1) Embryo lethality during the pre-implantation.

2) Embryo lethality at organogenesis.

3) Embryo lethality in the foetal period.

Many mutations causing pre-implantational death appear to disrupt basic cellular functions such as RNA metabolism (Michaud et al., 1993; DeGregori et al., 1994), transmethylations (Miller et al., 1994), transcription regulation of gene expression (Spyropoulos & Capecchi, 1994) and movement of chromosomes from the mitotic spindle (Magnuson & Epstein 1984).

At the organogenesis stage the embryo is critically dependent on the formation and maintenance of a functioning yolk sac circulation. It has been noted that in *GATA-2*, *Rbtn2*, and some of the *Tgfb1* knockout mice (Tsi et al., 1994; Warren et al., 1994; Dickson et al., 1995) the yolk sac contains no blood and embryos die due to hematopoiesis deficiency. Other lethalities at organogenesis stage are due to failure to establish a chorioallantoic placenta (Yang et al., 1993).

Death in the early foetal period is mainly due to failure of either cardiovascular circulation or liver hematopoiesis, or of both. For example; *Sp* or *Nf1* knockout embryos die between 12-15 dpc due to cardiovascular failure (Franze 1989; Jacks et al., 1994) or *Rb*, *c-myc*, and *keratin 8* knockout embryos die in the early foetal period mainly due to failure of liver hematopoiesis (Lee et al., 1992; Jacks et la., 1992, Baribault et al., 1993; Mucenski et al., 1991).

To study other functional aspects of these developmentally vital genes that would occur at later stages of development can be approached by employing "the tissue-specific knockout" technique (Barinaga, 1994). The tissue-specific knockout technology allows to investigate the lack-of-function of particular genes after passing the early critical period when the genes are

needed. This method enables the expression of a transgene to be controlled, using both the cre and FLP recombinases (Kilby et al, 1993).

cre is the recombinase of the bacteriophage P1, a virus that infects the bacterium Escherchia Coli, and recognises a site called LoxP. During the infection, cre lines up LoxP sites of phage DNA and removes DNA between them, leaving one LoxP site behind. FLP is from a yeast plasmid, and recognises FRT sites. Applying conventional homologous recombination techniques, enables to insert a transgene containing loxP or FRT sites into ES cells, which are then used to create transgenic mice. Another transgenic line is generated which expresses cre or FLP. Once the cre and lox, or FLP and FRT lines are mated, activation of the recombinase results in excision of the segment of DNA between the recombinase recognition sites. Applying this technique enables to switch genes on or off. If the sites are introduced at either end of the whole construct including promoter, expression of *cre* results in excising the entire coding sequence and transgene would switch off, whereas in absence of cre, the transgene would be expressed normally.

Using traditional knockouts to study the function of DNA polymerase β, Gue et al., (1994) found that eliminating the gene in all the cells of the mouse's body would result in lethality at an early stage of development. Therefore, it would not be possible to investigate other aspects of the gene's function that would occur at later stages. To overcome this problem, the DNA polymerase gene in ES cells were flanked LoxP sites (Figure 1.3). Mice that have this engineered gene were perfectly normal, because they were able to make an active DNA polymerase β. To achieve cell-type specific inactivation, these transgenic mice were crossed with the transgenic mice in which the *cre* gene was expressed only in developing T cells. Surviving of the T cells of offspring indicated that polymerase was not absolutely needed throughout T cell development.

II:1.5.1.1 Transforming Growth Factor beta 3 (*Tgfb3*) knockout mice:

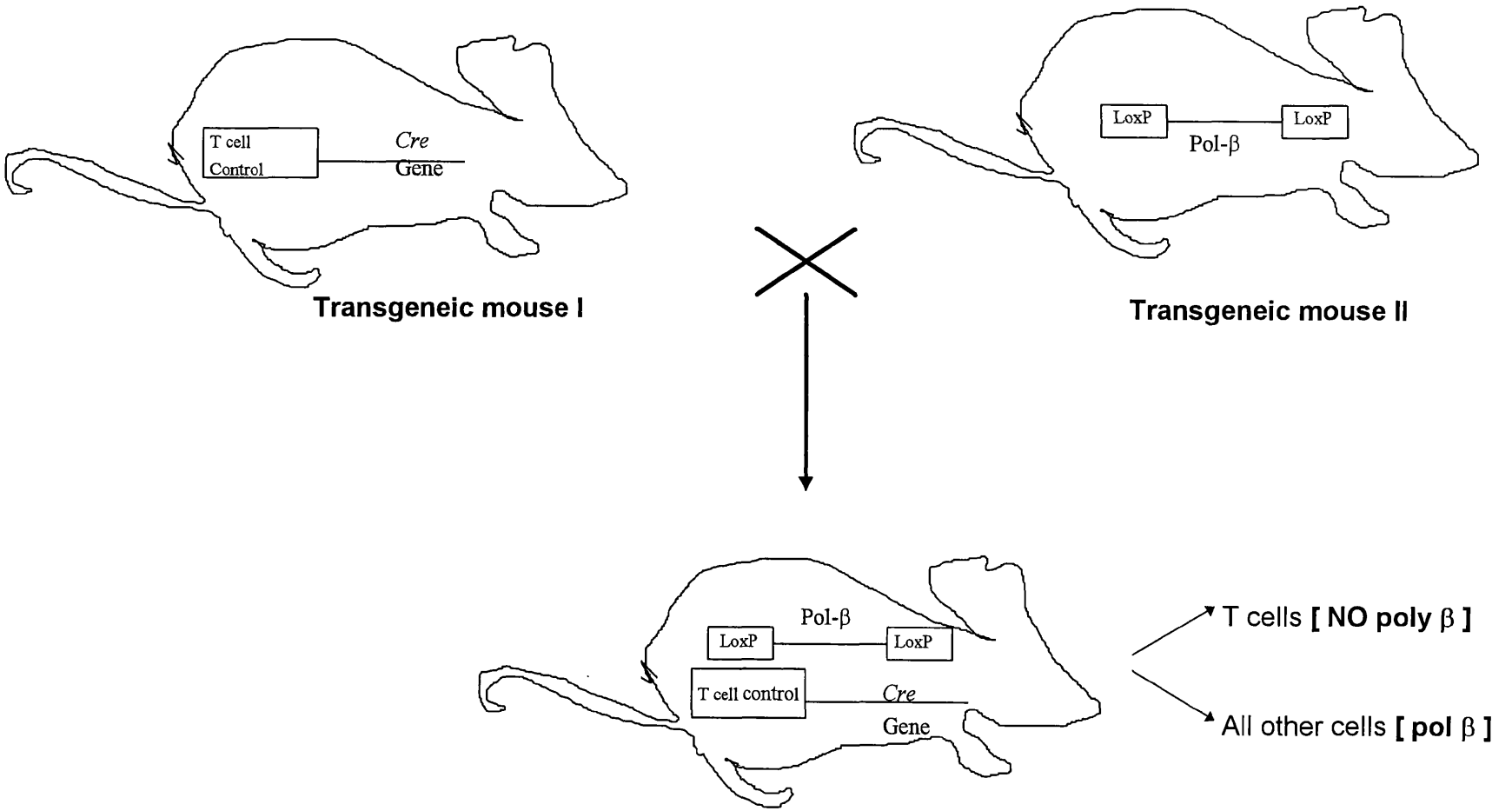


Figure 1.3 In transgenic mouse I, *Cre* gene is expressed only in developing T cells, and animal is perfectly normal. In transgenic mouse II, DNA polymerase β gene is flanked by LoxP sites, and animal is perfectly normal. In ofspring, only the T cells, express *Cre* gene which result in removing *Pol-β* gene in these cells. Thus no poly-β protein was made in the T cells.

The *Tgfb3* gene was mutated in ES cells by homologous recombination. The chimaeric mice produced by using *Tgfb3* mutated ES cells were mated with three different genetic backgrounds, i.e. CF-1, C57Bl/6, and 129/Sv. TGFB3(+/-) offspring showed no phenotypic difference compared to that of wild type offspring (Proetzel et al., 1995). To generate TGFB3(-/-) mutants, intercrosses between heterozygous animals were set up. TGFB3(-/-) pups developed to term but died shortly after birth. Embryonic analysis at different stages of development (10.5 dpc- 19.5 dpc) showed no statistically significant difference in the expected Mendelian ratio of 1:2:1 for wild type, heterozygous, and null embryos respectively. This indicated the presence of little or no prenatal lethality of TGFB3(-/-) mutants. Neonates lacking TGFB3 could not successfully suckle, and in their stomachs milk was not observed. These animals began gasping for air and subsequently became cyanotic and dehydrated just before death. All pups homozygous for TGFB3(-/-), suffered from cleft palate. In some of the TGFB3(-/-) neonates, the palatal cleft proceeded into the most anterior part of the palate, whereas in the rest the anterior segment appeared to be fused. Such variation in expressivity of the cleft palate phenotype observed among null pups has been suggested to be due to the effect of genetic background. The most severe cleft palate phenotypes were observed on the C57Bl/6 background. About 50% of the TGFB3(-/-) pups bred onto the C57Bl/6 genetic background exhibited a complete cleft palate, whereas only a very small percentage (2%) of TGFB3(-/-) pups bred onto the 129/Sv genetic background had a complete cleft palate.

Analysis of the lungs of TGFB3(-/-) mutants revealed a developmental delay, detectable as early as day E12.5. The number of epithelial tubules and primitive peripheral branches was reduced by 50% in TGFB3(-/-) mutants compared to that of wild type littermate controls. In neonates, the airway and terminal air spaces were grossly abnormal.

Analysis of other organs such as cartilage, bone, brain, skeleton and heart of TGFB3(-/-) embryos and neonates did not reveal any gross malformations. The lack of gross abnormality in other tissues could be due to compensation by other TGFB isoforms; i.e. TGFB1 and B2. However. expression studies on

TGFB3(-/-) embryos did not exhibit any significant difference in TGFB1 and B2 transcripts excluding the possible upregulation of these TGFB isoforms in the absence of TGFB3.

II: 1.5.1.2 Transforming Growth Factor beta type II receptor (*Tgfbr2*) knockout mice:

In order to breed *Tgfbr2* knockout mice, one allele of the gene was targeted in the ES cell line D3a2 by applying a homologous recombination approach. Targeted ES cells were injected into the blastocoel of C57Bl/6J embryos. Chimaeric males were mated with C57Bl/6J females (Oshima et al., 1996).

Tgfbr2(+/-) animals were developmentally normal and fertile. Tgfbr2(-/-) mutants were generated by heterozygous intercrossing. No Tgfbr2(-/-) animals developed to term, indicating that TBRII was vital during embryogenesis. Embryonic analysis revealed that Tgfbr2(-/-) embryos died by 13.5 dpc due to defects in hematopoiesis and vascular development. These findings indicate that signalling through TBRII was essential for hematopoiesis and vasculogenesis in the yolk sac. This phenotype has also been observed in some of the TGFB1(-/-) embryos dying *in utero* around 10.5 dpc (Dickson et al., 1995). Therefore, it could be suggested that TGFB1 signalling through TBRII is important for hematopoiesis and vasculogenesis in the yolk sac.

II: 1.5.1.3 Transforming Growth Factor beta 1 (*Tgfb1*) knockout mice:

Although there are some overlaps, the expression of each *Tgfb* gene is spatially and temporally distinct (Gatherer et al., 1990), so in order to investigate the role of TGFB1 and to differentiate its function during embryogenesis from other TGFBs (in mammals), gene "knockout" technology was employed. ES cell lines derived from 129/Sv blastocysts were subjected to homologous recombination to insert a *NEO* gene lacking a polyadenylation signal into the *Tgfb1* gene (Figure 1.4). The targeted ES cells were selected with neomycin and injected into C57Bl/6J/Ola blastocysts. These chimeric

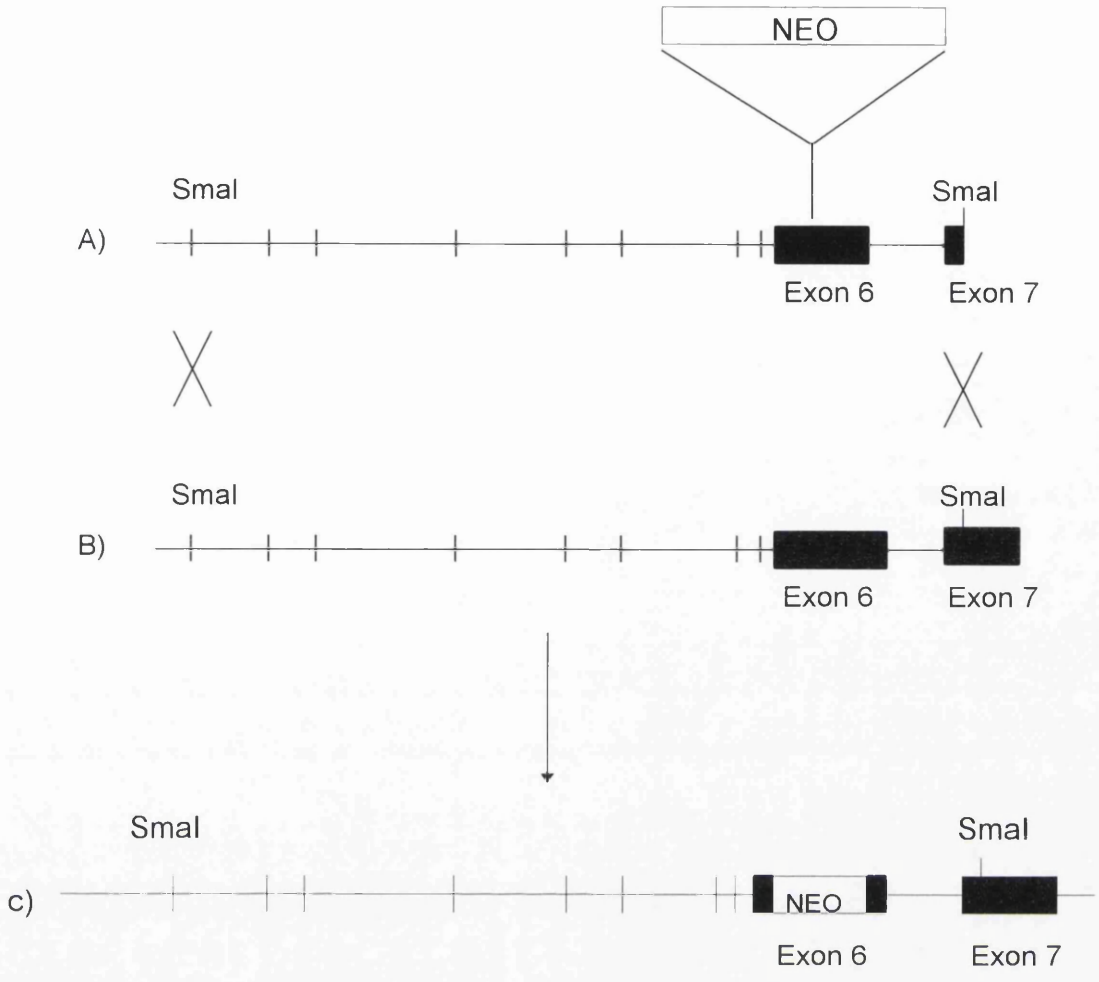


Figure 1.4 Targeted disruption of the murine *Tgfb1* gene in ES cells by homologous recombination.

A) Targeting construct consisting of a *Tgfb1* 4.0 kb SmaI genomic fragment containing exon 6 and a portion of exon 7 of the *Tgfb1* gene. A NEO gene lacking the polyadenylation signal was inserted into the BamH1 site in exon 6 of *Tgfb1*, 102 nucleotides from the N terminal of the mature peptide.

B) The map of the wild type Tgfb1 gene locuse.

C) Predicted structure of the disrupted *Tgfb1* allele.

blastocysts were incubated in a foster mother. The injected ES cells contributed to the germ cell lineage in the chimeric animals were allowed to pass the changes in the mouse genome to the next generation. Although there is no phenotypically obvious difference between a TGFB1(-/+) animal and a normal one (wild type), PCR techniques enable the mice carrying the mutated *Tgfb1* allele to be distinguished from wild type animals (Shull et al., 1992). Homozygotes for TGFB1(-/-) generated from intercrosses between TGFB1(-/+) animals were first described as developmentally normal animals, but they die at about 20 days postnatal with a severe inflammatory disease (Sull et al., 1992). An independent study of targeted disruption of *Tgfb1* exhibits the same inflammatory condition leading to organ failure and death by 3 weeks (Kulkarni et al., 1993).

Mild inflammatory infiltrates of the heart were seen as early as 5 days of age, with rapid spread to all organs by 14 days old. Inflammation became moderate to severe by 10 to 14 days (Boivin et al., 1995). Hyperproliferation of lymphoid cells was observed in TGFB1 deficient mice (Christ et al., 1994). Daily injection of synthetic fibronectin peptides reduced inflammatory cell infiltration of heart and lungs and modified the lethal wasting syndrome (Hines et al., 1994). Increased levels of major histocompatiblity class I and class II mRNA were found in TGFB1(-/-) mice, and may contribute to the inflammation/wasting disease of these mice. Therefore, TGFB1 deficient mice have been proposed as a model for human inflammatory disorders, such as autoimmune disease, transplant rejection, and graft versus host reactions (Shull et al., 1992).

Detailed analysis of the neonatal TGFB1 genotype ratios (+/+: +/- : -/-) from TGFB1(+/-) intercrosses demonstrated that only 50% of the TGFB1(-/-) embryos developed to term, while the rest died *in utero* due to defects in yolk sac vasculogenesis and hematopoiesis (Dickson et al., 1995). It has been suggested that dichotomy in TGFB1(-/-) lethal phenotypes is due to maternal TGFB1 rescue of some, but not all, TGFB1(-/-) embryos (Letterio et al., 1994).

II: 1.6 Phenotypic variations in Knockouts:

II: 1.6.1 Cystic fibrosis:

Cystic fibrosis (CF) is characterized by obstructive lung disease, elevated sweat electrolyte levels and exocrine pancreatic insufficiency (Welsh et al., 1995). The clinical variations observed in CF patients could be partly explained by the allelic heterogenity of the *CFTR* gene. However, the variation also occurs among patients of the same *CFTR* genotype, indicating the involvement of some other modifying factors (Rozmahel et al., 1996). To reveal more about CF disease, animal models have been generated by disrupting exon 1 of the mouse *Cftr* gene (Rozmahel et al., 1996). Different expressivity of the CF phenotype was observed among homozygous *Cftr* knockouts bred onto a mixed genetic background of 129/Sv and CD1 strains. The majority of the F2 homozygous *Cftr* knockout mice exhibited severe intestinal obstruction leading to death within the first week of life. However, ~30% of the F2 knockouts survived well past 6 weeks of age. The other group of animals, classified in terms of severity between these two classes, died by 5 weeks of age. Genetic analysis of animals has shown that genetic background plays an important role in the different expressivity of the phenotype among *Cftr* knockout animals. A genome wide search to identify modifier genes, conducted on F2 animals revealed a modifier gene located in the proximal region of mouse chromosome 7. The prolonged survival phenotype was contributed by a genetic locus from the CD1 background. Whereas most of the *Cftr* knockout animals dying within the first week inherited this region homozygous for 129/129 strain (Rozmahel et al., 1995).

II: 1.6.2 Epidermal Growth factor Receptor (*EGFR*):

The EGFR, which is a member of a family of tyrosine kinase receptors, is expressed on the trophectoderm of the blastocyst (Sibilia & Wagner., 1995). Due to its expression pattern, it has been suggested that EGFR may be important for embryo development. A gene targeting approach was utilised to examine the physiological functions of EGFR *in vivo* (Sibilia & Wagner., 1995;

Threadgill et al., 1995). Different expressivity of *Egfr* knockout phenotype in different genetic backgrounds has been observed. When the *Egfr* knockout allele was bred onto CF-1 genetic background, degeneration of the inner cell mass in null conceptuses led to death in the pre-implantation stage. The *Egfr* knockout allele bred onto a CD-1 genetic background developed to term and died by 3 weeks. The phenotypes observed in this group included abnormalities in skin, kidney, brain, liver, and gastrointestinal tract. EGFR(-/-) embryos bred onto a 129/Sv genetic background died at mid-gestation due to placental defects (Sibilia & Wagner., 1995; Threadgill et al., 1995). Different expressivity of the *Egfr* null phenotype in different genetic backgrounds indicates the involvement of some genetic factors modifying the phenotype. It remains to identify the modifier genes.

II: 1.6.3 Transforming Growth Factor beta 3:

As discussed in the previous section (1.6.1) TGFB3(-/-) animals exhibited the cleft palate phenotype with variable severity. The most severe cleft palate phenotypes were observed on the C57Bl/6 background, whereas on the 129/Sv genetic background the phenotype was much less severe. Therefore, it has been suggested that the variation in the expressivity of cleft palate phenotype observed among null pups could be due to the effect of some modifier genetic factors.

II: 1.7 The aim of the project:

II: 1.7.1 Deleterious effect of TGFB1(-/-) mutant in different genetic backgrounds.

The major aim of the project was to investigate the possible factor(s) involved in different expressivity of the TGFB1(-/-) phenotype reported in TGFB1(-/-) animals bred onto a mixed genetic background. As discussed above, targeted disruption of the *Tgfb1* gene does not necessarily lead to embryonic lethality (Shull et al., 1992; Kulkarni et al., 1993). On a mixed genetic background approximately 50% of TGFB1(-/-) conceptuses died

prenatally due to defects in yolk sac vasculogenesis and hematopoiesis (Dickson et al., 1995) whereas the other half were developmentally normal but died around three weeks *post-partum* as a consequence of massive multisystemic inflammation (Shull et al., 1992; Kulkarni et al., 1993). It has been suggested that dichotomy in TGFB1(-/-) lethal phenotype is due to maternal TGFB1 rescue of some, but not all, TGFB1(-/-) embryos (Letterio et al., 1994).

The project was mainly carried out to reveal whether there were any modifying factors discriminating between TGFB1(-/-) embryos dying from the yolk sac defect and those developing normally to term.

II: 1.7.2 Determining the effect of modifying factor(s) on expressivity of TGFB1(-/-) phenotypes during embryogenesis.

The absolute requirement to identify modifying factors by genetic analysis of inbred strains is to determine an unambiguous phenotype as a strain specific phenotype. To determine whether TGFB1(-/-) lethality was strain specific, the *Tgfb1* null allele was to be bred onto pure genetic backgrounds of either NIH/Olac or C57Bl/6J/Olac. The deleterious effect of the TGFB1(-/-) genotype was observed to be dependent mainly on genetic background. The second approach was to elucidate the nature of these differences in genetic background, which lead to variable expressivity. To achieve this, it was necessary to consider all potential factors which could have influenced the deleterious effect of the TGFB1(-/-) mutant. These factors could be classified as follows: 1) Non-embryonic factors, such as circulating maternal TGFB1 or the maternal immune system 2) Embryonic factors such as embryonic genetic background or 3) The combination of maternal and embryonic factors (additive or non-additive effect).

The results of this project would reveal hidden interactions of TGFB1 and other genetic and/or non-genetic factor(s) which could be important during development of the embryo and the other biological functions in which TGFB1 is implicated.

II: 1.7.3 Strategy:

To address the problem of different expressivity of TGFB1(-/-) phenotype in two different genetic backgrounds, various crosses between NIH/Ola and C57Bl/6J/Ola were to be set up to find out whether there was strain-specific expressivity of the TGFB1(-/-) phenotype and to then estimate the crude number and the inheritance pattern of genetic loci responsible for the different expressivity. It was estimated that one locus with a codominant pattern of inheritance was responsible for different expressivity of the TGFB1(-/-) phenotype.

The goal of the first stage in mapping modifying gene(s) was to link the locus (loci) to a defined sub-chromosomal interval. This approach was accomplished by typing a relatively small set of markers on a relatively small number of phenotypically typed animals. Due to the codominant behaviour of the modifying gene(s), the F2(NIH/Ola x C57Bl/6J/Ola) intercross animals were considered to be the most informative animals for genetic linkage analysis. 50 polymorphic DNA markers were utilised to initiate a genome-wide search by screening 50 TGFB1(-/-) neonates from an F1 intercross. More than 90% of the genome was screened for modifying gene(s) during this study. Four regions of the genome showed suggestive linkage ($P<0.05$) in the first screen. To confirm the linkages, 30 extra null animals were screened with the interested markers. To study on some candidate genes which were not mapped on the mouse genome, Southern blotting technique was utilised to map these candidate genes. Also some other candidate genes mapped in the vicinity of the region meeting the criterion of definitive linkage were examined by applying reverse transcriptase PCR to study their expression in both yolk sacs and embryos (9.5 dpc) from both NIH/Ola and C57Bl/6J/Ola strains. To study the possibility of polymorphism in cDNA of one of the candidate genes (*Fin13*), heteroduplex analysis and sequencing techniques were employed.

2: Materials and Methods

During this project, two different mouse strains; NIH/Ola and C57Bl/6J/Ola were utilised. To breed the *Tgfb1* null allele onto either NIH/Ola or C57Bl/6J/Ola genetic backgrounds, TGFB1(-/+) animals, which were mixture of three genetic backgrounds of 12.5% 129/Sv, 37.5% C57Bl/6J/Ola, and 50% NIH/Ola strains were utilised (Dickson et al., 1995). These animals were bred through four generations by Mrs. Frances Cousins onto either inbred NIH/Ola or inbred C57Bl/6J/Ola obtained from Harlan Olac Ltd., UK. In each generation, animals were genotyped for *Tgfb1* by performing polymerase chain reaction (PCR).

TGFB1(-/-) neonates used in this study were obtained from heterozygous x heterozygous matings [TGFB1(+/-) x TGFB1 (+/-)] of various combination of NIH/Ola and C57Bl/6J/Ola genetic backgrounds. Neonates were killed by cervical dislocation prior to DNA extraction.

2.1 Mouse Husbandry:

For each line, the male mice were mated with females to obtain embryos 9.5 days post coitum (dpc). Mice were housed in a conventional room (light 5am - 7pm). For these animals, noon on the day the copulation plug was found was taken as 0.5 dpc.

Two different lines of mice were used in this study; NIH/Ola and C57Bl/6J/Ola.

2.2 DNA extraction from tail tip biopsy:

1cm of tail was cut from a mouse (under general anaesthesia) or a neonate (killed prior to tail tipping) and placed in a 1.5ml autoclaved eppendorf tube which contained 0.7ml lysis buffer [100mM Tris-HCl pH 8.5, 5mM EDTA,

0.2% sodium dodecyl sulphate (SDS), 200mM NaCl, and 0.1mg/ml of freshly added Proteinase K]. The tube was incubated at 55-60°C in a water bath with agitation. Following complete lysis, the rack of tubes was shaken vigorously. The tube was then centrifuged in an eppendorf microcentrifuge at 14,000 rpm for 10 min. The supernatant was poured into a fresh 1.5ml autoclaved tube, and 0.7ml isopropanol added to the tube and mixed gently by inversion until precipitation was completed. The DNA was recovered by lifting the aggregated precipitate from the solution using a disposable yellow tip. Excess liquid was dabbed off and the DNA was dispersed in a sterile microfuge tube containing the required volume of 1 X TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0).

2.3 Screening animals for *Tgfb1*:

2.3.1 Screening animals for *Tgfb1* by polymerase chain reaction (PCR):

Three primer sets [Table 2.1 (95-98, TGFO, and PGK-1)] were utilised to amplify the *Tgfb1* wild type and Neo (null) alleles (Figure 2.1). These primers (prepared within the Department) required a deprotection stage, to remove the oligonucleotide from the columns [0.2μm (standard) purchased from Cruachem)], 2ml of ammonia solution was pushed through the column using a small syringe and the eluant pooled. The tube was incubated overnight at 55°C, and then the ammonia allowed to evaporate off in a fume hood. The concentration of the remaining primer solution was determined (ng/μl) by Spectrophotometer (Gene Quant II, Pharmacia Biotech) using Optical Density (260) (OD_{260} X diluted times X 33) and the required concentration was obtained by dilution in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). To amplify the *Tgfb1* wild type allele, 1μM from each of two primer sets (95-98), and to amplify the *Tgfb1* null allele, 1.3μM from each of primer sets (TGF-O and PGK-1) were added to the PCR solution containing 1x PCR buffer [(Cambio); 1.0M Tris-HCl pH 9.0 at 25°C, 0.4M ammonium sulphate], 0.6mM dNTPs, 1.5mM Mg^{2+}, 150-

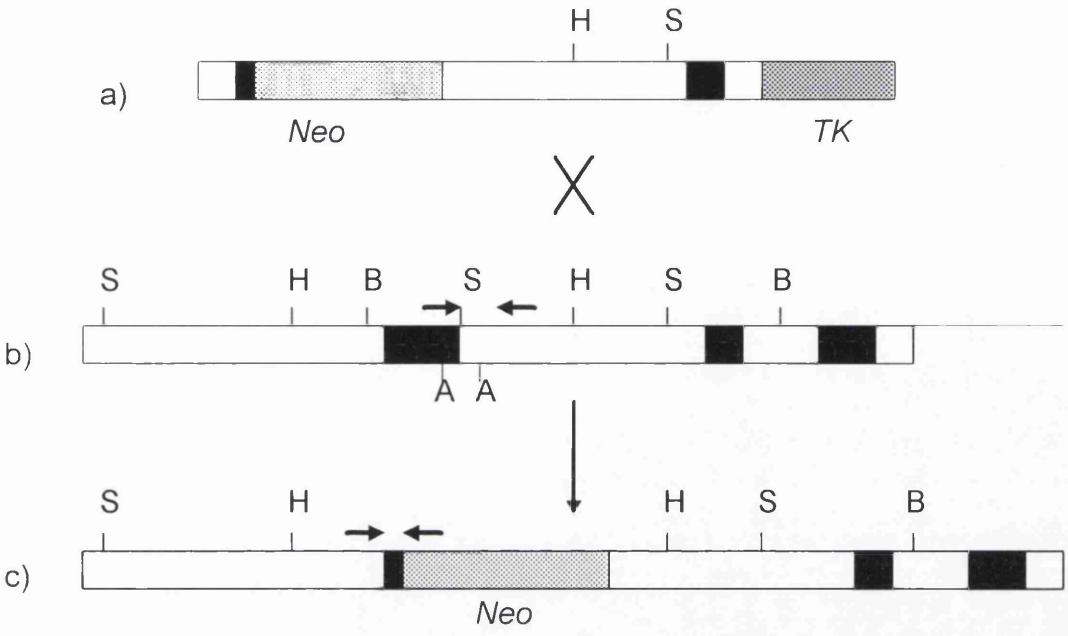


Figure 2.1: Targeted disruption of murine *Tgfb1* gene in ES cells by homologous recombination.

Schematic representations of: a) the targeting vector, b) normal allele of *Tgfb1*, and c) targeted *Tgfb1*.

Solid boxes indicate *Tgfb1* exons, open boxes show introns and the promoterless region. *Neo* and *TK* (hatched boxes) when expressed confer drug resistance and sensitivity, respectively, thereby enabling selection of transformed cells.

A 0.56 kb sequence was deleted between *Asp* 718 (A) sites and the Neo cassette inserted.

The location of primers used for amplification of normal and targeted alleles are shown by arrows.

S; *SacI*, H; *HindIII*, B; *BglII*, A; *Asp*.

200ng of genomic DNA, and 1 Unit Tf1 Thermostable DNA polymerase (Cambio) in a final volume of 25µl. The PCR reaction mixture was overlaid with 50µl mineral oil to prevent evaporation of solution during the PCR procedure. The PCR was performed under the following conditions for 30 cycles; 94°C (denaturing) for 1 min, 63°C (annealing) for 1.5 min, and 72°C (extension) for 2 min and it was terminated with 1 cycle 72°C (extension) for 10 min. By applying this protocol, animals were genotyped for both *Tgfb1* wild type and null alleles in a single combined PCR.

Table 2.1 : The sequences of different primer sets utilised for amplification of specific fragments of different genes screened during this project.

Name	5'..... 3' Sequence of Set Primer	Product Length	Annealing Tm.
FIN-A	723 ACA AAC CAG AGG ATG AAG 740		-
FIN-B	1048 GGA TGA CAA TAA CCG AAG 1031	326 bp	54°C
TGF-A	706 CGC CAT CTA TGA GAA AAC 723	-	-
TGF-B	1031 TCG CAC AAG AGC AGT GAG 1014	326 bp	55°C
IGFR-A	1700 ACT GTC CTG AAG ATG CTG C 1718	-	-
IGFR-B	2982 TTT CCG ATG CCT AAG ACC 2999	1300 bp	57°C
IGFR-C	6699 GCC CAA TGA CCA GCA TTT C 6717	-	-
IGFR-D	7453 TCT CAC CCT CCC TTT CCT TC 7434	755 bp	61°C
FGFR-A	2046 GTA TTC ACA GAG ACT TGG C 2064	-	-
FGFR-B	2254 GAC ACC AAA AGA CCA AAC 2237	209 bp	53°C
FGFR-C	299 AGT GCT TGT GTG TGC TGA GGT 319	-	-
FGFR-D	418 AAC TTG TCC ACT CTT CCC CAG 438	139 bp	55°C
GDH-A	TGA GTA TGT CGT GGA GTC TAC		-
GDH-B	GGC CAT GTA GGC CAT GAG GTC	720 bp	55°C
96	CGT GCG CCT GTC GCT TTC TG	-	-
97	GCG GAC TC TAT GCT AAA G	-	-
98	GGT CAC CCG CGT GCT AAT GG	-	-
95	GCG AGT AAG CCC ACT AGA G	625 bp	63°C
TGFO	AGG GAG CTG GTG AAA CGG AA	-	-
PGK-1	TCC ATC TGC ACG AGA CTA GT	375 bp	63°C

FIN(A,B), Fibroblast growth factor Inducible 13 (cDNA, EMBL Access No. Mmu42383); TGF(A,B), Transforming Growth Factor beta 1 (cDNA, EMBL Access No. M13177); IGFR(A,B,C,D), Insulin Like Growth Factor II Receptor(cDNA, EMBL Access No. MMU04710); FGFR(A,B), Fibroblast Growth Factor Receptor type III(cDNA, EMBL Access No. MusMFR3); FGFR(C,D), A Microsatellite located on the intron 4 of *FGFR3* gene (EMBL Access No. Musfgfr05.gbn); GDH(A,B), GAPDH (cDNA); 95-98, Transforming Growth Factor beta 1 (Genomic DNA) Wild type allele; TGF0, PGK-1, Transforming Growth Factor beta 1 (Genomic DNA) Null allele.

Table 2.2: The sequences of different primer sets utilised for the sequencing of the *FIN13* gene during this project.

Name	5'..... 3' Sequence of Set Primer	Product Length	Annealing Tm.
FN1A	11 CGAGATAAAGACAAAGTAGCAG 32		
FN1C	359 TCT TCA GCC TCT GAC AAG CT 378	**(nested)**	
FN1B	563 TCATCTCTTCATCATCATCC 544	**553**	**58°C**
FN2A	539 GAAGAGGATGATGATGAAGAG 559		
FN2C	711 TCT CCT ATG ACC ACA AAC 719	**(nested)**	
FN2B	1055 CCACAATGGATGACAATAAC 1036	**517**	**56°C**
FN3A	959 AATGTGATGAGCAAGCCAG 976		
FN3C	1101 GTACAGGGTGTGACAACATGAC 1122	**(nested)**	
FN3B	1481 TGAGCCCAAGGAGAGTAAG 1463	**523**	**59°C**

2.3.2 Detecting PCR products:

The PCR products were resolved by agarose gel electrophoresis. 10µl of the PCR product was mixed with 2µl of (6X) loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400; Pharmacia) in water] and loaded into the wells of a 1.5% agarose gel dissolved in either 1 X Tris-acetate/EDTA electrophoresis (TAE) buffer [0.04M Tris-acetate, 0.001M EDTA, pH8.0] or 1 X Tris-borate/EDTA electrophoresis (TBE) buffer [0.045M Tris-borate, 0.001M EDTA, pH8.0] containing 0.5µg/ml ethidium bromide. The gel was run in 1 X TAE or TBE buffer, at 100-120 Voltage for 30 min. The DNA bands were visualised under ultraviolet light and photographed by UVP Imagestore 5000.

2.4 Screening animals for microsatellites:

2.4.1 PCR protocol for screening microsatellites:

The PCR was performed in a final volume of 22μl containing; 3.5mM Mg^{2+}, 0.27mM dNTPs (dTTP, dATP, dCTP, dGTP), 1 x PCR buffer (Cambio), 1 Unit Taq polymerase enzyme (Cambio), 1μM from each of primer sets (150 primer sets used in this project were either from professor John Todd (Oxford) or professor Allan Balmain (Glasgow) or purchased from Genetic Research, or synthesised on an Applied Biosystems 391 DNA synthesiser within the Department), and 150-200ng of genomic DNA. The PCR reaction mixture was overlaid with 50μl mineral oil. The PCR was carried out under the following conditions with slight modifications for different primer sets: 94°C (denaturing) for 1 min; 55-60°C (annealing) for 1.5 min; 72°C (extension) for 1min. There were 30 amplification cycles and PCR was terminated with 1 cycle extension (72°C) for 10 min.

