UNIVERSITY OF CALGARY

Generating novel targeting vectors in vivo using phage-plasmid recombination.

by

M.W. Todd Unger

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

NOVEMBER, 2000

Summary

Sorsby Fundus Dystrophy (SFD) is a rare autosomal dominant degenerative disease of the retina usually caused by a single point mutation in the gene *tissue inhibitor of metalloproteinases-3 (TIMP3)*. As various aspects of the disease so closely resembles that of Age-Related Macular Degeneration (AMD), the most common cause of blindness in the western world, the study of SFD is regarded as a potential route by which to unlock part of the genetic basis of AMD. A mouse model of SFD would therefore be a most beneficial tool.

A targeting vector (TV) that could later be used for the generation of an SFD mouse was constructed using a novel approach. The TV was constructed in bacteriophage through two rounds of phage-plasmid recombination. In the first recombination event, a phage clone containing the desired region of mouse *Timp3* was passaged over cells that contained a novel plasmid, πSFDγ. This plasmid contained a small DNA fragment with the SFD mutation and a 50-bp region of homology to the cloned region of *Timp3*. Through homologous recombination, the entire plasmid was incorporated into the phage clone. In a subsequent step, the plasmid was able to revert out of the phage DNA. In a fraction of these reversion events, the mutation remained behind and the wild-type sequence reverted with the plasmid, displaying a powerful mutagenic system termed transplacement mutagenesis. In the next round of phage-plasmid recombination a large region of homology and the selectable marker *Hprt* were added by insertion into the TV.

The successful generation of a novel targeting vector using *in situ* phage-plasmid recombination demonstrates the power of this mutagenic strategy, the applications of which are not limited to gene targeting.

Acknowledgments

I gratefully acknowledge my supervisor, Derrick Rancourt, not only for giving me the opportunity to do research in his lab, but also for taking a sincere interest in the career goals of all of his graduate students.

I thank Maria Mosaico and Knut Woltgen for their help and valuable input. Maria, your incredible work ethic was inspiring to me; and Knut, even though you remain a lowly graduate student in the eyes of the University, you are in reality already a post-doc and have been functioning in that capacity for quite some time. Of course, you already knew that.

I thank Brenda Carson for teaching me how to handle mice, and Eileen Rattner for showing me how to handle the single-celled version of the same.

Thanks to all of the other citizens of the Rancourt Lab, both past and present. You put up with my bluntness just fine. I'm really impressed. I would have had very little patience for me if I were you. Also, I cannot imagine another lab having as many parties as we did. This was a really fun place to work. You are far too numerous to name here. In fact, there are more of you than I can shake a stick at, and believe me, I've occasionally wanted to shake a stick at some of you. Sorry, there's that bluntness again.

Maggie Naylor, you were a great sounding post. Having very little in common other than our repeated rejection by the elitist medical establishment, we sure made a great two-person support group.

Finally, I must thank my roommate Lucas Rosnau. What a strange fellow you are to actually keep the same hours as I. Even as I write this, you are sitting across from me at Denny's at 3:00am on a week night, working on your own thesis. How strange it that!?! I have not had a roommate whose company and conversation I have enjoyed more than yours. Thanks, big guy!

This thesis is dedicated to my parents,
Merv and **Margaret Unger**,

and to the memory of my good friend,
Darrell Bohn.

Table of Contents

List of Figures

List of Abbreviations

6-TG	6-thioguanine
AMD	age-related macular degeneration
BM	Bruch's membrane
cDNA	complementary deoxyribonucleic acid
CRD	cone-rod dystrophy
DNA	deoxyribonucleic acid
DNase	deoxyribonucleic acid nuclease
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ES	embryonic stem
FIAU	[1-(2'-deoxy-2'fluoro-β-D-arabinofuranosyl)-5-iodouracil]
G418	geneticin
GANC	gancyclovir
HAT	hypoxanthine, aminopterin, and thymidine medium
HPRT	hypoxanthine phosphoribosyl transferase
IPM	interphotoreceptor matrix
IPTG	isopropyl-β-D-thiogalactoside
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
neo	neomycin

OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
pgk	phosphoglycerate kinase
PNS	positive-negative selection
rap	recombination adept with plasmid
RC	replacement-condensation
RNA	ribonucleic acid
RNase	ribonucleic acid nuclease
RP	retinitis pimentosa
RPE	retinal pigment epithelium
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFD	Sorsby fundus dystrophy
spi	sensitivity to P2 interference
STGD	Stargardt disease
TIMP	tissue inhibitor of metalloproteinases
Timp3	mouse tissue inhibitor of metalloproteinases-3
tk	thymidine kinase
Tris	Tris (hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TV	targeting vector
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Genetic Attributes

X^{am}	gene X contains an amber mutation
X^{+}	wild-type
X°	gene X is null mutant
X^{-}	gene X is mutant
Y^{s}	exhibits sensitivity to Y
Y^{r}	exhibits resistance to Y

Units of Measure

bp	base pair
g	gram
kb	kilobase
l	liter
M	molar
mg	milligram
ml	milliliter
mM	millimolar
nm	nanometer
µg	microgram
µl	microliters
$^{\circ}C$	degrees Celsius

CHAPTER ONE: INTRODUCTION

Retina Overview

The function of the retina is to convert electromagnetic energy emitted by a visual image into neural signals, which are sent to the brain via the optic nerve. Several common major cell types make up the retina in all vertebrates. In the interior of the retina are the photoreceptors, also known as rods and cones (Figure 1a). The inner sections of the photoreceptors contain visual pigments, such as rhodopsin in the rods, that are necessary for phototransduction. The outer segments of the photoreceptors are in contact with retinal pigment epithelial (RPE) cells. Further outward from the RPE is the choriocapillaris, a single layer of capillaries that acts as the primary blood supplier for the retina (Figure 1b). Separating the choriocapillaris and the RPE is a thin, multi-layered meshwork of extracellular matrix (ECM) components known as Bruch's membrane (BM) (Hewitt and Adler 1989).

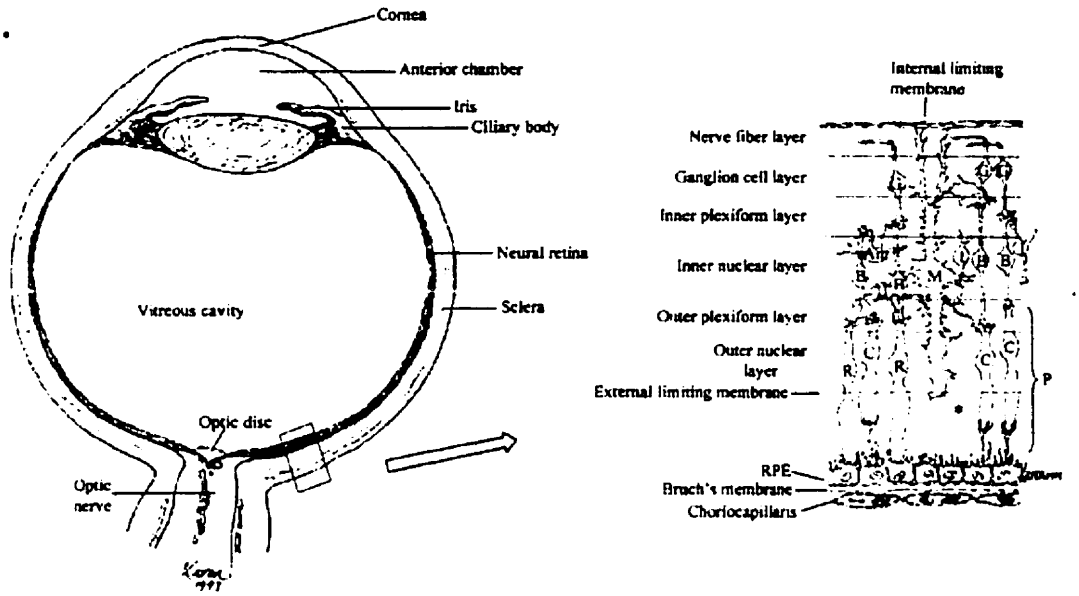
Retinal Pigment Epithelium

The RPE is a highly specialized, and metabolically highly active, main component of the blood-retinal barrier. Its polarization of function is demonstrated by its ability to transport nutrients and retinoids to the photoreceptors while also transporting wastes, water, and ions in the opposite direction (Hewitt and Adler 1989). Because outer segments of photoreceptors are constantly being shed, another role of the RPE is to remove this cellular debris via phagocytosis. Following ingestion, the waste products are degraded in lysozomes

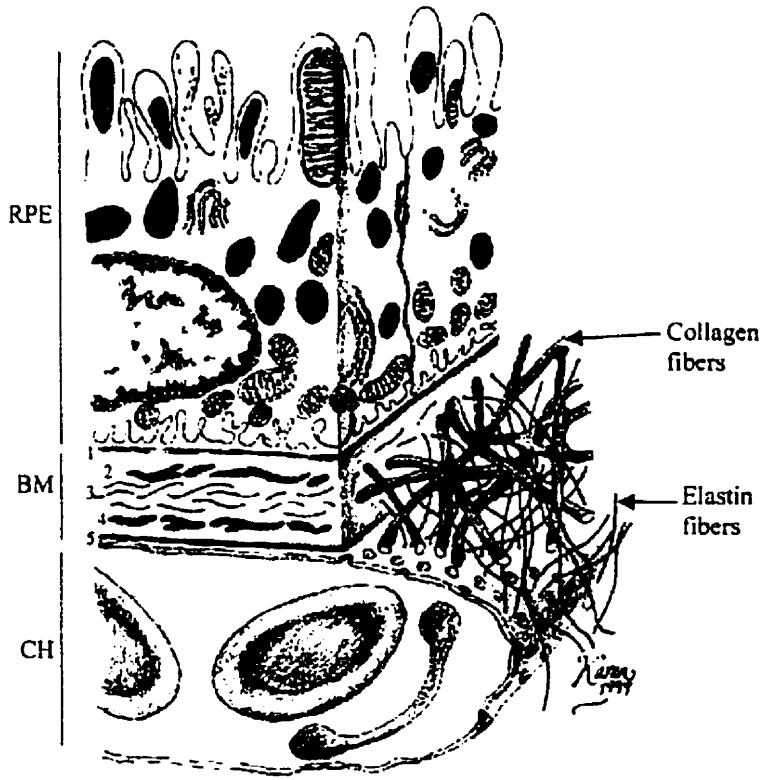
Figure 1. The human retina.

A. Schematic representation of a cross-section of the human eye, with a magnified view of the retina. The photoreceptor layer is indicated by "P". This layer is made up of both rod (R) and cone (C) photoreceptors, as well as an interphotoreceptor matrix (*). The more interior layers are involved in delivering the visual signal from the photoreceptors to the neural layer through specialized cells. The amacrine (Am), bipolar (B), horizontal (H), inner plexiform (I), Muller (M), and ganglionic (G) cells are all shown here.
B. A more magnified schematic representation of the outer retinal layers. Bruch's membrane (BM), the retina pigment epithelium (RPE) and the inner choroid layer (CH) are shown. The five layers of Bruch's membrane are indicated as follows: 1, RPE basement membrane; 2, inner collagenous zone; 3, elastic fiber area; 4, outer collagenous zone; 5, CH basement membrane. (Drawn by Dr Karen Yeow. Used with permission.)

A.