2.4.2 Detecting PCR products :

To screen the markers for polymorphisms between NIH/Ola and C57Bl/6J/Ola strains, the PCR products were run on a 3-3.5% low-melting-point agarose (NuSivEVE^R GTG, 50082, FMC BioProducts). However, most of the polymorphisms were only detectable on a non-denaturing polyacrylamide gel [19/1 (40%) acrylamide/N-N'-methylenebisacrylamide, 1 x TAE buffer, 0.7ml from 10% Ammonium persulphate,)] electrophoresis.

2.4.2.1 Casting 8% non-denaturing polyacrylamide gel:

Polymerisation was initiated by the addition of 35µl of N,N,N',N'-tetramethylethylenediamine(TEMED) to 100ml of 8% polyacrylamide solution. After polymerisation, which took about 1.5h, the comb was removed and the wells washed out with buffer and 10µl of the PCR product was mixed with (6X) loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400; Pharmacia) in water] and loaded into the wells. Electrophoresis occurred at 100V for 12h in 1 X TAE buffer.

2.4.2.2 Visualisation of DNA bands run on non-denaturing polyacrylamide gel electrophoresis:

DNA bands were visualised by applying a silver staining protocol. The gel was fixed for 10 min in fixation solution (10% ethanol, 0.5% Acetic Acid) then stained in 0.1% silver nitrate for 15 min with gentle shaking. To wash off extra silver nitrate, the gel was rinsed with two changes of distilled water. The gel was developed for 20 min with gentle shaking in 1.5% NaOH, and 0.1% Formaldehyde. DNA bands were stained light brown during this step. Staining was fixed for 10 min in 0.75% Na_2CO_3 and the gel was wrapped in a plastic sheet and stored in a dark place.

2.5 DNA purification for probe prepration & subcloning:

2.5.1 Purification of PCR product:

To purify DNA band, the Geneclean Kit (1001-400, Bio 101 Inc) was used according to manufacturer's protocol. To remove unincorporated nucleotides and primers from PCR solution, 3 volumes of 6M NaI and 20µl of Glassmilk were added to the solution and incubated in room temperature with gentle shaking. The solution was briefly centrifuged at full speed for 15 sec to pellet the Glassmilk which was washed 3x with 400µl New Wash (2.5% New

Wash, 50% Ethanol, 47.5% dH_2O, stored at -20°C). The Glassmilk was pelleted by brief centrifugation, the supernatant was removed and the pellet was dissolved in a required volume of TE buffer and incubated at 55°C in a water bath for 15 min. DNA was recovered by centrifugation in a microcentrifuge at full speed for 1 min and the supernatant containing the recovered DNA was transferred to a sterile microfuge tube and stored at -20°C until used. 1μl of DNA was tested on 1% agarose gel to check the efficiency of the recovery.

Alternatively to purify a PCR product, an equal volume of 3M ammonium acetate and 2 volumes of isopropanol alcohol was added to the PCR solution. The tube was quenched on ice for 10 min and DNA was precipitated by centrifugation at full speed for 10 min. The pellet was washed with 70% ethanol and dissolved in a required volume of TE buffer.

2.5.2 Purification of specific DNA band:

To purify a specific DNA band produced by either PCR or restriction enzyme digestion, the solution was run on 1% low-melting-point agarose (NuSivEVE^R GTG, FMC BioProducts) dissolved in TAE buffer. The specific DNA band was excised under UV (ultraviolet) light and placed in an eppendorf tube containing 3 volumes of 6M NaI. The tube was incubated for 5 min at 55°C on a water bath with agitation to dissolve the agarose gel covering the DNA band. 20μl of the Glassmilk was added to the tube and the purification continued as described before.

2.6 Southern blotting:

2.6.1 Digestion of genomic DNA:

7 to 10μg of genomic DNA was digested using the appropriate restriction endonuclease in a total volume of 40μl containing 1 x appropriate enzyme buffer (Gibco BRL) and 40 units of the specific enzyme (Gibco BRL). The digests were incubated for 12-16 h in a water bath set at the recommended temperature.

2.6.2 Agarose gel electrophoresis:

The DNA digests were resolved using a 20cm 0.8% agarose gel (FMC bioproducts) prepared in 1 x TAE buffer and containing 0.5μg/ml ethidium bromide. Samples were prepared by adding 8μl of (6x) agarose gel loading buffer to the 40μl digest. Samples were then loaded alongside 1kb size ladder in the gel and electrophoresed at 2 Volts/cm for 18 h. After electrophoresis the gel was visualised and photographed alongside a scale for reference under UV light. The gel was treated with depuration solution (0.25M HCl) for 10-15 min, rinsed in tap water, and then in denaturation solution (0.5M NaOH, 1.5M NaCl) for 30 min. After rinsing the gel in tap water, the gel was neutralised in neutralisation solution (3M NaCl, 0.5M Tris-HCl, pH7.4) for 30 min.

2.6.3 Southern blotting:

The apparatus was set up as described for Northern blotting (2.12.2).

2.6.4 Probe labelling:

The probe was radioactively labelled using the random primed DNA labelling kit (1004 760, Boehringer Mannheim,) according to the manufacturer protocol. 80-100ng of the probe was added to a mix of 2μl each of dATP, dGTP, and dTTP, 4μl of the reaction mix. The mixture was boiled for 5 min and

quenched on ice, then 5µl (50µCi) of α-^{32}P dCTP (Amersham), 2µl of Klenow enzyme, and up to 40µl dH_20 were added and mixed by pipetting. The tube was then incubated at 37°C for one h and then the reaction was stopped by addition of 2µl of 0.5M EDTA. To separate un-incorporated nucleotides from probe, Nick Columns Sephadex G-50 (Pharmacia Biotech) was utilised. The column was washed through with 2ml TE buffer. Then the sample was loaded and was washed through by adding 400µl TE buffer. A second 400µl aliquot of TE buffer was loaded and the elutant pooled. 2 µl of the solution was removed to determine the specific activity of the probe using a Texas Instruments scintillation counter. If the counting was found to be less than 10^8cpm/µg, the probe was discarded (the required counting was 3 x 10^7cpm/each hybridisation). Immediately before use, the probe was boiled at 100°C for 10 min to denature the DNA, then quenched on ice.

2.6.5 Southern blot Hybridisation:

QUIKHYB^R Hybridisation solution (201220, Stratagene) was utilised as a pre-hybridisation and hybridisation solution according to the manufacturer's protocol. The Hybond N filter and a fine mesh were soaked in tap water before rolling together and placing in a hybridisation bottle with 10-15mls of the Quick^R hybridisation solution. The bottle was incubated at 65°C for 15 min in an oven. The radioactive probe and salmon sperm DNA in a final concentration of 100µg/ml, were mixed and boiled for 10 min. The tube was quenched on ice for 5 min and added at 3 x 10^7cpm (per each hybridisation) to the Quick^R hybridisation solution. The hybridisation was continued at 65°C for 1 h.

2.6.6 Washing the filter after hybridisation:

After hybridisation, the hybridisation buffer was discarded and the filter was rinsed in 2 x SSC [Standard Saline Citrate (20 x SSC: 3M NaCl, 300mM Na citrate, pH7.4)], 0.1% SDS while inside the bottle. The filter was washed once by adding 10ml of 2 x SSC, 0.1% SDS to the bottle and returning it to the 65°C oven for 5-10 min. The filter was then taken out from the bottle and placed in a plastic tray with 0.5 x SSC, 0.1% SDS and washed by shaking at 65°C for a further 5 min. The filter was monitored using a series 900 mini-monitor and if a signal greater than 10-15cpm was detected, it was then washed at increasing stringency (65°C, 0.25 x SSC, 0.1% SDS) taking care not to over wash it.

2.6.7 Autoradiography:

The filter was exposed to a Kodak X-Omat autoradiographic film plus intensifying screens, and placed at -70°C. The time period before the film was developed depended on the signal counted by last monitoring.

2.6.8 Developing the X-Ray film:

The film was developed by using Fuji X-Ray Film Processor.

2.7 Chemical Cleavage of Mismatches (CCM):

To study the possibility of strain specific polymorphisms within the PCR products from *Igf2r* cDNA, the CCM method described by Cotton et al (1988) was used with some modifications. In this technique, DNA from one of the strains was allowed to form a duplex with a radiolabelled DNA from other strain (probe). Where mutations (polymorphism) were present, mismatches were

chemically modified and cleaved leading to detection of different-sized radioactive fragments. The protocol involved the following steps:

2.7.1 Preparation of test DNA

2.7.1.1 RT-PCR and DNA-PCR

Two RT-PCR segments (1.3 kb + 0.755 kb) amplified from the *Igf2r* cDNA (7.448 kb coding sequence, EMBL Access No. MMU04710) using two sets of primers (Table 2.1) and the method mentioned in 2.10.2. The PCR amplification was accomplished in a 50μl reaction mix using standard PCR protocol (2.10.2) with 30 cycles and a final 5 min extension step at 72°C.

2.7.1.2 Identification of PCR products

10μl aliquots from the PCR products were electrophoresed on 1% agarose gels in 1 X TAE buffer with 0.5μg/ml ethidium bromide. They were visualised with a UV transilluminator to confirm the size of the band. The remaining 40μl PCR products were cleaned (2.5) for use in CCM.

2.7.1.3 Preparation of labelled probes:

A probe is a PCR product obtained from one of the strain's DNA taken as a control (NIH/Ola or C57Bl/6J/Ola). Internal labelling of the PCR was carried out by direct incorporation 2μCi [α-^{32}P] dCTP in the PCR reaction mix. PCR was carried out in 50μl reaction mix using standard PCR conditions as for the non-radiolabelled one. The labelled probe was purified (2.5) and its specific activity was determined (approximately 10^7cpm/μg).

Alternatively, 5' end labelling of the probe was performed by mixing 100ng of the probe DNA (after Geneclean™ purification), 10μCi [γ-^{32}P] ATP, 1 X One-Phor-All buffer plus (10X ,Pharmacia Biotech, 100mM Tris-acetate, 100mM Magnesium acetate, 500mM potassium acetate), 10 Units of T4 polynucleotide kinase FPLC pure (Pharmacia Biotech) and up to 10μl ddH_2O. The mixture was incubated at 37°C for 45 min then stored at -20°C until used.

2.7.2 Formation of hybrids (heteroduplexes):

To minimise the formation of probe homoduplexes, hybridisation between probe and target was set up in which the target DNA was present in 10 to 20 fold molar excess over probe DNA. Approximately 5ng of probe DNA per target sample per modification reaction was required. 9 volumes of the solution containing [1 X hybrid buffer (0.3M NaCl, 0.1M Tris-HCl, pH8.0) and the appropriate quantity (≈10 ng/test DNA sample) of labelled probe in $T_{0.1}E$ buffer (10mM Tris-HCl, pH7.4, 0.1mM EDTA)] was added to one volume of test DNA (100-150ng) in a sterile eppendorf tube (1.5ml), topped with 50μl mineral oil and then placed in a boiling water bath for 5 min. Immediately, after boiling, the tube was incubated at 65°C in a water bath for 5-16 h to allow hybrid formation to occur. The tube was then quenched on ice and the aqueous phase (containing the hybrids) was divided equally between two siliconized eppendorf tubes containing 3μl of 20mg/ml glycogen (Boehringer-Mannheim) and 750μl of the stop/precipitation mix (63mM Na acetate, 20μM EDTA, 80% ethanol). The tube was vortexed for 1 min prior to incubation on dry-ice for 10 min. By centrifugating the solution at 14000 rpm for 10 min, DNA was pelleted which was then washed in 70% ethanol, air dried and resuspended in 7μl $T_{0.1}E$ buffer. The samples were used the same day.

2.7.3 Mismatch analysis

2.7.3.1 Chemical modification using Hydroxylamine and Osmium tetroxide.

20μl of 6.5M hydroxylamine hydrochloride (Sigma) [(Hydroxylamine; 1.39g, ddH_2O 1.6ml , Diethylamine (BDH, 10341) (approx. 1.5ml) until pH 6 , stored at 4°C up to one week)] was added to 7μl hybrid (2.7.2), mixed well and incubated at 37°C for 2 h. 18μl of freshly made solution of osmium tetroxide (Aldrich, 25,175-5) and pyridine (BDH, AnalaR 10225 4L) [6.75μl pyridine, 1.5μl of 4% osmium tetroxide, and 154μl $T_{0.1}E$ buffer, prepared on ice] was added to the 7μl hybrid (2.7.2), mixed well and incubated at 37°C for 2 h. Modification reactions were stopped and precipitated by adding 750μl stop/precipitation solution to each tube, mixed well and quenched on dry ice for 10 min. DNAs were pelleted by centrifugation at 14000 rpm for 10 min, then washed in 70% ethanol and air dried.

2.7.3.2 Piperidine cleavage of the chemically modified mismatches:

The air-dried pellets (2.7.3.1) were resuspended in 50μl of 1M freshly made piperidine (Fluka, 80640) by vortexing, and incubated at 90°C in a water bath for 30 min. The tubes were quenched on ice and precipitated by addition of 750μl stop/precipitation solution as described before. The pellets were then washed in 70% ethanol, air dried, resuspended in 10μl formamide loading buffer (95% deionized formamide, 10mM EDTA, 10mg/ml bromophenol blue and 10mg/ml xylene cyanol) and denatured by heating at 95°C for 5 min prior to run in 6% denaturing polyacrylamide gel (1 x TEA buffer, 6% polyacylamide, 7M urea (25.2 gr/60 ml of 6% polyacrylamide solution), amonium persulfate 0.7%, TEMED) cast in a sequencing gel apparatus. The gel was electrophoresed at 2000 volts for appropriate time (depending on the size of DNA band examined). Then it was transferred onto a 3 MM Whatman paper,

covered with Saran nylon, dried in a vacuum gel dryer, and exposed to Kodak X-Omat AR film using a cassette with intensifying screens for 12 to 16 h at -40°C.

2.8 Heteroduplex Analysis:

Several mutation scanning methods have been developed. Heteroduplex analysis is used to detect mismatches between double-stranded DNA containing one wild type strand and a complementary strand with an altered nucleotide sequence. Heteroduplex analysis is simple to perform; does not require complex chemical or temperature gradient; and bands can be generally be detected without radioactive labels. During this protocol, PCR products of a test sample and a wild type control are mixed, heated to denature the double-stranded DNA (dsDNA), and allowed to reanneal at a lower temperature. If the primary sequence of the sample and control differ, 50% of the reannealing dsDNA will be heteroduplex DNA.

2.8.1 Denaturing double stranded DNA fragments:

Two PCR products (amplified by utilising either of three set primers presented in the table 2.2) from *Fin13* gene originated from NIH/Ola and C57Bl/6J/Ola strains were mixed in 1:1 ratio. The mixture was overlaid by mineral oil and denatured by heating at 95°C for 5 min.

2.8.2 Re-annealing:

The denatured mixture (2.8.1) was allowed to cool down from 95°C to 35°C over a period of 30 min.

2.8.3 MDE gel preparation & electrophoresis condition:

The MDE (Mutation Detection Enhancement) gel (Flowgen) was utilised to detect the possible heteroduplex DNA bands. 1x MDE gel (1x MDE, 0.6x TBE, Urea 15g/100ml of solution, 0.7% ammonium persulphate, TEMED) was prepared and electrophoresed (20 V/cm) in non-denaturing conditions.

2.8.4 Silver staining:

DNA bands were visualised by applying the silver staining protocol as described previously (2.4.2.2).

2.9 Sequencing:

2.9.1 Sequencing cDNA

Direct sequencing of the Fin13 cDNA was carried out using the dideoxy chain terminating method (Sanger et al., 1977) using the Sequenase™ Version 2.0 sequencing kit (Amersham).

2.9.1.1 RT-PCR

RT-PCR was carried out to amplify *Fin13* cDNA by utilising the primer sets (table 2.2) as described in section 2.12.

2.9.1.2 Asymmetric PCR:

Single-stranded templates required for sequencing were generated by asymmetric PCR. 10µl of the gel-eluted double-stranded PCR product was used as a template for asymmetric PCR, where one primer was 50 to 100 times less concentrated than the other (2µM) in a total volume of 50µl. The other

components were the same as for symmetric PCR but the number of cycles was increased to 40 to 45 cycles as there is a linear increase in the amount of PCR products due to the limiting concentration of one primer. The asymmetric PCR product yield was monitored by agarose gel electrophoresis as described previously (2.3.2).

The asymmetric PCR products were purified by adding an equal volume of 4M ammonium acetate and 2 volumes of isopropanol. The mixture was incubated at room temprature for 10 min, centrifuged at full speed for 10 min in a microcentrifuge, washed in 70% ethanol and resuspended in 7μl of dH_2O.

2.9.1.3 Sequencing reaction protocol:

Sequencing was performed according to the manifacturer's instructions. 7μl of the single-stranded template was mixed with 2μl of 5x Sequenase™ reaction buffer (to give a final concentration of 40mM Tris-HCl pH 7.5, 20mM $MgCl_2$, 50mM NaCl) and 1pmol of the appropriate sequencing primer (the primer diluted 50 to 100 times during asymmetric PCR).

2.9.1.3.1 Denaturing double-stranded templates & annelaing:

The mixture (2.9.1.3) was denatured by incubating at 65°C for 2 min and then allowed to cool slowly to less than 35°C over a period of 30 min.

2.9.1.3.2 Labeling reaction:

The mixture was then immediately placed on ice and to it was added 1μl of 0.1M DTT, 2μl of a 1:5 dilution of labelling mix (7.5μM of each dNTP except dATP), 0.5μl of [α-^{35}S]dATP (1000 Ci/mmol), 1μl of Mn buffer (0.15M sodium isocitrate, 0.1M $MnCl_2$) if sequences close to the primer are to be read, and 2μl

(1.5 units) of a 1:8 dilution of T7 DNA polymerase [25µl of Sequenase™ Version 2.0, 13 units/µl; diulted with 25µl of inorganic pyrophosphatase (5 units/ml in 10mM Tris-HCl pH 7.5, 0.1mM EDTA, 50% glycrol) and 150µl of glycrol enzyme diultion buffer (20mM Tris-HCl pH 7.5, 2mM DTT, 0.1mM EDTA, 50% glycerol)]. The mixture was incubated at room temprature for 2 to 5 min.

2.9.1.3.3 Termination reaction:

While cooling (during section 2.9.1.3.1), four tubes (for each of the termination mixtuers) for each sample were labelled, 2.5µl of each termination mixture (contating 80µM of each dNTP and 8µM of each ddNTP) was transferred to the appropriate tube and pre-warmed at 42°C. 3.5µl of labeling mixture from section 2.9.1.3.3 was added to each termination mix at 42°C and incubated at 42°C for a further 5 min after which 4µl of formamide-dye stop solution (95% formamide, 2mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to terminate the reaction. The reaction could be stored at -20°C until required.

2.9.1.4 Denaturing gel electrophoresis:

2.9.1.4.1 Gel electrophoresis reagents and running conditions:

Because the Sequenase™ enzyme was diluted in glycerol-containing buffer, the buffer used in the polyacrylamide gel should be a glycerol tolerant gel buffer (20X TTE: 216g Tris base, 72g taurine, 4g $Na_2EDTA.2H_2O$ and made up to 1 lit.). The gel used to run the sequencing reactions was an 8% denaturing polyacrylamide gel (8% polyacrylamide, 1X TTE buffer, 7M ((25.2g/100ml)) Urea, 0.7% ammonium persulphate, TEMED). The gel was pre-warmed to 50°C before samples denatured for 5min at 75°C to be loaded. The

gel was electrophoresed at 50°C for 2 to 4 hours, after which it was transferred onto a 3MM paper, dried in a vaccum gel drier (Bio-Rad) at 80°C for at least 30min and exposed for 16 to 24 hours to KODAK X-Omat AR film in a cassette using intensifying screens at -20°C.

2.9.2 Sequencing plasmid (insertion):

To sequence the PCR product inserted in a vector (described in section 2.10.2), the plasmid was extracted as described in section 2.10.1.4.

2.9.2.1 Denaturing:

4μg of plasmid disolved in 20μl dH_2O was denatured by adding 2μl of denaturing solution (2M NaOH, 2mM EDTA) and incubated 5 min at room temperature.

2.9.2.2 Neutralisation:

The reaction from 2.9.2.1 was neutralised by adding 3μl 3M sodium acetate (pH 5.5) and 7μl dH_2O.
In order to precipitate DNA, 75μl of 100% ethanol was added and the mixture was incubated on dry ice for 5 min. The DNA was precipitated by centrifugation in a microcentrifuge for 5 min at a full speed. The supernatant was discarded and the pellet was washed with 70% ethanol, then dried and dissolved in 7μl dH_2O.

2.9.2.3 Annealing, labeling & termination:

The annealing, labelling and termination procedure was carried out as described previuosly (2.9.1).

2.10 Subcloning:

During this project two fragments were subcloned. A small fragment of Fgfr3 probe already cloned on a plasmid was subcloned for the purpose of expression studies. Also a RT-PCR product (523bp, 959-1481) from *Fin13* gene was subcloned into a plasmid for the purpose of expression studies.

2.10.1 Subcloning plasmid:

2.10.1.1 Ligation:

The plasmid DNA (MomFgfr3,Ornitz et al., 1992) and the pBluescript SK(+/-) phagemid were separately linearized by digestion with two different restriction enzymes (HindIII and XhoI) and run on 1% agarose gel alongside a marker. The appropriate bands were excised, purified (as described previously, 2.5) and dissolved in an appropriate volume of TE buffer to give 20-50ng/μl.
The ligation was initiated by addition of insert DNA and vector DNA in a 9:1 (weight : weight) excess to vector DNA, to ligation buffer (Gibco-BRL, 250mM Tris-HCl pH7.6, 50mM MgCl2, 5mM ATP, 5mM DTT, 25%(w/v) polyethylene glycol-8000) and 1μl of T4 DNA ligase (Gibco-BRL) to give a final volume of 10μl. The solution was incubated at 16°C for 12-16 h. Control ligations; ligation mixture without insert DNA, ligation mixture without plasmid, and ligation mixture without enzyme were also carried out simultaneously.
Half of the ligation mixture was used for transformation.

2.10.1.2 Preparation of competent bacterial cells:

DH5α bacteria cells (Gibco BRL) were used to generate competent bacterial cells during this project. The bacterial cells were inoculated from a glycerol stock into a 5ml of L Broth [1% bacto-tryptone (0123-17-3, DIFCO), 1% NaCl, 0.5% bacto-yeast extract (0127-17-9, DIFCO), pH7.0 adjusted by NaOH, autoclaved) and incubated at 37°C for 12-16 h with shaking (200 rpm). 1ml of the over/night culture was inoculated into 100ml LB medium and incubated for 3 hours at 37°C with shaking (200 rpm).The cells were pelleted in a 30-ml sterile tube by centrifugation at 1500 rpm for 10 min at 4°C, resuspended in 1ml filtered $CaCl_2$ (100mM), and quenched on ice for 15 min. The solution was recentrifuged at 1500 rpm for 10 min at 4°C, the supernatant was removed and the pellet was resuspended in 1ml filtered $CaCl_2$ (100mM). These cells (competent cells) were used for transformation and could be stored at -70°C until required.

2.10.1.3 Transformation:

Transformation was initiated by adding 10μl of plasmid DNA (0.5μg/μl) to a sterile (1.5ml) eppendorf tube containing 100μl of competent bacteria cells. The bacterial cells were incubated on ice for 30 min, heat-shocked at 42°C for 90 sec, and quenched on ice for 30 min. 0.5 ml sterile L broth was added to the tube prior to incubate at 37°C for 50 min with shaking (200 rpm). 10-20μl of the solution was plated out onto L-agar plate (1% bacto tryptone, 1% NaCl, 0.5% bacto yeast extract, 1.5% agar, pH7.0), containing 20μg/ml appropriate antibiotic. 0.02% of X-gal (Gibco BRL, 5-bromo-4-chloro-3-indoyl-β-D-galactoside dissolved in dimethylformamide) was also added onto L-agar media when pBluescript SK(+/-) was used as a vector. The colonies of bacteria harbouring pBluescript SK(+/-) plasmid, produce blue colour in the presence of X-gal, whereas pBluescript SK disrupted by a insert DNA, was unable to produce blue coloured colonies.

The plates were incubated at 37°C for 16 h in an inverted position.

2.10.1.4 Harvesting and lysis of Bacteria:

A single appropriate bacterial colony was transferred into 2ml of LB medium containing the appropriate antibiotic in 15-ml sterile tube and incubated at 37°C for 12-16 h with vigorous shaking. By centrifugating the solution at 12000 rpm for 30 sec at 4°C the bacterial cells were pelleted and were then resuspended in 300µl ice-cold STET (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA, pH8.0) and re-centrifuged for 15 min at 4000 rpm at 4°C. The pellet was resuspended in 40µl of ice-cold solution I (50mM Glucose, 10mM EDTA, 25mM Tris pH8.0, autoclaved, 10mg/ml freshly added of lysosome) followed by an addition of 120µl of freshly prepared solution II (0.2M NaOH, 1% SDS). The tube was gently inverted for several times and incubated for 5-10 min at room temperature (R/T). 70µl of ice-cold solution III (3 M KOAC) was added to the tube prior to incubatation on ice for 10 min. The solution was centrifuged at 4000 rpm for 15 min at 4°C and the supernatant containing the plasmid DNA was filtered through gauze, mixed with 60% volume of isopropanol and stored at R/T for 10 min. By centrifugation for 15 min at 5000 rpm at R/T, the DNA was pelleted which was then washed with 70% ethanol, and dissolved in an appropriate volume of 1 x TE buffer (pH 8) containing 20µg/ml of DNase-free pancreatic RNase (R-4875, Sigma) (Pancreatic RNase was dissolved at a concentration of 10mg/ml in 10mM Tris-HCl (pH7.5), 15mM NaCl, and heated to 100°C for 15 min and allowed to cool slowly to room temperature and stored at -20°C). The plasmid DNA was stored at -20°C until required.

2.10.1.5 Confriming the insertion:

To confirm that the desired fragment was inserted, the vector was cut with appropriate restriction enzymes and run on an agarose gel to observe the expected bands. Also this approach enabled to find out the direction of insertion in vector for the expression purposes.

2.10.2 Subcloning PCR product:

In all PCRs set up during this project, Tf1 thermostable DNA polymerase (Cambio) was utilised which resulted in adding an extra A in 3' end of PCR products. Therefore, in order to clone PCR product, the pGEM easy vector system (Promega, A1360) was utilised. The vector supplied in this kit contained a 3' terminal thymidine in both ends which could ligate with the PCR products but not with each other.

2.10.2.1 PCR product:

The RT-PCR product (523 bp; 959-1481) amplified from *Fin13* gene by utilizing FN3 (A & B) set primers (table 2.2) was run on 1% low melting agarose gel. The expected fragment was cut and cleaned as previously described (2.5.2).

2.10.2.2 Ligation:

The appropriate amount of PCR product (25ng) was added to the ligation reaction [50ng vector, 1 X T4 DNA Ligase buffer, T4 DNA Ligase (3 Weiss units/μl)]. The reaction was mixed by pipetting and incubated at 4°C overnight.

2.10.2.3 Transfromation & Harvesting and lysis of Bacteria:

Transformation & harvesting and lysis of bacteria was carried out as described previously (2.10.1.3-4)

2.10.2.4 Confirming the insertion by applying PCR and sequencing:

In order to confirm the insertion in the vector, a PCR reaction (as described in 2.12.2) was set up and 3μl of the plasmid was used as a template. The expected size band (523bp) was observed.
Also applying the sequencing technique described in (2.10.2) confirmed that the expected PCR product was inserted.

2.11 RNA Analysis:

2.11.1 Collection of embryos for RNA extraction:

All tips, eppendorfs, and solutions (except those containing Tris, or organic solvents) were treated with 0.1% diethylpyrocarbonate (DEPC) for at least 12 hours at 37°C before sterilisation to prevent the degradation of the RNA by RNase (DEPC suspected to be a carcinogen and should be handled with care).

Pregnant females from timed matings were killed by cervical dislocation, and a vertical incision made through the body wall, enabling the uterus to be removed. Decidua were dissected from the uterus, placed 3-4 per dishes containing ice-cold, millipore-filtered Phosphate Buffered Saline (PBS) (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, pH7.2) and visualised using an Olympus SZH stereo microscope. The embryos were removed from their decidua using sterile watchmakers forceps. The RNA was extracted separately from the yolk sac and embryo. When dissected free of contaminating material, the tissues

were placed in sterile eppendorfs and snap-frozen in liquid nitrogen, then stored at -70°C until required.

2.11.2 Extraction of RNA from embryos and yolk sacs:

To extract the total RNA from 9.5 dpc embryos and yolk sacs, two methods were utilised as follow:

1) The method described by Chomczynski and Sacchi (1987) with slight modifications.

2) The method described by TRIzol™ Reagent manufacturer (Life Technologies).

2.11.2.1 The acid quanidinium thiocyanate phenol/chloroform extraction:

Before proceeding with RNA extraction, embryo and yolk sac were thawed on ice. 550µl of solution D [4mM guanidinium thiocyanate (50980, Fluka Biochemika), 25mM Sodium citrate pH7.0, 0.5% lauryl sarkosyl (Sigma) and freshly added 0.1mM β-mercaptoethanol (sigma)] was added to each tube and the disaggregation of the tissue aided by pipetting. 25µl 2M Sodium citrate pH4.0, 200µl water-saturated phenol, and 55µl chloroform were added separately with gentle mixing after each reagent. Once all three solutions were added, the tubes were vortexed for 10 sec before incubation on ice for 15 min. The samples were then microcentrifuged for 20 min at 14000 rpm at 4°C in a microcentrifuge. The top aqueous layer was transferred to a fresh 1.5ml eppendorf and 700µl of isopropanol added. The solution was gently mixed and placed at -20°C for at least 1 h to precipitate the RNA. The RNA was pelleted by microcentrifugation at 14000 rpm for 20 min at 4C°, and the supernatant was dabbed off. The pellet was redissolved in 100µl solution D, and an equal volume of isopropanol added before a further precipitation at -20°C for at least

an hour. The tubes were centrifuged for 10 min at 14000 rpm at 4°C to pellet the RNA, which was then washed 2 x in 70% ethanol, air-dried and resuspended in 5-10μl of DEPC-treated water and stored at -70°C until required.

2.11.2.2 TRIzol™ Reagent extraction:

TRIzol™ Reagent (15596-026, Life Technologies) is a ready-to-use reagent for isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is used as a modification to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). Before proceeding with RNA extraction, embryo and yolk sac were thawed on ice. To homogenize tissue samples, 1ml of TRIzol™ reagent was added per 50-100mg of tissue and the disaggregation of the tissue aided by pipetting. The homogenised samples were incubated for 5 min at room temperature (R/T) to permit the complete dissociation of nucleoprotein complexes. 20% of the initial used volume of TRIzol™ reagent, chloroform was added to the tubes. The tubes were vigorously shaked for 15 sec prior to incubate at R/T for 2-3 min. The tubes were centrifuged in a microcentrifuge at not more than 12000 g for 10 min at 4°C to pellet the RNA which was washed once with 75% ethanol then dissolved in 5-20μl of DEPC-treated water and stored at -70°C until required.

2.11.3 Determination of RNA concentration:

To determine the concentration of the RNA samples, an optical density (OD) reading at 260nm, using a dual beam spectrophotometer, was utilised. An O.D. reading of 1 corresponds to 40μg/ml of the RNA. The integrity of the RNA

was assessed by comparing the ratio of O.D. at 260/280nm. A good quality preparation should give a value of 2.

2.11.4 Qualitative assessment of the RNA:

The quality of the RNA was detected by running 1μl of the RNA on 1% denaturing agarose gel containing 16.6% formaldehyde and 1 x MOPS (10 x MOPS; 200mM MOPS Sodium salt, 50mM Sodium Acetate, 10mM EDTA, pH7.0). 50% formamide, 2.2M formaldehyde, and 1 x MOPS was added to 5μl of RNA (1μl RNA + 4μl ddH_2O) to a total volume of 10μl, and incubated at 55-60°C for 10 min before quenching on ice. 1μl of loading buffer (50% glycerol, 1mM EDTA pH8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to the sample and loaded into the wells. Electrophoresis occurred at 50-100 V for 1-2 h in 1 x MOPS.

To visualise ribosomal RNA under UV light, the gel was immersed in distilled water for 10 min to wash out the formaldehyde and stained for 5 min in 5μg/ml solution of ethidium bromide. The gel was de-stained in water for 2 to 12 h and viewed under the UV light to check the integrity of ribosomal RNA bands.

2.12 Amplification of specific mRNA by reverse-transcription (RT)-PCR:

2.12.1 Reverse Transcription (RT) from RNA to cDNA:

To synthesise first strand cDNA from total RNA, the SuperScript™ kit (18089-011 Gibco BRL) pre-amplification was utilised according to the manufacturer's protocol. 5μg RNA (total RNA extracted from embryo or yolk sac 9.5 dpc) was combined with 1μl oligo(dT) (0.5μg/μl) to a total volume of 12μl DEPC treated H_2O, and incubated at 70°C for 10 min and quenched on ice for at least 1 min. 8μl of a solution containing; 1 x PCR buffer (10 x; 200mM Tris-HCl pH8.4, 500mM KCl), 2.5mM Mg^{2+}, 0.5mM dNTPs, and 10mM DDT was

added to the tube and incubated at 42°C for 5 min, followed by addition of 200 units SuperScript II RT (200 units/μl). The RT reaction was initiated by incubation at 42°C for 50 min and terminated by heating at 70°C for 15 min. To remove RNA, 1μl of RNase H was added to the tube, incubated at 37°C for 20 min, and stored at -70°C until use.

2.12.2 Amplification of target cDNA (PCR):

PCR was initiated by addition of 1μl cDNA product from RT (2.12.1) to a fresh microeppendorf tube containing; 1 x PCR buffer (Cambio), 1.5mM Mg^{+2}, 0.2mM dNTPs, 0.5μM of each primer sets, and 2 units Taq DNA polymerase in a final volume of 50μl. The PCR solution was overlaid by 50μl mineral oil and the PCR was performed 25 cycles of: 94°C (denaturing) for 1 min, 53-61°C (annealing) (Table 2.1) for 1-2 min, and 72°C (extension) for 2 min. The PCR reaction was terminated with 1 cycle 72°C extension for 5 min.
And the PCR products were detected on an agarose gel.

2.13 Northern analysis:

During this project, Northern blotting was utilised to study the expression of *Igf2r* gene in 9.5 dpc embryos bred onto either NIH/Ola or C57Bl/6J/Ola strains. 9.5 dpc embryos were dissected and then snap-frozen in liquid Nitrogen. The total RNA was extracted as described before (2.11.3).

2.13.1 RNA gel electrophoresis:

8-10μg of the total RNA extracted from embryos was run alongside a 1kb size marker in 0.8% denaturing agarose gel in circulating 1 x MOPS at 50-60V for 12 h as described before (2.11.5).

2.13.2 Northern Blotting:

The gel was washed for 20-30 min in Milli-Q-water with 2-3 changes to wash out the formaldehyde. The marker lane was cut off and stained for 10 min in 1.5μg/ml ethidium bromide before de-staining in Milli-Q-water for 2-16 hours. The marker was then viewed on a UV transilluminator and photographed alongside a scale for reference. The rest of the gel was placed on a piece of 10 x Standard Saline Citrate (SSC) (20 x SSC; 3M NaCl, 300mM Na citrate, pH7.4) soaked 3 MM Whatman paper, which was acting as a wick to a reservoir of 10 x SSC. Hybond N paper was cut to the size of the gel and placed on top, and any trapped air bubbles smoothed out. Two pieces of dry 3 MM Whatman paper were placed on top of the Hybond N, and the area surrounding the gel was sealed with clingfilm. This was to prevent the evaporation of the 10 x SSC reservoir, and to ensure its passage only through the gel. A stack of paper towels were placed on top of the dry Whatman paper, followed by a small weight (1kg). The blot was left overnight, and the following day the RNA was fixed on to the filter by UV irradiation for 4 min. The filter was wrapped in clingfilm and stored at 4°C until use.

2.13.3 Probe labelling:

80-100ng of probe was labelled and cleaned by using Nick™ column as described before (2.6.4).

2.13.4 Northern Hybridisation:

The Hybond N filter and a fine mesh were soaked in 2 x SSC before rolling together and placing in a hybridisation bottle with 10-15mls of pre-

hybridisation buffer [5 x SSC, 1 x Denharts [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA (Bovine Serum Albumin, RNase/DNase free, 27-8914-02 Pharmacia Biotech)], 50% formamide, 0.5% SDS, 100μg/ml Salmon Sperm DNA] depending on the size of the filter. Incubation occured for 3-5 hours at 42°C. The radioactive probe was denatured and added at 3 x 10^7cmp to the hybridisation buffer (pre-hybridisation buffer with the addition of 10% dextran sulphate). This replaced the pre-hybridisation buffer, and incubation at 42°C continued for a further 16 hours.

2.13.5 Washing and autoradiographing the filter after hybridisation:

The filter was washed and autoradiographed as described before (2.6.6-8).

3: Results

The aim of this project was to investigate factors modifying the phenotypic outcome of TGFB1(-/-) animals described in an earlier study (Dickson et al., 1995). To investigate the effects that genetic background might have on prenatal lethality, the *Tgfb1* null allele was to be bred through four generation onto C57Bl/6J/Ola and NIH/Ola (by F.M. Cousins) to obtain mice which were ≈93% pure for each strain.

3.1 Genotyping animals for *Tgfb1* applying a single polymerase chain reaction (PCR) protocol:

A dual PCR approach was previously utilized to genotype animals for *Tgfb1* (Kulkarni et al., 1993). However, in this project a single combined PCR protocol was developed (figure 3.1). The advantages of this PCR protocol were as follows:

1) Half as many PCRs were required to screen animals for *Tgfb1*. The nature of this project required screening more than 1800 transgenic animals. Thus, decreasing the number of PCRs was important in terms of time and economical benefits.

2) In the dual reaction PCR approach, failure in either reaction could result in misgenotyping animals for *Tgfb1.* In the single combined PCR approach each reaction was an internal control for the other.

3.2 Phenotypic assessment:

In the previous study, using mixed genetic background animals (12.5% 129/Sv, 50% NIH/Ola and 37.5% C57Bl/6J/Ola), the phenotypes of TGFB1(-/-) embryos were classified into two groups; embryo lethality at 9.5 dpc (yolk sac phenotype) and survival to birth (Dickson et al., 1995). In this project survival to birth was utilized as the unambiguous criterion for animal classification to identify genetic and/or non-genetic modifying factors.

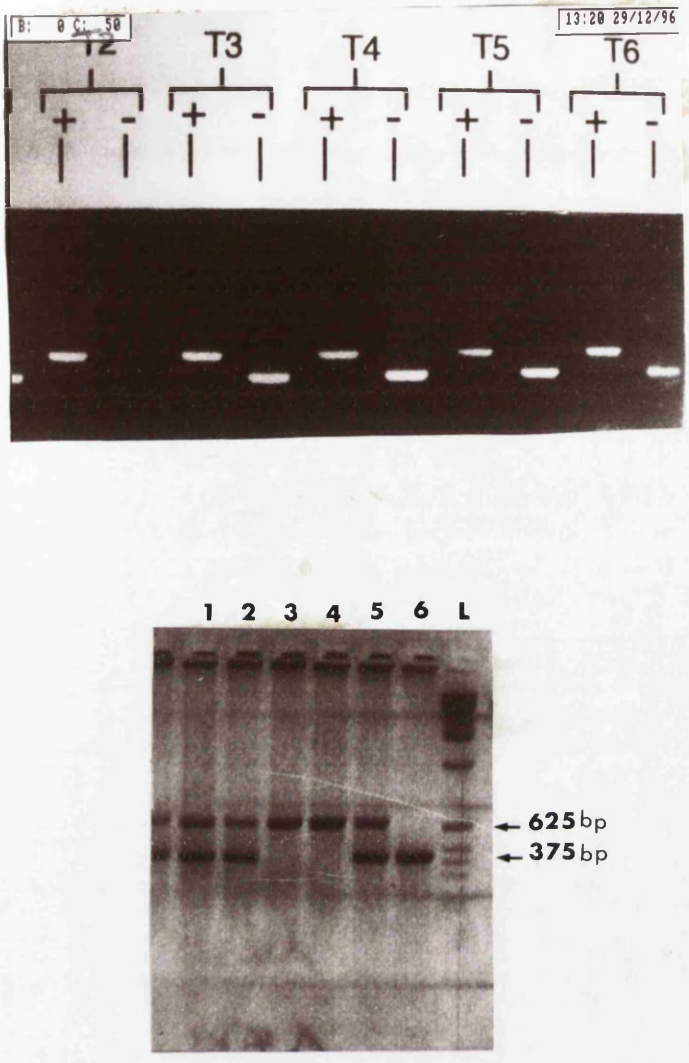


Figure 3.1: Genotyping animals for *Tgfb1* gene by application of PCR.

a) **Conventional PCR approach:** In this approach, a dual reaction PCR was utilised to genotype animals. In one of the PCR reactions, the wild type allele (625 bp) for *Tgfb1* gene was amplified and in the other reaction, the knockout allele (375 bp) was amplified. Animal T2= wild type, Animal T3= heterozygous, Animal T4= heterozygous, Animal T5= heterozygous, Animal T6= heterozygous.

b) **Single combined PCR approach:** In this project, a single combined reaction PCR was developed to genotype animals for the *Tgfb1* gene. The wild type allele (625 bp) and the knockout allele (375 bp) were amplified simultaneously in a single PCR reaction. Animal No. 1 is heterozygous, No. 2 is heterozygous, No. 3 is wild type, No. 4 is wild type, No. 5 is heterozygous, No 6 is null.

3.3 Estimation of the percentage of TGFB1(-/-) neonate survivals:

In order to generate TGFB1(-/-) animals, crosses between TGFB1(-/+) animals were set up. If there was no prenatal loss of TGFB1(+/-) and TGFB1(-/-), the Mendelian ratio of 1:2:1 for TGFB1(-/-), TGFB1(+/-) and TGFB1(+/+) animals would be expected. To avoid any interference by TGFB1(-/+) lethality which has been reported by previous researchers (Shull & Duchman., 1992, Dickson et al., 1995), the percentage survival (observed/expected) of TGFB1(-/-) embryos to birth was estimated as N° (-/-) / N° (+/+). The premise was that any possible factor leading to prenatal loss of TGFB1(+/+) embryos would have an equal effect on TGFB1(-/-) embryos.

To estimate the percentage survival of TGFB1(-/-) neonates to birth, all neonates were culled prior to the stage of postnatal death 3 weeks *post-partum* (Shull et al., 1992) and were screened for *Tgfb1* genotype. The data obtained from this screening are shown in table 3.1. The percentage of TGFB1(-/-) born to NIH/Ola genetic background was estimated to be 82% whereas that of born to C57Bl/6J/Ola was 0% which indicated that there were strain-specific modifying factor(s) determining survival versus pre-natal lethality of TGFB1(-/-) conceptuses.

To confirm strain-specific dependence of the TGFB1(-/-) phenotype, 2 x 2 contingency statistical analysis was performed (Appendix I-A, Table 1) assuming the null hypothesis for involvement of strain-specific factors on the phenotype (Table 3.1). The P-value ($<<10^{-5}$), by excluding the possibility of the null hypothesis, confirms that different expressivity of the TGFB1(-/-) phenotype was due to strain specific factor(s).

3.4 Possible factors involved in different expressivity of TGFB1(-/-) phenotypes:

The possible factors involved in modifying the phenotypic outcome of TGFB1(-/-) conceptuses may be categorized into three classes:

Table 3.1 The number of TGFB1 wild type and null neonates developing to term in two NIH X NIH and C57 X C57 crosses. To compare the number of TGFB1(-/-) neonates born to different strains, 2 X 2 contingency statistical analysis (Appendix I, Table 1) assuming the null hypothesis for involvement of strain-specific factors in the phenotype, was performed (Data extracted from table 3.2).

Group	TGFB1(+/+) neonates	TGFB1(-/-) neonates	Total
NIH/Ola	89	73	162
C57Bl/6J/Ola	68	0	68
Total	157	73	230
$\chi 2$	42.83		
P-value	$<<10^{-5}$		

1) Strain-specific role of TGFB1 in gametogenesis.

2) Strain-specific influence of maternal factors on the survival and development of TGFB1(-/-) conceptuses.

3) Cardinal effect of the genetic background of TGFB1(-/-) conceptuses on the phenotypic outcomes.

To investigate the involvement of these possible factors in the differential expressivity of TGFB1(-/-) phenotype, various combinations of intercrosses between NIH/Ola and C57Bl/6J/Ola were set up (Table 3.2). These results were analyzed in separate groups to evaluate the involvement of possible factors in the different expressivity of TGFB1(-/-) phenotype.

To investigate the possibility that there was selective failure or low transmission of the knockout allele (*Tgfb1*) through maternal gametes in C57Bl/6J/Ola strain, statistical analysis on the results of various crosses using C57Bl/6J/Ola females (Table 3.3.a) was performed. Assuming the involvement of *Tgfb1* gene in transmission of maternal gametes and/or gametogenesis, one would expect to observe no significant difference in percentage of TGFB1(-/-) neonates born to females from the same genetic background in various crosses. However, the calculated χ^2 of 19.49, 9.44 and 31.7 (Appendix I-B, Table 1,2,3) for the data from table 3.3a,b and c, with the relevant P-value of 5.8×10^{-5} , 8×10^{-3} and 10^{-5},respectively,excluded this possibility.

The estimated χ^2 of 15.00 obtained from statistical analysis of various crosses using C57Bl/6J/Ola males (Table 3.4) with the relevant P-value of 5.5×10^{-4} also ruled out the possible involvement of *Tgfb1* gene in transmission of male gametes and/or male gametogenesis process.

To address the possible effect of maternal factors (such as maternal circulating TGFB1) on the phenotypic outcome of TGFB1(-/-) conceptuses, statistical analysis was carried out (Appendix I-B) on the results obtained from different breedings (Table 3.3, a,b,c). In this table, three different crosses were statistically analysed. In each group(a or b or c), three different crosses were compared with one another and despite the fact that all females were from the same genetic background, statistically significant deviation was observed in the

Table 3.2: Intercrosses between various combinations of NIH/Ola and C57Bl/6J/Ola , and the number of TGFB1 transgenic animals born to each cross. These results were analyzed in separate groups to evaluate the involvement of possible factors in the different expressivity of TGFB1(-/-) phenotype.

Cross	TGFB1(-/-)	TGFB1(+/-)	TGFB1(+/+)
NIH x NIH	73	129	89
NIH x F1	14	52	21
F1 x NIH	85	235	118
F1 x F1	80	530	190
NIH x C57	15	90	50
C57 x NIH	9	50	25
C57 x F1	3	50	29
F1 x C57	9	163	75
C57 x C57	0	122	68

Table 3.3: The number of TGFB1$^{-/-}$ embryos developing to term in different crosses using females from the same genetic background (Data extracted from table 3.2). The calculated χ^2 and the relevant P-values are shown (statistical analyses are demonstrated in Appendix I-B).

Cross	TGFB1 (-/-) embryos	TGFB1 (+/+) embryos	Percentage of nulls survived
a) Females from C57Bl/6J/Ola			
C57 X C57	0	68	0%
C57 X F1	3	29	10.3%
C57 X NIH	9	25	36%
χ^2	**19.49**		
P-value	**5.8×10^{-5}**		
b) Females from NIH/Ola			
NIH X NIH	73	89	82%
NIH X F1	14	21	66.66%
NIH X C57	15	50	30%
χ^2	**9.44**		
P-value	**8.9×10^{-3}**		
c) Females from F1 (NIH X C57)			
F1 x NIH	85	118	72%
F1 x F1	80	190	42%
F1 x C57	9	75	12%
χ^2	**31.7**		
P-value	**$<<10^{-5}$**		

Table 3.4: The number of TGFB1(-/-) embryos developing to term in different crosses using males from C57Bl/6J/Ola genetic background (Data extracted from table 3.2). The calculated χ^2 and the relevant P-value are shown.

Cross	TGFB1 (-/-) embryos	TGFB1 (+/+) embryos	Percentage of nulls survived
males from C57Bl/6J/Ola			
C57 X C57	0	68	0%
F1 x C57	9	75	12%
NIH x C57	15	50	30%
χ^2	**15.00**		
P-value	**5.5×10^{-4}**		

number of TGFB1(-/-) embryos developing to term. These results excluded the involvement of maternal factors in different expressivity of TGFB1 null phenotypes in two strains. However, the possible effect of maternal factors in the survival of TGFB1(-/+) to birth which could have a similar influence in both strains, remained to be elucidated.

3.5 Candidate gene approach:

Concurrently with the collection of DNA samples for a full genome search, the candidate gene approach was taken, to investigate possible genetic factors involved in determining different expressivity of TGFB1(-/-) phenotype. In a preliminary study, carried out on a small number of TGFB1(-/-) animals bred onto mixed genetic backgrounds, suggestive linkage of TGFB1(-/-) survival with markers tightly linked to Insulin like growth factor type II receptor (*Igf2r)* was shown (Bonyadi, Msc thesis, 1994). Therefore, further investigation of this gene was pursued to strengthen or exclude the linkage data, certain studies were carried out on *Igf2r* to investigate.

3.5.1 Insulin like growth factor type II receptor (*Igf2r*):

The following investigations were carried out on the *Igf2r* gene:
1) *Igf2r* gene expression study.
2) Investigation on 2.05kb of the cDNA of *Igf2r* gene.

1) *Igf2r* expression study:

Igf2r expression was investigate in 9.5 dpc embryos bred onto two genetic backgrounds, i.e. NIH/Ola and C57Bl/6J/Ola strains. Embryos were dissected, total RNA was extracted, and checked for quality and quantity on a denaturing agarose gel (Figure 3.2). Applying Northern blotting, the gene expression level of *Igf2r* at 9.5 stage was compared between embryos from NIH/Ola and C57Bl/6J/Ola strains (figure 3.3). As the results indicate, at 9.5 dpc stage of embryo development there was no significant difference in the expression of *Igf2r* gene between two strains; NIH/Ola and C57Bl/6J/Ola.

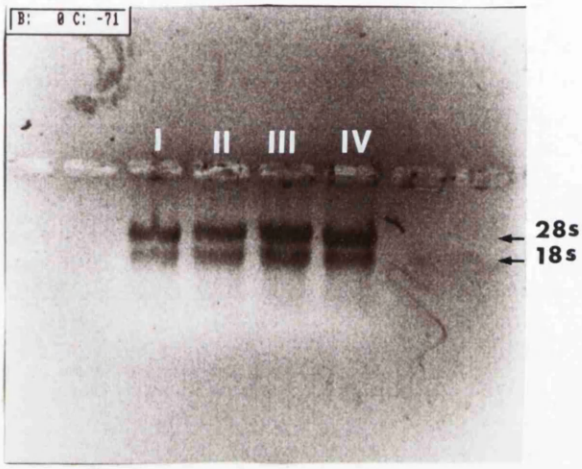


Figure 3.2: Qualifying and quantifying RNAs extracted from 9.5 dpc embryos on a denaturing agarose gel: RNAs from whole embryos were extracted and run on a denaturing agarose gel. In each lane, 2μl of the RNA extracted from either 9.5 dpc embryos from NIH/Ola (lane I, II), or from 9.5 dpc embryos from C57Bl/6J/Ola strains were run on a denaturing agarose gel. The density of 28s rRNA and 18s rRNA was used to check the quantitiy and quality of RNAs.The concentration of RNAs in all lanes of denaturing agarose gel is the same.

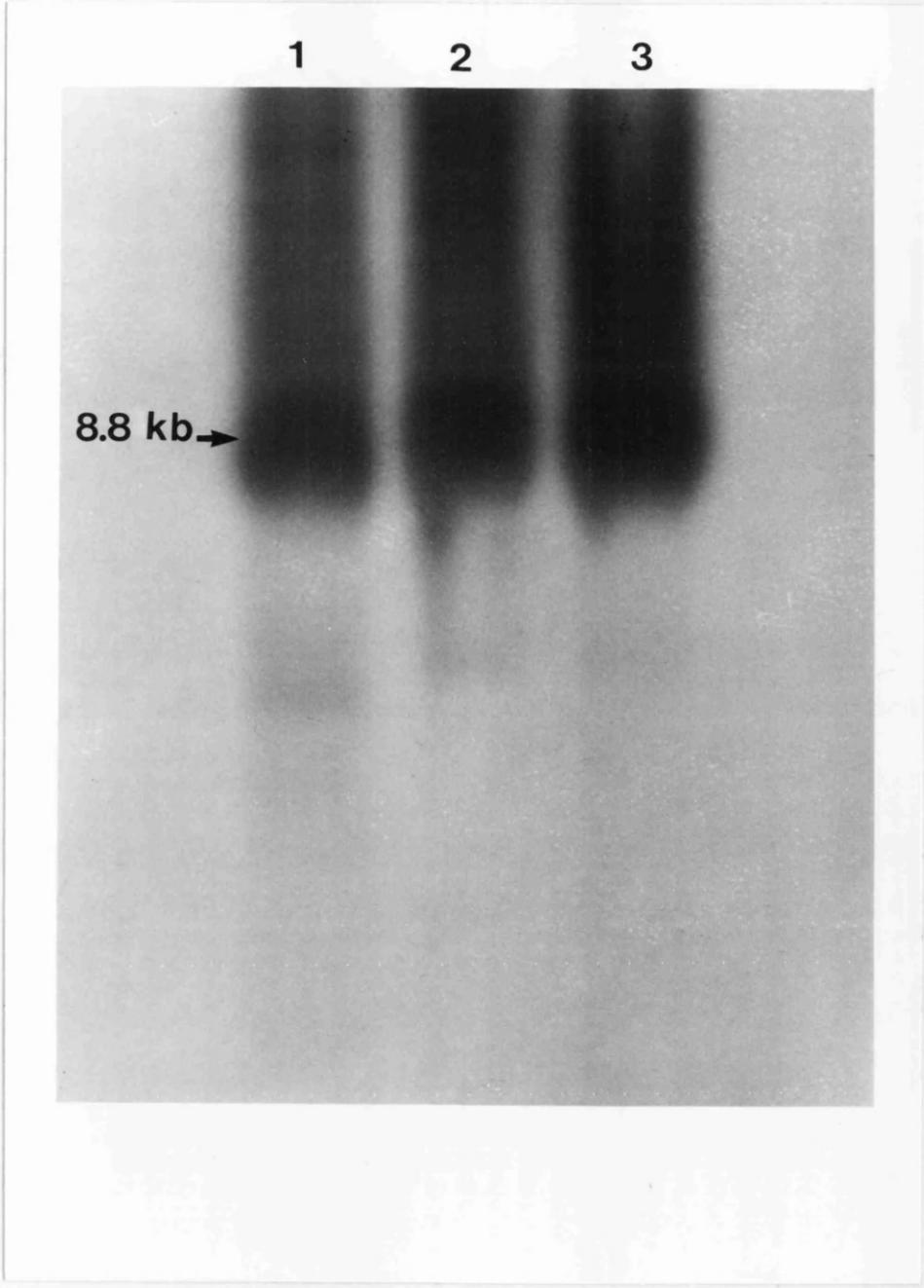


Figure 3.3: Northern Blotting of *Igf2r*. The expression of *Igf2r* gene was examined at the 9.5 dpc stage in embryos of the mouse strains NIH/Ola (lane 1), C57Bl/6J/Ola (lane 2) and 129/Sv (lane 3). No significant difference is observed in the expression of the *Igf2r* gene at the 9.5 dpc stage between embryos from NIH/Ola, C57Bl/6J/Ola and 129/Sv strains. The full length of mRNA for *Igf2r* gene is 8877 bp (Embl access number is: MMU04710).

2) Investigation of the cDNA of *Igf2r* gene.

To investigate the possibility that strain specific polymorphisms are present in the protein encoded by *Igf2r*, 0.755 (6699→7453) + 1.3 (1700→2982) Kb of the mRNA were amplified by applying RT-PCR (Figures 3.4 a,b) on RNA from the two strains, NIH/Ola and C57Bl/6J/Ola. These regions were subjected to Chemical Mismatch Cleavage (CMC) analysis (figures 3.5 a,b). Since the sensitivity of CCM is about 90% (Cotton et al., 1988), from figures 3.5a, and b, it could be concluded that the coding sequence for *Igf2r* in the regions under investigation is identical (with 90% certainty).

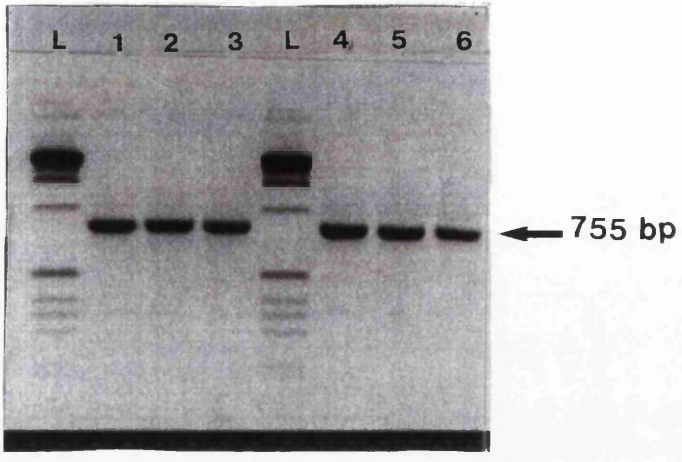
Prior to completion of the screening of the entire coding sequence of *Igf2r*, results obtained from new breedings dismissed the possible involvement of the *Igf2r* in the survival of homozygous *Tgfb1* null embryos, thus this aspect of the project was discontinued.

3.5.2 Evidence for exclusion of imprinted genes:

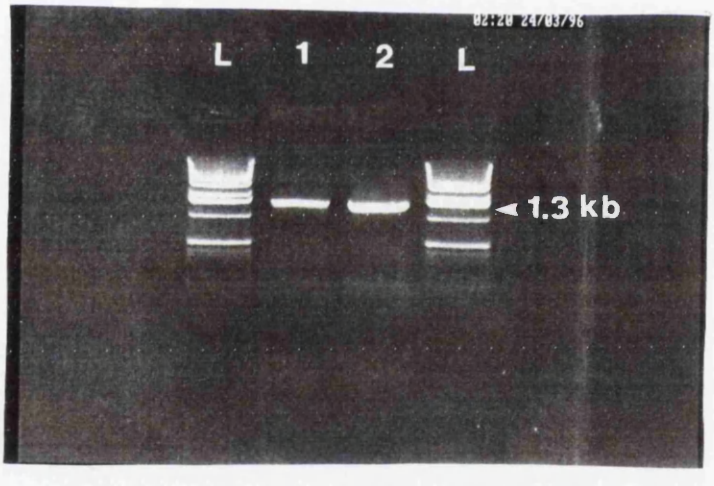
In this study, the possible involvement of imprinted genes in which expression of the genes dependent on parent-of-origin, was carefully examined. The data obtained from various reciprocal crosses (Table 3.7), excluded the possible involvement of any imprinted genes (e.g. *Igf2r* gene which is pateranally imprinted) in different expressivity of the TGFB1(-/-) phenotype.

3.6 Estimating the number of gene(s) involved in determining differential survival of TGFB1(-/-) neonates:

Before embarking on a detailed mapping project, it was useful to derive an estimate of the number of segregating genes involved in the expression of the trait under analysis. If we assume that a single codominant gene with two alleles explains the pattern of survival, then the maximum likelihood estimates



(a)



(b)

Figure 3.4: RT-PCR products of the coding region of the *Igf2r* gene.

a) A 755 bp fragment from coding region of the *Igf2r* gene was amplified by applying RT-PCR. RNA was extracted from 9.5 dpc NIH/Ola embryos (lanes 1, 2, 3) or C57Bl/6J/Ola embryos (lanes 4, 5, 6). L; 1-kb ladder.

b) A 1.3 kb fragment from coding region of the *Igf2r* gene was amplified by applying RT-PCR. RNA was extracted from NIH/Ola embryos (lane 1) or C57Bl/6J/Ola embryos (lane 2). L; 1-kb ladder.

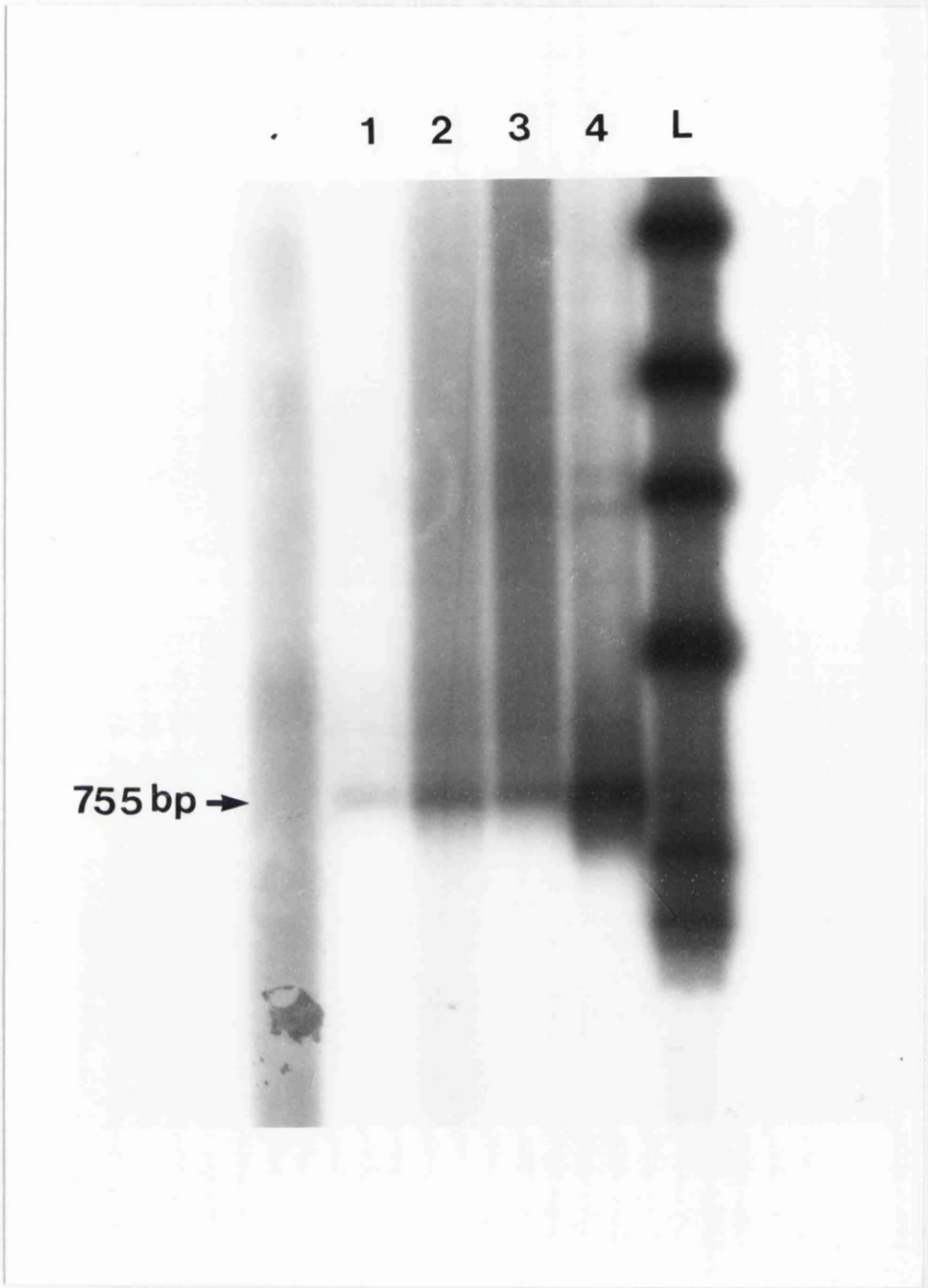


Figure 3.5: Chemical Mismatch Cleavage analysis on the coding region of *Igf2r* gene.

a) RT-PCR products (755 bp) of *Igf2r* cDNAs from NIH/Ola and C57Bl/6J/Ola strains were subjected to chemical mismatch cleavage analysis. No polymorphism in this region of *Igf2r* cDNA is observed between NIH/Ola and C57Bl/6J/Ola strains. **Lane 1**; The hybrid (i.e. NIH / C57) was treated with hydroxylamine, **Lane 2**; The control treated with hydroxylamine (i.e. C57), **Lane 3**; The hybrid (i.e. NIH / C57) was treated with osmium tetroxide, **Lane 4**; The control (i.e. C57) treated with osmium tetroxide, **L**; 1-kb ladder.

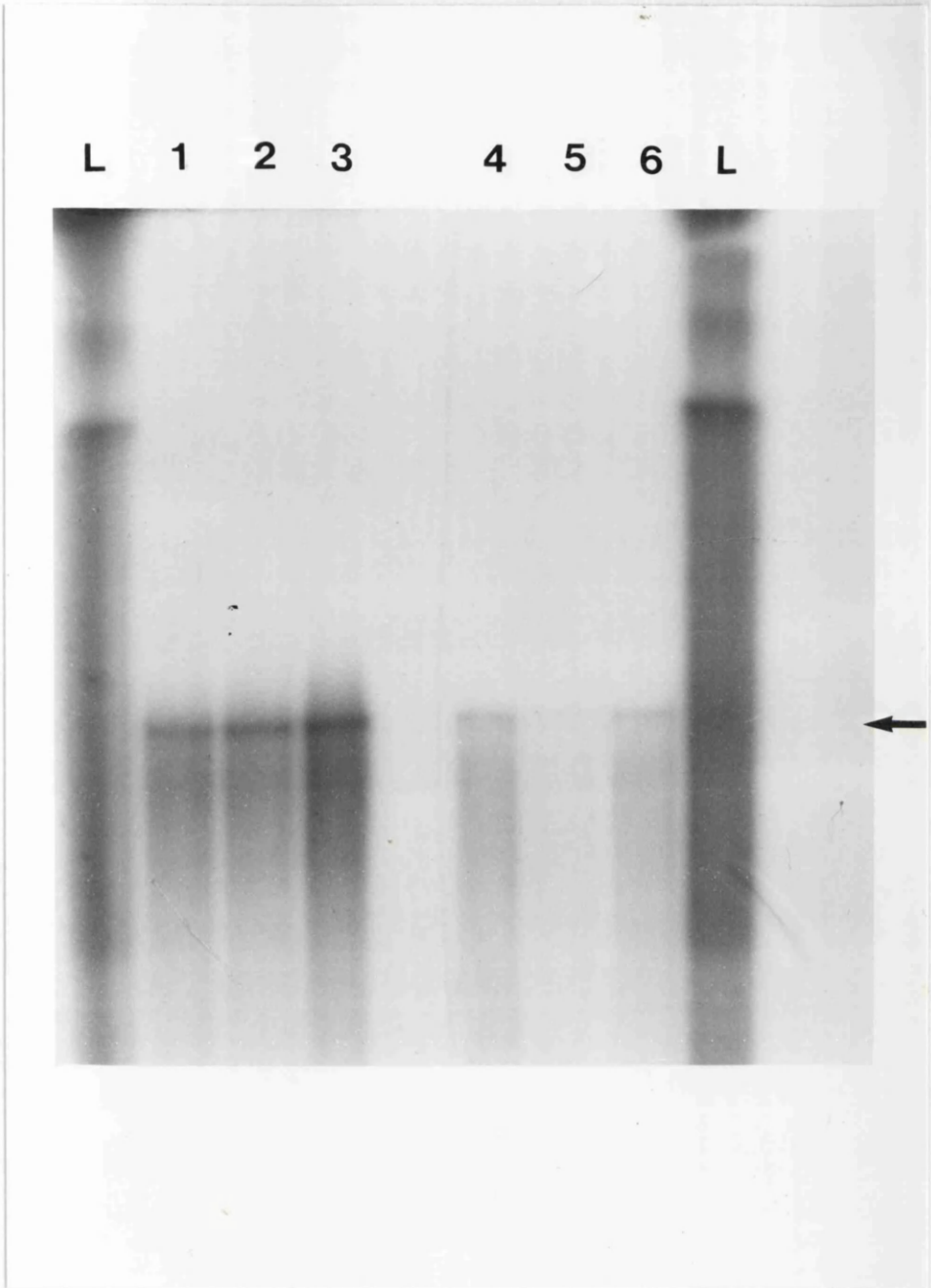


Figure 3.5: Chemical Mismatch Cleavage analysis on the coding region of *Igf2r* gene.

b) RT-PCR products (1.3 kb) of *Igf2r* cDNAs from NIH/Ola and C57Bl/6J/Ola strains were subjected to chemical mismatch cleavage analysis. No polymorphism in this region of *Igf2r* cDNA is observed between NIH/Ola and C57Bl/6J/Ola strains. **Lanes 1, 2**; The hybrid (i.e. NIH / C57) was treated with hydroxylamine, **Lane 3**; The control treated with hydroxylamine (i.e. C57), **Lane 4, 5**; The hybrid (i.e. NIH / C57) was treated with osmium tetroxide, **Lane 6**; The control (i.e. C57) treated with osmium tetroxide, **L**; 1-kb ladder.

for the survival rates for N/N, C/C, and N/C genotypes are 86%, 0%, and 32% respectively. These estimates take into account the varying numbers of pups as well as attempting to allow for the missing pups. The observed and predicted relative survival rates are shown in table 3.5. The relevant P-value is more than 0.05 (table 3.5), therefore we could conclude that the data were adequately modeled by a single codominant gene with two alleles (NIH, C57). In this model, F1 animals showed incomplete dominance (codominant trait) of the C57 allele, and mostly they were poised between lethality and survival.

3.6.1 Towards genetic mapping of loci modifying TGFB1(-/-) phenotype:

An important choice upon beginning a new linkage study is whether to screen F1 backcross animals or F1 intercross. In this study, due to the codominant nature of the locus (loci), and to procure more informative animals and high-resolution mapping for the purpose of positional cloning, the intercross approach was applied for the linkage analysis.

3.7 Sex-linked genes:

The X Chromosome mostly comprises genes involved in sex-linked traits. The X chromosome constitutes at least 5% of the whole genome. To study the possible involvement of any sex-linked genes, further detailed analysis of the intercross data was required.

A): The percentage of TGFB1(-/-) male and female survivals:

The ratio of male to female TGFB1(-/-) survivals in different crosses (table 3.6) indicates that there was no significant difference between different crosses which excludes involvement of major X-linked genes in the different expressivity of TGFB1 null phenotypes.

B): The percentage of TGFB1(-/-) null neonates in reciprocal crosses:

Table 3.5: Presentation of the observed and predicted relative survival rates in different crosses (Data extracted from table 3.2), assuming that a single gene with two alleles explains the pattern of survival.

Cross	(Number) TGFB1 (-/-) neonates	(Number) TGFB1 (+/+) neonates	(Percent.) Observed (-/-) neonates	(Percent.) predicted (-/-) neonates	(Number) expected (-/-) neonates
C57 x C57	**0**	68	0%	0%	**0**
NIH x NIH	**73**	89	82%	86%	**76.54**
F1 x NIH	**99**	139	71.2%	59%	**82.01**
F1 x C57	**12**	104	11.5%	16%	**16.64**
F x F1	**80**	190	42%	37.5%	**71.25**
χ^2	**6.051**				
P	**0.195**				

Table 3.6: Displaying the number of males and females born to different crosses (Data extracted from table 3.2) and the calculated Chis Square and the pertinent P-value.

Cross	Number of TGFB1(-/-) Males	Number of TGFB1(-/-) Females
NIH x NIH	27	33
NIH x F1	47	37
NIH x C57	10	14
χ^2	**2.38**	
P-value	**0.1**	

Table 3.7: Presentation of the number of TGFB1(-/-) born to reciprocal crosses (Data extracted from table 3.2) and the calculated χ^2 and the relevant P-value (2 X 2 contingency table was applyed for statistical analysis, Appendix I-A).

Cross	TGFB1(-/-)	TGFB1(+/+)	Percentage of TGFB1(-/-)
NIH x F1	14	21	66.6%
F1 x NIH	85	118	72%
χ^2	**0.04**		
P-value	**0.85**		
NIH x C57	15	50	30%
C57 x NIH	9	25	36%
χ^2	**0.13**		
P-value	**0.72**		
C57 x F1	3	29	10%
F1 x C57	9	75	12%
χ^2	**0.03**		
P-value	**0.85**		

Assuming the involvement of sex-linked loci in the phenotype, significant deviation between the percentage of TGFB1(-/-) neonates in reciprocal crosses would result. The results of reciprocal crosses between NIH/Ola and C57Bl/6J/Ola presented in table 3.2 are analyzed statistically (table 3.7). As results in table 3.7 indicate there was no significant differences in the number of TGFB1(-/-) survivals between reciprocal crosses. Therefor, these results verified the exclusion of the involvement of major sex-linked gene in different expressivity of the TGFB1(-/-) phenotype.

3.8 Genome screening (Autosomal chromosomes):

In this study, 150 microsatellites scattered throughout the genome were screened for polymorphisms between NIH/Ola and C57Bl/6J/Ola strains (Appendix II-C). In terms of the location and being polymorphic, only 55 markers were informative for linkage analysis in this project. Moreover, two Restriction Fragment Length Polymorphism's (RFLPs) for two important candidate genes, transforming growth factor type II receptor (*Tgbr2*) and plasminogen activator inhibitor type I (*PlanH1*), not previously mapped in the murine genome, were identified and utilised in this research (section). These RFLPs were also applied to map genes in the mouse genome.

3.8.1 Number of animals and markers required for preliminary study:

The first stage in the linkage approach was mapping to a sub-chromosomal interval. An optimal strategy to obtain high-resolution linkage mapping is to select animals with a specific phenotype, to choose an adequate number of affected animals, and to develop a framework map anchored by previously well-mapped loci spaced uniformly throughout the entire genome.