Cornea
Anterior chamber
Iris
Ciliary body
Neural retina
Vitreous cavity
Sclera
Optic disc
Optic nerve
Internal limiting membrane
Nerve fiber layer
Ganglion cell layer
Inner plexiform layer
Inner nuclear layer
Outer plexiform layer
Outer nuclear layer
External limiting membrane
RPE
Bruch's membrane
Choriocapillaris
P

B.


RPE
BM
CH
Collagen fibers
Elastin fibers

within the RPE cells. It is assumed that the resulting degradation products that are not recycled are passed through the BM and passively diffused into choroidal circulation. As well as transporting macromolecules and breaking down cellular debris, the RPE also acts as a cellular factory, synthesizing and secreting ECM molecules in a polarized fashion. While several types of ECM molecules are supplied by the RPE basally to the BM, a completely different complement of ECM molecules is secreted apically into the interphotoreceptor matrix (IPM) surrounding the photoreceptors. It is believed that the discharge of cytoplasmic material by the RPE into the BM is the means by which the RPE achieves cytoplasmic renewal, a mechanism required in non-dividing yet metabolically active cells (Ishibashi, et al., 1986).

Choriocapillaris

As the main supplier of nutrients to the retina, the capillaries of the chorion exhibit one of the highest rates of blood flow in the body. In fact, these capillaries have more blood flowing through them, per tissue gram, than any other vasculature in the body (Guyer, et al., 1989). The vessels are large (up to 60 μm in diameter) with very thin walls, and circulate blood through the choroid, the RPE, and the inner nuclear layer of the retina. Forming a single layer immediately outward from the BM and the RPE, the capillary walls facing the BM contain many fenestrations with covering diaphragms. Endothelial protrusions from the

capillaries extend into BM. The primary function of these processes is believed to be to add physical stability to the cells (Hageman and Kelly 1983).

Bruch's Membrane

Bruch's membrane is a five-layered sheet of connective tissue interposed between the RPE and its major source of nutrition, the choriocapillaris. It is composed mainly of basement membrane and ECM elements (Bressler, et al., 1988; Weber, et al., 1994). In children, the BM is 2 μm thick centrally, but its thickness increases with age, becoming up to 4 μm later in life. As well as acting as an attachment site for the RPE, the BM also serves as a semi-permeable membrane through which nutrients pass to the RPE and photoreceptors, and cellular debris passes in the opposite direction to the choriocapillaris (Bok 1985; Lyda, et al., 1957). The five structurally distinct layers of the BM include the basement membrane of the RPE, an inner collagenous zone, an elastic zone, an outer collagenous zone, and finally an outer basement membrane bordering the epithelial cells of the choriocapillaris (Hogan and Alvarado 1967). Although collagen fibres are present in all five layers of the BM, collagen types I, III, IV, V, and VI each seem to be more dominantly represented in particular layers. Heparin sulphate and chondroitin/dermatin sulphate also constitute a large part of the BM meshwork. While heparin sulphate is more concentrated in the RPE basement membrane, chondroitin/dermatin sulphate is located more prominently within the other four layers of the BM (Hewitt, et al., 1989).

Permeability of the BM has been shown to be dependent on pH and concentrations of salts and glucose. Also, at physiological conditions the BM is negatively charged, a characteristic that may impede the passage of negative macromolecules (Hewitt and Newsome 1985; Lyda, et al., 1957). A modification to any one of the structural or physiological characteristics of the BM may drastically alter its diffusion properties and indirectly affect the RPE and outer retina.

In Sorsby fundus dystropy (SFD) patients, the most consistent histopathology is the presence of a thick layer of abnormal material between the inner collagenous zone of the BM and the RPE basement membrane (Capon, et al., 1989).

Inherited Ocular Diseases

Over the past few years there has been a remarkable increase in understanding regarding the etiology of retinal dystrophies. At the time of this writing, more than 104 genetic loci have been mapped, and the underlying gene defects in over 45 ocular disorders have been identified (Zack, et al., 1999; www.RetNet). Dominant macular dystrophies such as cone-rod dystrophy (CRD), macula dystrophy, pattern dystrophy, and central areolar choroidal dystrophy have been found to be related to mutations in the *peripherin/RDS* gene. The *rhodopsin* and *peripherin/RDS* genes have also been shown to be involved in classical forms of autosomal dominant retinitis pigmentosa (ADRP). Autosomal recessive RP can be due to a null mutation in the *rhodopsin* gene, the gene encoding a subunit of the

rod cGMP phosphodiesterase, or a gene encoding a subunit of the rod cGMP-gated channel. Mutations in the *RPGR* (retinitis pigmentosa GTPase regulator) gene are associated with X-linked RP. Another X-linked gene, *XLRS1*, is associated with X-linked juvenile retinoschisis. Mutations in *ABCR*, a gene encoding a retina-specific ATP binding protein, have been linked to recessive Stargardt's disease (STGD) and age-related macular degeneration (AMD) (Zack, et al., 1999). Recently, this gene was also implicated in autosomal recessive RP and CRD (Cremers, et al., 1998).

Molecular and cellular concepts of pathogenesis are being developed as the genetic basis of each disease is uncovered. This, in turn, has led to a renewed interest in therapeutic strategies.

Age Related Macular Degeneration

Of all known heritable degenerative diseases of the eye, the class of disorders known as the macular dystrophies is the most prevalent. The most common of these diseases in the Western world is AMD, which usually manifests itself at a late age through the loss of photoreceptor function (Zack, et al., 1999).

In non-exudative AMD, vision loss occurs gradually due to the subretinal accumulation of drusen (extra-cellular deposits) in the BM, and subsequent atrophy of the RPE. In exudative AMD, choroidal neovascularization into the BM contributes to more rapid and severe vision loss. In both cases, an initial

histopathology at the level of the BM leads to a loss of photoreceptor function (Curcio, et al., 1996;Green and Enger 1993;Polkinghorne, et al., 1989).

Because it is the most common retinopathy in developed countries, much importance has been placed on unlocking the genetic basis of AMD. However, several factors have limited the progress of this effort. Due to the late onset of the disease, it is difficult to obtain large pedigrees. Studies thus far indicate that the molecular basis of AMD is much more complex than a simple monogenic mutation, and there is also strong evidence that environmental conditions play a role in the disease (Zack, et al., 1999). In order to better understand AMD, it may prove beneficial to study retinopathies similar to AMD, whose genetic causes are more fully understood. Sorsby fundus dystrophy is one such disease.

Sorsby Fundus Dystrophy

SFD is a rare but well-documented macular dystrophy in which the symptoms begin to appear in the third or fourth decade in humans. It is a fully penetrant autosomal dominant disorder, the symptoms of which include nutritional night blindness (Jacobson, et al., 1995) and central vision loss as a result of subretinal neovascularization and atrophy of the choriocapillaris, RPE and retina (Sorsby 1949). Delayed dark adaptation and rhodopsin regeneration have also been observed in Sorsby patients (Steinmetz, et al., 1992).

Role of Bruch's Membrane in SFD

The impaired metabolic activity of the choriocapillaris and the RPE which leads to the visual complications is thought to be due to accumulating deposits of lipid-containing material in the BM. Because the BM, along with the RPE, functions in a regulatory capacity in the transport of ions, nutrients, and metabolites between the choriocapillaris and the subretinal space, BM is important for the normal maintenance of retinal photoreceptors (Fariss, et al., 1997).

It should be noted that the accumulation of drusen and lipids in the BM is a normal result of the aging process (Sarks, et al., 1999). As the BM ages, its meshwork of collagens becomes less soluble, and its non-collagenous makeup is also significantly altered (Karwatowski, et al., 1995). This results in a natural thickening of the BM (Hogan and Alvarado 1967), and possibly a change to its electrochemical properties that inhibits the passage of some types of molecules (Hewitt, et al., 1989).

In SFD and AMD, all of the histopathological features of normal aging are manifested, but with a much greater severity (Yoo and Adamis 1998). The lipid-rich deposits that build up in the BM of SFD and AMD patients thicken the BM significantly more than in normal aging, and increase the hydrophobicity of the membrane. Also, the drusen that accumulate are structurally and chemically different than in the normal eye (Okubo, et al., 1999). These factors are thought to interfere with the passage of nutrients and debris between the choriocapillaris and the RPE. This ultimately leads to the starvation and subsequent degeneration of

functional retinal photoreceptors (Steinmetz, et al., 1992). Jacobsen et al (1995) showed evidence that the subretinal deposits act as a diffusion barrier for entry of vitamin A into the photoreceptors, leading to nutritional night blindness.

In addition to the accumulation of drusen and lipids, new blood vessels from the choroid typically invade Bruch's membrane in SFD patients, often rupturing to cause a hemorrhagic retinopathy. Cultured human RPE cells are known to release anti-angiogenic substances (Glaser, et al., 1985), so it is quite possible that this neovascularization could be the choroidal response to a dysfunctional RPE (Capon, et al., 1989).

Aside from the aforementioned histopathology of SFD, there is also a wide spectrum of other less consistent features of the disease. For instance, choroidal atrophy is a well-documented clinical feature of SFD, although it is only observed in a small proportion of SFD patients (Polkinghorne, et al., 1989).

TIMP3

Sorsby fundus dystrophy has been found to be caused by a point mutation in the gene *tissue inhibitor of metalloproteinases-3* (*TIMP3*), which has been mapped to chromosome 22q13 (Apte, et al., 1994; Gregory, et al., 1995). It is clear that *TIMP3* must have a critical function in the eye because, not only does it have a role in SFD; overexpression of *TIMP3* has been observed in degenerating retinas of simplex retinitis pigmentosa patients (Jones, et al., 1994; Jomary, et al., 1997).

Overview of TIMPs and MMPs

The complete picture of what TIMPs do and how they work is not yet clear. What is known about this family of secreted proteins is that they work in a regulatory capacity against matrix metalloproteinases (MMPs). MMPs are a family of zinc binding endopeptidases that includes collagenases, gelatinases, and stromelysins (Matrisian 1992). The ECM must undergo continuous remodeling. For this to be possible, MMPs and other proteinases are involved in the continual degradation of the ECM structural molecules (Matrisian 1990). However, the activity of MMPs must not be left unchecked. Functioning as regulators of MMPs, TIMPs bind to and inhibit the activity of MMPs in a one to one stoichiometry (Apte, et al., 1995). The balance that is achieved by these special classes of proteins is of vital importance to various physiological processes including embryonic development, connective tissue remodeling, wound healing, glandular morphogenesis, and angiogenesis. Also, several human degenerative diseases have been linked to imbalances in the regulation of MMPs and TIMPs. These diseases include rheumatoid arthritis (Pelletier, et al., 1990), pulmonary emphysema (D'Armiento, et al., 1992), liver fibrosis (Milani, et al., 1994), and atherosclerosis (Henney, et al., 1991). As well, elevated levels of MMPs have been implicated in playing a role in ECM degradation during metastasis (Mignatti, et al., 1986), while increased TIMP levels have been demonstrated to suppress tumor invasion and metastasis (Hicks, et al., 1984; Schultz, et al., 1988; Khokha, et al., 1989). More recent studies have shown evidence that indicates TIMPs may

play a role in cell growth, differentiation, signalling, and migration (Birkedal-Hansen 1995; Denhardt, et al., 1993). These same studies suggest that in addition to acting as inhibitors of MMPs, TIMPs may have additional functions that are independent of MMPs.