3.8.1.1 The phenotypic assessment taken in this project was previously discussed (3.2).

3.8.1.2 Number of animals and markers:

To obtain a high-resolution map with both an average crossover resolution of 0.1 cM and an average marker density of one per cM, one would have to analyse 500 intercross animals for segregation at 1500 marker loci (spanning 1500 cM) which would require 750,000 independent typings. Therefore in this study a much more efficient approach was taken by dividing the protocol into two separate stages. The goal of the first stage was to link the locus (loci) to a defined sub-chromosomal interval. This approach was accomplished by typing a relatively small set of markers on a relatively small number of phenotypically typed animals. To develop a framework map which covers the entire genome, it was critical to calculate the minimum number of anchor loci sweeping the entire genome by screening a minimum number of phenotypically typed animals. This calculation was based on the length of the swept radius. The swept radius, the distance over which linkage can be detected between any marker and a test locus typed in a set number of animals was defined originally in terms of map distances (Carter & Falconer 1951).
With 100 backcross samples, the framework swept radius is at least 0.23 recombinant fraction (rf) (Carter & Falconer 1951, Frankel et al., 1990). To a first approximation, each intercross sample is equivalent to two backcross samples. Thus, a swept radius of 0.23 rf can be obtained with 50 intercross samples. A further increase in the critical number of samples (45-50) provide only a marginal increase in the distance that is swept. In this study, as it is shown on table 3.8 and the figures presented in Appendix II-B, by applying 54 informative markers conducted on 50 intercross samples, about 90% of the genome was swept for a major modifying gene.

3.9 Statistical Methods applied in this project:

3.9.1 Null hypothesis:

In statistical analysis of data, it was important to predict the null hypothesis, which means that alleles at different genes would assort independently, leading to a 1:1 ratio of gametes with parental or recombinant combinations of alleles. In this study, by applying a null hypothesis in the analysis of each marker, it became possible to apply a statistical test to

Table 3.8: The number of markers per chromosome utilized to screen 48-80 F2 (NIH/Ola X C57Bl/6J) designed panel and the approximate percentage of the genome scanned in this research. Swept radius is taken 0.23 rf.

Chromosome	Number of animals	Number of Markers	Length (cM) of the chromosome	Length (cM) of the chromosome not swept	Percentage of the chromosome swept.
1	48-50	4	116.61	0 cM	**100 %**
2	48-50	2	97.34	29 cM	**70 %**
3	48-80	2	68.62	4 cM	**93 %**
4	48-50	4	76	0 cM	**100 %**
5	80	10	87.32	0 cM	**100 %**
6	48-50	3	64.5	0 cM	**100 %**
7	48-50	1	70.9	3.98 cM	**94 %**
8	48-50	3	76.4	0 cM	**100 %**
9	48-50	5	72.37	0 cM	**100 %**
10	48-50	2	74.74	0 cM	**100 %**
11	48-50	2	86.	14 cM	**83 %**
12	48-80	4	62.75	0 cM	**100 %**
13	48-50	2	63.45	0 cM	**100 %**
14	48-50	4	62.27	0 cM	**100 %**
15	48-50	2	63.97	0 cM	**100 %**
16	48-50	3	55.76	0 cM	**100 %**
17	48-50	4	52	0 cM	**100 %**
18	48-50	1	40.6	0 cM	**100 %**
19	48-50	3	59.1	0 cM	**100 %**

determine whether the data actually observed were significantly different from the expected outcomes for no linkage. The expected outcome for each marker in this project in an F2 intercross was 25%, 50% and 25% for homozygosity for the NIH/Ola allele, heterozygosity (NIH/C57), and homozygosity for the C57Bl/6J/Ola allele respectively.

3.9.2 The χ^2 test for intercross data:

The standard method to evaluate whether non-Mendelian recombination results are statistically significant is the method of χ^2. Upon calculating a value for χ^2 , it is possible to determine the likelihood that an observed set of data represents a chance deviation from the values predicted by a particular hypothesis. This determination provide information to reject or accept the hypothesis that is being tested.

The general form of the χ^2 statistic is defined as follows:

$$\chi^2 = \sum_{i=1}^{n} \frac{[\text{Observed} - \text{Expected}]^2}{\text{Expected}}$$

Where n are potential outcome classes. Quick examination of the equation, shows that the χ^2 value is inversely related to the goodness-of-fit between the experimental results and the null hypothesis being tested. The size of the data set is important when the χ^2 test is applied. It can not be applied to very small data sets, which are defined as those in which 20% or more of the outcome classes have expected values that are less than five (Cochran, 1954).

3.10: Genome-wide search by applying microsatellites or restriction fragment length polymorphisms (RFLPs):

3.10.1: Applying markers tightly linked to the candidate genes:

Prior to screening the whole genome using markers scattered randomly throughout the genome, several important candidate genes already mapped on the mouse genome were investigated for their possible implication in the

different expressivity of the TGFB1(-/-) phenotype. To achieve this purpose, markers tightly linked to the candidate genes were utilized.

3.10.1.1: TGFB isoforms:

To investigate the possibility of functional redundancy with other TGFB isoforms in the two different strains; NIH/Ola and C57Bl/6J/Ola, informative markers linked to TGFB2 and TGFB3 located on chromosomes 1 (101.5 cM) and 12 (41cM) respectively, were selected (Appendix II-B). Performing linkage studies conducted on 48-50 TGFB1(-/-) survivals by applying the linked markers (Appendix III), excluded any possible linkage of the TGFB isoforms with TGFB1(-/-) survivals.

3.10.1.2 Endoglin (Eng):

Due to histological similarities seen in HHT1 patients which have allelic variation in the *ENG* gene (McAllister et al., 1994) and the TGFB1(-/-) embryos dying due to yolk sac phenotype (Dickson et al., 1995), the *Eng* gene was chosen as a candidate gene in this study. The segregation of the gene was to be followed among TGFB1(-/-) neonates in F2 intercross. To achieve this purpose, a tightly-linked-marker (Qureshi et al.,1995) was selected (Appendix II-B) and studied on 48-50 TGFB1(-/-) survivals. As the result indicates (Appendix III), there was no significant segregation distortion from 1:2:1 for homozygous NIH, heterozygous, and homozygous C57 alleles of the marker respectively. Thus, it excludes the involvement of *Eng* or any linked gene to the different experssivity of TGFB1(-/-) phenotype.

3.10.2: Applying RFLPs for screening unmapped candidate genes:

3.10.2.1 Transforming growth factor beta type II receptor(*Tgfbr2*):

The TBR2 is a receptor for the ligands TGFB1, TGFB2 and TGFB3 (Wrana et al., 1994). Its absence result in lethality of embryo in uterus at 9.5-11.5 dpc due to deficiency in haematopoiesis and yolk sac vasculagenesis

(Oshima et al., 1996). To investigate the possible involvement of *Tgfbr2* in different expressivity of TGFB1 null phenotype, a 1.7-kb coding region cDNA probe was utilised to identify informative RFLPs between NIH/Ola and C57Bl/6J/Ola strains. BamH1 digestion of genomic DNA revealed an RFLP that was 14 kb in NIH/Ola and 8 kb in C57Bl/6J/Ola. A 317-nt BglII-SacI fragment hybridised with the BamH1 RFLP was designed (Figure. 3.6). Probe for *Tgfbr2* was used to probe DNA samples from 40 F2 (NIH x C57) TGFB1(-/-) null survivals, to study the segregation of RFLPs among survivals (table 3.9). Significant deviation from the expected distribution was not observed among TGFB1(-/-) survivals. Therefore, the possible implication of *Tgfbr2* gene in different expressivity of the TGFB1(-/-) phenotype, was ruled out.

3.10.2.2 Plasminogen activator inhibitor type I (PlanH1):

It has been proposed that PlanH1 involves in the activation of latent TGFB. And its implication in a number of vascular diseases has been reported (Grainger et al., 1993 and 1994). To investigate the possible implication of PlanH1 in phenotypic outcomes of TGFB1 null conceptuses, a murine *PlanH1* coding region cDNA was utilized to identify informative RLFPs between NIH/Ola and C57Bl/6J/Ola strains. Different enzyme-digested-DNAs from C57Bl/6J/Ola and NIH/Ola were analyzed by Southern blot hybridization for informative RLFPs, using the full length murine PlanH1 coding region cDNA (520 bp). Estimated fragments of 4 kb and 4.3 kb were detected in BamH1-digested NIH/Ola and C57Bl/6J/Ola DNAs, respectively (figure 3.7).
Applying full length murine *PlanH1* cDNA, 48-50 TGFB1 null survivals were screened for distribution of *PlanH1* alleles (table 3.10). As analysis of the obtained data (table 3.10) indicates, no significant deviation from 1:2:1 for homozygous NIH, heterozyogus and homozyogus C57 alleles respectively was observed among TGFB1(-/-) neonates. Therefore, the possible involvement of *PlanH1* in different expressivity of TGFB1(-/-) phenotype, was excluded.

3.10.3 Genome- Wide search by applying random selected markers:

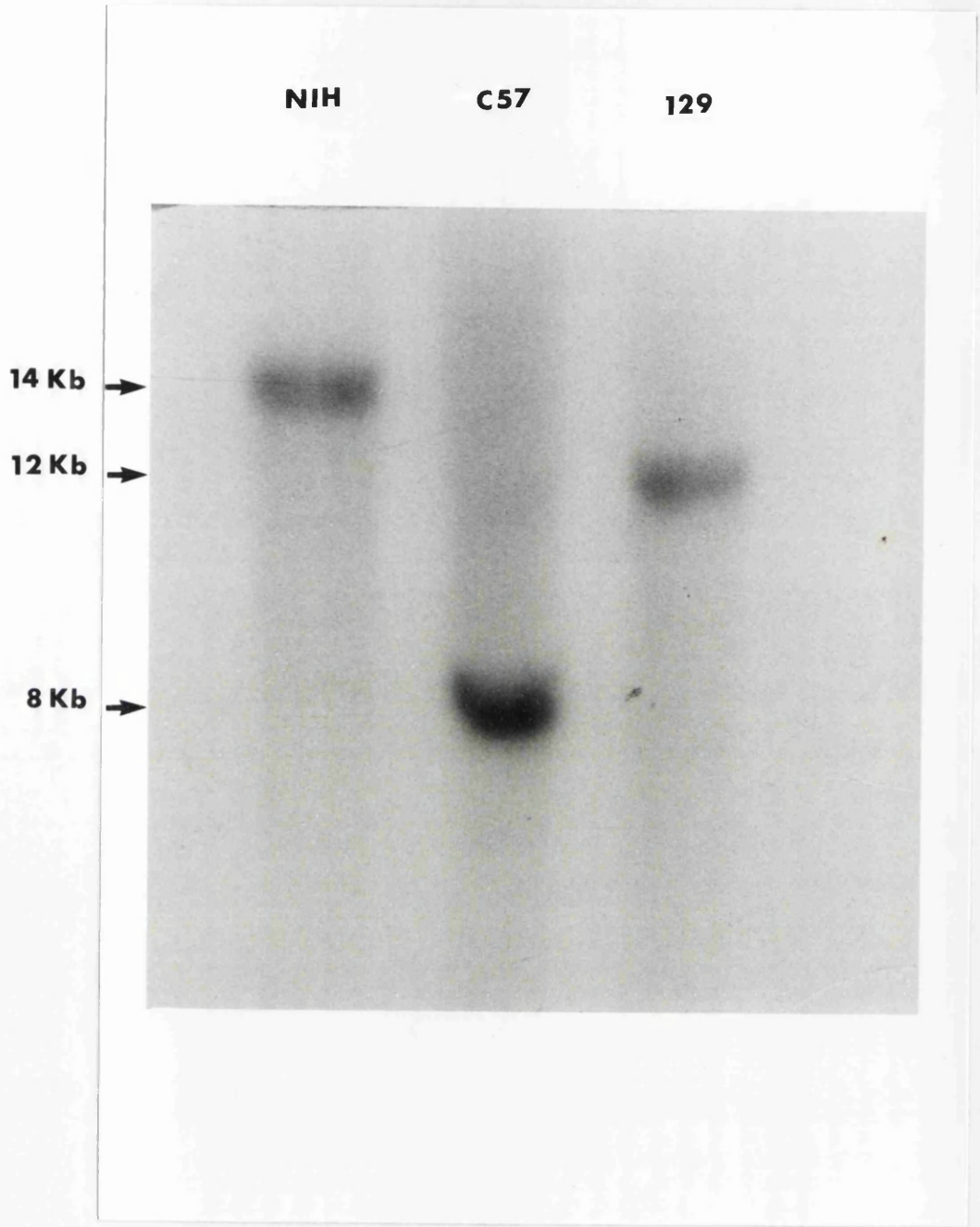


Figure 3.6: Southern Blotting analysis of the *Tgfbr2* gene from NIH/Ola and C57Bl/6J/Ola strains. 317 bp BglII-SacI fragment from the coding region of the gene was used as a probe. BamH1 digestion of genomic DNA revealed an RFLP that was 14 kb long in NIH/Ola and 8 kb in C57Bl/6J/Ola, and 12 kb in 129/Sv strain.

Table 3.9: Presentation of the segregation of RFLPs for *Tgfbr2* gene among 40 F2 (NIH X C57) TGFB1(-/-) animals developing to term.

Probe	Total TGFB1 (-/-) neonates	N / N	N / C	C / C
Tgfbr2	40	12	14	14
χ^2	3.8			
P-value	0.149			

Table 3.10: Presentation of the segregation of RFLPs for *PlanH1* gene among 48 F2 (NIH X C57) TGFB1 (-/-) animals.

Probe	Total TGFB1 (-/-) neonates	N / N	N / C	C / C
PlanH1	48	15	26	7
χ^2	**3.2**			
P-value	**0.2**			

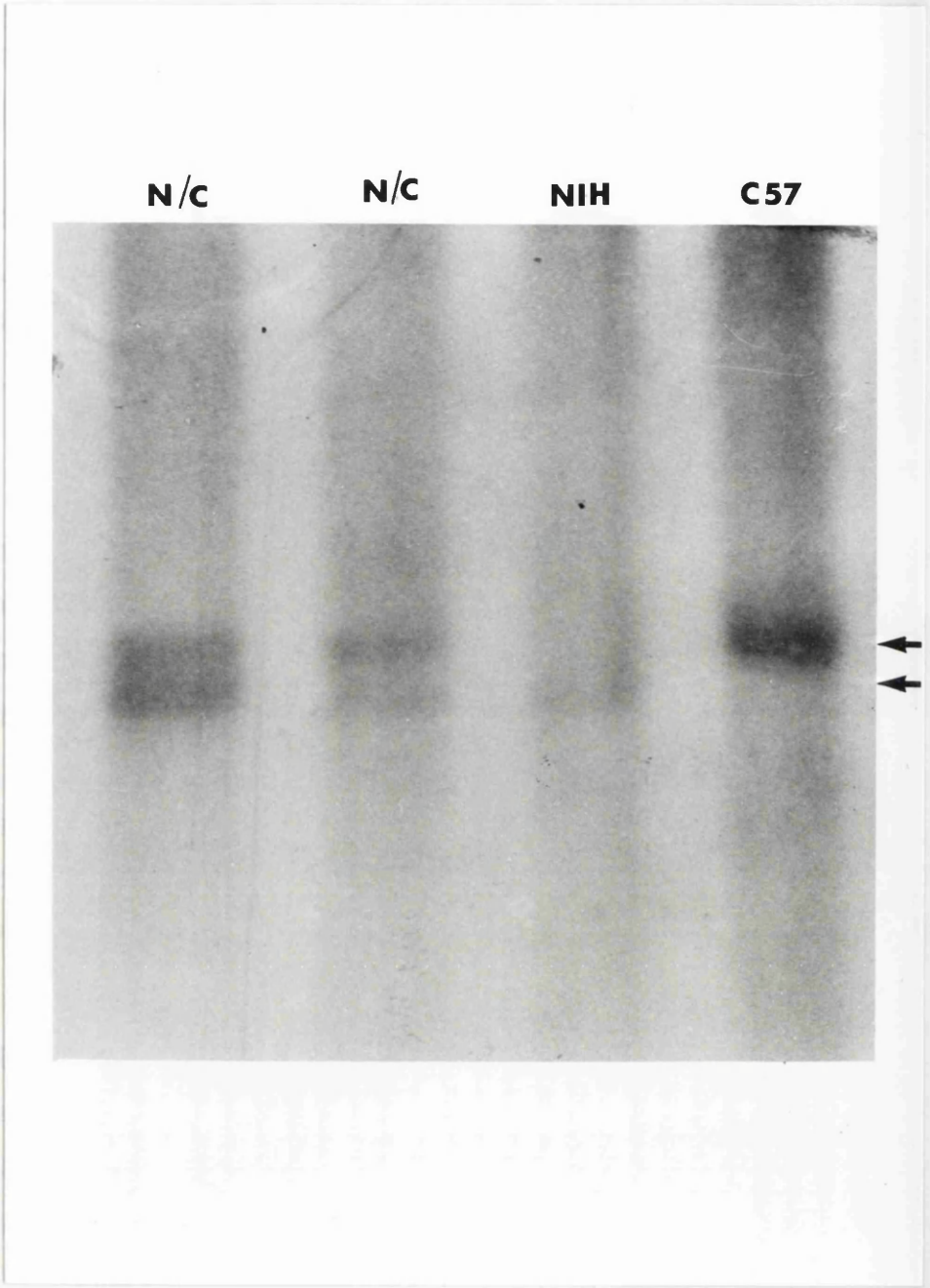


Figure 3.7: Southern Blotting analysis on *PlanH1* gene from NIH/Ola and C57Bl/6J/Ola strains. Full length of the coding region of the gene was used as a probe. The fragments were approximately 4 kb and 4.3 kb in the BamHI-digested NIH/Ola and C57Bl/6J/Ola DNAs, respectively.

Most of the markers used in this project were chosen on the basis of the previously published data that they were more informative between C57Bl/6J/Ola intra-strain and other intra-strains such as Ob, Cast, C3h, Dba, Balb, Akr, Non, Nod, Lp. However, there was no information about the size of markers on NIH/Ola strain. 150 markers were screened between NIH/Ola and C57Bl/6J/Ola. 55 informative markers in terms of being polymorphic and covering maximum space of genome were selected (Appendix II-C, III). Most of the polymorphisms were small and detectable only by running on 8% polyacrylamide gel electrophoresis (Figures 3.8).

Genetic linkage analysis was conducted on 48-50 TGFB1(-/-) neonates born to F2 (NIH/Ola X C57Bl/6J/Ola) crosses by applying 54 informative markers. In the first screen conducted on 48-50 TGFB1(-/-) nulls, four regions of the genome (D3Mit6, D5Mit268, D5Mit188, D12Mit46) showing suggestive linkage ($P \geq 0.05$) were focused for further linkage analysis. Two of the regions (D5Mit268, D5Mit188) were located on the same chromosome. Thus, there was a possibility that linkage in one of the regions resulted in segregation distortion of the other region. To estimate the distance of these markers from each other, Kosambi's formula (equation 3), or MAP_{MAKER} programme could be applied.

$$m_k = 0.25 [\ln(1+2r) - \ln(1-2r)] \qquad \text{(equation 3)}$$

By applying MAP_{MAKER} program, it was revealved that D5Mit188 was located in a region 41 cM distal to that of D5Mit268. In order to evaluate the independent support for the linked major modifier defined by D5Mit268 following formula (Martin Farral, Unpublished) was applied:

$$\left.\begin{aligned} f_{cc} &= g_{cc}(1-r)^2 + g_{cN}(r-r^2) + g_{NN}r^2 \\ f_{cN} &= 2g_{cc}(r-r)^2 + g_{cN}(1-r)^2 + 2g_{NN}(r-r^2) \\ f_{NN} &= g_{cc}r^2 + g_{cN}(r-r^2) + g_{NN}(1-r)^2 \end{aligned}\right\} \text{ equations (4)}$$

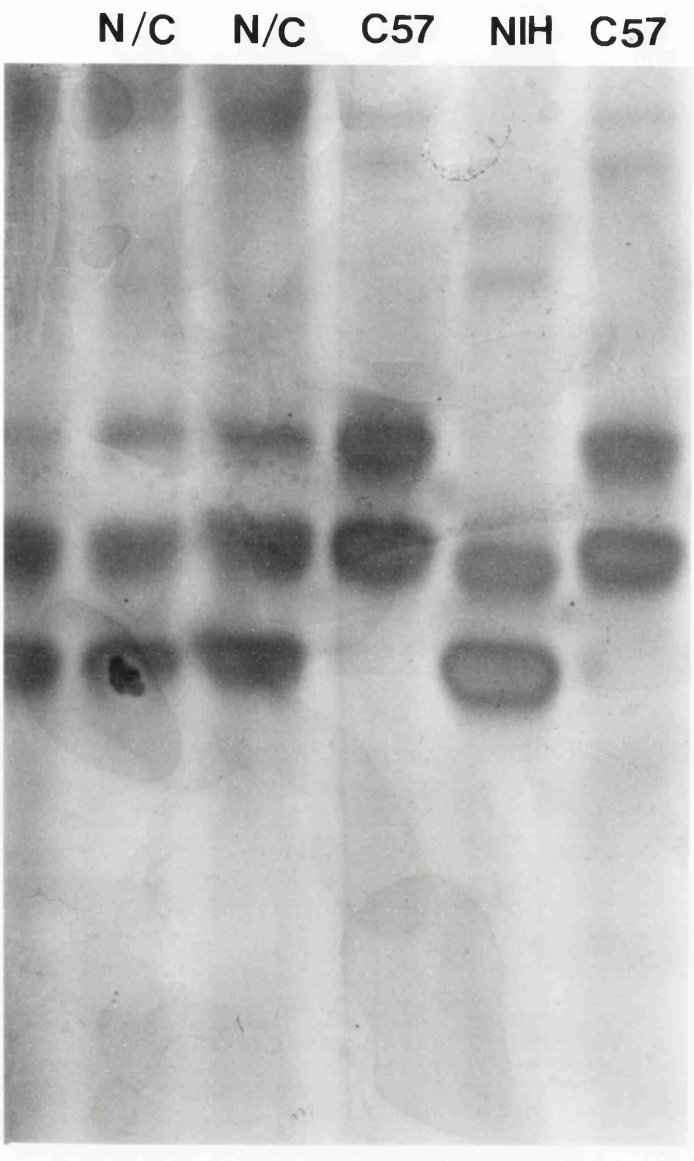


Figure 3.8: Detecting polymorphism between microsatellites from NIH/Ola and C57Bl/6J/Ola strains. Electrophoresis was carried out on an 8% polyacrylamide gel. Length polymorphism in D2Mit83 marker was less than 8bp between NIH/Ola and C57Bl/6J/Ola strains which was detectable on an 8% polyacrylamide gel.

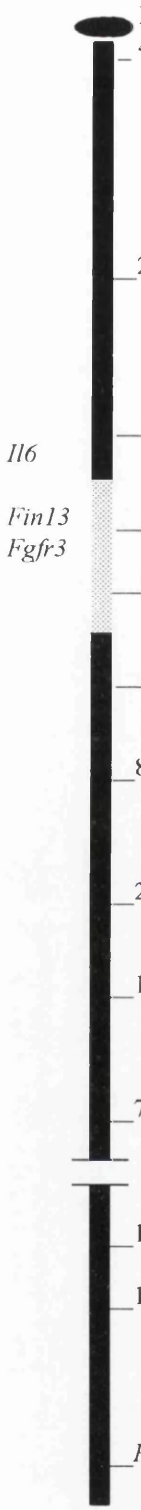
Where f_{CC}, f_{CN}, and f_{NN} are the expected genotype frequencies at a linked locus and g_{CC}, g_{CN} and g_{NN} are the observed genotype frequencies at the modifying genes (when r=0, then $f_{CC}=g_{CC}$, $f_{CN}=g_{CN}$, and $f_{NN}=g_{NN}$).

By solving the equations (4) for the observed frequencies (g_{CC}, g_{CN}, and g_{NN}) of D5Mit268, the conditional genotype frequencies expected (f_{CC}, f_{CN}, and f_{NN}) at D5Mit188 were calculated. The expected genotype frequencies are f_{CC}=18%, f_{CN}= 49%, and f_{NN}=33% which are not significantly different from the observed frequencies (P>0.05). Therefore, segregation distortion of D5Mit188 was most likely due to the affect of the segregation distortion of D5Mit268 locus not due to the possible modifying gene(s) linked to D5Mit188.

With P-value 0.05 as providing evidence for linkage in this experiment, the expected proportion of false positive loci was 60%. Therefore, in the first screen only one marker out of three independent markers showing a P-value of 0.05 was expected to be a truly linked locus. To confirm or dismiss linkage of each of four loci which appeared to show linkage, an additional 30 neonatal TGFB1(-/-) intercross DNA samples were analysed for markers on chromosomes 3, 5 and 12. P-values at the loci on chromosomes 3 and 12 remained only suggestive ($\cong$ 0.05). However, the support for linkage was strengthened at D5Mit268, surpassing the stringent linkage criteria ($\leq 10^{-5}$) (Figure 3.9).

3.10.3.1 Screening control animals for D5Mit268 maker:

In this study only one group of animals [TGFB1(-/-)] was available for linkage analysis i.e. those that survived to birth. Therefore, it was necessary to evaluate the observed linkage of chromosome 5 (D5Mit268) and provide evidence that the observed segregation distortion of the marker was genuinely due to a TGFB1 modifying gene(s) not due to any unknown possible factor. To address this possible problem, 50 wild type animals [TGFB1(+/+)] from the same cross as the TGFB1(-/-) pups were screened for segregation of D5Mit268 marker (Appendix IV). No segregation distortion (P>0.05) was observed among control animals, confirming that the linkage of the marker on TGFB1(-/-) animals must be due to a *Tgfb1* modifying gene.



Mit	cM	N/N	N/C	C/C	No. of Animals
49		18	23	8	49
	18.6				
251		17	27	5	49
	8.0				
76		34	39	7	80
Il6					
	3.3				
Fin13 268		37	35	8	80
Fgfr3	1.3				
75		35	37	8	80
	3.3				
267		33	39	8	80
	3.0				
80		21	23	5	49
	7.4				
233		20	21	8	49
	5.2				
197		20	22	7	49
	8.6				
7		21	21	7	49
	15.2				
188		31	37	12	80
	2.5				
161		26	28	9	63
	17.3				
Planh1		15	26	7	48

Figure 3.9: Map of chromosome 5 showing the location of the major modifying gene and the proximity of candidate genes. The position of the informative Mit markers used for mapping are shown to the right of the chromosome, and the map distances (cM) are indicated between each pair of markers (not drawn to scale). Map distances were calculated from the data using MAP-MAKER. Note that D5Mit268 was previously incorrectly mapped (Research Genetics), its true map location being between D5Mit76 and D5Mit267. The genotype numbers (CC:CN:NN) of the F2 neonates for each Mit marker is shown to the right of the figure. Note that 8/80 TGFB1 (-/-) mice are homozygous CC over the region containing the major modifier gene.

3.10.3.2 Genotypic Risk Ratio (GRR):

GRR was initially defined by Risch et al (1993) as the ratio of disease risk in the backcross generation, compared with that in the high-risk parental strain. In this study, the formula of GRR for the backcross generation was modified for an intercross generation (Appendix II-D) and it was defined to be the ratio of survival in the F2 generation, compared to the NIH/Ola (N/N) parental strain. From the data in table 3.2, the overall GRR_{F2} in the F2 generation is therefore equal to 42% / 82% = 51.2%.

The GRR_{F2} contributed by a specific modifying gene can be estimated from the strength of linkage between a marker and the modifying gene (equations 2 and 6, Appendix II-D). By substituting the observed genotype frequencies for D5Mit268, the estimated GRR_{F2} for the chromosome 5 major modifying gene was 54%. Risch et al (1993) proposed two multilocus genetic models, an additive model in which the effects at one modifying gene may substitute for another and a multiplicative, epistatic model of gene interaction. Under an additive model, where the overall GRR_{F2} {expressed as $[(1 : GRR_{F2}) - 1] = [(1 : GRR_1) - 1] + [(1 : GRR_2) - 1]$ } is a sum of contributions from individual modifying genes, the chromosome 5 modifying gene explains 89% of the overall GRR_{F2}. Under a multiplicative model, where the overall GRR_{F2} is the product of contribution from modifying genes (expressed as: $GRR_{F2} = GRR_1 \times GRR_2$) the chromosome 5 modifier explains 92% of the overall GRR_{F2}. Therefore, it could be concluded that the chromosome 5 modifying locus accounts for almost all of the genetic variability in the lethal embryonic phenotype.

3.11 Mapping order for the MIT markers on chromosome 5.

The mapping order and estimated map distance (cM), for the MIT markers located between centromere and D5Mit80 marker on mouse chromosome 5 was reported (Chromosome Commitee report 1996) as follow: D5MIT49 (8.6 cM)-D5MIT251 (6.7 cM)-D5MIT76 (0 cM)-D5MIT75 (1.3 cM)-

D5MIT267 (0.4 cM)-D5MIT268 (1.3 cM)-D5MIT80, whereas, in our designed panel, for the same markers the following order and estimated map distance (cM) was found: D5MIT49 (18.6 cM)-D5MIT251 (8.0 cM)-D5MIT76 (3.3 cM)-D5MIT268 (1.3 cM)-D5MIT75 (3.3 cM)-D5MIT267 (3.0 cM)-D5MIT80. On the basis of these results, D5Mit76 maps to a region 1.9 cM proximal to that of D5Mit75, whereas these markers previously were not mapped separately. Also, in this designed panel, D5Mit268 previously mapped on a region 0.4 cM distal to that of D5Mit267, maps to a region 4.7 cM proximal to that of D5Mit267 (figure 3.9).

3.12 The order of the candidate genes on the centromeric region of chromosome 5:

Three important candidate genes; Fibroblast growth factor receptor 3 (*Fgfr3*), Interleukin 6 (Il6), and Fibroblast growth factor inducible gene 13 (*FIN 13*), map in the region showing definitive linkage. However, the positions of these genes on mouse chromosome 5 relative to that of MIT markers presently mapped has not been reported. Therefore, in an attempt to map the candidate genes relative to MIT markers utilised in the designed panel, the following investigations were carried out:

3.12.1 A tandom repeat on *Fgfr3* intron:

A tandom repeat located in intron 4 (336....380nt) of *Fgfr3* gene was already reported (Avivi et al., 1992). A primer set (Table 2.1,FGFR-C,D) was designed to screen the microsatellite for the possiblity of polymorphism between NIH/Ola and C57Bl/6J/Ola strains. However, there was no size polymorphism between NIH/Ola and C57Bl/6J/Ola strains.

3.12.2 RFLPs:

In an attempt to find any possible RFLP for *Fgfr3* gene, the following restriction enzymes were utilized to digest DNA samples from NIH/Ola and

C57Bl/6J/Ola strains; ScaI, BglI, BglII, ClaI, EcoRI, EcoRV, PstI, XhoI, SalI, XbaI, KpnI, ApaI, HinfI, HindIII. The digested-DNAs were hybridized with an *Fgfr3* cDNA probe (full length). However, no informative RFLP was observed between NIH/Ola and C57Bl/6J/Ola strains.

3.12.3 The microsatellites tightly linked to *Il6*:

D5Nds8 maps to 17 cM of mouse chromosome 5, has been reported to be tightly linked to *Il6* (Breen et al., 1994). Amplified PCR of this marker between NIH/Ola and C57Bl/6J/Ola strains did not show any size polymorphism.

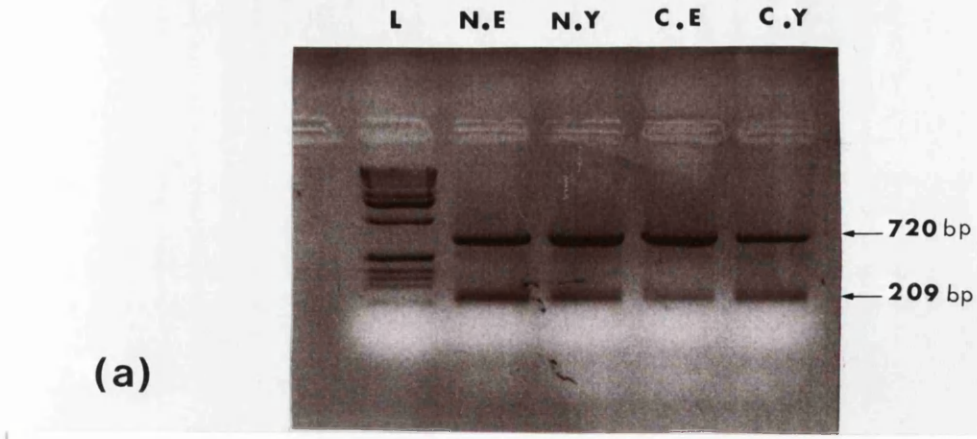
A marker for *Il6* gene reported by Jacob et al (1993) was screened for polymorphism between NIH/Ola and C57Bl/6J/Ola. The size of band was reported 125 bp in C57Bl/6J/Ola strain. The same size was observed in NIH/Ola strain. Another marker for Il6 reported by the same group, was screened between NIH/Ola and C57Bl/6J/Ola. No size polymorphism between NIH/Ola and C57Bl/6J/Ola strains was observed. The size of the marker on C57Bl/6J/Ola strain is 80 bp.

3.13 Studies carried out on Candidate genes:

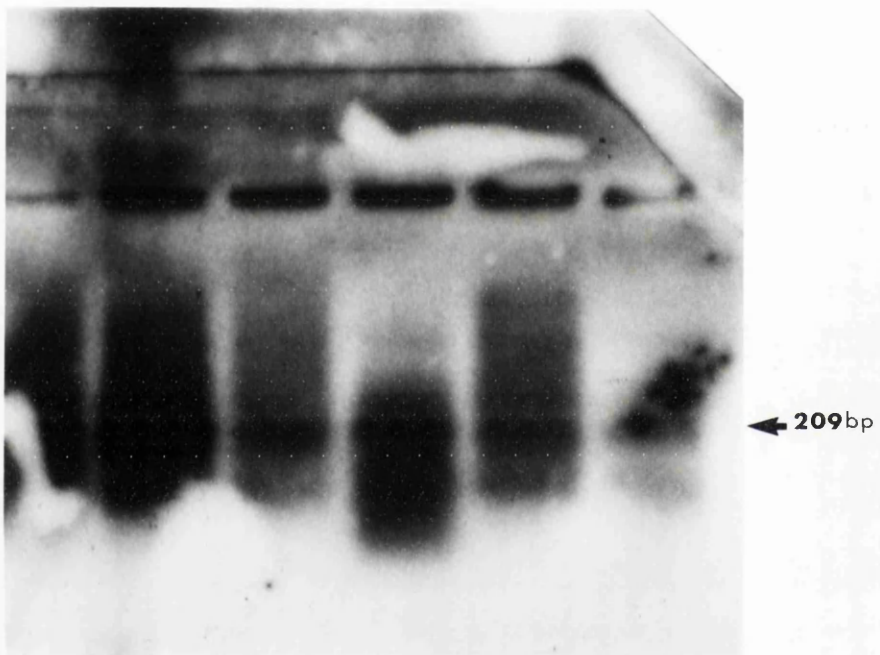
3.13.1 Expression Studies of candidate genes:

3.13.1.1 *Fgfr3*

Fgfr3 expression throughout the embryonic development (9.5-14.5 days pc) and into the early stages of fetal growth (14.5-16.5 dpc) has been reported. Its expression declined during late fetal development and then appeared to increase in the newborn (peters et al., 1993). However, there was no report about the expression of *Fgfr3* in yolk sac. To investigate the possibility of strain specific expression of *Fgfr3* at 9.5 dpc stage of embryo development, its expression was investigated by applying RT-PCR and utilizing designed primer set (Table 2.1, FGFR-A,B) from the coding sequence of *Fgfr3* (Figure 3.10 a). To confirm that the fragment amplified by RT-PCR was complementary to *Fgfr3* gene, the PCR product was blotted onto a N hybond membrane and hybridized



(a)



(b)

Figure 3.10: Expression studies on *Fgfr3* by applying RT-PCR.

a) The expression of *Fgfr3* in both yolk sac and embryo (9.5 dpc) of different strains. A single combined reaction PCR was developed to study the expression of *Fgfr3*. The 209 bp fragment is amplified from the coding region of the *Fgfr3* gene and 72o bp fragment was amplified from *GAPDH* gene which is used as a control. *Fgfr3* is expressed at 9.5 dpc stage in both yolk sac and embryo from NIH/Ola and C57Bl/6J/Ola strains. L; 100 bp ladder, N; NIH/Ola, C; C57Bl/6J/Ola, E; Embryo, Y; Yolk sac.

b) Confirmation that the PCR product is from *Fgfr3* gene. The PCR product was blotted onto a N hybond membrane and hybridised with a full length *Fgfr3* cDNA probe. The observation of the expected fragment size confirms that the PCR product is transcribed from the *Fgfr3* gene.

with a full length *Fgfr3* cDNA probe (Figure 3.10 b). The figure 3.10 b confirms that the PCR product was transcribed from *Fgfr3* gene.
As it is illustrated on figure 3.10 a, *Fgfr3* was expressed in 9.5 dpc embryos and yolk sacs in both strains.

3.13.1.2 *Fin13*:

To investigate the expression of *Fin13* in 9.5 dpc embyos and yolk sac, in both strains, a RT-PCR was performed by utilising a designed set primers (table 2.1) from coding sequences of the gene (Figure 3.11).
The possibility that the expression of *Fin13* was strain specific at 9.5 dpc embyo, is excluded. However, these results do not rule out the possiblity of different expression of FIN13 in TGFB1(-/-) embryos.

3.13.1.3 *Tgfb1*:

To investigate the expression of *Tgfb1* at 9.5 dpc embryos and yolk sacs in both strains, a set of primers was designed to perform RT-PCR from coding region of *Tgfb1* gene (Figure 3.12).
As it is illustrated in figure 3.12, *Tgfb1* expressed at 9.5 dpc stage in embryos and yolk sacs in both strains. Therefore, the possiblity of strain specific expression of *Tgfb1* was excluded.

3.13.2 Studies (heteroduplex and sequencing) on coding region of *Fin13* gene:

In an attempt to investigate the possiblity of polymorphism in *Fin13* cDNA between NIH/Ola and C57Bl/6J/Ola strains, 1074 bp length (nt. 11→563, nt. 959→1481) of the cDNA was amplified by utilising the designed primer sets (FN1A-B & FN3A-B, Table 2.2). The RT-PCR products were subjected to the heteroduplex anaylsis (HA) (figure 3.13). As it is illustrated in the figure there is no polymorphism detectable by HA in the regions under investigation between NIH/Ola and C57Bl/6J/Ola strains.

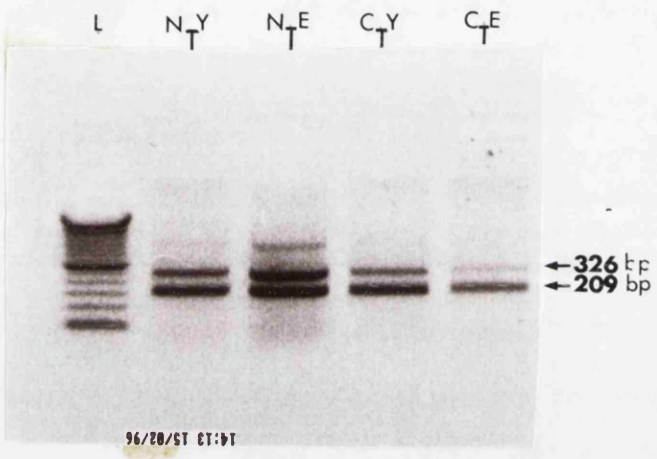


Figure 3.11: Expression studies on *Fin13* gene by applying RT-PCR. The expression of *Fin13* in both yolk sac and embryo (9.5 dpc) of different strains. A single combined reaction PCR was developed to study the expression of *Fin3*. The 326 bp fragment is amplified from the coding region of the *Fin13* gene and 209 bp fragment was amplified from *Fgfr3* gene which is used as a control. *Fin13* is expressed at 9.5 dpc stage in both yolk sac and embryo from NIH/Ola and C57Bl/6J/Ola strains. **L**; 100 bp ladder, **N**; NIH/Ola, **C**; C57Bl/6J/Ola, **Y**; Yolk sac, and **E**; Embryo.

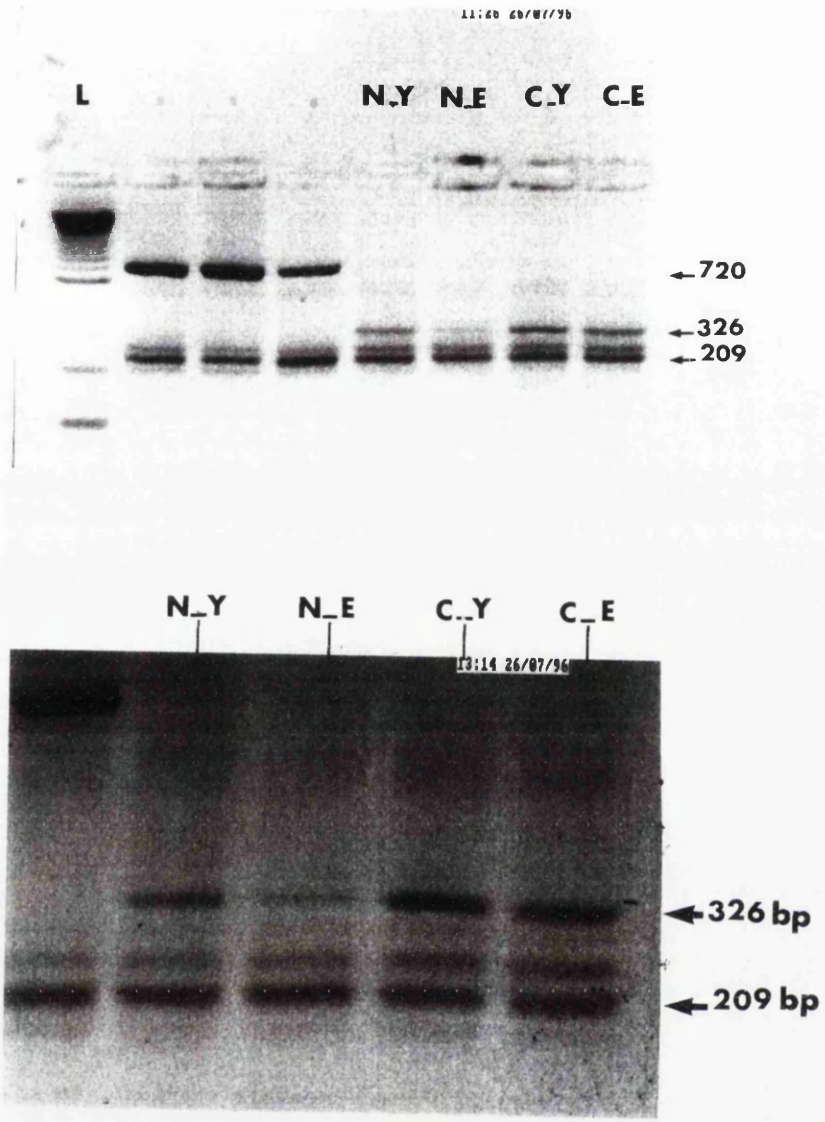


Figure 3.12: Expression studies on *Tgfb1* gene by applying RT-PCR. The expression of *Tgfb1 gene* in both yolk sac and embryo (9.5 dpc) of different strains. A single combined reaction PCR was developed to study the expression of *Tgfb1*. The 326 bp fragment is amplified from the coding region of the gene and 209 bp fragment was amplified from *Fgfr3* gene which is used as a control. *Tgfb1* is expressed at 9.5 dpc stage in both yolk sac and embryo from NIH/Ola and C57Bl/6J/Ola strains. L; 100 bp ladder, N; NIH/Ola, and C; C57Bl/6J/Ola, Y; Yolk sac, E; Embryo.

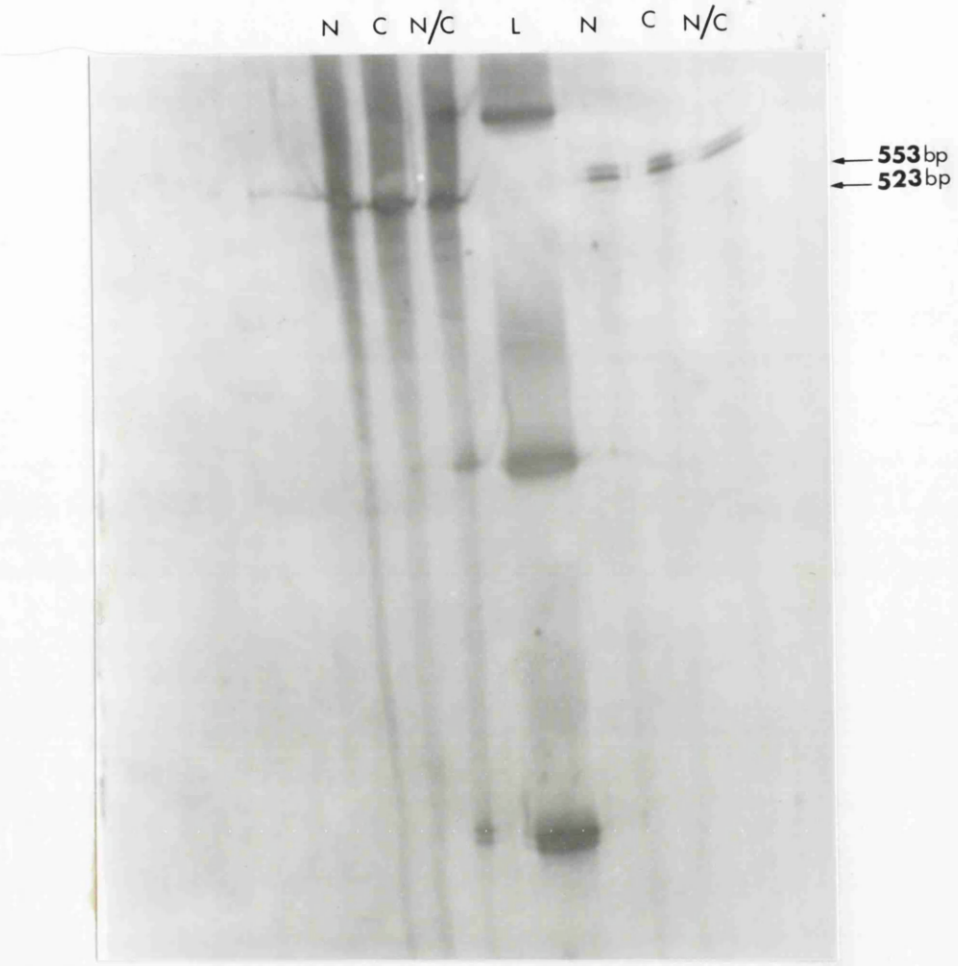


Figure 3.13: Heteroduplex analysis (HA) on *Fin13* gene. In an attempt to investigate the possible polymorphism in the coding region of the *Fin13* gene between NIH/Ola and C57Bl/6J/Ola strains, the RT-PCR products (553bp + 523 bp) from coding (and some untranslated) regions of the gene was amplified by utilising the designed primers (FN1A-B & FN3A-B, Table 2.2). The RT-PCR products were subjected to heteroduplex analysis. It can be seen that, there is no detectable polymorphism between the fragments from NIH/Ola and C57Bl/6J/Ola. **N**; NIH/Ola, **C**; C57Bl/6J/Ola, **L**; 1-kb ladder.

911 bp of the cDNA (table 3.11) (from coding and some non-coding sequence region of the gene) was sequenced (figure 3.14) to investigate possible polymorphism in the protein between NIH/Ola and C57Bl/6J/Ola strains. No polymorphism was detected in the regions under investigation.

One of the RT-PCR products (nt. 959→1481) from *Fin13* gene amplified by utilising the designed primer set (FN3A-B, Table 2.2) was subcloned for further expression studies by other people in the group. The cloning was confirmed by utilising 2μl of the plasmid as a template. The expected size of the PCR product was observed (figure 3.15) confirming the insertion of the expected PCR product in the plasmid. Sequencing the insert (figure 3.14) verified the insertion of the expected PCR product.

3.14 TGFB1(+/-) embryo lethality:

TGFB1(+/-) lethality was suggested to be due to reduced TGFB1 levels (Dickson et al., 1995). During this project, to investigated the possibility of strain specific lethality of TGFB1(+/-) embryos, statistical analysis was performed on the data obtained from NIH/Ola and C57Bl/6J/Ola crosses (Appendix IB, Table 4). As result indicate, there is no strain-specific factor responsible for TGFB1(+/-) embryo lethality in NIH/Ola and C57Bl/6J/Ola strains.

3.15 Genomic mapping of Murine Transforming Growth factor beta type II receptor (*Tgfbr2*) gene.

Using somatic cell hybridisation and fluorescence in situ hybridisation, *TBRII* has been mapped to human chromosome 3p22 (Mathew et al., 1994) in a region syntenic with mouse chromosomes 6 (49-53 cM) and distal 9 (55-74 cM) (Figure 3.16).

A 1.7-kb coding region cDNA probe was cloned by RT-PCR (Cui et al., 1995). This full length cDNA was utilised to identify informative restriction fragment length polymorphism (RFLPs) among NIH/Ola, 129/Sv, and

Table 3.11: The regions sequenced by applying different set primers of *Fin13* cDNA.

Regions sequenced (cDNA)	bp sequenced (bp)
36 → 181	145
301 → 434	133
571 → 690	119
735 → 940	205
955 → 1075	120
1138 → 1260	122
1375 → 1442	67

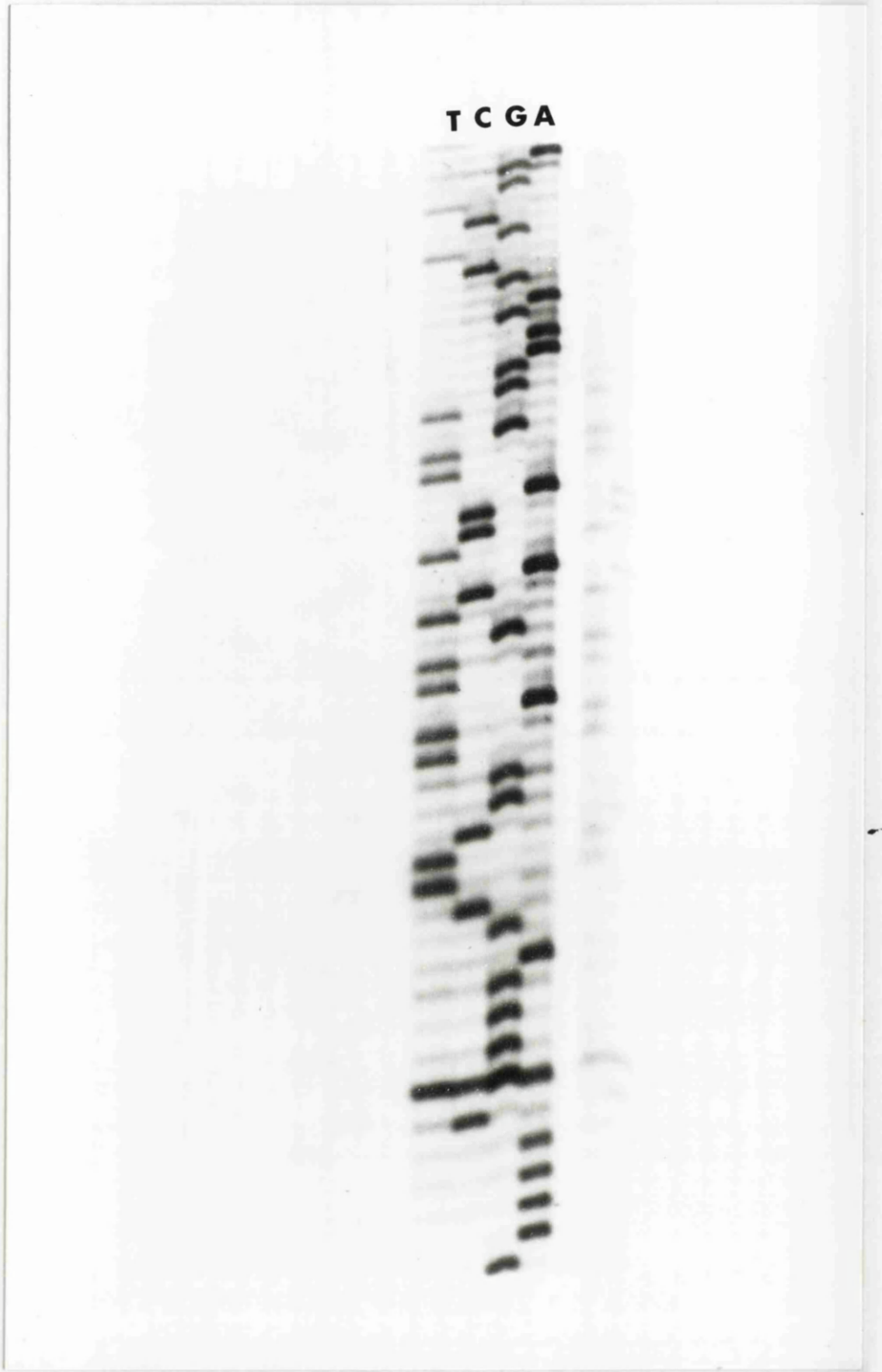


Figure 3.14: Direct sequencing of cDNA of *Fin13* gene from NIH/Ola and C57Bl/6J/Ola strains.

d) 47 bp fragment sequenced between nucleotides 1017 - 1064. **T**;Thyamine, **C**; Cytosin, **G**; Guanin, and **A**; Adenosin.

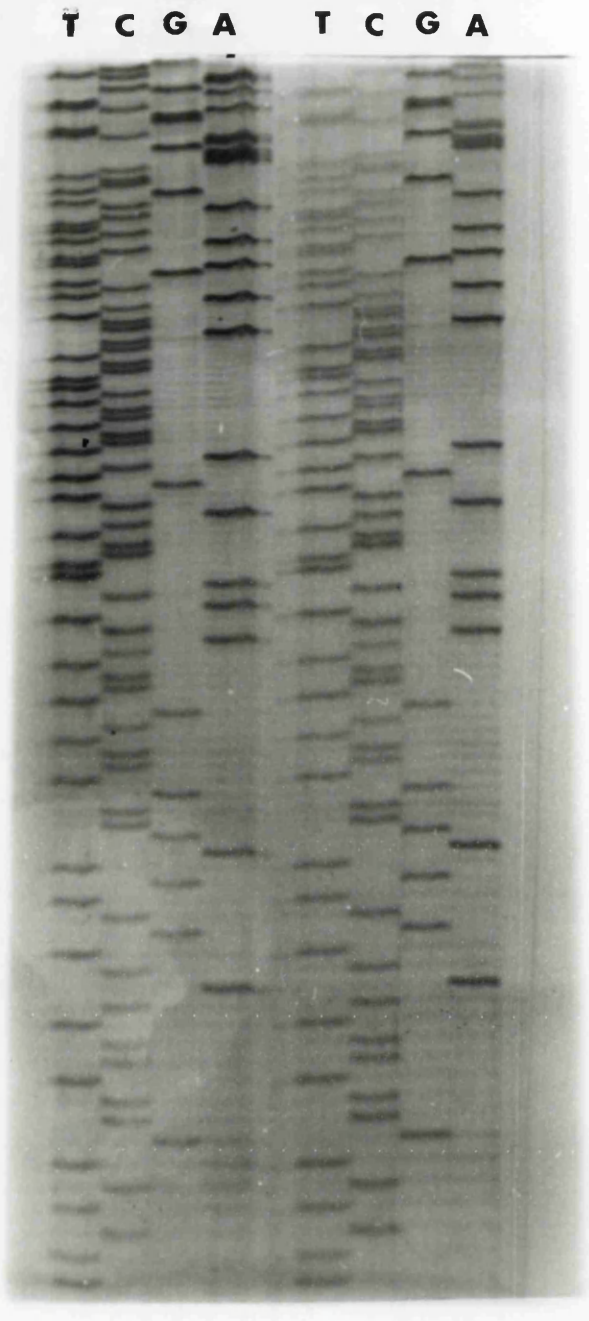


Figure 3.14: Direct sequencing of cDNA of *Fin13* gene from NIH/Ola and C57Bl/6J/Ola strains.

c) 93 bp fragment sequenced between nucleotides 414 - 507. **T**;Thyamine, **C**; Cytosin, **G**; Guanin, and **A**; Adenosin.

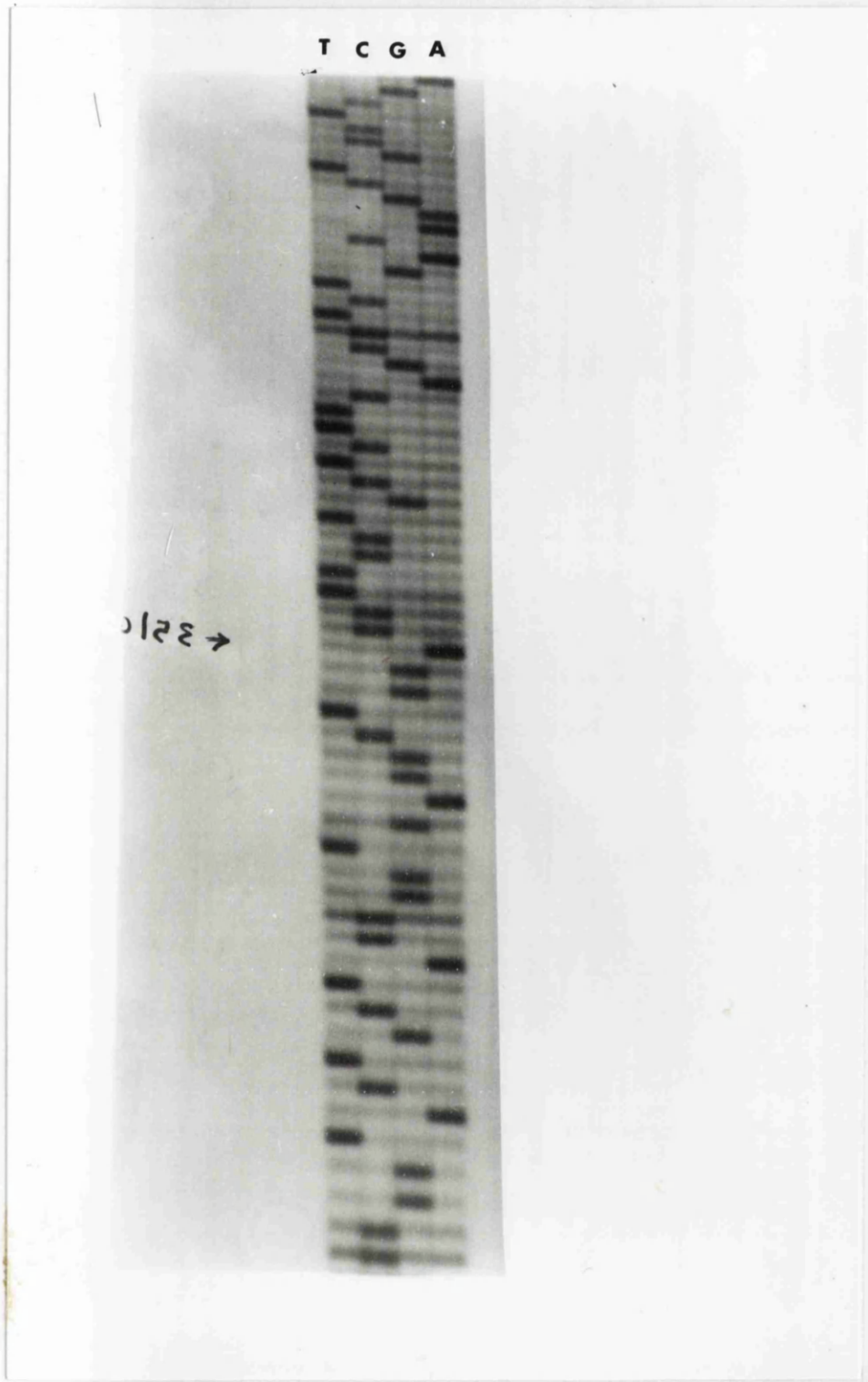


Figure 3.14: Direct sequencing of cDNA of *Fin13* gene from NIH/Ola and C57Bl/6J/Ola strains.

b) 57 bp fragment sequenced between nucleotides 322 - 379. **T**;Thyamine, **C**; Cytosin, **G**; Guanin, **A**; Adenosin.

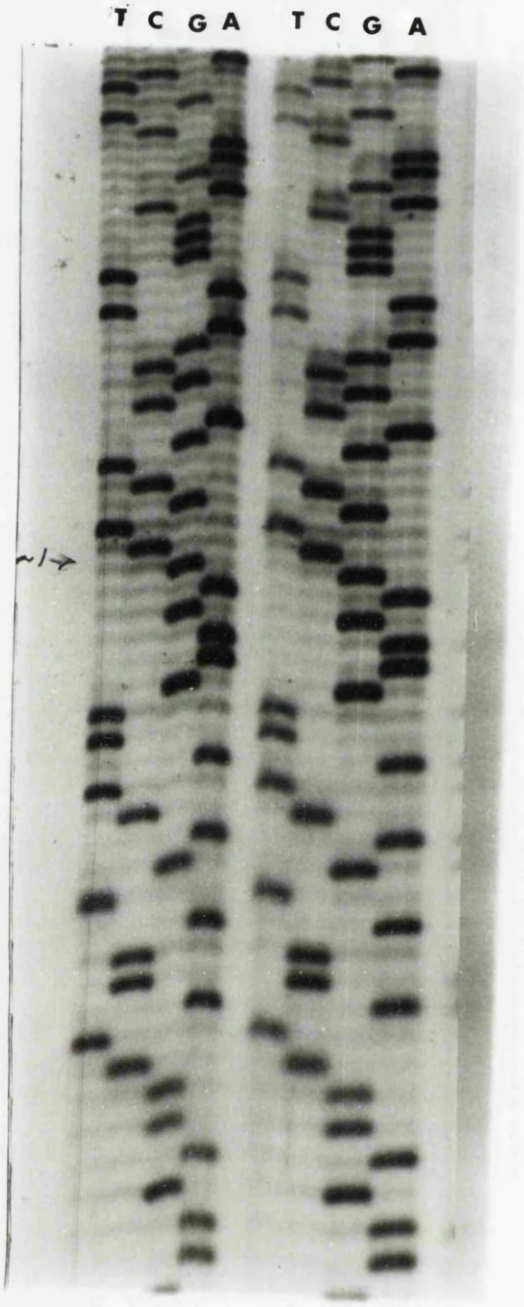


Figure 3.14: Direct sequencing of cDNA of *Fin13* gene from NIH/Ola and C57Bl/6J/Ola strains. Several regions of cDNA of *Fin13* gene were amplified by utilising three primer sets and three nested primers (table 2.2). Afterward, the amplified regions were subjected to the sequencing technique.

a) 52 bp fragment sequenced between nucleotides 73 - 125. **T**;Thyamine, **C**; Cytosin, **G**; Guanin, and **A**; Adenosin.

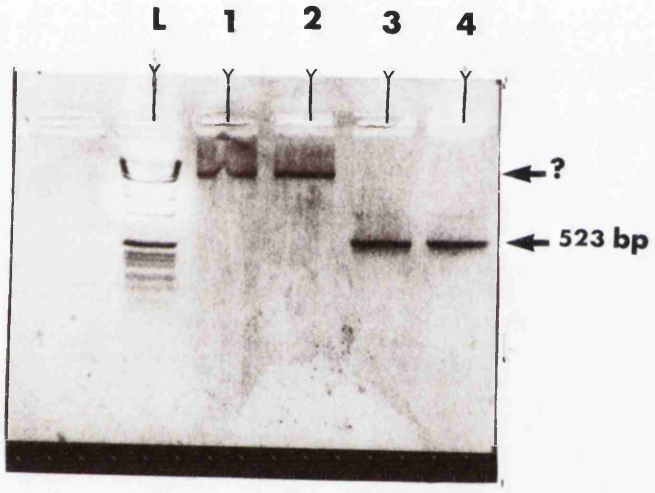


Figure 3.15: Confirmation of subcloning (*Fin13*) by applying PCR. A 523 bp RT-PCR product of *Fin13* was cloned into a expression plasmid. Four different clones were picked up and the plasmid was extracted. Utilising these plasmids as templates in PCR revealed that two of the clones (**lanes 3, 4**) contained the expected RT-PCR product (523 bp) whereas the other two clones (**lanes 1, 2**) contained longer RT-PCR products which could be due to recombination or insertion of non-specific RT-PCR products. **L**; 1-kb ladder.

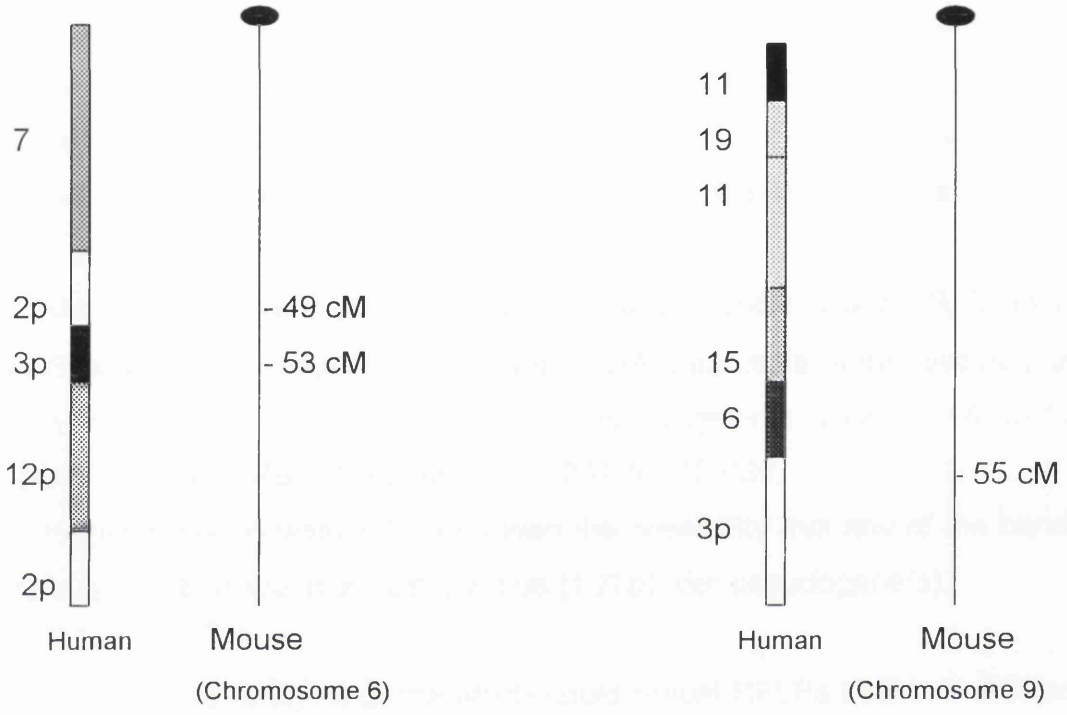


Figure 3.16: Homologous regions of human chromosome 3p, on mouse chromosomes 6 and 9 (human-mouse homologue).

C57Bl/6J/Ola. Genomic DNAs were digested with six different enzymes (BclI, BglII, EcoRV, XbaI, HindIII, BamHI) and subjected to Southern blot analysis. They were hybridised with full length 1.7-kb *Tgfbr2* cDNA. Only BamHI-digested genomic DNA, revealed different bands among different strains (table 3.12, Figure 3.17). There was a possibility that this RFLP among the strains was due to hybridisation of the probe (1.7-kb) with pseudogenes. To address this problem, the cDNA (Figure 3.18) was digested with different enzymes to obtain more specific fragments of the probe and also a 5' *Tgfbr2* probe (1-676) (Cui et al, 1995), represented as P1 in figure 3.18. These were utilised for detailed examination of RFLPs revealed in BamHI-digested DNAs. Using P1 as a probe for hybridisation of BamHI-digested DNA's, revealed the following DNA fragments; 4, 7.5, 14 kb for NIH/Ola, 4, 6, 8 kb for C57Bl/6J/Ola, and 4, 7.5, and 12 kb for 129/Sv (Table 3.13, Figure 3.19).

BamHI-digested DNAs from different strains, was hybridised with P3, 3' 557nt *SacI-EcoRI* probe (1160-1717). Different DNA fragments were revealed as shown in Table 3.14 and Figure 3.20. These fragments were; 8, 14 kb for NIH/Ola, 8 kb for C57Bl/6J/Ola, and 4.5, 12 kb for 129/Sv.

Results presented in table 3.13, excluded the possibility that any of the bands were due to hybridisation of *Tgfbr2* probe (1.7kb) with pseudogene(s).

In order to design a probe which could reveal RFLPs between different strains of NIH/Ola, C57Bl/6J/Ola, and 129/Sv, a BamHI-restriction map of TβRII genomic structure between different strains of NIH/Ola, C57Bl/6J, and 129/Sv was inferred by detailed examination of the DNA bands observed by applying different regions of the *Tgfbr2* cDNA probe for hybridisation (Figure 3.21, Table 3.15). On the basis of these results, a probe (P2) was designed which enabled an RFLP distinguished. Table 3.16 and figure 3.6 show DNA fragments revealed by applying P2 (843-1160) probe for hybridisation of BamHI-digested DNAs. This RFLP for the *Tgfbr2* gene enabled the segregation of the gene in 50 F2 (NIH/Ola x C57Bl/6J/Ola) animals to be followed in order to localise the *Tgfbr2*.

As discussed before, *TGFBR2* was mapped to human 3p22 in a region syntenic with mouse chromosomes 6 (49-53) and distal 9 (55-74). 12

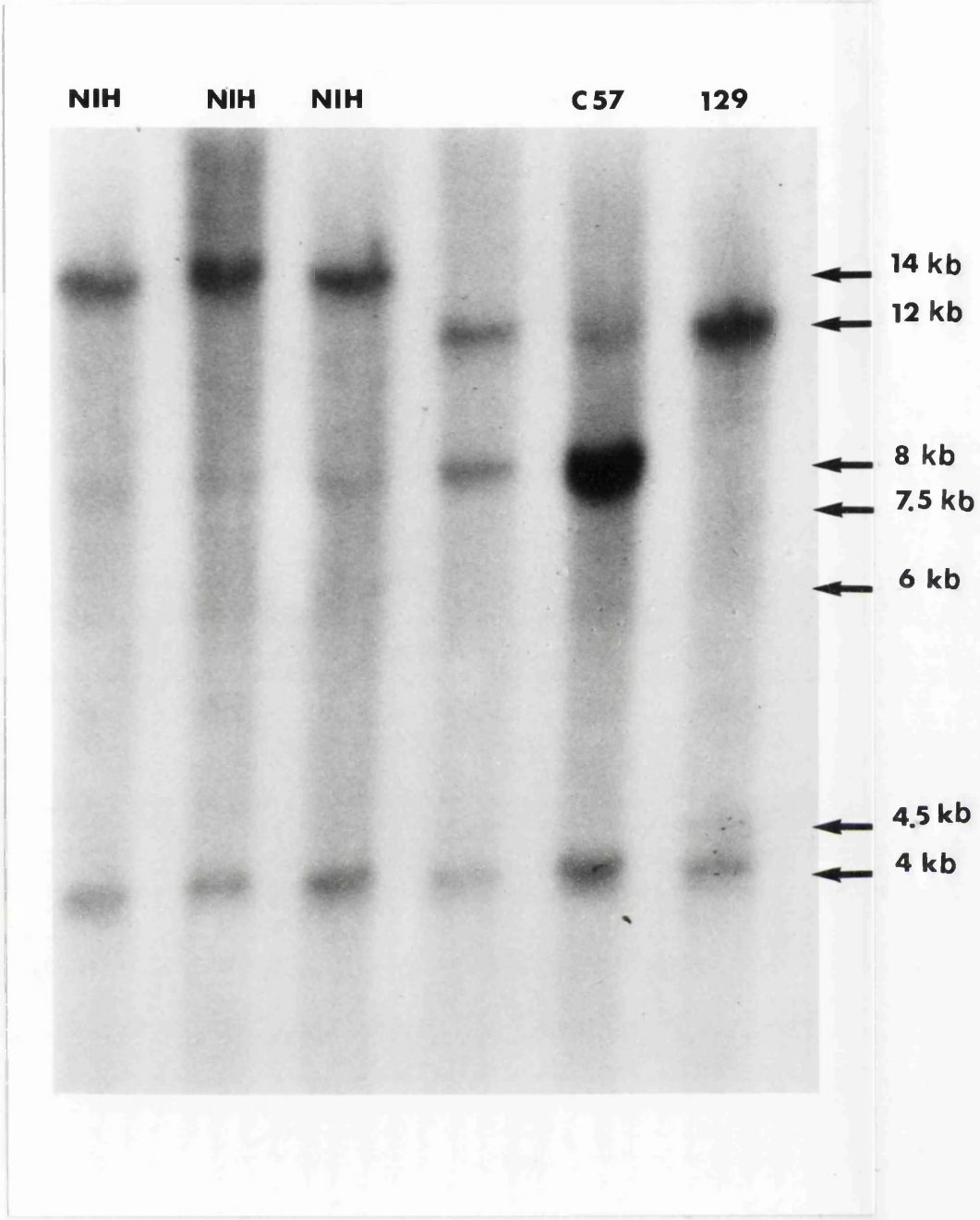


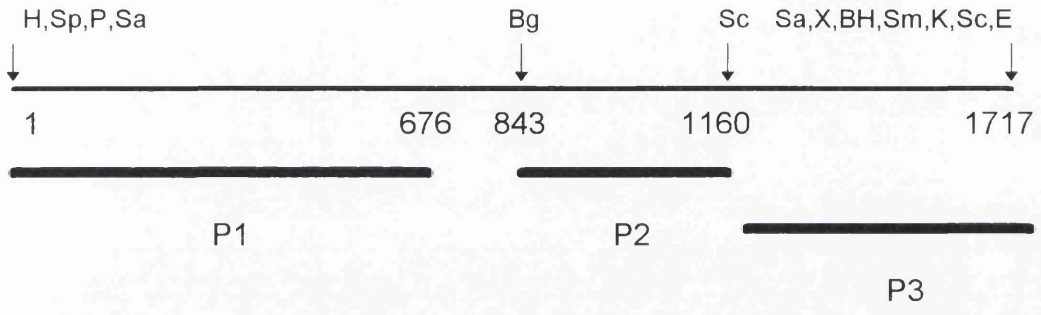
Figure 3.17: Southern Blotting analysis of the *Tgfbr2* gene from NIH/Ola, C57Bl/6J/Ola, and 129/Sv strains. Full length fragment (1.7 kb) from the coding region of the gene was used as a probe to reveal RFLPs between all strains under investigation; NIH/Ola, C57Bl/6J/Ola, abd 129/Sv. BamH1 digestion of genomic DNA revealed different size of fragments between the mouse strains as follows: **NIH:** 4, 7.5, 8, and 14 kb. **C57:** 4, 6, 8, and 12 kb. **129:** 4, 4.5, and 12 kb.