Characterization of TIMP3

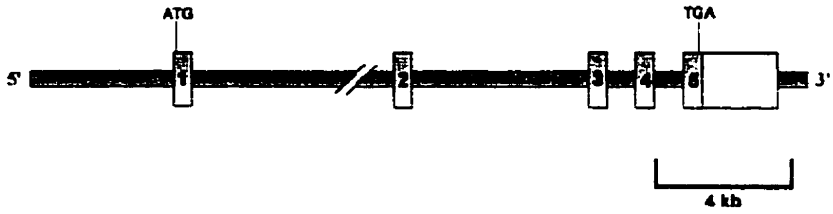
Originally identified in the lab of Dr. Dylan Edwards at the University of Calgary, TIMP3 is a predominantly matrix-associated protein (Leco, et al., 1994), which distinguishes it from the three other known TIMPs (Goldberg, et al., 1990; Ward, et al., 1991; Greene, et al., 1996). The *TIMP3* gene is at least 30 kilobases in length due to its large introns, and is encoded by five exons (Figure 2a) (Apte, et al., 1995). The human *TIMP3* mRNA translates into a peptide of 188 amino acids. The structure of the TIMP3 protein is likely similar to that of the rest of the TIMP family proteins due to the presence of twelve conserved cysteine residues that form six disulfide bonds within the peptide chain (Figure 2b) (Williamson, et al., 1990).

TIMP3 mRNA expression in the eye is localized to the RPE and ciliary epithelium, and has not been observed at any other site. This sets *TIMP3* apart from other genes that cause human retinal disease by preferential expression in the photoreceptors (Della, et al., 1996). Recently, TIMP3 has been determined, by immunolabelling, to be an ECM component of the BM (Fariss, et al., 1997). It is quite probable that the cellular source of TIMP3 localized to BM is the RPE.

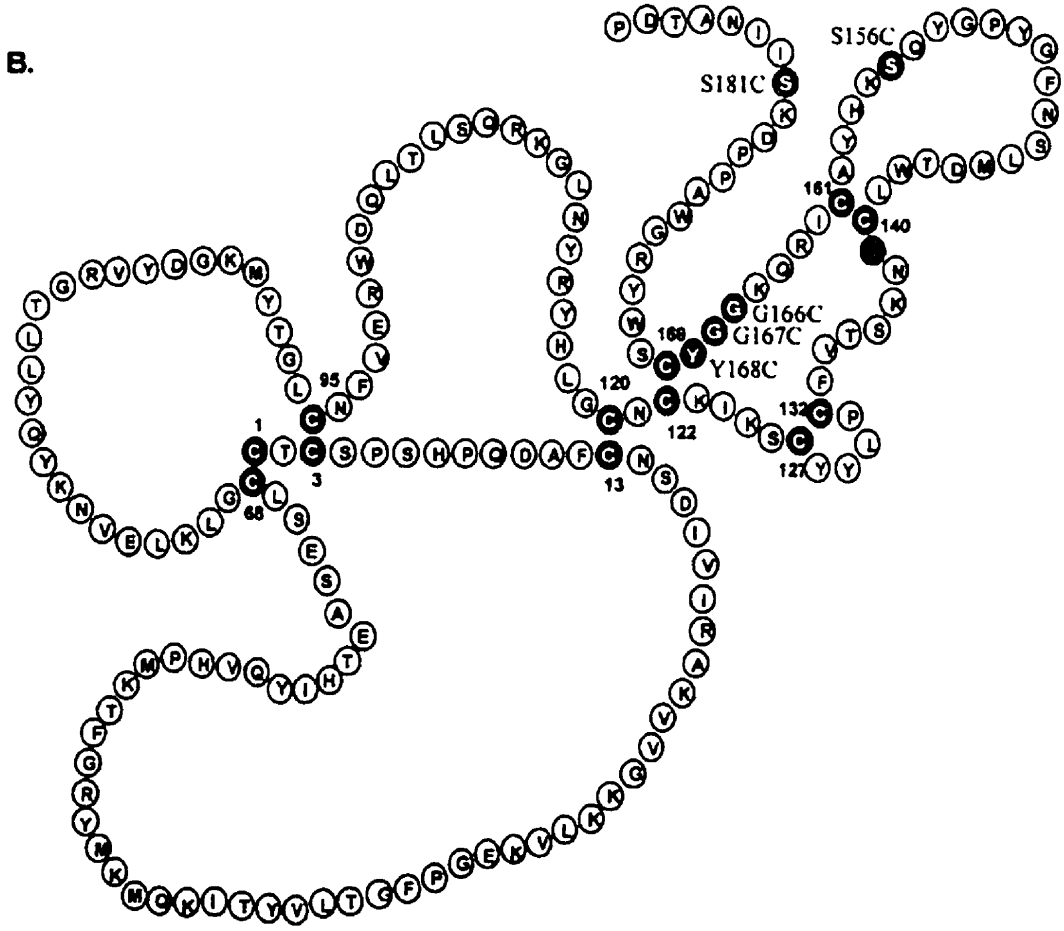
Figure 2. Structure of *Timp3* gene and TIMP3 protein.

A. The Timp3 gene spans over 30 kb of mouse chromosome 10. Exons are numbered and represented by light blue boxes. The size of exon 5 varies, depending on polyadenylation signal usage. This is represented by a violet box immediately downstream of the stop codon. A break in the sequence of intron 1 is indicated by double slashes. Translation start (ATG) and stop (TGA) codons are also shown. B. Schematic representation of human TIMP3 protein, based on the structure of TIMP1. Conserved cysteine residues are shown in blue. Amino acid residues shown in green are known Sorsby fundus dystrophy mutation sites where the wildtype residue in each case is changed to a cysteine. The recently observed SFD-causing mutation at residue 139 (indicated in red) changes the residue from wildtype glutamic acid to a stop codon (Adapted from drawing by Dr. Karen Yeow. Used with permission.)

A.


ATG
TGA
5'
3'
1
2
3
4
5
4 kb

B.


S156C
S181C
G166C
G167C
Y168C
95
120
122
132
127
1
3
13
60
140
161
169

Function of TIMP3

Like other members of the TIMP family of proteins, the functional pathways of TIMP3 are not yet clear. What is known is that it does play intrinsic roles in various systems. For instance, TIMP3 serves as a regulator within the decidualizing murine uterus to restrict invasion of trophoblast cells to the site of implantation (Leco, et al., 1996). In another system TIMP3 has been demonstrated to prevent uncontrolled growth of human colon carcinoma cells by protecting TNF-α receptors from the proteolytic activity of MMPs, thus allowing the preserved TNF-α p55 signaling pathway to kill the cells (Smith, et al., 1997). It has also been shown, *in vitro* and *in vivo*, that TIMP3 is an effective inhibitor of endothelial cell migration and angiogenesis (Anand-Apte, et al., 1997).

TIMP3 Mutations in SFD Patients

Tyr168Cys and Ser181Cys substitutions in exon 5 of *TIMP3* were the first point mutations to be identified in SFD patients (Weber, et al., 1994; Carrero-Valenzuela, et al., 1996), but soon after this Gly167Cys (Jacobson, et al., 1995), Ser156Cys (Felbor, et al., 1995), and Gly166Cys (Felbor, et al., 1997) mutations to *TIMP3* were observed in other SFD pedigrees. In SFD cases in which the Ser156Cys mutation is present, SFD symptoms are displayed 10-15 years earlier than in cases in which any of the other cysteine substitutions are involved. Although the average age of SFD symptom onset is variable from one mutation to

the next, what these mutations have in common in the majority of cases is that they affect the C-terminal region of the mature TIMP3 protein by changing one amino acid to a cysteine residue (Felbor, et al., 1996).

Recently, a novel *TIMP3* mutation was discovered in two families in Japan in which SFD was diagnosed (Tabata, et al., 1998). This is the first report of SFD in the Eastern world. Unlike the previously described point mutations, this case involves a single base insertion at the intron 4/exon 5 junction. This novel splice site mutation converts the consensus sequence CAG to CAAG in the splice acceptor site. Interestingly, the onset of SFD symptoms occurs at a significantly later age in these families. The average age for the rapid or gradual loss of central vision in this study was 67.4 years (Isashiki, et al., 1999).

Just this year, another novel *TIMP3* mutation was discovered in a family diagnosed with SFD (Langton, et al., 2000). The phenotype of this pedigree is identical to those in other SFD patients, and the age of onset is also about the same as in the cases with *TIMP3* point mutations. Unlike those mutations however, the mutation described here has a glutamic acid residue changed to a stop codon, resulting in a truncated TIMP3 protein that lacks a large portion of the C-terminal domain (Langton, et al., 2000).

Analysis of TIMP3 Mutations

Ser156Cys, Gly167Cys, Tyr168Cys, and Ser181Cys mutants were recently generated from human *TIMP3* cDNA . Analysis of these mutants by SDS-PAGE,

reverse zymography, and Western blotting revealed that the Ser156Cys and Ser181Cys mutants formed higher molecular weight aggregates. While all four of the mutants had a tendency to form aberrant protein-protein interactions and increased adhesiveness of the cells that expressed them, each of the mutants retained its inhibitory activity of gelatinases A and B, and remained localized to the extracellular matrix (Yeow, et al., 2000; Langton, et al., 1998). As well, it was found that, unlike the wild-type gene, these mutations cause TIMP3 to dimerize (Langton, et al., 2000).

The *TIMP3* mutations, as observed by Yeow et al (2000) and Langton et al (2000), do not seem to affect the MMP inhibitory activity of TIMP3, which remained confined to the ECM in all cases. It has been shown that TIMP3 is an inhibitor of angiogenesis, and neovascularization of the choriocapillaris into BM is one of the hallmarks of SFD. This suggests that vascular penetration into BM may be a consequence of TIMP3 dysfunction (Weber, et al., 1994). The recent discovery of mutant TIMP3's dimerization and its involvement in cell adhesion may shed more light on the biochemical origin of the SFD phenotype.

Developing Treatment Strategies

With the biochemistry of SFD becoming clearer, treatment strategies are being devised that would deal with the disease on a molecular level. Gene therapy is one such strategy. Although still in its infancy research-wise, gene therapy may have the potential to revolutionize the way we treat genetic diseases but, of course,

much more research must take place before gene therapy can be applied routinely in a clinical setting. In order for this type of research to be possible, animal models must be made available to be used as experimental subjects. It is this need for animal models that has driven the enormous growth in the field of transgenics in recent years, and has resulted in the generation of scores of transgenic animals, most commonly transgenic mice. In order for gene therapy research to continue for SFD, therefore, it is of vital importance that SFD mouse models be generated. Recently, mouse germline transmission of the Ser156Cys mutation was achieved, although phenotypic data from this model has not yet been published (Zack, et al., 1999). Models of the other SFD mutations would, along with the Ser156Cys mouse, contribute greatly to our understanding of the disease. Again, mouse models of SFD should also prove valuable for gaining insights into various aspects of prevalent AMD.

Transgenics via Embryonic Stem Cells

Transgenic mice have been produced using several methodologies. The first transgenic mouse strain was successfully generated when mouse embryos were exposed to infectious Moloney leukemia virus (Jaenisch 1976). Soon after this, the value of using mouse embryonic stem cells (ES cells) in transgenic experiments was discovered and the first ES cell lines were generated (Evans and Kaufman 1981; Martin 1981). The generation of *Hprt*-deficient mice from ES cell lines in which the hypoxanthine phosphoribosyl transferase (*Hprt*) gene was

disrupted through random methods of mutagenesis demonstrated that ES cells could remain viable and contribute to the formation of an animal, even after a genetic alteration (Hooper, et al., 1987; Kuehn, et al., 1987).