Table 3.12: Illustration of different size DNA fragments revealed when BamH1-digested DNAs were hybridised with the full length (1.7-kb) *Tgfbr2* probe.

Strain ↓ / Length of Band →		3 Kb	4 Kb	4.5 Kb	6 Kb	7.5 Kb	8 Kb	12 Kb	14 Kb
NIH/Ola		-	++	-	-	d	+	-	++
C57Bl/6J		-	++	-	d	-	++	d	-
129/sV		-	++	+	-	d	-	++	-
Mus. Spret.		d	++	-	d	-	++	-	-

"++" represents the presence of a very high density band whereas "+" stands for the presence of a band with weak density. "-" stands for not observing any band. "d" stands for dubious band.

Figure 3.18: Restriction Enzyme map of mouse *Tgfbr2* cDNA.



H: HindIII, Sp: SphI, P: PstI, Sa: Sal I, Bg: Bgl II, Sc: SacI, X: XbaI, BH: BamHI, Sm: SmaI, K: KpnI, E: EcoRI.
P1: Probe 1, P2: Probe 2, P3: Probe 3.

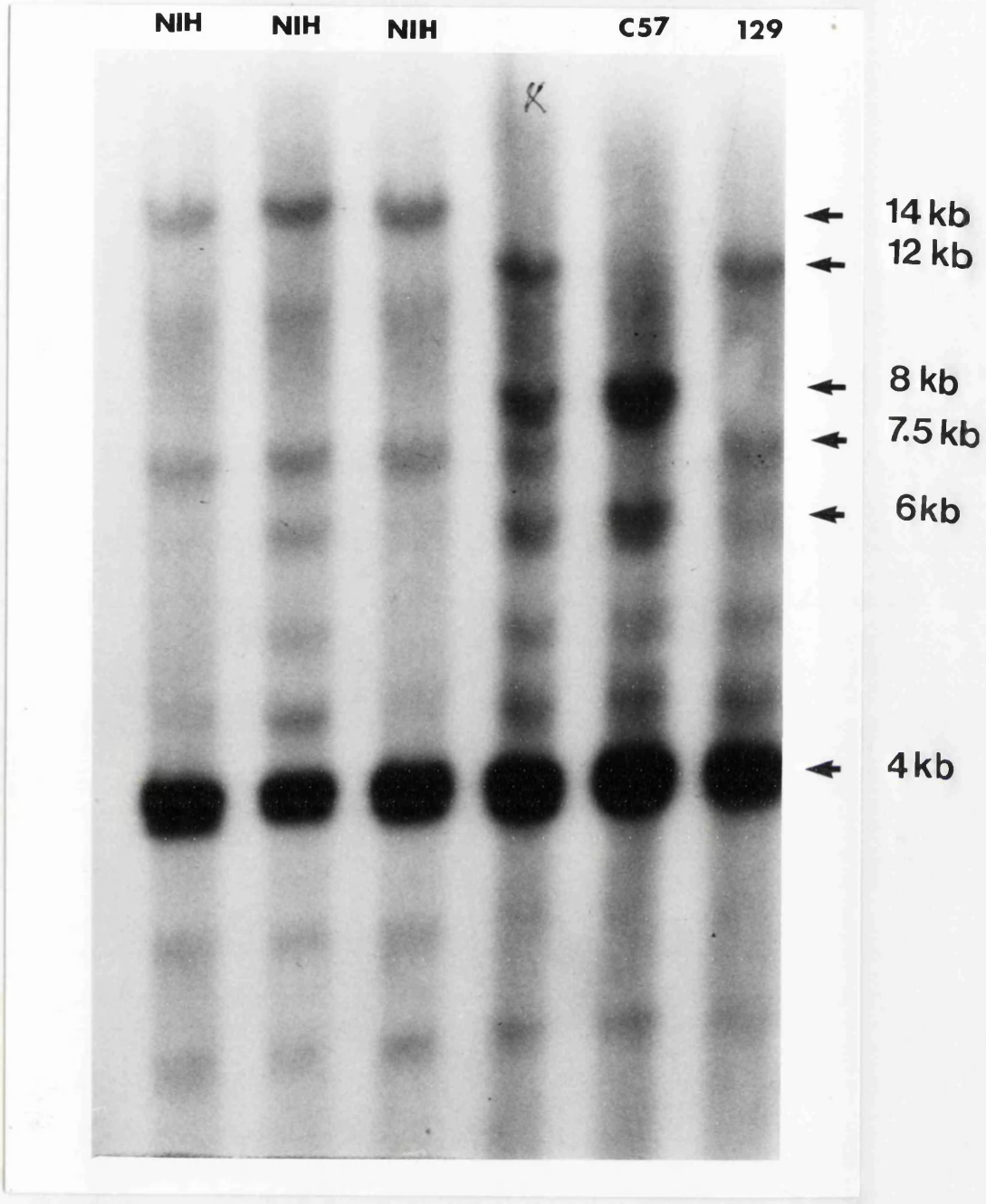


Figure 3.19: Southern Blotting analysis of the *Tgfbr2* gene from NIH/Ola, C57Bl/6J/Ola, and 129/Sv. 676 bp (1-676 nt) fragment (P1) from the coding region of the gene was used as a probe. BamH1 digestion of genomic DNA revealed the following fragments; **NIH:** 4, 7.5 and 14 kb. **C57:** 4, 6, and 8 kb. **129:** 4, 7.5, and 12 kb.

Table 3.13: Presentation of different size DNA fragments revealed when BamH1-digested DNAs were hybridised with the P1 probe (1-676 nt) *Tgfbr2* probe.

Strain ↓ / Length of Band →		3 Kb	4 Kb	4.5 Kb	6 Kb	7.5 Kb	8 Kb	12 Kb	14 Kb
NIH/Ola		-	++	-	-	+	-	-	+
C57Bl/6J		-	++	-	+	-	+	-	-
129/sV		-	++	-	-	+	-	+	-
Mus. Spret.		+	++	-	-	-	+	-	-

"++" represents the presence of a very high density band whereas "+" stands for the presence of a band with weak density. "-" stands for not observing any band.

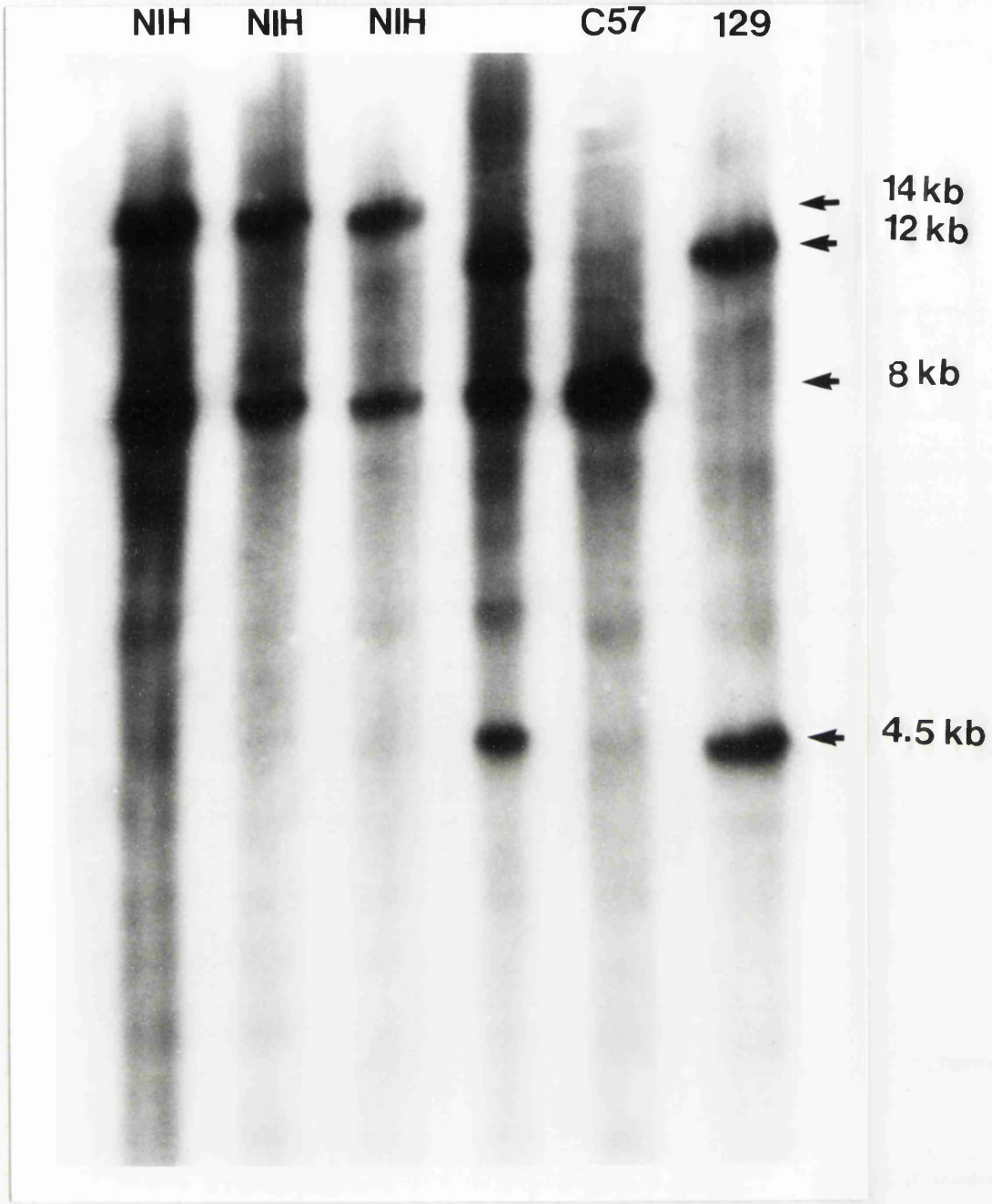


Figure 3.20: Southern Blotting analysis of the *Tgfbr2* gene from NIH/Ola, C57Bl/6J/Ola, and 129/Sv. 557 bp (1160-1717 nt) SacI-BamHI fragment (P3) from the coding region of the gene was used as a probe. BamH1 digestion of genomic DNA revealed the following fragments; **NIH:** 8 and 14 kb. **C57:** 8 kb. **129:** 4.5 and 12 kb.

Table 3.14: Presentation of different size DNA fragments revealed when BamH1-digested DNAs were hybridised with the P3 probe (1160-1717 nt) *Tgfbr2* probe.

Strain ↓ / Length of Band →	3 Kb	4 Kb	4.5 Kb	6 Kb	7.5 Kb	8 Kb	12 Kb	14 Kb
NIH/Ola	-	-	-	-	-	+	-	+
C57Bl/6J	-	-	-	-	-	+	-	-
129/sV	-	-	+	-	-	-	+	-
Mus. Spret.	-	-	-	+	-	-	-	-

"++" represents the presence of a very high density band whereas "+" stands for the presence of a band with weak density. "-" stands for not observing any band.

Figure 3.21: BamH1-restriction enzyme mapping of *Tgfbr2* genome in different strains. (small arrows represent the restriction enzyme sites for BamH1 enzyme)

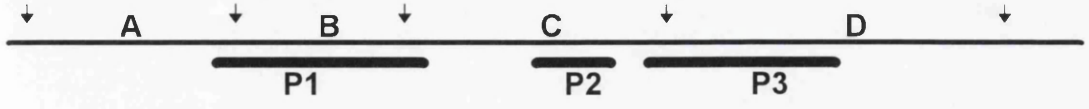


Table 3.15: Size of BamH1-restriction enzyme fragments of *Tgfbr2* genome inferred from revealed DNA fragments in different Southern blotting by applying different parts of *Tgfbr2* cDNA probe.

Strain	A (Kb)	B (Kb)	C (Kb)	D (Kb)
NIH/Ola	7.5	4	14	8
C57Bl/6J	6	4	8	8
129/sV	7.5	4	12	4.5
Mus. Spr.	3	4	8	6

Table 3.16: Presentation of different size DNA fragments revealed when BamH1-digested DNAs were hybridised with the P2 probe (843-1160) *Tgfbr2* probe.

Strain ↓	Length →	3 Kb	4 Kb	4.5 Kb	6 Kb	7.5 Kb	8 Kb	12 Kb	14 Kb
NIH/Ola		-	-	-	-	-	-	-	+
C57Bl/6J		-	-	-	-	-	+	-	-
129/sV		-	-	-	-	-	-	+	-
Mus. Spret.		-	-	-	-	-	+	-	-

"+" represents the presence of a band, whereas "-" stands for not observing any band.

microsatellite markers from syntenic regions were screened between NIH/Ola and C57Bl/6J/Ola strains. Four of the informative microsatellite markers (D6Mit25, D9Mit16, D9Mit150, D9Mit18) were selected from syntenic regions (table 3.17) and their segregation was followed in 50, F2 (NIH/Ola x C57Bl/6J) animals.

Segregation of the microsatellite markers in 50 F2 intercross animals were compared to that of *Tgfbr2* RFLPs. No linkage was found with D6Mit25, but linkage was found with markers on chromosome 9 (Figure 3.22)

By solving the equation (Standard Error, Appendix II-E) for data obtained from mapping *Tgfbr2*, standard errors (SE) were calcualted as follow (cM ± SE): Centromere-D9Mit16 (2.1 ± 0.014)-D9Mit150 (3.1 ± 0.02)-*Tgfbr2* (1 ± 0.0)-D9Mit18.

3.15.1 Uncloned mouse mutations:

Several uncloned mouse mutations map within the region of *Tgfbr2*, including *ky* (kyphoscoliotic degenerative muscle; 56 cM), *du* (ducky; 59-60 cM), *tip* (tippy; 63 cM), *sr* (spinner; 64 cM), *fd* (fur deficient; 48-54 cM), and *sch* (scant hair; 69-72 cM). In order to examine the possibility of a large deletion covering *Tgfbr2* in any of these mutant mice, DNAs were obtained from Jackson labs. Southern blot analysis was performed using HindIII-digested DNAs from mice homozygous for *du* (Green 1989, Snell 1995) and homozygous for *tip* (Green 1989, Lane 1984) (Figure 3.23).
As illustrated in figure 3.23, the possibility of a large deletion covering the *Tgfbr2* gene within either of these mutants was excluded. However, it is now known that nullizygous *Tgfbr2* mice are not viable (Oshima et al., 1996), and any of the mutant mice listed above might involve more subtle molecular pathologies within the *Tgfbr2* gene.

3.16 Genomic mapping of Murine Plasminogen activator inhibitor type I (*PlanH1*) gene.

Table 3.17: Microsatellite markers located on mouse chromosomes 6 and 9, in a syntenic region with human 3p22.

Marker	Chromosome	cM	Informative between NIH & C57
D6Mit13	**6**	**49**	–
D6Mit25	**6**	**50**	+
D6Mit197	**6**	**50**	–
D6Mit9	**6**	**36.5**	–
D6Mit19	**6**	**33.5**	–
D9Mit19	**9**	**71**	–
D9Mit152	**9**	**70**	–
D9Mit82	**9**	**71**	–
D9Mit151	**9**	**72**	–
D9Mit18	**9**	**67**	+
D9Mit16	**9**	**57**	+
D9Mit150	**9**	**59**	+

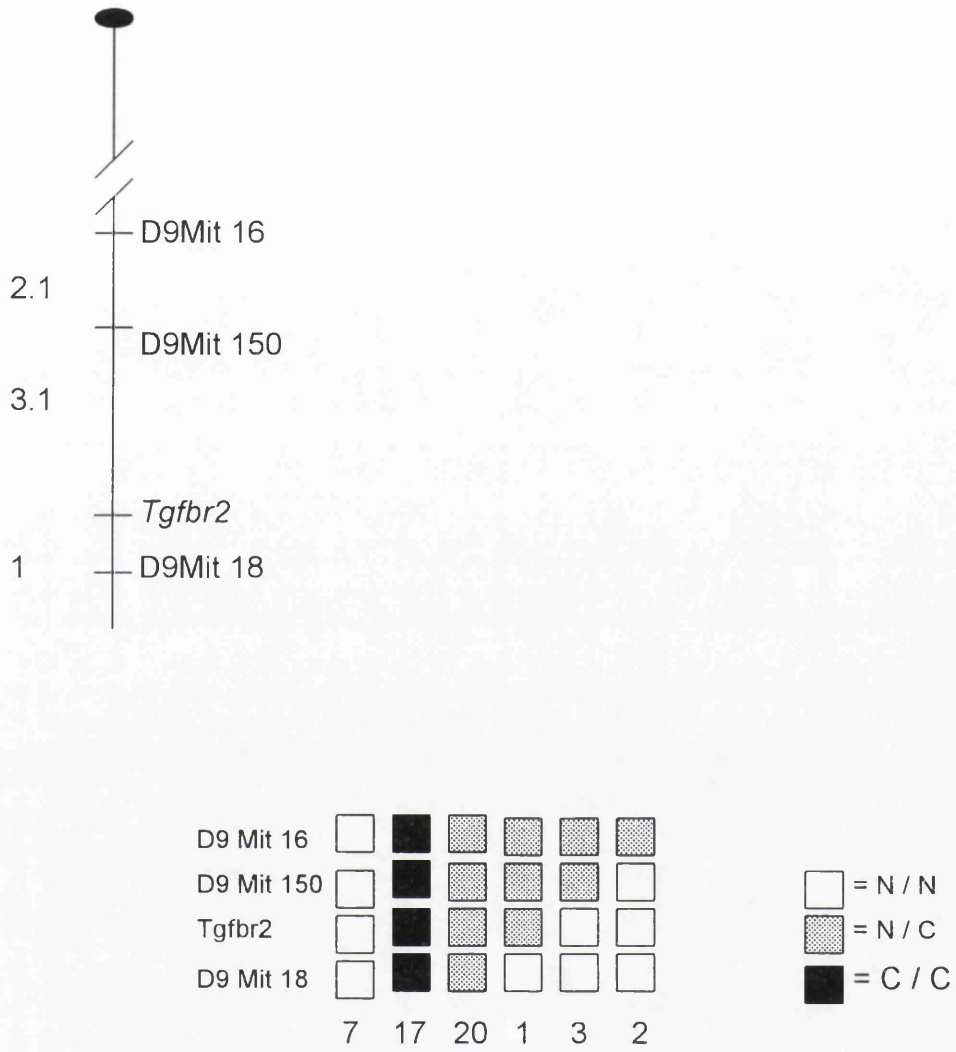


Figure 3.22: *Tgfbr2* maps to distal chromosome 9 (59-67 cM). (up)

Partial linkage map of chromosome 9, showing the position of *Tgfbr2* relative to D9 Mit 16, D9 Mit 150, D9 Mit 18. Recombination distances between loci are shown to the left of the chromosome. (below) Allelic segregation of *Tgfbr2* and D9 Mit 18 in the 50 (NIH/Ola X C57Bl/6J/Ola) intercross mice, using the BglII-SacI *Tgfbr2* probe. N, NIH allele; C, C57Bl/6J/Ola allele. The number of animals exhibited each allelotype are shown below each column.

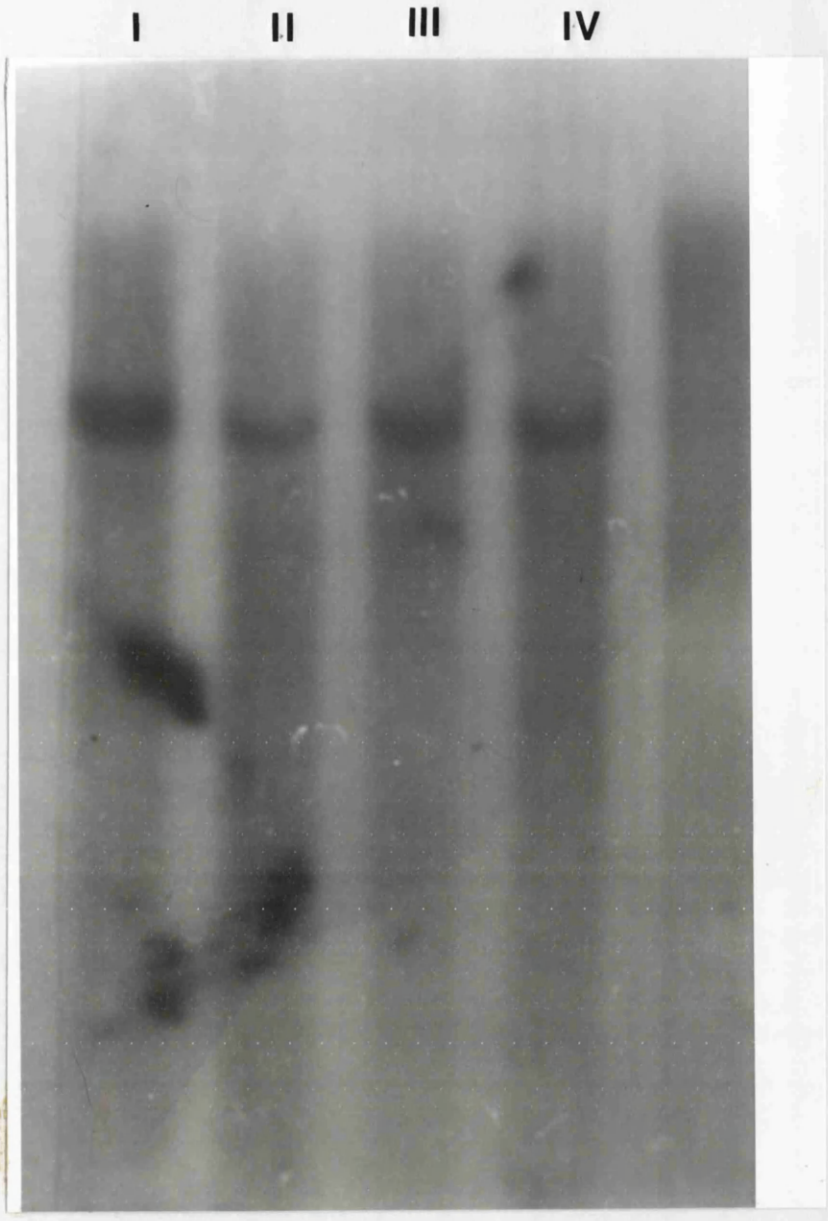


Figure 3.23: Southern Blotting analysis of the *Tgfbr2* gene from himozygous *tip* and homozygous *du* mutant mice. 317 bp BglII-SacI fragment from the coding region of the gene was used as a probe to investigate the possible large deletion of the *Tgfbr2* gene in the mutant *tip* and *du* mice. Southern blot analysis was performed using HindIII-digested DNA from mice homozygous for *du* (Line I), wild type (Line II), homozygous for *tip* (Line III), and wild type (Line IV). All lines are showing the same size which exclude the possible large deletion of the gene in these mutant mice.

The *PAI1* gene has been assigned to human 7 cen-q32 by Southern blot analysis of a panel of human/mouse somatic cell hybrids and utilising florescent in-situ hybridisation (FISH), it was further localised to 7q21.3-q22 (Klinger et al., (a)1987;(b)1987). This region is syntenic with three regions of the mouse genome, the centromeric (1-20 cM) and distal (70-88 cM) region of chromosome 5 and the centromeric region of chromosome 6 (cent.-15 cM) (figure 3.24). To map *PlanH1* on the mouse genome, a murine *PlanH1* coding region cDNA (Lund et al., 1996) was utilised to identify informative restriction fragment length polymorphism (RFLPs) between NIH/Ola and C57Bl/6J. BamH1-digested DNAs hybridised with full length PlanH1 probe (520 bp) revealed RFLPs among NIH/Ola, C57Bl/6J/Ola, and 129/sV (Table 3.18, Figure 3.7). DNAs from *Mus. Spretus* were either purchased from Jax. Lab. (USA) or given by Professor A. Balmain's research group (Beatson Institute, UK). BamH1-digested DNAs from two *Mus. Spretus* animals originated from USA and UK, hybridised with full length PlanH1 cDNA revealed a RFLP between *Mus. Spretus* from USA and *Mus. Spretus* from UK (Figure 3.25).

To localise the murine *PlanH1* gene, 48 progeny derived from matings of (C57Bl/6J/Ola x NIH/Ola) F2 mice were utilised to examine the segregation of *PlanH1* RFLPs. Informative microsatellite markers from mouse chromosomes 5 and 6 (Appendix II-C) located in the syntenic region with human chromosome 7q21.3-22, were selected (D6Mit74, D5Mit49, D5Mit251, D5Mit233, D5Mit7, D5Mit188, and D5Mit161) to screen the 48 progeny derived from matings of (C57Bl/6J/Ola x NIH/Ola) F2.

The mapping of *PlanH1* gene was accomplished by searching for concordant segregation on designed panel of 48 F2 (NIH/Ola x C57Bl/6J) animals between *PlanH1* RFLP's and markers previously mapped on syntenic regions of human (7q21.3-22) on mouse chromosomes 5 and 6.

No linkage was observed with D6Mit74, which has already been mapped to the centromeric area (11 cM) of chromosome 6. However, linkage was obvious with some markers on chromosome 5 (Figure 3.26). Recombination fractions were calcualted by applying the computer program, MapMAKER vers. 3.0 b (Lander

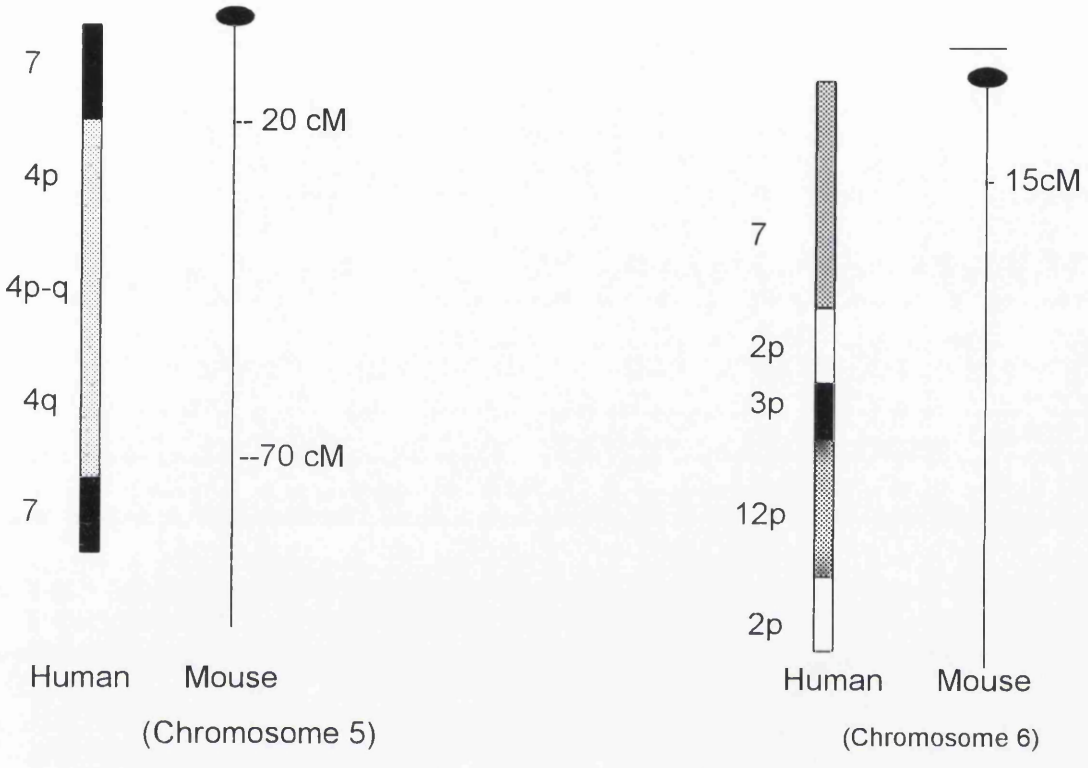


Figure 3.24: Syntenic regions of human chromosome 7q21, on mouse chromosomes (human-mouse homologue).

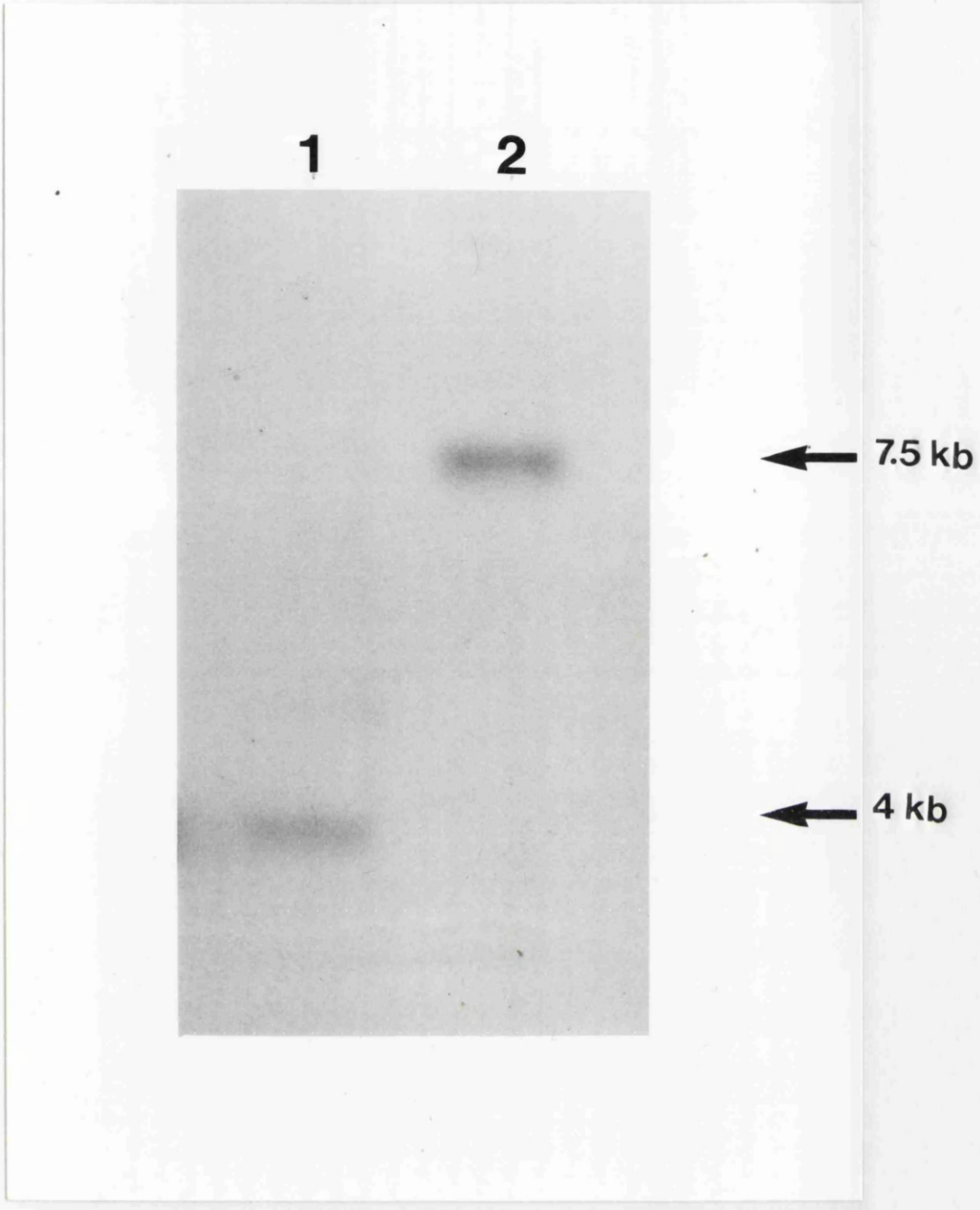


Figure 3.25: Southern Blotting analysis on *PlanH1* gene between *Mus Spretus* strains origenated from the UK and USA. Full length of the coding region of the gene was used as a probe. The fragments were approximately 4 kb and 7.5 kb in the BamHI-digested DNAs from *Mus Spretus* (USA) (lane 1), and *Mus Spretus* (UK) (lane 2), respectively.

Table 3.18: Presentation of the size of RFLPs in different strains revealed by hybridisation of BamH1-digested-DNAs with full length *PlanH1* probe.

Strain	Estimated Size of Band
NIH/Ola	4 Kb
C57Bl/6J	4.3 Kb
129/sV	4 Kb
Mus. Spretus (UK)	7.5 Kb
Mus. Spretus (USA)	4 Kb

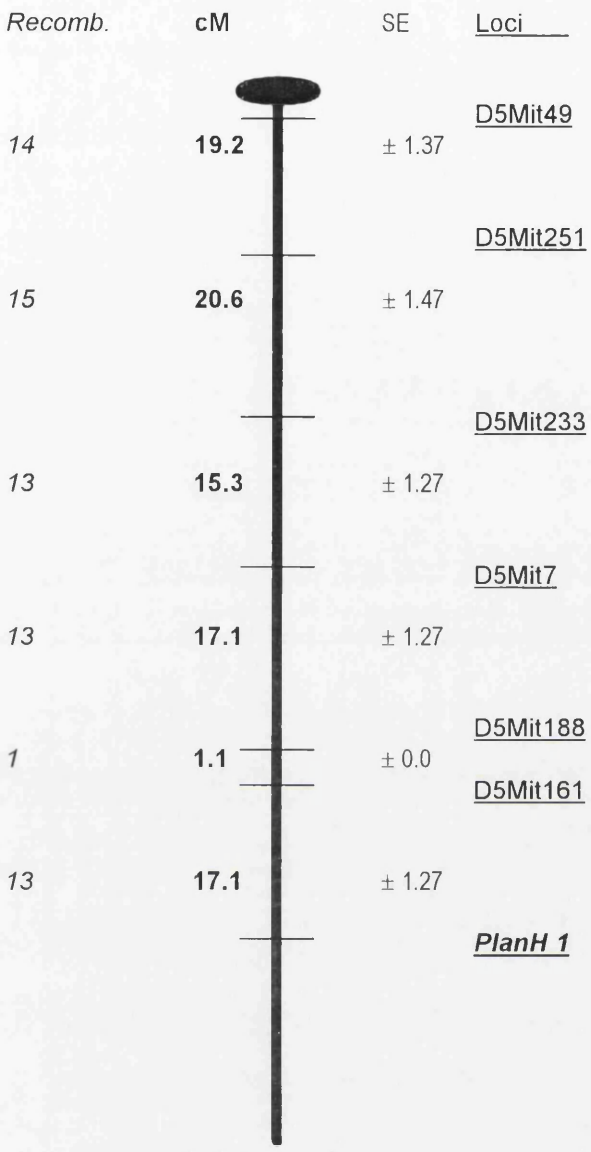


Figure 3.26: The *PlanH1* RFLPs segregation in the 48 F2 (NIH/Ola x C57Bl/6J) intercross mice compared with that of microsatellite markers (D5Mit49, D5Mit251, D5Mit233, D5Mit7, D5Mit188, D5Mit161, D6Mit74). These markers were selected from the regions of mouse chromosome 5 and 6 which show synteny with human 7q21.3-q22.

et al., 1987). Standard error was calculated by applying SE equation (Appendix II-E).

4: Discussion

4.1 TGFB1 knockout animals:

The observation of at least two different phenotypes of TGFB1(-/-) conceptuses bred onto the mixed genetic background (NIH/Ola, C57Bl/6J/Ola, and 129/Sv), gave rise to the notion of the possible involvement of modifying factor(s). The presence of maternal TGFB1 in TGFB1(-/-) embryos has been demonstrated. It has been suggested that dichotomy in TGFB1(-/-) lethal phenotypes is due to maternal TGFB1 rescue of some, but not all, TGFB1(-/-) embryos (Letterio et al., 1994). Therefore, it was an absolute requirement to define the observed traits as strain-specific phenotypes and to then to attempt identify the modifying factors influencing in the expressivity of TGFB1(-/-) phenotype.

A previous study in which different expressivity of TGFB1(-/-) phenotype was observed, *Tgfb1* null allele was bred onto three different genetic backgrounds; 50% NIH/Ola, 37.5% C57Bl/6J/Ola, and 12.5% 129/Sv. In this project, in order to study the possible influence of genetic background in the phenotype, the *Tgfb1* knockout allele was bred through four generations onto either NIH/Ola or C57Bl/6J/Ola inbred strains. The selection of these two strains was on the basis of the following facts:

1) In independent studies carried out by Mr. Peter Kerr and Dr. Wei Cui, it was noticed that on a 75% NIH/Ola genetic background a high percentage of TGFB1(-/-) animals developed to term.

2) C. Biron and H. Su had bred null allele onto C57Bl and found that out of 300 animals no TGFB1(-/-) animal developed to term.

3) 129/Sv strain contributed 12.5% to the mixed genetic background animals showing different expressivity of TGFB1(-/-), whereas that of NIH/Ola and C57Bl/6J/Ola strains was 50% and 37.5% respectively. Therefore, due to less contribution of 129/Sv strain to the mixed genetic background, it was excluded from this study.

4.2 Phenotypic assessments:

To understand the biology and pathological processes, and moreover to investigate the factors involved in any phenotype such as IDDM or NTD (neural tube defects), it has been necessary to carry out a full and objective phenotypic assessment to distinguish and classify animals into two or more classes. Therefore, according to conventional linkage analysis, animals had to be classified into at least two classes on the basis of their phenotypes. Upon starting this project two phenotypes; yolk sac phenotype and survival to birth, were identified on the mixed genetic background.