Derived from the inner cellular mass of mouse blastocysts, ES cells have been demonstrated to be a most convenient route by which to introduce exogenous DNA sequences into the germ line. Accumulating evidence suggests that gene targeting is much more efficient in ES cells than in other cell types (Arbones, et al., 1994). As well, ES cells are a valuable tool in gene targeting because they have been demonstrated to be pluripotent both *in vitro* and *in vivo*, and under stringent conditions they can be maintained in culture indefinitely in an undifferentiated state (Evans and Kaufman 1981; Martin 1981).

Considerations in Working with ES Cells

In working with ES cells, the stringency of the cell culture conditions cannot be overemphasized because it is crucial to maintain the cells in an undifferentiated state and to preserve a normal karyotype. To ensure the cell line retains the potential to contribute to the generation of a chimeric mouse, regular karyotyping is important. Targeted ES cells must be diploid, and the presence of forty autosomal chromosomes and an X and Y chromosome must be observed (Hogan, et al., 1994). Another factor of vital importance is the number of times ES cells are passaged. The more the cells are passaged, the greater the tendency for natural selection to favour tumorigenic ES cells with higher growth rates

(Baribault and Kemler 1989). As well, high passage ES cells are differentially methylated, a factor that may contribute to undesirable phenotypic variation in the developing mouse (Dean, et al., 1998).

DNA Delivery into ES Cells

Several approaches exist by which to deliver exogenous DNA through the membranes of ES cells. These methods include electroporation (Potter, et al., 1984), calcium phosphate precipitation (Gossler, et al., 1986), retroviral vectors (Robertson, et al., 1986), and microinjection (Zimmer and Gruss 1989). The most commonly used of these approaches is electroporation, a process that causes pores to form in the cells' membranes by applying a high voltage electric pulse to the cells. Exogenous DNA that is present in the cell suspension is then able to pass through the pores into the cells. The electric pulse usually results in the death of a high percentage of cells, and transformation efficiencies are quite low (Wassarman and DePamphilis 1993). By incorporating selectable markers into the mutagenic DNA beforehand, the low number of successfully targeted cells can be easily identified and enriched for. To increase the number of successfully targeted cells, site-directed mutations are possible, which take advantage of homologous recombination (Smithies, et al., 1985). In using homologous recombination, the length of homology is another variable that can be optimized in order to increase the efficiency of gene targeting in ES cells (Deng and Capecchi 1992).

Widespread Utility of ES Cells

Another reason ES cells are highly advantageous is that they can be screened for desired genetic alterations before being reintroduced into mouse blastocysts. Once reintroduced into blastocysts, ES cells are able to contribute efficiently to chimera formation (Figure 3), including contributions to the germ line (Bradley, et al., 1984; Gossler, et al., 1986).

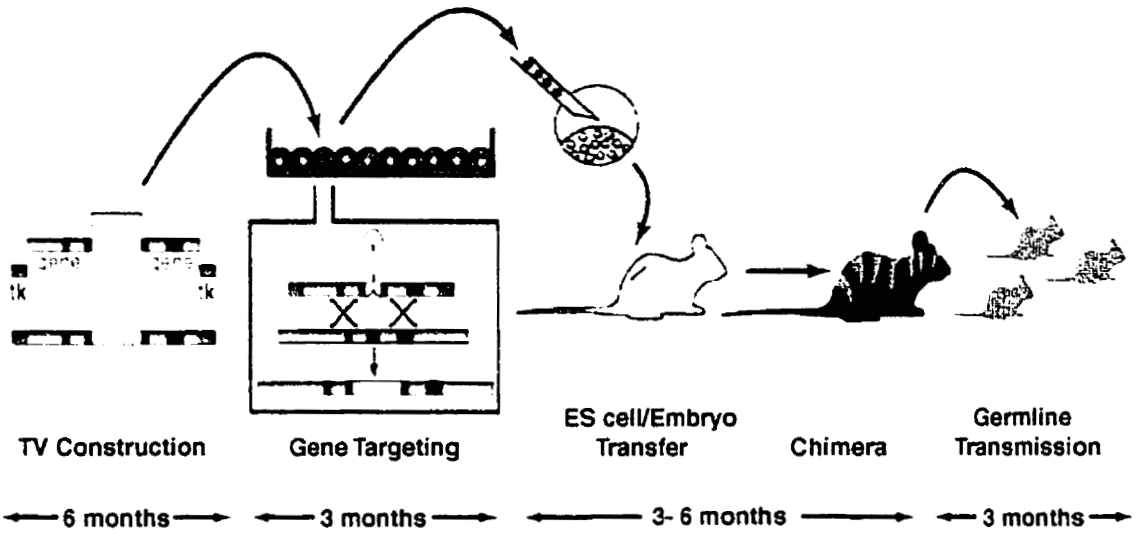
Since the utility of ES cells in transgenics was first discovered in 1981, scores of transgenic murine models have been generated using this powerful approach. As well, ES cell lines have now been established for other mammalian species, including rabbit (Schoonjans, et al., 1996), pig (Wheeler 1994), and monkey (Thomson, et al., 1995). More recently, the establishment of five distinct human ES cell lines was reported (Thomson, et al., 1998). As well as opening the door to a plethora of ethical considerations, these cell lines will potentially provide a new window through which to study various aspects of human development and disease.

Targeted Mutagenesis

Targeted mutagenesis is the process by which any type of mutation is delivered to a specific site in the genome. This targeted event occurs through homologous recombination, between the mutagenic DNA and a specific locus on chromosomal DNA, using the cell's own molecular machinery. Homologous recombination in mammalian cells occurs at a much lower frequency than in yeast

Figure 3. Generation of transgenic mice.

Formation of transgenic mice from mouse ES cells. The rate-determining step in generating transgenic mice is often the construction of the targeting vector. Once the targeting vector is complete, gene targeting experiments are conducted on mouse ES cells. Successfully targeted cells (blue circles), identified as such in selective liquid medium, are microinjected into mouse embryos, which are then transferred into surrogate mothers of a different murine line. Chimeric progeny are identified by abnormal color patterns in their coats. If the chimeric mouse has germline chimerism, it will produce offspring of two different coat colors.


tk
tk
TV Construction
Gene Targeting
ES cell/Embryo
Transfer
Chimera
Germline
Transmission
6 months
3 months
3- 6 months
3 months

and bacteria, and for every homologous recombination event there are approximately 1000 instances of random integration (Thomas and Capecchi 1987; Ng and Baker 1999; Thompson, et al., 1989). In spite of this, homologous recombination remains the only method by which to target specific genomic loci.

Being that there are numerous applications for gene targeting, a variety of strategies have been developed to specifically serve the purposes of any given application. Such strategies include the simple silencing of genes, upregulation of genes, swapping related genes, implementing conditional expression of genes, and even attaching reporter genes to genes whose expression would be otherwise unobservable. For each strategy, a specialized type of targeting vector (TV) is required.

Gene Knockouts/Knock-ins

The most commonly used TVs serve to simply prevent expression of the gene of interest. If the targeted mutation effectively generates null alleles and silences expression of the target gene, the process is referred to as a "knockout," be it via an additional or a deletional mutation. A "knock-in" involves adding a gene or changing an allele through the addition of exogenous DNA. The purpose of a knock-in could be to generate a gain of function mutation or to exchange functional domains from related genes (Hasty and Bradley 1993). Models for genetic diseases can be generated via knock-ins or knockouts, depending on the molecular basis of the disease.

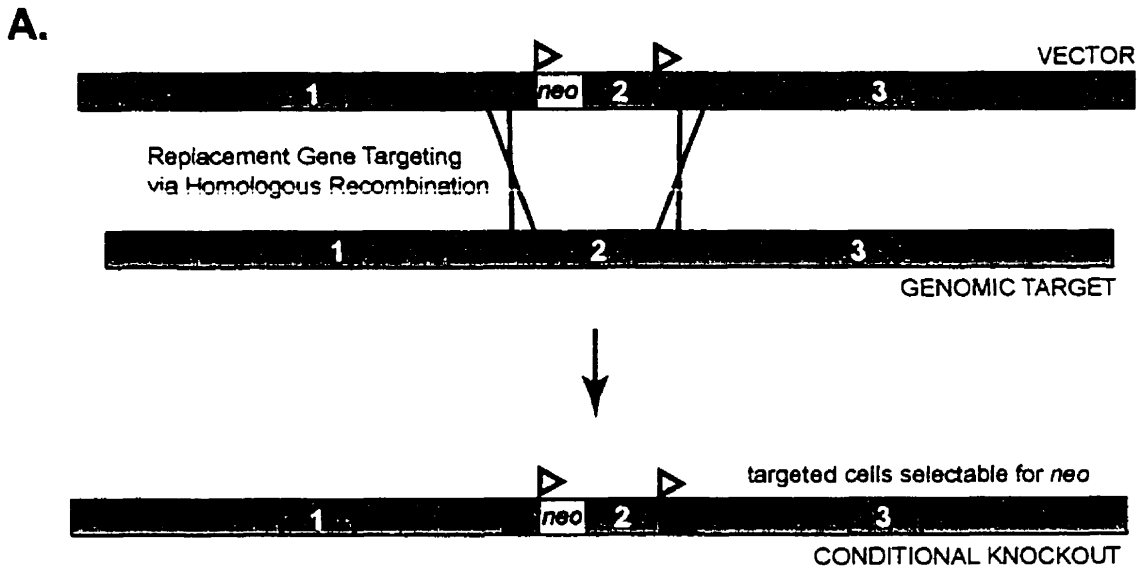
Simple gene knockouts are not always the best route by which to study gene function, however, because they often result in an embryonic lethal phenotype. Although this may shed light on a gene's importance in prenatal pathways, further analysis of the gene in adults is not possible (Copp 1995). To get around this problem, other methods of gene alteration have been developed that allow the investigator to control the temporal and spatial expression of genes of interest.