4.2.1 Yolk sac phenotype:

Ideally, DNA would have been analysed from both categories or phenotypes observed, by extracting DNA from embryos of known phenotype at 9.5 dpc i.e. normal and abnormal but prior to prenatal death. However, due to the difficulty of dissecting and accurately scoring very small 9.5 dpc embryos, the variable expressivity of the yolk sac phenotype, and the limiting quantities of DNA available, this did not seem like an ideal option. Furthermore, at the time, there was no information about the stage of TGFB1(-/-) embryo lethality bred onto C57Bl/6J/Ola strain. Latest studies has shown that in C57Bl/6J/Ola genetic background, a significant fraction of TGFB1(-/-) embryos tend to die before the midgestation stage (Rusholme PhD Thesis., 1997).

4.2.2 Survival to birth:

It was noticed that in NIH/Ola strain TGFB1(-/-) neonates tend to develop to term normally whereas in C57Bl/6J/Ola strain all of the conceptuses were dying *in utero*. This implied that modifying factors in the NIH/Ola strain could rescue TGFB1(-/-) conceptuses from prenatal lethality but not in C57Bl/6J/Ola strain. Therefore, utilising only animals which survived to birth would provide large quantities of DNA from an unambiguous phenotype. This would overcome the problems of different expressivity of the yolk sac

phenotype and the availability of limiting quantity and quality of DNA. However, by taking survival to birth as the criterion for classification, only one group of animals could be accessible for the purpose of linkage analysis. The implication was that more matings needed to be set up to obtain sufficient TGFB1(-/-) neonates for analysis [only 42% survival of TGFB1(-/-)] . In practise, the need to generate more TGFB1(-/-) neonates was not a problem since mating pairs produced new litters every three weeks, and each dam was capable of generating several litters. In contrast, if embryos had been harvested, each dam would only have provided one litter for analysis.

4.2.3 Control animals:

In conventional crosses set up for the purpose of identifying genetic factors involved in complex traits such as IDDM or NTD, affected versus unaffected animals have been studied as controls for each other. Therefore, the percentage of affected animals in various crosses between susceptible and resistance parental strains could be estimated to derive an approximate number of the loci involved in the phenotype. In this study, because of the constraints discussed above (4.2.2), only one group of TGFB1(-/-) animals was selected to study, therefore, it was necessary to choose an alternative group of animals as a control. To generate TGFB1(-/-) animals, intercrosses between TGFB1(+/-) animals were set up. The Mendelian ratio of 1:2:1 for TGFB1(-/-), TGFB1(+/-) and TGFB1(+/+) animals respectively was expected. TGFB1(+/-) lethality was observed in both pure (93.7%) NIH/Ola and C57Bl/6J/Ola strains without statistically significant differences between each other, indicating the involvement of non-strain specific factors in lethality of heterozygous embryos. Moreover, according to the previous study, the level of maternal circulating TGFB1 could be important in influencing prenatal survival of TGFB1(+/-) embryos (Dickson et al., 1995). Therefore, to avoid any interference by TGFB1(+/-) embryo lethality, the percentage of which is probably due to non-genetic factors, the percentage survival (observed/expected) of TGFB1(-/-) embryos to birth was estimated as N^{o} (-/-) / N^{o} (+/+). The premise was that any

possible factor leading to prenatal loss of TGFB1(+/+) embryos would have an equal effect on TGFB1(-/-) embryos.

4.3 Strain-dependent expressivity of TGFB1(-/-) phenotype:

Observing 82% of TGFB1(-/-) embryos developing to term in NIH/Ola genetic background but non in C57Bl/6J/Ola genetic background gave rise to the notion that different expressivity of TGFB1(-/-) phenotype was due to strain specific modifying factor(s). In NIH/Ola strain 18% of the TGFB1(-/-) embryos died in mid-gestation stage from defective yolk sac vasculogenesis and in C57Bl/6J/Ola strain TGFB1(-/-) conceptuses tend to die prior to mid-gestation stage (Rusholme, PhD Thesis., 1997). The stage in which C57Bl/6J/Ola TGFB1(-/-) conceptuses died yet remains to be revealed.

4.4 Possible factors involved in different expressivity of TGFB1(-/-) phenotypes:

In studies carried out to identify susceptible loci for a variety of genetically disorders such as IDDM or NTD, adult animals have been utilised. Therefore, in these investigations the major factor(s) involved in resistance or susceptibility of animals to the trait was solely due to the genetic background of animals and/or the respond of genetic factors toward environmental factors. However, in this project the major objective of the study was to study developmental outcome of TGFB1(-/-) embryos which could profoundly be influenced by maternal factors as well as embryonic factors. Therefore, despite the fact that different expressivity of the TGFB1(-/-) phenotype was strain-dependence, it was an absolute requirement for linkage analysis to differentiate between maternal strain specific (or non-embryonic) factors and embryonic strain-specific factors.

In the project, the possible strain-specific modifying factors involved in modifying the phenotypic outcome of TGFB1(-/-) conceptuses could be categorised into three classes as follows:

1) Strain-specific role of TGFB1 in gametogenesis.

2) Strain-specific influence of maternal factors such as maternal source of TGFB1 or the maternal immune system on the survival and development of TGFB1(-/-) conceptuses.

3) Cardinal effect of the genetic background of TGFB1(-/-) conceptuses on the phenotypic outcome.

Also, there could be the possibility of additive or non-additive interactive effects of these factors in the different expressivity of TGFB1(-/-) phenotype.

To accomplish the indirect investigation in clarifying the involvement of either of these possible factors in the different expressivity of TGFB1(-/-) phenotype, various combinations of inter-crosses between NIH/Ola and C57Bl/6J/Ola were set up and statistically analysed.

4.4.1 Viability of gametes carrying null *Tgfb1* allele:

The selective failure or low transmission of mutant alleles is a rare event. The best model in the mouse is that of t-haplotypes that cause a low transmission of the homologous wild type (+) chromosome from male but not female. Sperm bearing complete t-haplotypes are preferentially transmitted during fertilisation from heterozygous t/+ males, often in excess of 95% relative to their (+) bearing meiotic partner. It has been reported that sperm from t-bearing males have an approximate two-to four fold increase in beta 1,4-galactosyltransferase (GlaTase) activity, a cell surface protein that mediates sperm binding to the egg zona pellucida (Shur & Scully, 1990).

There is much evidence indicating the role of TGFB1 in reproductive function, with effects ranging from an influence on germ cell migration during embryogenesis, to modulation of ovarian and testicular function in the adult (Godin and Wylie, 1991). TGFB1 has been localised in both sperm and ovum (Chu et al., 1996) and its expression in embryonic and adult testis and all ovarian cell types has been reported (Derynck et al., 1988; Miller et al., 1989 a, b; Watrin et al., 1991). Based on previously published data (Shull & Doetschman., 1994) and confirmed during this project, it is apparent that there is a loss of TGFB1(+/-) embryos. This could raise the possibility that TGFB1

function may be important in haploid germ cell function such that in the absence of a functional *Tgfb1* gene, germ cell function or survival is impaired, leading to a reduction in the frequency of conceptions involving the mutant germ cells. This possibility could explain the reductions in both the heterozygous and homozygous mutant offspring relative to the wild type. However, in this study, the possibility of selective failure or low transmission of *Tgfb1* knockout allele through maternal and/or paternal gametes in C57Bl/6J/Ola strain was ruled out by observing statistically significant differences in the number of TGFB1(-/-) survivals from various crosses using C57Bl/6J/Ola either as females or males. Moreover, the possibility of failure or low transmission of the *Tgfb1* null allele should have changed the Mendelian ratio of 1:2 for TGFB1(+/+) and TGFB1(+/-) offsprings in C57Bl/6J/Ola crosses but not in NIH/Ola crosses whereas there was no significant differences between these two strains in the percentage of TGFB1(+/-) neonates developing to term normally.

4.4.2 Maternal factors:

4.4.2.1 Maternal circulating TGFB1:

The main purpose of gene knockout technology is to remove a protein and examine the outcome *in vivo*. However, in the case of secreted proteins maternally-acquired protein could interfere with interpretations. It has been demonstrated that maternal TGFB1 can cross the placenta and its contribution to survival of TGFB1(-/-) embryos has been suggested (Letterio et al., 1994). Moreover, it has been shown that addition of TGFB1 to the culture medium significantly increases the percentage of embryos developing into blastocyst without increasing the number of cells per blastocysts (Paria & Dey., 1990) indicating the possible involvement of the environmental TGFB1 in development of embryos. Survival of more than 80% of the TGFB1(-/-) bred onto NIH/Ola strain but non in C57Bl/6J/Ola strain, raised the possibility that the level of maternal circulating TGFB1 could differ and result in different expressivity of TGFB1(-/-) phenotype between two strains.

4.4.2.2 The maternal immune system:

There is much evidence that pregnancy loss may be immunologically mediated. Failure of the maternal immune system to actively support the pregnancy may be responsible for its demise. Some mouse models have an unusual high rate of fetal resorption; 30% as compared to 5% in other matings. The first mouse model mentioned by Clark et al., (1980) includes CBA/J female mice mated to DBA/2J males. In the CBA/J x DBA/2J system, abortion occurs at four to six days after implantation and is followed by specific changes in the immune profile of CBA/J mothers. However, it is important to bear in mind that in inbred crosses, e.g. NIH/Ola or C57Bl/6J/Ola strains, due to the fact that all animals are genetically identical, expressed antigens by embryos which belong to both maternal and paternal major histocompatibility complex (MHC) do not represent a potential target for the maternal immune system.

It has been shown that some cytokines produced by both T cells and non-T cells (IL-3, TGFB, IL-4, IL-10) favour fetal survival and growth whereas other cytokines such as IFN-γ, TNF-β and TNF-α can rather compromise pregnancy (Piccinni & Romagnani., 1996). Therefore, it raised the notion that TGFB1(-/-) embryos could represents a potential target for the maternal immune system in C57Bl/6J/Ola genetic background but not in NIH/Ola strain (polymorphism in the maternal immune systems between two strains).

The possible involvement of all maternal factors in different expressivity of the TGFB1(-/-) phenotype was studied indirectly by statistical analysis of the number of TGFB1(-/-) neonates developed to term in various reciprocal crosses between two strains; NIH/Ola and C57Bl/6J/Ola. The lack of statistical significant difference in the number of TGFB1(-/-) survivals in reciprocal crosses, ruled out the involvement of any possible maternal factor in different expressivity of the TGFB1(-/-) phenotype.

4.4.3 Embryonic strain specific factor:

The only major factor(s) contributed ~80% to the different expressivity of the TGFB1(-/-) phenotype has been clearly demonstrated to be embryonic strain specific factor(s). These genetic factor(s) in NIH/Ola but not in C57Bl/6J/Ola could either compensate for the absence of TGFB1 function during early stage of embryogenesis or enable the embryo to respond to maternal source of TGFB1.

4.5 Estimating the number of gene(s) involved in the survival of TGFB1(-/-) neonates:

Before embarking on a detailed mapping project, it was useful to derive an estimate of the number of segregating genes involved in the expression of the trait under analysis. In complex cases of inheritance, the derivation of such an estimate is not possible. However, a crude estimate of the *minimum* number of genes involved can be made with a sufficient number of backcross animals if a discrete phenotype is evident which shows an absolute requirement for alleles at multiple unlinked loci from the affected parent. An estimation of gene number in this situation is trivial because the expression of the variant phenotype is absolutely correlated with the presence of a parental strain genotype at involved loci. The probability of this occurrence is $(0.5)^n$, where n is the total number of loci required for expression. Thus, if the observed proportion of affected animals is approximately 25%, this would imply the action of two required genes [i.e. $(0.5)^n=0.25 \Rightarrow n=2$].

Sometimes a single polymorphic locus with a high degree of variable expressivity can give rise to phenotypic variation. However, if a single locus is responsible for the entire genetic contribution to different traits between two inbred strains, this would almost certainly become apparent in the second generation of either an F1 backcross or F1 intercross breeding protocol. The estimated number of loci involved depends critically on the assumed model of recessive epistasis. In fact, if the loci do not act in a purely recessive fashion (i.e. if heterozygotes can also be affected) then the above procedure can significantly underestimate the number of loci involved.

To estimate the number of loci involved in the survival of TGFB1(-/-) neonates, analysis of data obtained from F1 (NIH x C57) crosses was required. Figure 4.1, shows idealised distributions for the expression of a discontinuous trait in two inbred strains and the F1 hybrid between them. In this example, the levels of expression for two inbred parental population (NIH , C57) are separated from each other according to the percentage of null pups bred onto NIH/Ola or C57Bl/6J/Ola genetic backgrounds which developed to term normally. Three examples of F1 distribution are shown. The first F1 distribution would be expected with a trait controlled by a series of C57 alleles that all show complete dominance to their corresponding NIH alleles. The third F1 distribution would be expected with a trait controlled by NIH and C57 alleles that all show strict semidominance. The second F1 distribution would be expected with more complex scenarios including incomplete dominance skewed toward one parent. Since each population is genetically homogenous, variation in expression must be due to environmental effects or chance!.

In this study, TGFB1(-/-) phenotype expressivity in F1 was 32%. This expression pattern follows the second F1 distribution in which incomplete dominance skewed towards the C57 parent. It could be interpreted to indicate that at least one codominant acting gene is involved or that several interacting genes with codominant outcome trait are involved, making it that 32% of the F1 null embryos rescue from yolk sac phenotype (Rusholme PhD Thesis., 1997) while the rest die *in utero*. As mentioned above, the observed variation in expression in genetically homogenous TGFB1(-/-) embryos must be due to environmental factors. In this project the best candidate for environmental factor was the level of maternal circulating TGFB1.

To estimate the number of loci, several models could be proposed and the simplest model which fits the observed data obtained from different backcrosses and intercross could be suggested. The simplest model which fits the data, is a single gene model with two alleles. If we assume that a single gene with two alleles explains the pattern of survival, then the maximum likelihood estimates for the survival rates for N/N, C/C, and N/C genotypes are

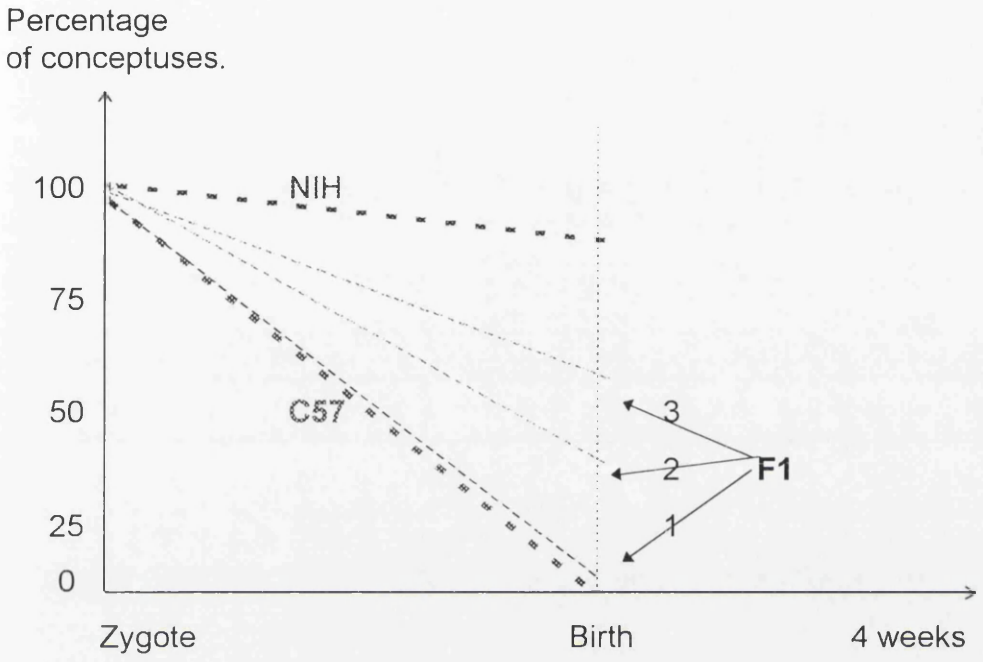


Figure 4.1: Illustration of distribution for the expressivity of TGFB1 null conceptuses in two inbred strains and the three idealized distribution of F1 hybrids between them.

86%, 0%, and 32% respectively. These estimates take into account the varying numbers of pups as well as attempting to allow for the missing pups. In this model, F1 animals showed incomplete dominance of the C57 allele, and mostly they were poised between lethality and survival.

4.6 Screening the genome:

Due to the fact that TGFB1(+/-) parents bred onto either NIH/Ola or C57Bl/6J/Ola strains were only ~93% pure, not all markers were informative in every animal. Therefore, uninformative animals for some of the markers were excluded from the statistical analysis.

During this project three approaches were utilised to perform linkage analysis on different loci:

1) Performing linkage analysis on candidate loci not mapped previously.

2) Linkage analysis carried out on candidate loci mapped previously.

3) Linkage analysis performed on unknown loci (genome-wide search).

4.6.1 F1 backcross or F1 intercross animals:

Upon beginning the linkage analysis in this study, either of crosses; F1 intercross or F1 backcross, could be utilised based on the fact that F1(NIH/Ola x C57Bl/6J/Ola) males were fertile. However, it should be borne in mind the possibility that in some complex traits due to epistatic effects among the involved loci, some of the loci literally vanish between different crosses of the same parents. For example, in the epileptic EL mouse model, dominant alleles at EL1 and EL2 have major effects on seizure frequency in an (ABP x EL)F1 x ABP backcross, but negligible effects in the intercross (ABP x EL)F2 (Rise et al., 1991; Frankel et al., 1995). Conversely, EL5 had a large effect in an intercross (EL x DDY)F2 but none in the corresponding backcross to DDY (Frankel et al., 1995). A combination of backcross and intercross analysis would be ideal to check for the presence of all possible genes.

In this study, due to the codominant nature of the locus (loci), and to procure more informative animals and high-resolution mapping for the purpose of positional cloning, the F1 intercross approach was applied for the linkage analysis.

4.6.2 Imprinted and sex-linked genes:

Genomic imprinting, inactivation of maternal (e.g. *snrpn*)(Leff et al., 1992) or paternal (e.g. *Igf2r*)(Barlow et al., 1991) alleles of certain genes during gametogenesis, has been increasingly recognised as an important component of gene regulation (Surani et al., 1984; Barlow et al., 1991; Leff et al., 1992; DeChiara et al., 1991; Bartolome et al., 1991). During this study, the involvement of imprinted and/or sex-linked genes in the different expressivity of TGFB1(-/-) phenotype was investigated. The involvement of imprinted and/or sex-linked genes in any phenotype (trait) would result the observation of different percentages of animals developing the disease (or phenotype) in reciprocal crosses between different strains. The absence of significant difference in reciprocal crosses between NIH/Ola and C57Bl/6J/Ola excluded this possibility. Also based on these results, the possible involvement of *Igf2r* in the survival of TGFB1(-/-) conceptuses which was suggested in a preliminary study carried out on a small number of TGFB1(-/-) animals from a mixed genetic background (Bonyadi, Msc Thesis, 1994), was ruled out. Moreover, the lack of any statistically significant difference in survival of male and female offsprings [i.e. TGFB1(-/-) neonates] verified the exclusion of any major sex-linked gene involved in the different expressivity of TGFB1(-/-) phenotype. Therefore, the X chromosome constituting at least 5% of the whole genome was excluded from the genome-wide screening.

4.6.3 Candidate genes:

Prior to start genome-wide search and as part of the genome-wide search, the linkage of a few important candidate genes which were directly or indirectly involved in modifying TGFB1 functions, were investigated. The

linkage of some candidate genes which had already been mapped to the mouse genome including *Tgfb* isoforms (*Tgfb2* and *3*) and *endoglin* was carried out by utilising tightly linked polymorphic markers (i.e. microsatellites). To study the linkage of the other candidate genes; *Tgfbr2* and *PlanH1*, not previously mapped to the mouse genome, an informative RFLPs approach was applied. However, observing no linkage between either of these genes and the survival of TGFB1(-/-) in F2 intercrosses ruled out the possible involvement of these genes in the different expressivity of TGFB1(-/-) phenotype.

4.6.4 Genome-wide search:

As tools for genetic mapping, markers which can be typed in large numbers of animals with minimal amounts of sample material by PCR and referred to as microsatellites (Litt and Luty., 1989) are considerably more useful than standard RFLPs and minisatellites. These powerful new markers, microsatellites, and the availability of dense genetic linkage maps of them makes feasible to wide range of studies, including positional cloning of monogenic traits, genetic dissection of polygenic traits, construction of genome-wide physical maps, rapid marker-assisted construction of congenic strains, and evolutionary comparisons (Copeland et al., 1993).

During this project these highly polymorphic markers were utilised to reveal the genetic factor(s) involved in different expressivity of TGFB1(-/-) phenotype. Due to the fact that there was no information about the size of these markers in NIH/Ola strain, 150 microsatellites showing high polymorphism between other published intrastrains and C57Bl/6J/Ola strain, were selected. These microsatellites scattered throughout the (autosomal) genome were screened for polymorphisms between NIH/Ola and C57Bl/6J/Ola strains and in terms of the location and being polymorphic, only 55 markers were informative for linkage analysis in this project. In order to cover the entire genome during a genome-search the following factors are very critical:

1) The number and the location of markers.

2) The number of animals to be screened.

3) The P-value accepted as an evidence for linkage especially for a whole genome search.

4.6.4.1 Number of animals and markers:

The first step in the first stage of the protocol was to develop a framework map that was "anchored" by previously well-mapped loci spaced uniformly throughout the entire genome. To accomplish this task most efficiently, it was critical to calculate the minimum number of anchor loci required to develop this low-resolution, but comprehensive, map. This calculation was based on the length of the " swept radius " which is the distance over which linkage can be detected between any marker and a test locus when both are typed in a set number of offspring generated with a defined breeding protocol (Carter & Falconer, 1951). The corresponding framework swept radius associated with 94 samples from backcross for each anchor locus is about 24 cM (Frankel et al., 1990; Lee M. Silver., 1995). To a first approximation, each intercross sample is equivalent to two backcross samples. Thus, a swept radius of ~0.23 rf can be obtained with 50 intercross samples. A further increase in the critical number of samples (45-50) provide only a marginal increase in the distance that is swept.

4.6.4.2 The P-value for genome-wide search:

The P value is the probability that a statistic as large as or larger than the observed one would occur by chance. The P-value can be determined after computing the χ^2 in the intercross approach. P-value taken as evidence for linkage is very critical, especially for a whole genome search. Accepting a P-value of 0.05 as providing evidence for linkage means that 5% of the unlinked loci will show a false positive result. In this study, the experimental design was such that linkage was detectable out to 0.23 rf on both sides of an unmapped locus. As shown in equation (1), where 1500 (cM) is the total genome length, 3.0 markers out of 100 distributed randomly across the genome should actually

be in linkage with any particular test locus, in the whole genome search approach.

$$\frac{23 + 23}{1500} = 0.03 \quad [1]$$

Thus, in this example, 8 positive markers would be expected but only 3 would be genuinely linked. This means that a P-value 0.05 only provides a probability of linkage of 40% (Bayesion analysis). To increase this probability, it has been recommended to take a P-value of $\approx 10^{-5}$ as criterion for definitive linkage and a P-value of 0.05 only as a suggestive linkage (Lander & Kruglya, 1995).

54 informative markers scattered randomly throughout the genome were utilised to screen 48-50 TGFB1(-/-) neonates. Only three independent regions on chromosomes 3, 12, and 5 showed linkage with P value of less than 0.05. Another region on chromosome 5, located in ≅40 cM of the region showing linkage, also showed linkage with P value of less than 0.05. Statistical analysis performed on these two regions suggested that linkage in one of the regions could be due to the influence of the linkage in other region. As discussed above, in this study, P value of 0.05 provide a probability of linkage of 40%, therefore, only one of the three regions was expected to be a truly linked locus. To confirm or dismiss linkage of each of four loci which appeared to show linkage, an additional 30 neonatal TGFB1(-/-) intercross DNA samples were analysed for markers on chromosomes 3, 5 and 12. P-values at the loci on chromosomes 3 and 12 remained only suggestive (≅ 0.05). However, the support for linkage was strengthened at marker located on chromosome 5, D5Mit268, surpassing the stringent linkage criteria ($\leq 10^{-5}$).

4.6.4.3 Control animals:

In conventional linkage studies, animals are classified into at least two phenotypic or physiologically distinctive groups. Linkage analysis is normally performed on both classes. Therefore, observing a linkage in one of the groups but not in the other group confirms that linkage is genuinely due to the interested phenotype (or trait) not due to other unknown strain specific factors. The segregation distortion of few markers in crosses between wild type strains has been observed (e.g. F1(B6 x D2) x D2 backcrosses (Corcus, unpublished

1997). There are some possible explanations for this type of segregation distortion as follows:

1- Genotyping errors: One allele may be amplified preferentially, perhaps due to mismatches at the primer binding sites in one strain versus the other. A lower annealing temperature sometimes will enable both alleles to be amplified. In this case, several other closely linked markers would not show deviation from expected genotype frequencies.

2- Segregation distortion: The marker is linked to a gene that is subject to selection at the gamete or embryo level. If this is true, then several closely linked markers should also show similar distortion.

3- Chance (False positive): This depend on that which P-value taken as an evidence for linkage in a whole genome search.

In this study, 50 wild type [TGFB1(+/+)] animals born from the same F2 litters were genotyped by the marker showing linkage. Linkage at the marker was not observed in F2 wild type animals, excluding the possibility that linkage could be artefactual.

4.6.4.4 Genetic Risk Ratio (GRR) and proposed genetic models:

Insight into the contribution of the chromosome 5 modifier gene to the overall genetic variability segregating in the F1 intercross may be gained by adopting the approach of Risch and colleagues (1994). Genetic risk ratio (GRR) has been defined as the ratio of disease risk in the backcross generation, compared with that in the high-risk parental strain. In this study, the formula of GRR for the backcross generation was modified to that of an intercross generation. It is convenient to define the GRR to be the ratio of survival in the F2 generation, compared to the NIH/Ola parental strain. The overall GRR_{F2} in the F2 generation is estimated to be 51.2%.

An estimate of the GRR_{F2} assuming that there is no recombination between the marker and the modifier gene is equal to 54%. Risch et al (1994) proposed two multilocus genetic models, an additive model in which the effects at one modifier gene may substitute for another and a multiplicative, epistatic model of gene interaction. Under an additive model, where the overall GRR_{F2} expressed

as [(1 : GRR_{F2}) - 1] = [(1 : GRR_1) - 1] + [(1 : GRR_2) - 1] } is a sum of contributions from individual modifier genes, the chromosome 5 modifier gene explains 89% of the overall GRR_{F2}. Under a multiplicative model, where the overall GRR_{F2} is the product of contribution from modifier genes expressed as: (GRR_{F2} = GRR_1 X GRR_2) the chromosome 5 modifier explains 92% of the overall GRR_{F2}. Therefore, it could be concluded that the chromosome 5 modifier locus is a major modifier of embryonic lethality.

4.6.4.5 The stage of TGFB1(-/-) embryonic lethality in different genetic backgrounds:

An independent study carried out on the stages of TGFB1(-/-) embryo lethality on both NIH/Ola and C57Bl/6J/Ola strains (Rusholme PhD Thesis., 1997) revealed that null embryo lethality (18% of the total nulls) in NIH/Ola genetic background was due to yolk sac phenotype whereas in C57Bl/6J/Ola strains almost all of the nulls die prior to this stage. According to that study, TGFB(-/-) embryos bred onto F1(NIH X C57) genetic background tend to survive up to 8.5 dpc, but only 32% of them develop to term normally and the rest die due to yolk sac phenotype. Those results verifies the results obtained during this project.

4.7 Candidate genes located in the region with definitive linkage:

Three important candidate genes; interleukin 6 (*IL6*), fibroblast growth factor receptor 3 (*Fgfr3*), and fibroblast growth factor inducible gene 13 (*Fin13*) map to the region showing linkage with TGFB1(-/-) survivals. *Il6* maps 2.2 cM proximal to *Fgfr3* (Avraham et al., 1994). However, neither of the genes have been mapped in comparison to MIT markers. The data based on integration of independent studies (Jax lab.) and data produced during this project, the order of genes and markers could be inferred as follow:
D5Mit251-Il6-D5Mit76-D5Mit268-D5Mit75-D5Mit267. *Fgfr3* and *FIN13* genes map in a region very close to that of D5Mit76. Therefore, it could be inferred

that the possible implication of *Fgfr3* and *Fin13* in different expressivity of TGFB1(-/-) phenotype is more than that of *Il6*.

4.7.1 Fibroblast growth factor receptor 3 (*Fgfr3*):

The fibroblast growth factor receptors (FGFRs) are high affinity receptors for the large family of fibroblast growth factors (FGFs). FGFs are involved in a variety of activities including embryonic pattern formation, mitogenesis, angiogenesis, and wound healing. FGFRs contain an extracellular domain with 3 immunoglobin-like domains, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (Givol & Yayon, 1992).

A complex pattern of binding specificities between FGFs and FGFRs is further complicated by the generation of multiple isoforms from a given receptor gene by alternative splicing and use of alternative start sites (Bottaro et al., 1990). Unlike FGFR1 and FGFR2 which bind both acidic FGF (FGF1) and basic FGF (FGF2), FGFR3 binds preferentially aFGF and showed approximately 20-fold lower affinity for bFGF (Ornitz & Leder., 1992). The immunoglobin III domain in the extracellular region of FGFR3 differs from those in FGFR1 and FGFR2 as a result of alternative splicing (Avivi et al., 1993). FGFR3 mRNA was abundant in the intestine, lung, kidney, and bone growth plates (Partanen et al., 1991). *Fgfr3* was cloned from the K562, myeloid cell line, which suggests a possible role in hematopoietic development (Keegan et al., 1991).

The gene for achondroplasia (ACH), inherited as an autosomal dominant with essentially complete penetrance was assigned to 4p16.3 by linkage analysis (Le Merrer et al., 1994; Velinove et al., 1994; Francomano et al., 1994). Causative mutations were identified by the candidate gene approach in the gene for *FGFR3* (Shiang et al., 1994; Rousseau et al., 1994; Bellus et al., 1995). Nucleotide 1138 of the *FGFR3* gene is the most mutable nucleotide discovered to date. This mutation resulted in the substitution of an arginine residue for a glycine at position 380 of the mature protein, which is in the transmembrane domain of FGFR3. Mutations in the extracellular domains of FGFR3 have been reported in Thanatophoric displasia (TD). TD resembles homozygous achondroplasia in some respects (Tavormina et al., 1995; Rousseau et al., 1996). Naske et al (1996) studied the effect of the ACH and

TD mutations on the activity and regulation of FGFR3 by transient transfection of NIH 3T3 and Baf3 pro-B cells with mutant *FGFR3* cDNA. They showed that each of the mutations studied constitutively activates the receptor, as evidenced by ligand-independent receptor tyrosine phosphorylation and cell proliferation. Moreover, the mutations responsible for TD were more strongly activating than the mutation causing ACH, providing Naski et al (1996) with a biochemical explanation for the observation that the phenotype of TD is more severe than that of ACH.

Colvin et al (1996) reported studies in mice made FGFR3 deficient by targeted disruption in the *Fgfr3* gene by knockout technique. Fgfr3(-/-) mice had phenotypic effects to bones that arise by endochondral ossification, i.e., increased length of the vertebral column and long bones had occurred. It has been proposed that one of the functions of FGFR3 is to limit osteogenesis. The recessive loss-of-function mutation in Fgfr3(-/-) mice produces a phenotype that is the opposite of that observed in ACH and TD.

Peter et al (1993) by applying *In situ* hybridisation studies have shown that *Fgfr3* in common with *Fgfr1* and *Fgfr2*, was expressed prenatally in the germinal epithelium of the neural tube. The nature of the *Fgfr3* gene as a growth factor receptor and its map location made it possible candidate for the different expressivity of TGFB1(-/-) phenotype. Therefore, during this project the expression of *Fgfr3* was examined in both 9.5 dpc embryo and yolk sac. The possibility of strain specific expression of the gene between two mouse strains; NIH/Ola and C57Bl/6J/Ola strains, was excluded by observing the expression of the gene in both wild type embryo and yolk sac in either of the strains. However, this study could not rule out the possibility of different expression of the gene in TGFB1(-/-) conceptuses bred onto different genetic background due to fact that in C57Bl/6J/Ola strain null embryo were dying in very early stage of embryogenesis.

4.7.2 Fibroblast growth factor inducible gene 13 (*Fin13*):

Guthridge et al (1996) has shown that *Fin13* is one of the 21 late-induced genes from an fibroblast growth factor-4 (FGF-4) transformed NIH3T3

cell line. 6 fold Induction of mRNA for *Fin13* was determined in NIH3T3 cells following FGF-4 stimulation. Sequencing of *Fin13* and translation of a putative open reading frame revealed that it would encode a protein of 392 amino acids. The expression of *Fin13* transcript was demonstrated in 10.5 dpc. embryos which became down-regulated thereafter. Due to map location of the gene and its nature to be highly induced by fibroblast growth factor-4, made it possible candidate in this study. This gene maps to a region 4.4 cM distal that of Il6 (proximal mouse chromosome 5). In this study, the expression of *Fin13* transcript in 9.5 dpc both embryo and yolk sac was examined. Studies on wild type 9.5 dpc embryos bred onto NIH/Ola and C57Bl/6J/Ola excluded the possibility of strain specific expression of this gene in 9.5 dpc embryo and yolk sacs. However, comparative studies on TGFB1(-/-) embryos bred onto two different strains due to the fact that null embryos bred onto C57Bl/6J/Ola strain die prior to 9.5 dpc stage, was not practical.

To study the possibility of polymorphism in the protein encoded by *Fin13* gene from NIH/Ola and C57Bl/6J/Ola strains, the coding sequence of the gene was subjected to heteroduplex and direct sequence analysis. The examined regions between two mouse strains are shown to be identical.

4.8 TGFB1(-/-) embryonic lethality in 129/Sv genetic background:

During this project it has been demonstrated that genetic factors clearly influence the frequency of prenatal lethality of TGFB1(-/-) neonates with the C57Bl/6J/Ola allele of chromosome 5 predisposing to prenatal lethality. However the NIH/Ola allele of this chromosome tends to rescue null embryos from prenatal lethality. It could be interesting to study the effect of a 129/Sv genetic background in expressivity of the TGFB1(-/-) phenotype.

When Shull et al (1992) generated TGFB1(-/-), one copy of the *Tgfb1* gene was disrupted in mouse embryonic stem (ES) cells derived from the 129/Sv strain. ES cells containing the disrupted *Tgfb1* allele were then injected into C57Bl/6J mouse blastocysts. TGFB1(+/-) animals that transmitted the ES cell-derived dominant agouti coat colour and the disrupted *Tgfb1* allele to their progeny were intercrossed to generate TGFB1(-/-) animals (shull et al., 1992).

From intercrosses of these animals, which were a mixture of 129/Sv and C57Bl/6J genetic backgrounds, a total number of 806 live offspring were yielded (Shull & Doetschman., 1992). Of these, 280 were homozygous wild-type, 413 were heterozygous, and 113 were homozygous for the disrupted *Tgfb1* allele (Shull & Doetschman, 1992). Significant deviation from the Mendelian ratio of 1:2:1 was clearly observed among these numbers. These numbers revealed that only 40% of TGFB1(-/-) and 73.7% of TGFB1(+/-) neonates developed to term normally whereas the rest died *in utero*. On the basis of this data, the percentage of 129 /Sv strain contributed to the TGFB1(-/-) animals is not clear. However, observing 40% survival of TGFB1(-/-) conceptuses bred onto a mixture of 129/Sv and C57Bl/6J strains give rise to the notion that the modifying gene originated from 129/Sv strain, despite that from C57Bl/6J, could rescue TGFB1(-/-) conceptuses from embryo lethality. To find out the penetrance of modifying gene originated from 129/Sv strain, it is required further experiments.

4.9 TGFB1(+/-) embryo lethality in different crosses:

~20% of TGFB1(+/-) embryo lethality on a mixed genetic background was already reported (Shull & Doetschman, 1992; Dickson et al., 1995). Further investigation by Dickson et al (1995), showed that 20% loss of TGFB1(+/-) conceptuses was rectified when 2 cell-stage preimplantation embryos from TGFB1(+/-) cross underwent gestation in the recipient TGFB1(+/+) mother. They also noticed that in a TGFB1(+/-) mother and TGFB1(+/+) father cross the frequency of TGFB1(+/-) survival was less compared to that of a reciprocal cross; TGFB1(+/+) mother and TGFB1(+/-) father. The only difference in these reciprocal crosses was whether female was TGFB1(+/-) or TGFB1(+/+). These results indicate that TGFB1(+/-) conceptuses which have the reduced level of endogenous TGFB1 are dependent to the maternal source of TGFB1 and there is a connection between the maternal level of TGFB1 and the percentage of TGFB1(+/-) survivals. In the TGFB1(+/-) cross when mother is TGFB1(+/-) and has the reduced level of TGFB1, the maternal TGFB1 is not adequate to compensate the reduced level

of endogenous TGFB1 in all TGFB1(+/-) embryos. This may be the reason for the high frequency of TGFB1(+/-) embryo lethality in the TGFB1(+/-) mother and TGFB1(+/+) father cross.