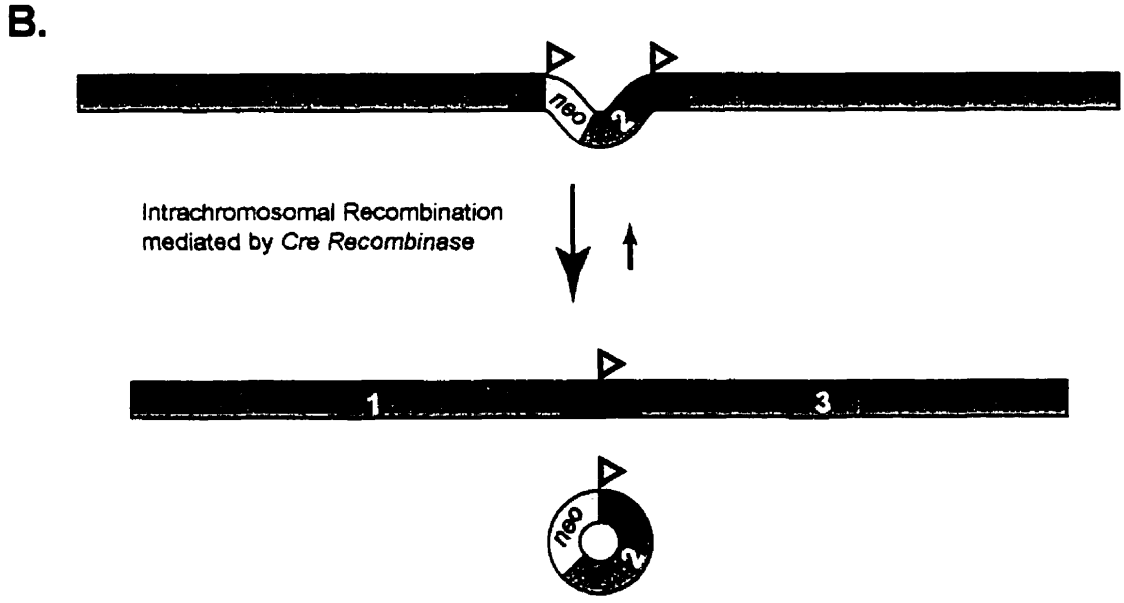
Cre-loxP Conditional Mutations

Conditional mutations can be targeted to genes, whereby a gene is expressed only at a certain time, under certain conditions, or in a specific tissue. For example, in cases where a particular mutation has proven to possess an embryonic lethal phenotype, the mutation can be suppressed throughout the developmental stages of the organism, and then triggered at a later stage. In recent years, the Cre-loxP recombination system derived from coliphage P1 (Sternberg and Hamilton 1981) has been a popular tool for knockout and knock-in experiments. Relying on the Cre protein's ability to efficiently promote recombination of DNA at *lox*P sites (Sauer and Henderson 1988), genomic DNA flanked by two directly repeated *lox*P sites (floxed) can be excised with Cre expression (Figure 4). In addition, the opposite reaction can be used for integrative recombination; plasmid DNA containing one *lox*P site can be inserted into a *lox*P site on a chromosome (Araki, et al., 1997).

Figure 4. Gene targeting using Cre-loxP.

Schematic flow diagram of Cre-loxP gene targeting system. A) In order to conditionally knock out a gene (as indicated in black with hypothetically numbered exons in blue), the gene is targeted via homologous recombination by a targeting vector (shown in red) bearing a selectable marker, flanked by two *lox*P sequences (shown as yellow triangles). Following double-crossover homologous recombination, the endogenous target is replaced. Successfully targeted cells are then selected for the presence of the marker (on this occasion, *neo* can be selected for on G418 medium). Expression of the targeted gene is transiently maintained. B) To disrupt expression of the targeted gene, Cre recombinase is introduced. Cre expression initiates a recombination event between the directly repeated *lox*P sequences, resulting in excision of the DNA fragment they are flanking. One *lox*P remains behind in the genomic sequence.


A.
VECTOR
neo
1
2
3
Replacement Gene Targeting
via Homologous Recombination
1
2
3
GENOMIC TARGET
targeted cells selectable for neo
neo
1
2
3
CONDITIONAL KNOCKOUT


B.
neo
2
Intrachromosomal Recombination
mediated by Cre Recombinase
1
3
neo
2

Reporter Genes

Besides using TVs to alter the expression of a given gene, they can also be used to enable investigators to study the spatial and temporal expression patterns of genes. This is done by targeting a reporter gene to replace a portion of the amino acid-coding sequence of the endogenous gene of interest. By positioning a gene such as *lacZ*, followed by an SV40 poly(A) tail, in frame with the targeted gene the expression of the gene can be observed as indicated by the visible expression of *lacZ* in fixed cells (Guillemot, et al., 1994). To monitor gene expression in living cells, the reporter gene encoding green fluorescent protein (GFP) can be used (Ikawa, et al., 1995).

Insertion and Replacement TVs

Replacement TVs are designed to replace homologous gene sequences with a selectable mutation, often a *neo* cassette, which is most usually positioned to disrupt an exon (Thomas and Capecchi 1987; Hasty, et al., 1991a). Encoding a bacterial aminoglycoside phosphotransferase, the *neo* gene confers upon cells resistance to the antibiotic G418 (Thomas and Capecchi 1986). The intention of using this type of TV is usually to generate null alleles. Such TVs require that regions of homology be present on each side of the mutation in order for accurate targeting to occur. Homologous recombination then occurs either by double reciprocal recombination or gene conversion (Deng and Capecchi 1992). Only the

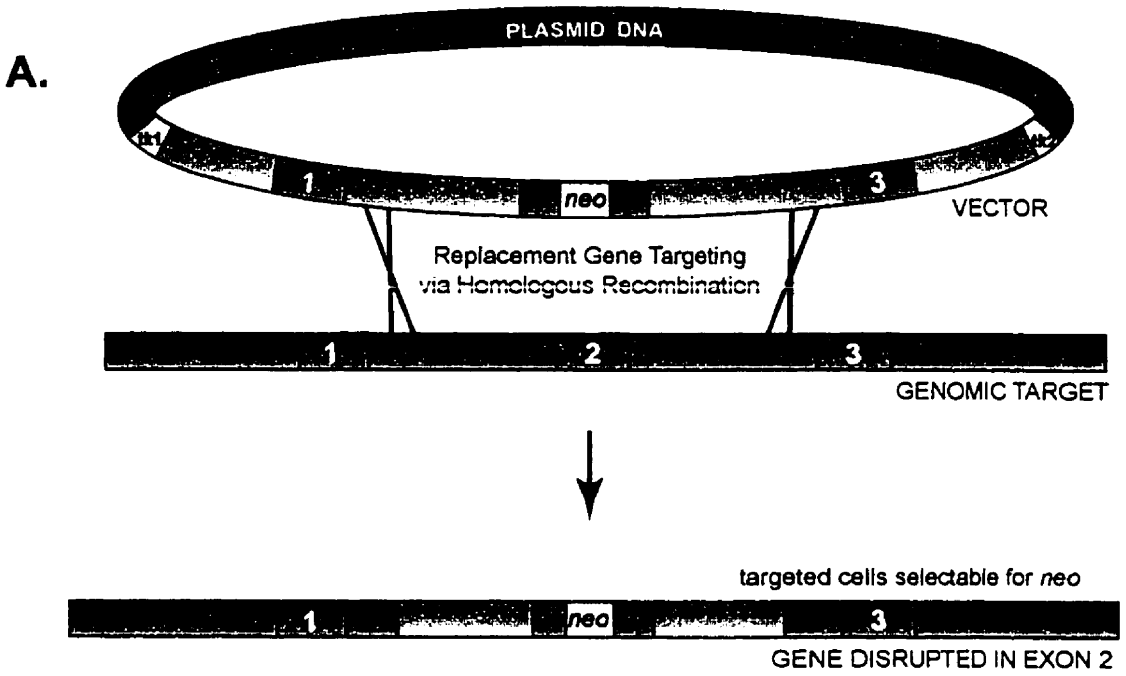
region of homology on the replacement vector and the mutation within it replace the homologous region of the targeted locus (Figure 5a).

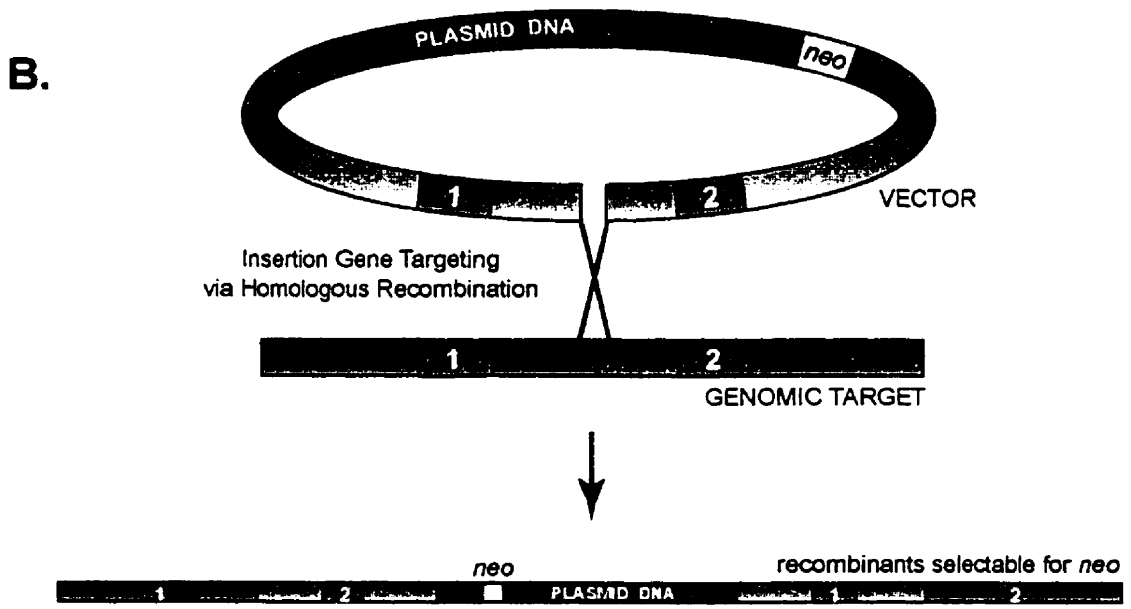
Insertional TVs have been used both for gene disruption (Thomas and Capecchi 1987) and gene correction (Doetschman, et al., 1988). A typical insertion vector will contain a single region of homology to allow for a single crossover event to occur between the TV and the targeted locus. After a double-strand breakage within the region of homology the entire TV is inserted into the targeted locus, resulting in a duplication of homology separated by the linearized plasmid DNA and a positive selection marker, often *neo* (Figure 5b). Such a tandem duplication is unstable and will often result in a reversion of the locus to its wild-type state (Thomas, et al., 1992).

For both insertion vectors and replacement vectors, targeting efficiency has been found to be about the same. However, this is not always the case. One report demonstrates that certain chromosomal loci are successfully targeted at much higher frequencies with one type of vector over the other, suggesting that DNA sequence or chromatin structure may play a role in limiting particular targeting pathways (Hasty, et al., 1994).

Figure 5. Replacement and insertion gene targeting vectors.

Flow diagrams of gene targeting using replacement and insertion targeting strategies. A. A typical replacement targeting vector contains a selectable marker (in this case *neo*), flanked by large regions of homology (light blue) to the target gene (black). Hypothetical exons are represented in dark blue boxes. Homologous double crossover recombination results in replacement of the endogenous target sequence with the homologous exogenous sequence bearing the selectable marker. Here, cells in which the target gene has been successfully disrupted (in exon 2) can be selected for on G418 medium. B. Insertion vectors contain a single large region of homology to the target gene. The selectable marker lies outside of the homologous region. Successful targeting consists of an homologous single crossover recombination event. This results in an insertion of the entire targeting vector into the chromosome, creating a duplication of homology that is separated by plasmid DNA and the selectable marker.


A.
PLASMID DNA
tk1
tk2
1
neo
3
VECTOR
Replacement Gene Targeting
via Homologous Recombination
1
2
3
GENOMIC TARGET
targeted cells selectable for neo
1
neo
3
GENE DISRUPTED IN EXON 2


B.
PLASMID DNA
neo
1
2
VECTOR
Insertion Gene Targeting
via Homologous Recombination
1
2
GENOMIC TARGET
neo
recombinants selectable for neo
1
2
PLASMID DNA
1
2

Targeting Efficiency

In both insertion and replacement TVs, targeting efficiency is influenced by both the length of homology and the degree of polymorphic variation between the vector and its targeted sequence (te Riele, et al., 1992). Targeting efficiency increases exponentially as the extent of homology increases, from 2 kb to 10 kb. Efficiency seems to peak at 14 kb of sequence homology (Deng and Capecchi 1992). Also, it has been found that targeting efficiency is up to five times higher when the homologous DNA in the TV is derived from the same mouse strain as the targeted ES cells (Deng and Capecchi 1992). Even so, the frequency of random integration of the exogenous DNA remains about three orders of magnitude higher than the gene-targeting frequency (Ng and Baker 1999). To overcome this problem, selection systems have been developed to identify the rare homologous recombination events. To enrich for recombinants that have undergone a successful insertion or replacement event, a positive selection marker, such as *neo*, is required. In order to target exogenous DNA to a locus that is just downstream of a promoter, a promoterless *neo* is used in the TV. This ensures that *neo* will only be expressed as a selectable marker when the TV delivers the DNA to such a site.

Limitations of Traditional Targeting Strategies

The problem with traditional insertion and replacement vectors is that the addition of a selectable marker into the genomic locus may potentially deregulate the transcription of neighbouring genes, thereby making the interpretation of the mutant phenotype more difficult. Similarly, when using the Cre-loxP recombinase system to excise a selectable marker flanked by two *lox*P sites, one *lox*P site remains behind. The presence of this *lox*P site may have an adverse effect on expression of the gene, and make phenotypic interpretation difficult. Also, this system requires a step in which Cre is delivered into the cells in order for the targeted DNA to be excised. Cre delivery could be adenovirus-mediated, or by crossing the 'floxed' mouse line with mice expressing Cre in a precise manner spatially and temporally. Both strategies have problems. Adenoviral vectors display different infectivity rates in different tissues, and they often elicit an immunogenic response (Kass-Eisler, et al., 1994). Using Cre mice to cross-breed with floxed mice is often a burden on manpower, finances, and facilities because a separate mouse line must be created for every spatial or temporal manner of *Cre* expression that is desired.

Hit-and-Run Technique

While much can be learned and accomplished by adding deleterious exogenous sequences into genomic targets, much more potential lies in the ability

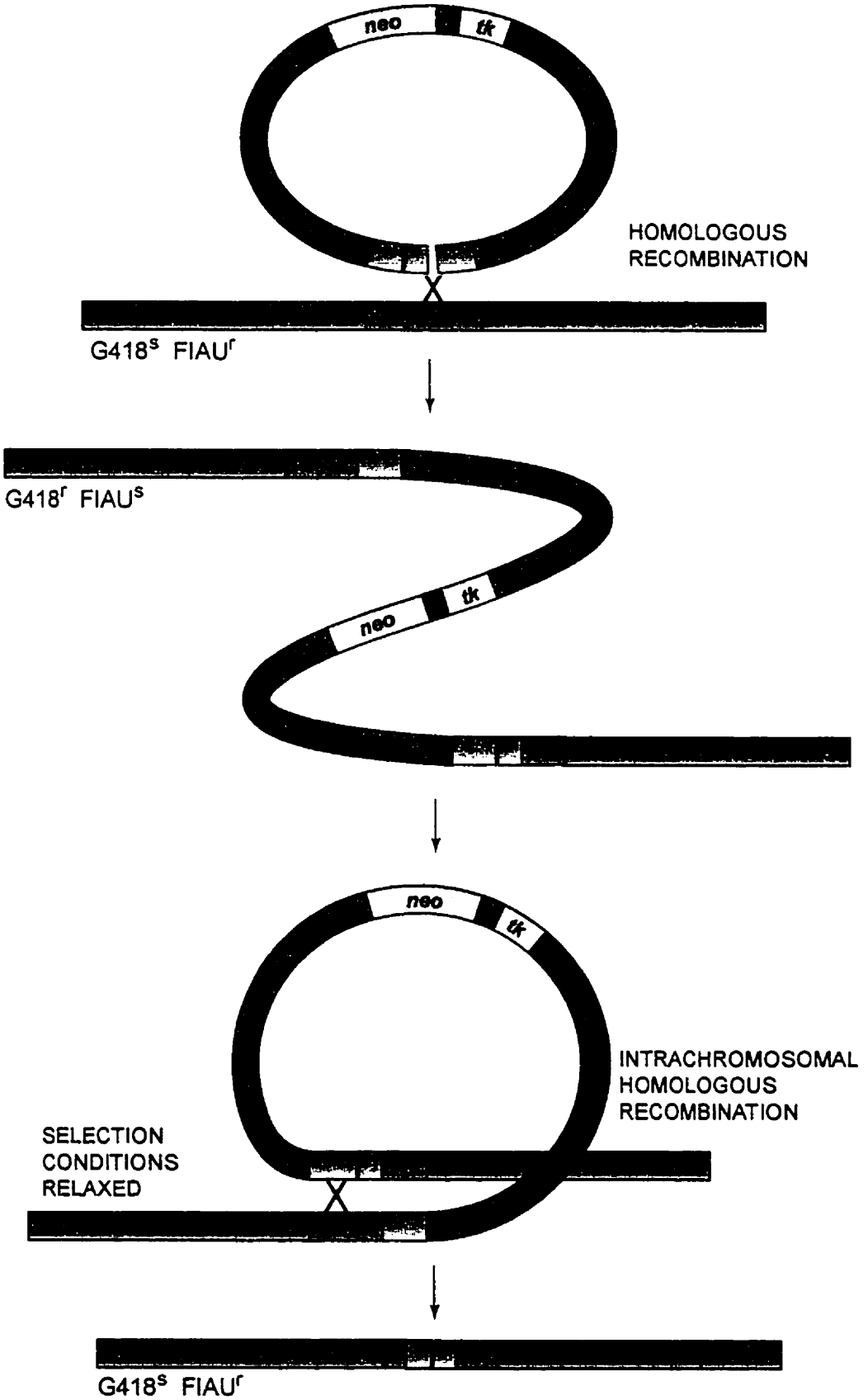
to target subtle mutations to specific loci without leaving a positive marker behind.

A method termed the 'hit-and-run' procedure accomplishes this objective using insertion vectors, because it makes possible the generation of ES cells with subtle site-specific mutations, leaving no selectable marker behind (Hasty, et al., 1991b). Originally used in yeast, this procedure involved two recombination events: the integrative transformation of yeast chromosomal DNA with plasmid DNA, followed by the excision of the plasmid from the transformants, leaving the mutation behind (Winston, et al., 1983). At the time these experiments were being performed on yeast, it was thought that this technique would be limited in application to only yeast and bacteria. More recently, however, the hit-and-run procedure has been used successfully by Hasty et al (1991b) to deliver a subtle site-specific mutation into the *Hox-2.6* locus in mouse ES cells (Figure 6). Hasty's group constructed a plasmid insertion vector with a small mutation inside a large region homologous to the target DNA. *Neo* and *tk* genes were located outside of the homologous region for selection purposes. *Tk*, the Herpes Simplex Virus *thymidine kinase* gene, was used here as a negative selectable marker because it confers FIAU sensitivity to cells.

The insertion vector was linearized within the homologous region before being electroporated into mouse ES cells. Transformed ES cells were selected for the presence of *neo* in media containing G418. The presence of *neo* was evidence of insertion of the TV into the genome via single-crossover recombination.

Figure 6. Hit-and-run gene targeting.

Flow diagram of hit and run gene targeting strategy. A plasmid targeting vector (red) bears both positive (*neo*) and negative (*tk*) selectable markers, as well as a region of homology (light blue) to the target sequence (dark blue). The homologous region of the vector contains a subtle mutation, indicated here as a green line. A double-strand break is made within the homologous sequence before the vector is introduced into ES cells. Through single-crossover recombination, the entire vector is then incorporated into the genomic target, and can be selected for on G418 medium. Due to the duplication of homology in the targeted DNA, when selection conditions are relaxed, the vector is able to excise via intrachromosomal recombination. Cells in which reversion of the plasmid vector has taken place may be selected for the absence of *tk* on FIAU medium. Targeted cells that have undergone condensation of the targeting vector will, in most cases, retain a variable portion of the homologous sequence in place of the endogenous DNA. If the reversional recombination event occurs upstream of the subtle mutation, the mutation remains behind in the target locus.


neo
tk
HOMOLOGOUS
RECOMBINATION
G418^{s} FIAU^{r}
G418^{r} FIAU^{s}
neo
tk
neo
tk
INTRACHROMOSOMAL
HOMOLOGOUS
RECOMBINATION
SELECTION
CONDITIONS
RELAXED
G418^{s} FIAU^{r}

Selection conditions were relaxed to allow the plasmid TV to recircularize and revert out of the chromosomal DNA through single-crossover intrachromosomal recombination. Depending on which side of the mutation the single-crossover event occurred, the mutation would either be excised along with the plasmid or be left behind in the targeted locus. Chromosomes having undergone reversion were selected for by the absence of *tk* in media containing FIAU. The resulting colonies were screened by southern analysis for the presence of a unique *Nhe*I restriction site generated by the mutation.

Although the hit and run procedure provides a means by which to introduce subtle site-specific mutations into the genome, it is not a popular approach simply because investigators have preferred using replacement vectors over insertion vectors.

Positive-Negative Selection (PNS)

The value of including both positive and negative selectable markers in TVs is well-demonstrated by the hit-and-run method of targeted mutagenesis, where the negative marker serves to select against cells whose genome retains the undesirable plasmid DNA incorporated during the insertional event.

In replacement TVs, *tk* is also commonly used as a negative selectable marker. However, instead of selecting against cells whose genomes have not undergone a reversion of plasmid DNA, *tk* is used in replacement TVs to select against random integrants, which outnumber homologous recombinants by several

orders of magnitude. Also, *tk* selects against targeted events in which concatamerized TVs are inserted into the genome by double-crossover homologous recombination. This is a major reason why replacement vectors are preferred by a majority of investigators.

Common Usage of PNS in Replacement TVs

In a common gene targeting strategy, HSV*tk1* and HSV*tk2* are constructed into a targeting vector, flanking the region of homology so that during the process of homologous recombination both cassettes are lost, being outside of the homologous region. The occurrence of intermolecular recombination is reduced because the *tk* cassettes are not homologous to each other (Hasty and Bradley 1993). Incorporation of *tk* into the genome is indicative of a random non-homologous recombination event (Capecchi 1989). The presence of this gene makes cells sensitive to the antiviral drugs GANC and FIAU, both of which block DNA replication, leading to cell death. Therefore, growing cells in GANC or FIAU media selects against tk^+ transformed cells. If cells are transfected with a replacement TV that contains *neo* within its homologous region and *tk* adjacent to it, cells in which only homologous double-crossover recombination occurs can be selected for in medium that contains both G418 and FIAU.

A problem still remains with insertion TVs because *tk* cannot be used to select against random integration as it can in replacement TVs. On the other hand, unlike replacement TVs, the hit-and-run method of insertional targeted

mutagenesis provides a means by which to deliver a subtle mutation to the targeted locus. An ideal gene targeting system would be able to deliver a subtle mutation to a specific locus while at the same time selecting against random integrants.