During the present project, it has been demonstrated that the TGFB1(+/-) embryo lethality occurs in both NIH/Ola and C57Bl/6J/Ola strains with no significant difference. Therefore, it could indicate the similar level of maternal TGFB1 in both C57Bl/6J/Ola and NIH/Ola strains. Unpublished data from Dr. Akhurst's Lab suggesting that C57Bl/6J/Ola and NIH/Ola have similar TGFB1 circulation levels verifies this suggestion.

4.10 Genetic mapping:

A genetic map is simply a representation of the distribution of a set of loci within the genome. There are three distinct types of genetic maps that can be derived for each chromosome in the genome. The three types of maps; linkage, chromosomal, and physical are distinguished both by the methods used for their derivation and the metric used for measuring distances within them.

4.10.1 Linkage maps:

"Linkage maps" referred to as a "recombination map", can only be constructed for loci that occur in two or more heritable forms or alleles. These maps are generated by counting the number of offspring that receive either parental or recombinant allele combinations from a parent that carries two different alleles at two or more loci. Analysis of this type of data allows one to determine whether loci are linked to each other and if they are, their relative order and the relative distances that separate them. Distances are measured in centiMorgans, with one centiMorgan equivalent to a crossover rate of 1%. Therefore, the linkage map is based on classical breeding analysis.

4.10.2 Chromosome maps:

The chromosome (cytogenetic) map is based on the karyotype of the genome. All mouse chromosomes are defined at the cytogenetic level according to their size and banding pattern. In this map, positions are indicated with the use of band names. Several different approaches are utilised to generate chromosome maps.

1-Somatic cell hybrid lines contain only portions of the mouse karyotype within the milieu of another species' genome. By correlating the presence or expression of a particular mouse gene with the presence of a mouse chromosome or subchromosomal region in these cells, one can obtain a chromosomal or subchromosomal assignment.

2-The second approach can be used in those special cases where karyotypic abnormalities appear in conjunction with particular mutant phenotypes. When the chromosomal lesion and the phenotype assort together, from one generation to the next, it is likely that the former causes the latter. When the lesion is a deletion, translocation, inversion, or duplication, one can assign the mutant locus to the chromosomal band that has been disrupted.

3-In situ hybridisation: This method enables to visualise the location of the corresponding sequence within a particular chromosomal band directly.

4.10.3 Physical maps:

All physical maps are based on direct analysis of DNA, and distances between and within loci are measured in basepairs (bp), kilobasepairs (kb), or megabasepairs (mb).

During this project a linkage map approach was utilised to map two important candidate genes; *Tgfbr2* and *PlanH1* on mouse chromosomes,.

4.10.4 Transforming growth factor beta type II receptor (Tgfbr2):

4.10.4.1 RFLPs:

Utilising 1.7 kb cDNA of *Tgfbr2* as a probe, variety of different bands were revealed between BamH1 digested DNAs from NIH/Ola, C57Bl/6J/Ola, 129/Sv, and *Mus Spretus* strains. RFLPs observed between these strains could have been due to hybridisation of the probe with pseudo genes. To address this problem, the full length cDNA was digested into small fragments which were then used as probes. Utilising small digested fragments of cDNA as probes, resulted only selective hybridisation of bands. Therefore, it was confirmed all RFLPs observed between different strains belonged to *Tgfbr2* gene and the possible interference of any pseudo gene was ruled out.
Detailed examination of the DNA bands observed when applying different regional *Tgfbr2* cDNA probes for hybridisation enabled the design of a probe which distinguished RFLPs of *Tgfbr2* gene among different strains of NIH/Ola, C57Bl/6J, 129/sV, and *Mus. Spretus.*

4.10.4.2 Human-mouse homology:

Mathew et al (1994) used somatic cell hybrids and fluorescence *in situ* hybridisation to map *TGFBRII* to human chromosome 3p22 in a region syntenic with mouse chromosomes 6 (49-53) and distal 9 (55-74). During this project by utilising the designed probe, this gene was mapped on distal end of mouse chromosome 9. The order of the mapped gene in compare to MIT markers mapped previously was calculated as follow (cM ± SE): Cenrtromere-D9Mit16 (2.1 ± 0.014)-D9Mit150 (3.1 ± 0.02)-*Tgfbr2* (1 ± 0.0)-D9Mit18.

4.10.4.3 Mutant mice.

Several uncloned mutations map to the region of *Tgfbr2* gene including *Ky* (kyphoscoliotic degenerative muscle), *duc* (ducky), *pcy* (polycystic kidney disease), *tip* (tippy), *sr* (spinner), *fd* (fur deficient), and *sch*(scant hair). During this project, two uncloned mutants; ducky and tippy, were examined for the possibility of large deletion of *Tgfbr2* gene within them.

4.10.4.3.1 Ducky:

The ducky mutation arose spontaneously in a non-inbred stock. Homozygous show a waddling or reeling gait and tendency to fall to one side. They are slightly smaller than normal and may occasionally have seizures. Viability is somewhat less than normal. Males living to maturity may be fertile, but are poor breeders. Females rarely breed (Snell 1955). This mutation which shows recessive pattern of inheritance maps to distal end of chromosome 9 (Kingsley et al., 1989). Southern blot analysis, performed using HindIII-digested DNA from mice homozygous for *duc* has excluded the possibility of a large deletion of *Tgfbr2* within this mutant.

4.10.4.3.2 Tippy:

This mutation arose in a linkage cross in 1977. Mice homozygous for *tip* are small, cannot stand or walk without falling over, and generally die by a bout 3 weeks postpartum (Green 1989; Kingsley 1992). The gene is on chromosome 9 near ducky and shows recessive pattern of inheritance (Lane 1984). Southern blot analysis, performed using HindIII-digested DNA from mice homozygous for *tip* has excluded the possibility of a large deletion of *Tgfbr2* within this mutant.

However, it has been recently shown that *Tgfbr2* null embryos die by 13.5 dpc. due to yolk sac hematopoiesis and vasculogenesis deficiency (Oshima et al., 1996) and any of the mutant mice listed above might involve more subtle molecular pathologies within the *Tgfbr2* gene.

4.10.4.4 *Tgfbr2* gene as a candidate gene for cancer:

Studies carried out in susceptibility to skin carcinogenesis has revealed that there are two different stages of initiation and promotion. Linkage studies on a genetic model developed for the study of promotion susceptibility utilising relatively sensitive DBA/2 and relatively resistant C57Bl/6 mice has suggested the involvement of genes mapping to chromosomes 2, 9, and 11 in promotion

susceptibility. Further study supported the linkage of a promotion susceptibility locus to mouse chromosome 9 (D9Mit53) (Angel et al., 1996). This marker maps close to a region in which *Tgfbr2* gene maps. Therefore, *Tgfbr2* gene could be a good candidate gene for the skin carcinogenesis promotion susceptibility.

4.10.5 Plasminogen activator inhibitor type I (*PlanH1*)

In an attempt to map the *PlanH1* gene on mouse genome, polymorphic RFLPs was observed between two mouse strains by applying the *PlanH1* cDNA as a probe. The *PAI1* gene has already been mapped to human chromosome 7q21.3→q22 (Klinger et al., 1987). Therefore, markers from the syntenic region were selected and the segregation of markers and the RFLP were followed among 48 F2 intercross animals. The murine *PlanH1* has been mapped in the distal region of chromosome 5, which is syntenic with human chromosome 7q21-q22.

4.11 Conclusion:

During this project it has been shown that the TGFB1(-/-) phenotype depends on the genetic background of the conceptus. In NIH/Ola strain, null conceptuses tend to develop to term normally whereas in C57/6J/Ola strain almost all of the nulls die *in utero.* A major codominant modifying gene of embryo lethality was mapped to proximal mouse chromosome 5, using a genome scan for non-Mendelian distribution of alleles in TGFB1(-/-) neonatal animals which survive prenatal lethality. This gene accounts for around three quarters of the genetic effect between mouse strains.

Expression studies of three candidate genes; *Tgfb1, Fin13,* and *Fgfr3* carried out on 9.5 dpc wild type embryos and yolk sacs bred onto either NIH/Ola or C57/6J/Ola strains, excluded the possibility of strain specific expressivity of the genes in a specific stage of embryogenesis.

Applying heteroduplex and direct sequencing approaches, excluded the possibility of polymorphism in the protein encoded by *Fin13* gene between NIH/Ola and C57Bl/6J/Ola strains.

During this project also Transforming Growth Factor Beta type II receptor (*Tgfbr2*) and Plasminogen activator inhibitor type I (*PlanH1*) genes were mapped on the proximal ends of mouse chromosomes 9 and 5, respectively. Following the mapping *Tgfbr2* on the mouse genome, two uncloned mutant mice; Tippy and Ducky, which map in the vicinity of the *Tgfbr2* location were examined by application of Southern blotting for the possibility of a large deletion in *Tgfbr2* gene in either of these mutants. Observing no differences in the size of Southern blot bands between wild type and homozygous mice (in both Tippy and Ducky mice) the possibility of large deletion of *Tgfbr2* in these mutants has been excluded.

4.12 From Linkage to Locus for Polygenic Traits:

Putting aside speculation about possible candidate genes, it is important to consider the general issue of how to proceed from the initial detection of linkage to the definitive identification of a gene contributing to a polygenic trait. Cloning mammalian genes based solely on position remains a daunting task even for genes controlling simple Mendelian traits; it has been accomplished in very few cases to date. Nonetheless, genomic mapping tools are improving and the task may well became more tractable. In anticipation of continued improvement, an approach to cloning the modifier gene based on position is outlined:

1- It is important to confirm the linkage, by repeating the precise cross used initially.

2- If linkage can be confirmed, a small region containing the modifier locus (ML) allele from C57 strain could be moved genetically into NIH strain by successive backcrosses to create congenic strains differing only in the region of the ML. To ensure the introgression of the ML allele from C57 strain, progeny that have inherited alleles from C57 strain at a pair of genetic markers flanking the ML should be selected. To accelerate the removal of C57 strain elsewhere in the genome, individuals who have fortuitously lost more than the

expected 50% proportion of C57 strain alleles per generation could be selected. In practice, such selection can roughly halve the number of backcross generations required of introgression (Paterson et al., 1988). Once congenic strains have been established, the percentage of TGFB1 nulls developing to term in intercrosses of these congenic animals could be compared to that of C57 cross to find out if there is any other loci involved in different expressivity of the TGFB1(-/-) phenotype.

3- Using the congenic strains, the ML could be mapped more closely by arranging a backcross to collect individuals recombinant for a pair of flanking markers. Using a denser map of genetic markers, the recombination breakpoints could be identified and the ML could be mapped relative to these by testing the recombinant progeny.

4- when the region has been adequately narrowed, a physical map could be constructed to identify genes in the region. Because MLs seem likely to involve subtle mutations rather than gross gene disruption, the mutations themselves may be difficult to find. Instead, one may need to select and test plausible candidate genes in the region.

5- To prove that a candidate gene corresponds to the trait, homologous recombination could be utilised to construct gene knockouts in strains carrying a dominant allele (which can then be tested for loss of dominance at the ML) or to substitute the allele in C57 strain into NIH strain and demonstrate that the ML effect has been transferred as well.

Appendix:

Appendix I-A

Table 1: The format of 2 X 2 contingency table by the use of symbols (Wardlaw, 1992).

Group	TGFB1 (+/+) neonates	TGFB1 (-/-) neonates	Total
A	a	b	(a + b)
B	c	d	(c + d)
Total	(a + c)	(b + d)	N

The formula (1) to calculate Chis Square:

$$\chi^2 = \frac{N\{|ad - bc| - \frac{1}{2}N\}^2}{(a+b) \times (c+d) \times (a+c) \times (b+d)} \quad \textbf{(equation 1)}$$

Appendix I-B

Table 1: Observed and expected numbers for the TGFB1(-/-) and TGFB1(+/+) embryos born to C57Bl/6J females mated to males with different genetic backgrounds (Data extracted from table 3.2), and calculation of χ^2.

Cross	Observed numbers (O)	Expected numbers (E)	$\frac{(O-E)^2}{E}$
TGFB1(-/-) embryos born to (C57 X C57)	0	{ (0+3+9)/ 134 } x {(68 + 0)} = **6.089**	**6.089**
TGFB1(+/+) embryos born to (C57 X C57)	68	{(68+29+25)/134} x (68+0)=**61.91**	**0.599**
TGFB1(-/-) embryos born to (C57 X F1)	3	{ (0+3+9)/ 134 } x {(3+29)} = **2.865**	**0.006**
TGFB1(+/+) embryos born to (C57 X F1)	29	{(68+29+25)/134} X (29+3)=**29.13**	**0.0005**
TGFB1(-/-) embryos born to (C57 X NIH)	9	{ (0+3+9)/ 134 } X {(9+25)} = **3.044**	**11.653**
TGFB1(+/+) embryos born to (C57 X NIH)	25	{(68+29+25)/134} X (25+9)= **30.95**	**1.143**
Total	**134**	**133.993**	**χ^2= 19.49**

Chis Square test was performed (discussed in section 3.10.2) on each group of nulls and wild types separately. To calculate the expected numbers for each group of wild types, the total number of wild types (68+29+25), was divided by the total number of animals under statistical analysis [i.e. (68+29+25) : 134]. This ratio was multiplied to the total number of animals (nulls and wild types) in each cross, to find the estimated number of wild types in that cross [e.g. in C57 x C57 cross, this ratio was multiplied to (68+0)]. The same approach was utilised to find the estimated number of nulls in each cross. The total χ^2 is 19.49. Assuming the null hypothesis, the relevant P-value with 2 d.f. is equal to 5.8×10^{-5}.

Table 2: Observed and expected numbers for the TGFB1(-/-) and TGFB1(+/+) embryos born to NIH/Olac females mated to males with different genetic backgrounds (Data extracted from table 3.2), and calculation of χ^2.

Cross	Observed numbers (O)	Expected numbers (E)	$\frac{(O-E)^2}{E}$
TGFB1(-/-) embryos born to (NIH X NIH)	73	63.05	**1.57**
TGFB1(+/+) embryos born to (NIH X NIH)	89	98.93	**0.99**
TGFB1(-/-) embryos born to (NIH X F1)	14	13.62	**0.01**
TGFB1(+/+) embryos born to (NIH X F1)	21	21.37	**0.006**
TGFB1(-/-) embryos born to (NIH X C57)	15	25.30	**4.19**
TGFB1 (+/+) embryos born to (NIH X C57)	50	39.69	**2.67**
Total	**262**	**261.96**	χ^2=9.44

Chis Square test was performed (discussed in section 3.10.2) on each group of nulls and wild types separately. To calculate the expected numbers for each group (i.e. nulls and wild types), the same approach as table 1 (appendix I-B) was taken. The χ^2 is 9.44. Assuming the null hypothesis, the relevant P-value with 2 d.f. is equal to 0.008 (<0.05).

Table 3: Observed and expected numbers for the TGFB1(-/-) and TGFB1(+/+) embryos born to F1 (NIH x C57) females mated to males with different genetic backgrounds (Data extracted from table 3.2), and calculation of χ^2.

Cross	Observed numbers (O)	Expected numbers (E)	$\frac{(O-E)^2}{E}$
TGFB1(-/-) embryos born to (F1 X NIH)	85	63.41	**7.35**
TGFB1(+/+) embryos born to (F1 X NIH)	118	139.5	**3.31**
TGFB1(-/-) embryos born to (F1 X F1)	80	84.34	**0.22**
TGFB1(+/+) embryos born to (F1 X F1)	190	185.65	**4.35**
TGFB1(-/-) embryos born to (F1 X C57)	9	26.24	**11.32**
TGFB1(+/+) embryos born to (F1 X C57)	75	57.75	**5.15**
Total	**557**	**556.899**	**χ^2 = 31.70**

Chis Square test (discussed in section 3.10.2) was performed on each group of nulls and wild types separately. To calculate the expected numbers for each group (i.e. nulls and wild types), the same approach as table 1 (appendix I-B) was taken. The sum of χ^2 was equal to 31.70. Assuming the null hypothesis, the relevant P-value with 2 d.f. is less than 10^{-5}.

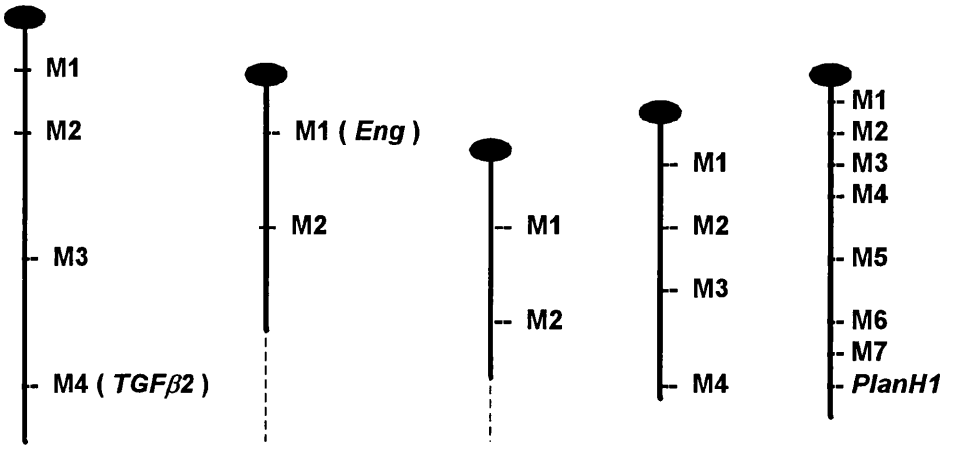
Table 4: Observed and expected numbers for the TGFB1(+/-)and TGFB1(+/+) embryos born to either NIH/Ola or C57Bl/6J/Ola strains (Data extracted from table 3.2), and calculation of χ^2.

Cross	Observed numbers (O)	Expected numbers (E)	$\frac{(O-E)^2}{E}$
NIH/Ola TGFB1(+/+)	89	83.8	
NIH/Ola TGFB1(+/-)	129	134	
C57Bl/6J/Ola TGFB1(+/+)	68	73.1	
C57Bl/6J/Ola TGFB1(+/-)	122	116.8	
Total	408	407.9	χ^2=1.0966

Chis Square test (discussed in section 3.10.2) was performed on each group of nulls and wild types separately. To calculate the expected numbers for each group (i.e. hets and wild types), the same approach as table 1 (appendix I-B) was taken. The sum of χ^2 was equal to 1.0966. Assuming the null hypothesis, the relevant P-value with 1 d.f. is 0.2950.

Appendix II-B

Location of the markers utilised to screen the whole mouse genome. The position of some candidate genes screened during this project are shown. Continous lines show the regions of the chromosomes swept by the markers.



Chrom. 1

M1; MIT318, M2; MIT76.
M3; MIT387, M4; MIT403.

2

M1; MIT83,
M2; MIT38.

3

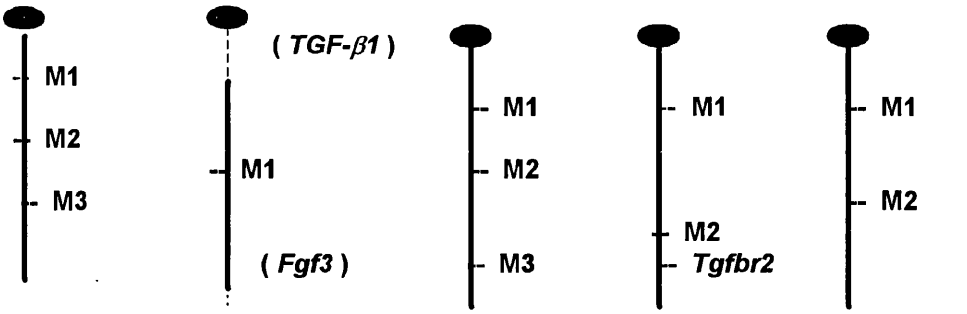
M1; MIT 6,
M2; MIT106,

4

M1; MIT192,
M2; MIT178,
M3; MIT175,
M4; MIT170.

5

M1; MIT49,
M5; MIT80
M7; MIT188



Chrom. 6

M1; MIT74,
M2; MIT213,
M3; MIT25.

7

M1; MIT284,

8

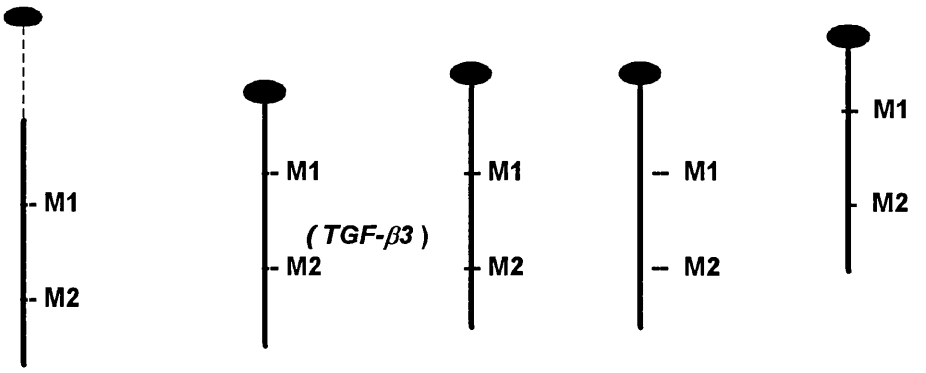
M1; MIT190.
M2; MIT9,
M3; MIT166.

9

M1; MIT268
M2; MIT16

10

M1; MIT20
M2; MIT95



Chromos: 11 **12** **13** **14** **15**

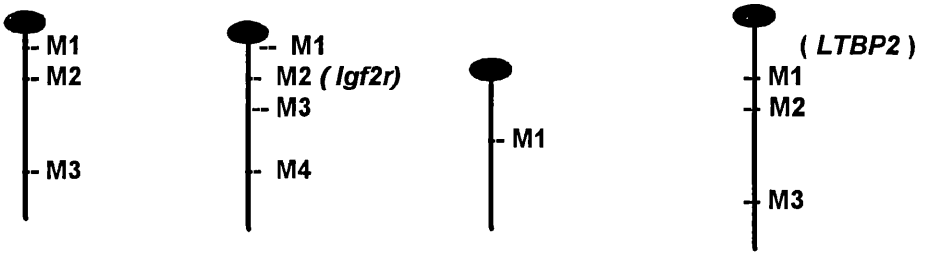
M1; MIT5, M2; MIT258.

M1; MIT46, M2; MIT8.

M1; MIT63, M2; MIT290.

M1; MIT141, M2; MIT35.

M1; MIT220 M2; MIT159.



Chrom: 16 **17** **18** **19**

M1; MIT181, M3; MIT114.

M1; MIT43, M3; MIT21, M4; MIT93.

M1; MIT150.

M1; MIT31, M2; MIT19, M3; MIT103.

Appendix IIC:

Table showing markers used to screen NIH/Ola and C57Bl/6J/Ola strains for polymorphism.

Chromosome:	Marker:	Polymorphism:	Location:
Chromosome 1	D1 Mit 318	129=NIH<C57	17 cM
Chromosome 1	D1 Mit 403	NIH<C57	98 cM
Chromosome 1	D1 Mit 387	NIH<C57	66 cM
Chromosome 1	D1 Mit 60	?	60 cM
Chromosome 1	D1 Mit 451	129<=NIH=C57	82 cM
Chromosome 1	D1 Mit 76	NIH >> C57	33 cM
Chromosome 1	D1 Mit 236	NIH << C57	25.9 cM
Chromosome 1	D1 Mit 446		75.7 cM
Chromosome 1	D1 Mit 102		77.9 cM
Chromosome 2	D2 Mit 98	129=NIH=C57	52 cM
Chromosome 2	D2 Mit 7	129=NIH<C57	28 cM
Chromosome 2	D2 Mit 83	129<NIH<C57	17 cM
Chromosome 2	D2 Mit 368	129=C57>NIH ?	28 cM
Chromosome 2	D2 Mit 38	NIH>>C57	45 cM
Chromosome 2	D2 Mit 244		34.2 cM
Chromosome 2	D2 Mit 243		34.2 cM
Chromosome 3	D3 Mit 38	NIH=?129>>C57	50 cM
Chromosome 3	D3 Mit 6	NIH<C57	18 cM
Chromosome 3	D3 Mit 106	129=NIH<<C57	41 cM
Chromosome 4	D4 Mit 192	129=NIH>C57	9 cM
Chromosome 4	D4 Mit 175	NIH>>C57	42 cM
Chromosome 4	D4 Mit 9	NIH=C57>129	33 cM
Chromosome 4	D4 Mit 129	129<NIH<C57	64 cM

Chromosome 4	D4 Mit 192	129?NIH>>C57	9 cM
Chromosome 4	D4 Mit 170	NIH>>C57=?129	63 cM
Chromosome 4	D4 Mit 178	NIH >> C57	27 cM
Chromosome 4	D4 Mit 80		28 cM
Chromosome 5	D5 Mit 147	129<C57=NIH	5.6 cM
Chromosome 5	D5 Mit 135	129>C57=NIH	29.4 cM
Chromosome 5	D5 Mit 25	129=C57=NIH	47.4 cM
Chromosome 5	D5 Mit 66	129=NIH>C57	13.5 cM
Chromosome 5	D5 Mit 234	129<C57=NIH	28.3 cM
Chromosome 5	D5 Mit 83	129>NIH=C57	28 cM
Chromosome 5	D5 Mit 250	129=C57=NIH	5.6 cM
Chromosome 5	D5 Mit 7	129=NIH<C57	33.9 cM
Chromosome 5	D5 Mit 251	129=C57>NIH	9 cM
Chromosome 5	D5 Mit 23	129=C57>NIH	38.4 cM
Chromosome 5	D5 Mit 103	NIH=C57=129	3 cM
Chromosome 5	D5 Mit 233	129=C57<NIH	20 cM
Chromosome 5	D5 Mit 265	NIH=C57	68 cM
Chromosome 5	D5 Mit 188	129=?NIH<<C57	53 cM
Chromosome 5	D5 Mit 24	C57<129<NIH	47 cM
Chromosome 5	D5 Mit 6	129=C57=NIH	23.9 cM
Chromosome 5	D5 Mit 73	129<C57=NIH	9 cM
Chromosome 5	D5 Mit 10	129=C57=NIH	38.4 cM
Chromosome 5	D5 Mit 76	129=C57<NIH	15.8 cM
Chromosome 5	D5 Mit 49	129=C57<NIH	0 cM
Chromosome 5	D5 Mit 109	129=C57=NIH	26 cM
Chromosome 5	D5 Mit 268	NIH<<C57=129	18 cM
Chromosome 5	D5 Mit 4	129=C57=NIH	15.8 cM
Chromosome 5	D5 Mit 44	129=C57<NIH	13.5 cM
Chromosome 5	D5 Mit 1	NIH=C57>129	3 cM

Chromosome 5	D5 Mit 82	129>C57=NIH	27.2 cM
Chromosome 5	D5 Mit 80	129=C57<NIH	18 cM
Chromosome 5	D5 Mit 197	129=C57<<NIH	27.2 cM
Chromosome 5	D5 Mit 5	129=C57=NIH	27.2 cM
Chromosome 5	D5 Mit 77	129=C57=NIH	16 cM
Chromosome 5	D5 Mit 267	129<<C57<<NIH	*******
Chromosome 5	D5 Mit 180	129=C57=NIH	5 cM
Chromosome 5	D5 Mit 251	129=C57>NIH	9 cM
Chromosome 5	D5 Mit 34	129=C57=NIH	84 cM
Chromosome 5	D5 Mit 126	NIH > C57	16 cM
Chromosome 5	D5 Mit 75	NIH << C57	16 cM
Chromosome 5	D5 Mit 182	NIH = C57	17 cM
Chromosome 5	D5 Mit 354	NIH = C57	17 cM
Chromosome 6	D6 Mit 9	NIH=C57>129	36 cM
Chromosome 6	D6 Mit 74	NIH<<C57=?129	11 cM
Chromosome 6	D6 Mit 19	NIH<C57>>129	33 cM
Chromosome 6	D6 Mit 13	NIH=C57=129	63 cM
Chromosome 6	D6 Mit 25	129<NIH<C57	50 cM
Chromosome 6	D6 Mit 70	NIH<<C57=?129	34 cM
Chromosome 6	D6 Mit 69	NIH=?129<<C57	35 cM
Chromosome 6	D6 Mit 324		30 cM
Chromosome 6	D6 Mit 213	NIH << C57	30 cM
Chromosome 7	D7 Mit 109	NIH=C57=129	64 cM
Chromosome 7	D7 Mit 284	NIH=129>>C57	44 cM
Chromosome 8	D8 Mit 16	NIH=C57	7 cM
Chromosome 8	D8 Mit 3	NIH=C57<<129	7 cM
Chromosome 8	D8 Mit 13	NIH<<C57<<129	69 cM
Chromosome 8	D8 Mit 166	NIH=129>>C57	57 cM
Chromosome 8	D8 Mit 9	NIH<C57	36 cM

Chromosome 8	D8 Mit 8	NIH=C57	35 cM
Chromosome 8	D8 Mit 63	NIH#C57	14 cM
Chromosome 8	D8 Mit 226		23 cM
Chromosome 8	D8 Mit 190	NIH << C57	23 cM
Chromosome 9	D9 Mit 286	129=NIH<<C57 ?	17 cM
Chromosome 9	D9 Mit 150	NIH<<C57<129	59 cM
Chromosome 9	D9 Mit 18	NIH>C57=129	67 cM
Chromosome 9	D9 Mit 286	NIH=?129<<C57	23 cM
Chromosome 9	D9 Mit 82	NIH=C57	74 cM
Chromosome 9	D9 Mit 16	NIH<<C57=129	57 cM
Chromosome 9	D9 Mit 19	NIH=C57=129	71 cM
Chromosome 9	D9 Mit 17	C57=129	62 cM
Chromosome 9	D9 Mit 9	C57<129	48 cM
Chromosome 9	D9 Mit 4	C57<129	29 cM
Chromosome 9	D9 Mit 2	C57=129	17 cM
Chromosome 9	D9 Mit 104		34 cM
Chromosome 9	D9 Mit 174		32 cM
Chromosome 10	D10 Mit 20	NIH<C57	23 cM
Chromosome 10	D10 Mit 21	NIH=C57	39 cM
Chromosome 10	D10 Mit 95	NIH=?129<<C57	50 cM
Chromosome 10	D10 Mit 32	NIH=C57	32 cM
Chromosome 10	D10 Mit 95	C57>>NIH=?129	50 cM
Chromosome 10	D10 Mit 15	NIH<C57	23 cM
Chromosome 10	D10 Mit 21	NIH=C57	39 cM
Chromosome 10	D10 Mit 15	NIH<C57	23 cM
Chromosome 10	D10 Mit 20	NIH<C57	23 cM
Chromosome 10	D10 Mit 30	NIH=C57	21 cM
Chromosome 10	D10 Mit 42	NIH > C57	40.5 cM
Chromosome 10	D10 Mit 117		43.9 cM

Chromosome 11	D11 Mit 258	NIH>>129>>C57	67 cM
Chromosome 11	D11 Mit 5	NIH=129<<C57	37 cM
Chromosome 11	D11 Mit 151	NIH<C57<<129	10 cM
Chromosome 11	D11 Mit 110		17.9 cM
Chromosome 11	D11 Mit 308		19 cM
Chromosome 12	D12 NDS 2	NIH>C57	62 cM
Chromosome 12	D12 Mit 8	129>NIH>C57	60 cM
Chromosome 12	D12 Mit 46	C57>>NIH	17 cM
Chromosome 12	D12 Mit 77	NIH>C57	44 cM
Chromosome 12	D12 Mit 101	NIH=129=C57	47 cM
Chromosome 12	D12 Mit 154	NIH#C57	18 cM
Chromosome 12	D12 Mit 46	NIH=?129<<C57	15 cM
Chromosome 12	D12 NDS 11	NIH=129>C57	7 cM
Chromosome 12	D12 Mit 101	NIH=129=C57	47 cM
Chromosome 12	D12 Mit 5	NIH>129	33.9 cM
Chromosome 12	D12 Mit 68	NIH<C57	23 cM
Chromosome 13	D13 Mit 290	C57.1>C57.2>NIH	46 cM
Chromosome 13	D13 Mit 63	NIH>C57	20 cM
Chromosome 13	D13 Mit 119	NIH=129=C57	20 cM
Chromosome 14	D14 Mit 141	NIH=?129<<C57	19.2 cM
Chromosome 14	D14 Mit 140	NIH=?129<<C57	16.9 cM
Chromosome 14	D14 Mit 239	NIH<C57	46.5 cM
Chromosome 14	D14 Mit 61	NIH=C57	21.4 cM
Chromosome 14	D14 Mit 35	NIH<?C57	48.8 cM
Chromosome 15	D15 Mit 159	NIH<<C57	46 cM
Chromosome 16	D16 Mit 114	NIH<<C57	35 cM
Chromosome 16	D16 Mit 181	NIH>C57	3.3 cM
Chromosome 16	D16 Mit 146	NIH=C57>129	19 cM
Chromosome 16	D16 Mit 57	NIH=C57	23 cM

Chromosome 16	D16 Mit 181	NIH>C57	3 cM
Chromosome 16	D16 Mit 58		25.8 cM
Chromosome 16	D16 Mit 59	NIH >> C57	26.9 cM
Chromosome 17	D17 Mit 16	NIH<C57=129	8 cM
Chromosome 17	D17 Mit 93	NIH>C57	41 cM
Chromosome 17	D17 Mit 43	NIH>129>C57	1 cM
Chromosome 17	D17 Mit 47		12 cM
Chromosome 17	D17 Mit 13		10 cM
Chromosome 17	D17 Mit 48		1 cM
Chromosome 17	D17 Mit 21		10 cM
Chromosome 18	D18 Mit 209	NIH=C57>129	27 cM
Chromosome 18	D18 Mit 150	NIH>C57>129	17 cM
Chromosome 18	D18 Mit 66		0 cM
Chromosome 18	D18 Mit 4		38 cM
Chromosome 19	D19 Mit 92	NIH=C57	60 cM
Chromosome 19	D19 Mit 22	NIH=C57=129	13 cM
Chromosome 19	D19 Mit 28	NIH<C57=129	14 cM
Chromosome 19	D19 Mit 103	NIH>>C57	40 cM
Chromosome 19	D19 Mit 31	NIH>C57=129	13 cM

Appendix II-D:

Modification of GRR for backcross (Risch et al., 1993) to GRR for intercross.

GRR was defined as the ratio of disease risk in the backcross generation with that in the high-risk parental strain.

$$GRR = \frac{\frac{1}{4}F1 + \frac{1}{2}F2 + \frac{1}{4}F3}{F3} \quad (1)$$

$$GRR = \frac{1 + \frac{F1}{F3} + \frac{2F2}{F3}}{4} \quad (2)$$

Where in this research, F1 denotes the penetrance (proportion of animals affected) of the CC genotype, F2 denotes the penetrance (proportion of animals affected) of the NC genotype, and F3 denotes the penetrance (proportion of animals affected) of the NN genotype.

The GRR for a given locus can be estimated from the observed genotypes (e.g. at the closest linked marker) among affected animals. If a total of A affected animals is observed in the intercross generation, It would be expected that the proportion (formula 3) would be homozygous CC, the proportion (formula 4) would be heterozygous NC, and the proportion (formula 5) would be homozygous NN.

$$\frac{F1}{F1 + F2 + F3} \quad (3)$$

$$\frac{2F2}{F1 + F2 + F3} \quad (4)$$

$$\frac{F3}{F1 + F2 + F3} \quad (5)$$

By solving the equation (2) for the number of animals (equations 3, 4, 5) the following formula (6) was obtained.

$$GRR = \frac{1 + \frac{A1}{A3} + \frac{A2}{A3}}{4} \quad (6)$$

Where A1 = the number of (C/C) animals, A2 = the number of (C/N) animals, and A3 is the number of (N/N) animals.

Appendix II-E

$$SE = \left[\frac{x(1-x)}{n} \right]^{\frac{1}{2}}$$

Where SE is " standard error", "n" is the number of meiosis, and "X" is the recombination fraction. This formula was applyed to find standard error in estimation of distance of the markers and genes utilised in the designed panel.

Appendix III

Performing linkage studies conducted on 48-50 TGFB1(-/-) survivals by applying the linked markers to some candidate genes.

candidate gene	linked marker	Chromosome	χ^2	P-Value (2 d.f)
Tgfb2	D1Mit403	1	0.45	>0.05
Eng	D2Mit83	2	2.59	>0.05
fgf3	D7Mit284	7	1.79	>0.05
Tgfb3	D12Mit8	12	4.49	>0.05
Igf2r	D17Mit16	17	1.0	>0.05
Ltbp2	D19Mit31	19	2.79	>0.05

Appendix IV

In order to evaluate the observed linkage of chromosome 5 (D5Mit268) and provide evidence that the observed segregation distortion of the marker was only due to TGFB1 modifying gene(s) not due to any unknown possible factor, 50 wild type animals [TGFB1(+/+)] from the same cross as TGFB1(-/-) were screened for D5Mit268 marker.

Animal	Marker	N/N	N/C	C/C	χ^2	P-Value
TGFB1(+/+)	D5Mit268	14	24	12	0.24	>0.05

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