HPRT as a PNS Marker

The *HPRT* gene is a housekeeping gene involved in the salvage pathway for purine synthesis in mammalian cells (Melton, et al., 1986). In humans, *HPRT* expression is elevated in brain relative to other tissues, and a severe neurological disorder, Lesch-Nyhan syndrome, results when *HPRT* is not sufficiently expressed in brain tissue. *Hprt*-deficient mouse embryonic stem cells were initially generated in order to produce a mouse model of this human disease. It was demonstrated from this experiment that mutant mouse strains could be developed from cell clones selected *in vitro* (Kuehn, et al., 1987).

The mouse *Hprt* gene, in conjunction with *Hprt*-deficient ES cells, is now used commonly in gene targeting as a selectable marker system because it offers several advantages. It is located on the X chromosome, which makes it hemizygous in male ES cells. In addition, the structure of the gene is well-characterized (Melton, et al., 1984). Another enormous advantage of using *Hprt* as a selectable marker is that it is one of the few genes that can be used for both positive and negative selection (Szybalska and Szybalska 1962). When *Hprt*-deficient ES cells are transfected with the wildtype *Hprt* gene, cells expressing the exogenous gene can be selected for on HAT medium (containing hypoxanthine,

aminopterin and thymidine). Cells losing *Hprt* gene expression can be selected for on 6-thioguanine medium.

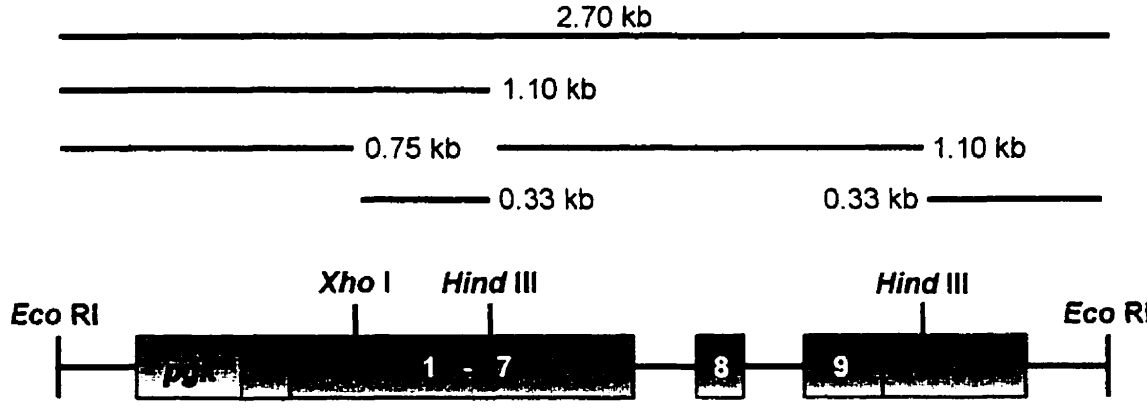
In gene targeting experiments using ES cells, it is important that the exogenous DNA be as small as possible, so that the vector will be able to carry it. The mouse *Hprt* gene is quite large, extending over 30 kb of the mouse X chromosome and containing nine exons (Melton, et al., 1984). Like other housekeeping genes, *Hprt* lacks CAAT and TATAA boxes; however, an essential 420-bp sequence within the first intron, 230 bp downstream from the transcription site, has been found to be an integral part of the *Hprt* promoter. An *Hprt* minigene (pDWM1), which is only 2.8 kb in length, has been constructed (Figure 7) (Magin, et al., 1992). To partially overcome the requirement for the promoter element within intron 1, a *pgk* promoter was added to the minigene in the plasmid pBTII SK^+. Effective expression of this minigene has been demonstrated in *Hprt*-deficient ES cells.

In Vitro Mutagenesis

Before any gene targeting experiments can be performed, a suitable vector containing the desired mutation and markers must be constructed. A genomic library must first be screened in order to obtain a clone that is homologous to the region of the gene that is to be targeted. The desired mutation must then be delivered into the cloned genomic DNA, traditionally through *in vitro* mutagenesis.

Figure 7. *Pgk-Hprt* minigene.

Scaled map of *Hprt* minigene with *pgk* promoter. Normally extending over 30 kb in the mouse genome, all nine exons of the gene, shown here in dark blue, are condensed within a region of about 1.5 kb. The *pgk* promoter is indicated in a light blue box. Black boxes represent untranslated regions. The entire 2.7-kb minigene is easily isolated from pBT/PGK-HPRT (Magin, et al., 1992) using restriction endonuclease *Eco*RI.


2.70 kb
1.10 kb
0.75 kb
1.10 kb
0.33 kb
0.33 kb
Xho I
Hind III
Hind III
Eco RI
Eco RI
1 - 7
8
9

Common Approaches to Mutagenesis

There are many methods of *in vitro* mutagenesis that are currently being used, most of which require an oligonucleotide bearing the desired mutation and a plasmid vector containing the genomic clone. The majority of these methods involve the use of PCR. In a relatively simple approach, the oligonucleotide is used as a primer together with a universal primer. PCR is then carried out on the plasmid vector, and the oligonucleotide is incorporated into the genomic region of the plasmid. The trouble with this system is the low fidelity of PCR itself.

In another approach, a uracil-containing DNA template is obtained from phages grown in *dut*$^-$ *ung*$^-$ host cells (Kunkel 1985). The mutation-bearing oligonucleotide is annealed to the template, followed by polymerization and ligation into a closed circle. The plasmid is transfected into *ung*$^+$ cells to select against non-mutated uracil-containing DNA. While this method and variations of it offer a higher fidelity of mutagenesis than the PCR approach, some difficulty remains with having to transfer the rebuilt fragment into a TV.

Limitations Inherent in Plasmid Vectors

The construction of plasmid TVs is both awkward and time-consuming. The genomic region inserted into the plasmid vector must be sufficiently large to ensure efficient targeting to the homologous region of chromosomal DNA within the ES cell. Homologous recombination within mammalian cells is relatively rare

and, as previously mentioned, requires a much greater degree of homology than in bacterial cells. Inserting a large genomic fragment into the plasmid vector creates several obstacles that are not easily overcome. Incorporation of positive and negative selection markers into the plasmid is required to construct a working TV; however, with a large genomic region in the plasmid, the availability of unique restriction sites is limited, making it more difficult to insert markers into the vector. As well, a large genomic region within a plasmid is often unstable and prone to rearrangements. Due to these difficulties, plasmid TVs often take up to six months to complete (Thomas and Capecchi 1987). A mutagenesis system in which a mutation could be transferred *in situ* into a TV would be more desirable.

TV Construction via Phage-Plasmid Recombination

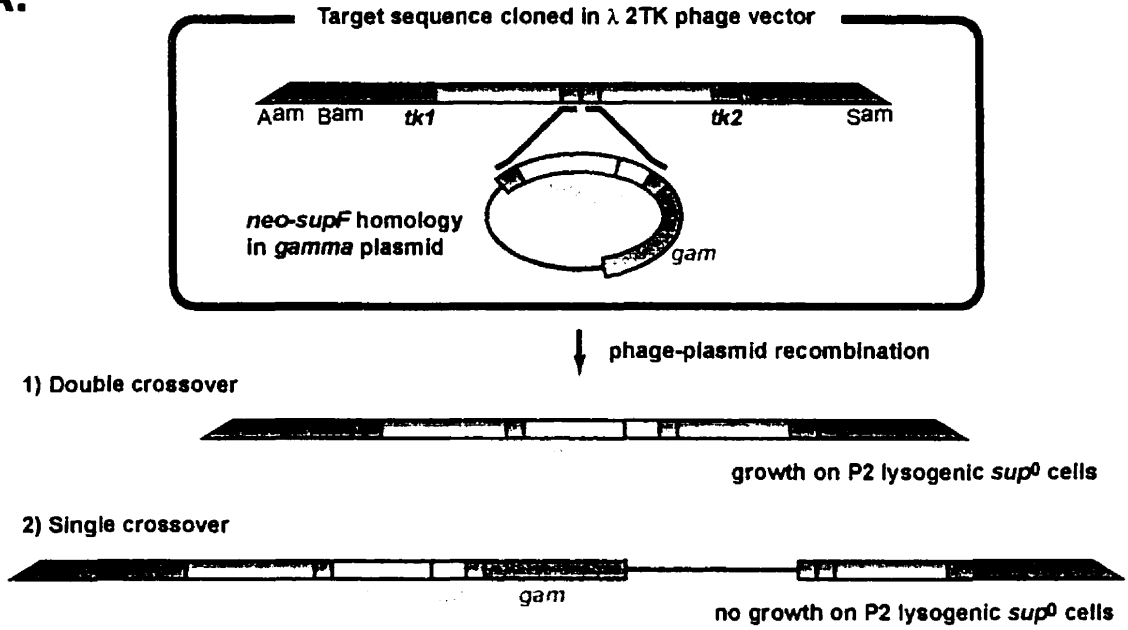
To overcome the problems encountered when using plasmid TVs, a novel approach has been devised in which TVs are constructed in a recombination-proficient bacteriophage, λ2TK (Tsuzuki and Rancourt 1998). The advantage λ phage provides is that it is able to maintain large unstable genomic regions that are otherwise prone to rearrangement, thus allowing for quicker and easier TV construction.

In one experiment, Tsuzuki and Rancourt (1998) targeted *neo* to a specific site within a λ2TK TV via homologous recombination (Figure 8a). To accomplish this, *neo* and *supF* were inserted into the homologous sequence within a KS*gam*

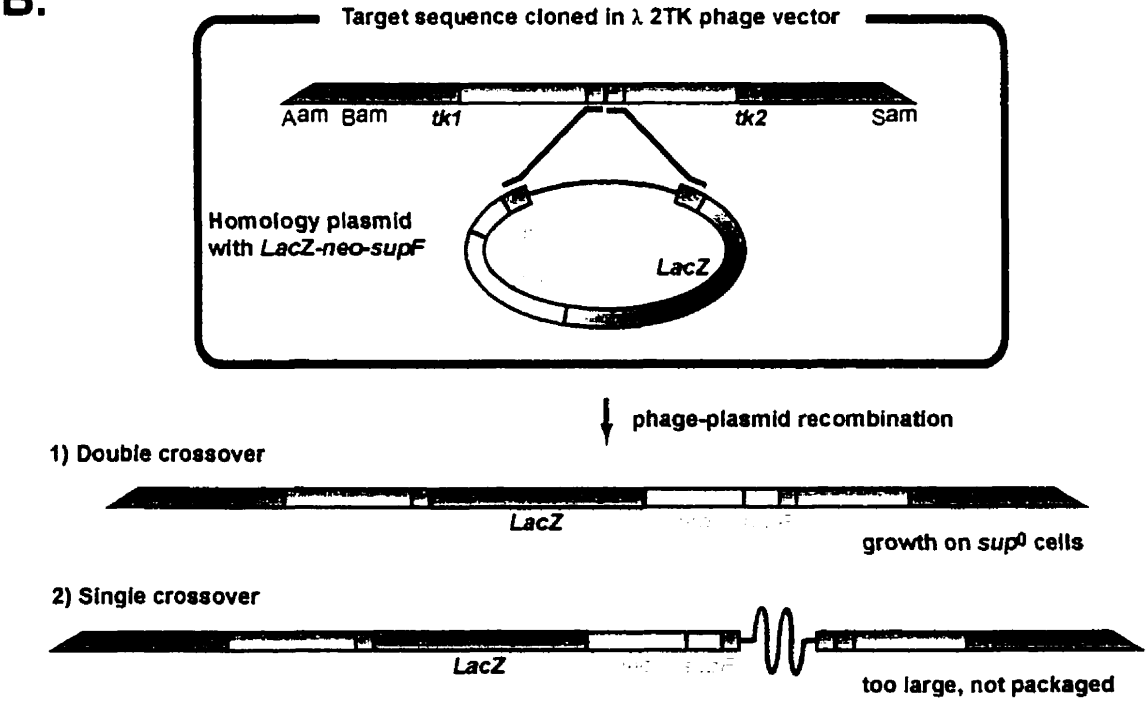
Figure 8. Targeting vectors in bacteriophage λ.

Flow diagrams showing generation of replacement targeting vectors in bacteriophage λ. A. A plasmid contains a region homologous to the target sequence in the phage clone. Homology here is represented by light blue boxes. The region of homology in the plasmid is separated by selectable markers *neo* and *supF* (orange). Outside of the homology is the negative selectable marker *gamma* (pink). Double crossover recombinants are selected for on a P2 lysogenic *supF*° host. Such a host selects for the presence of *supF* and selects against *gamma*, which would be present after a single crossover event. The completed replacement targeting vector is designed to replace its genomic target with a gene that is disrupted by *neo* and *supF*, which are used to select for such a targeting event. B. A large plasmid contains homology to the locus of interest in the phage clone. In this case, the homologous region is separated by *neo*, *supF*, and the relatively large marker *LacZ* (green). The selectable markers are all incorporated into the phage clone via double crossover recombination, effectively disrupting the target locus. Recombinants are then selected for on a *supF*° host. Due to the large size of the mutagenic plasmid, single crossover plasmid insertions into phage DNA are selected against simply by their inability to be packaged.

A.


Target sequence cloned in λ 2TK phage vector
Aam Bam
tk1
tk2
Sam
neo-supF homology
in gamma plasmid
gam
phage-plasmid recombination
1) Double crossover
growth on P2 lysogenic sup0 cells
2) Single crossover
gam
no growth on P2 lysogenic sup0 cells

B.


Target sequence cloned in λ 2TK phage vector
Aam Bam
tk1
tk2
Sam
Homology plasmid
with LacZ-neo-supF
LacZ
phage-plasmid recombination
1) Double crossover
LacZ
growth on sup0 cells
2) Single crossover
LacZ
too large, not packaged

plasmid. *SupF* is a tRNA suppresser of amber mutations, which operates by inserting a tyrosine residue at the UAG site. When using a strategy where λ2TK phage is involved, *supF* is required for lytic growth of the phage on any *supF*⁻ host strain because λ2TK contains A^{am}, B^{am}, and S^{am}. The phage may acquire *supF* through a recombination event with a plasmid carrying the *supF* gene; therefore, the ability of λ2TK to display lytic growth on a *supF*° host is evidence that such an event has taken place. Thus, *supF* is useful in this situation as a positive selectable marker.

The λ *gamma* gene was positioned next to the region of homology in the plasmid to act as a negative selectable marker because it can be selected against on any P2 lysogenic host via spi (sensitivity to P2 interference) selection. Phages carrying the *gamma* gene will not grow on a P2 host due to the spi activity of the host. *Gamma* was positioned outside of the homologous sequence in the plasmid so that it could only be incorporated into the TV through single-crossover recombination. Because the purpose of the experiment was to deliver *neo* to a specific site within the λ2TK TV via double-crossover recombination, *gamma* was used to select against single-crossover recombinants that result in a duplication of homology within the phage TV. Using this bacteriophage PNS system, only double-crossover recombinants are able to grow on *supF*⁻ P2 lysogenic host cells. Another factor used to discriminate against integrated single-crossover recombinants in this system was the size of the plasmid (Figure 8b). By using a

relatively large plasmid, single-crossover recombinants were selected against simply because, by incorporating the plasmid DNA, they fell outside of the size limitation of packageable DNA (Tsuzuki and Rancourt 1998).

When the contruction of a replacement TV in λ2TK was completed, the phage arms were removed and the TV electroporated into ES cells. This system was shown to be effective in disrupting the *cRABPI* locus in mouse ES cells (Tsuzuki and Rancourt 1998).

Objectives and Rationale

The purpose of this research was to construct a novel targeting vector in bacteriophage λ. Although not included in this thesis, successfully targeted ES cells would subsequently be used to generate a mouse model of Ser181Cys SFD. To accomplish this goal, it was my intention to demonstrate a novel two-step mutagenesis methodology that would result in the generation of the completed TV. The first step was the novel introduction of the point mutation into the phage vector *in situ*. The second step involved the addition of other necessary components for the functionality of this novel gene targeting system.

CHAPTER TWO: MATERIALS & METHODS

Materials

Nucleic Acids

Plasmid vectors used in subcloning experiments were πAN13 (ATCC; Lutz, 87) and pGEM®-7Zf (Promega). A 2.8-kb mouse *Hprt* minigene with a *pgk* promoter (Magin, et al., 1992) was provided in pBTII SK$^-$ by Dr. David Melton (University of Edinburgh).

Bacterial Cell Lines

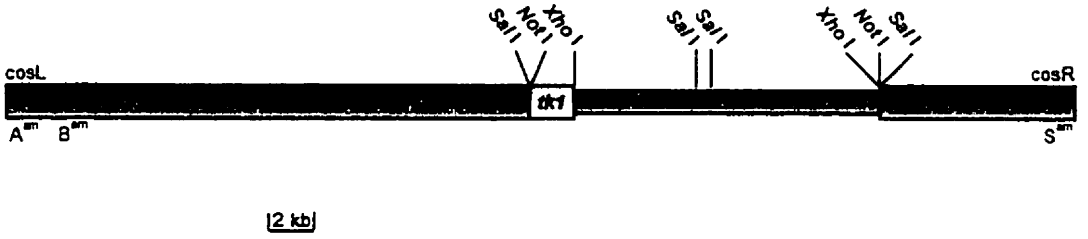
Escherichia coli strains used as plating cells were DH5α (rec$^-$, *lacZ*ΔM15), LG75 (*rec*$^-$, *sup*°, *lacZ*^{am}), MC1061[p3] (*rec*$^-$, *sup*°), LE392 (*rec*$^-$, *supE*, *supF*) and its P2 lysogenic derivative P2392. LG75 and MC1061[p3] were provided by Dr D. M. Kurnit (University of Michigan, Ann Arbor, MI). LE392 and P2392 were obtained from Stratagene (La Jolla, CA).

Mouse ES Cell Genomic Library

The mouse genomic library used in this project was constructed by Dr. Gerard Bain (University of Calgary) in bacteriophage λTK1 (Figure 9). The library was made from DNA isolated from undifferentiated embryonic stem cells (strain R1). Average insert size for the library was approximately 12 kb.

Figure 9. Bacteriophage λTK1 vector.

Schematic representation of bacteriophage λTK1. A 14-kb stuffer region in the phage contains *Sal*I restriction endonuclease sites, and is flanked on each end by an *Xho*I restriction endonuclease site. The HSV-*tk1* gene is located between the stuffer region and the cosL arm of the phage. All other notable restriction sites are indicated.


Sal I
Not I
Xho I
Sal I
Sal I
Xho I
Not I
Sal I
cosL
tk1
cosR
A^am
B^am
S^am
2 kb

Methods

Preparation of Plating Cells

LE392, LG75, and P2392 were each prepared by streaking onto an NZY plate (0.94 g/l $MgCl_2$, 5 g/l NaCl, 5 g/l yeast extract, 10 g/l select peptone 140, 15 g/l agar) from a bacterial stock and incubating overnight at 37°C. An LB plate (5 g/l NaCl, 5 g/l yeast extract, 10 g/l select peptone 140, 15 g/l agar) containing the antibiotic kanamycin was streaked with an MC1061[p3] bacterial stock and incubated overnight at 37°C. Single colonies were picked from each plate and grown at 37°C in 50 ml of their respective media broths, supplemented with 0.2% maltose, to an OD of 1.0 at A_{600} ($8x10^8$ cells/ml). Cells were pelleted by centrifugation and then resuspended in 20 ml of 10 mM $MgSO_4$ and stored at 4°C.

Frozen permanent stocks of each bacterial strain were prepared by splitting 3 ml of an overnight culture into two 2 ml screw-cap Eppendorf™ tubes with 7% DMSO, and storing the tubes at –80°C.

Large-Scale Phage Preparation

Large-scale phage preparations were carried out using a standard protocol (Sambrook, et al., 1989). Plating cells were infected with phages at phage to bacteria ratios of 1:100 and 1:200. After incubating these infections for twenty minutes at 37°C to allow phages to adsorb to bacteria, the phage-bacteria solutions were added to 400 ml of pre-warmed NZY media in large flasks. Flasks were then

shaken vigorously at 37°C until cell lysis was evident. Lysis, observed as clumps of cellular debris floating in the media, usually took six to nine hours to achieve. Once the cells were lysed, 1.5 ml of chloroform and 23.4 g of NaCl were added to each flask, and the flasks were returned to vigorous shaking at 37°C for 30 minutes, to ensure that all cells were destroyed and all phage particles released into the media.

After pelleting the bacterial debris by centrifugation at 8000 rpm for 10 minutes, the phage-containing supernatant was transferred to 500 ml centrifuge bottles. To precipitate the phage particles, 100 g/l of polyethylene glycol (PEG) was added and mixed. The bottles were then stored, either in ice for one hour, or at 4°C overnight. After this, the precipitated phage particles were pelleted at 8000 rpm for twenty minutes. The supernatants were removed, and the pellet was resuspended in 4 ml of TM buffer (50mM Tris pH 7.5, 10mM MgSO4) in order to allow the phage particles to go into solution. To remove insoluble debris from the solution, an equal volume of chloroform was added, and the mixture vortexed. The mixture was centrifuged at 8000 rpm for twenty minutes, and the aqueous phase was saved. A cesium chloride gradient was then set up for each sample by first adding 0.75 g/ml CsCl to each solution, and sealing the solutions in polyallomer tubes. These tubes were then centrifuged at 68,000 rpm for a minimum of four hours at room temperature. After this, phage particles in each sample were visible as a faint dark band in an otherwise clear and colourless gradient. This band was removed with an 18-guage needle and loaded directly

into dialysis tubing. Dialysis was performed at least three times in a 1000-fold volume of TM buffer, allowing each round of dialysis to continue for at least three hours. After dialysis, the purified intact phage were titered and stored at 4°C.

Phage DNA Preparation

In order to extract DNA from purified phage particles, the phages were first 'cracked' by adding an equal volume of phenol, followed by SDS to a final concentration of 0.02%. This mixture was inverted on a rocker for forty minutes, after which it was centrifuged for ten minutes. The aqueous phase was removed directly to dialysis tubing, and dialyzed three times against a 1000-fold volume of TE buffer (10mM Tris pH 7.5, 1mM EDTA). A spectrophotometric measurement was then taken on the dialyzed DNA at 260 nm wavelength. From this reading, the concentration of the DNA suspension was calculated (an OD of 1 corresponds to 50 μg/ml of double-stranded DNA). Purified DNA was stored at -20°C.

Small-Scale Phage DNA Preparation Using Liquid Lysis

Small-scale phage DNA preparations were performed for a quick analysis of phage clones. A single phage plaque was removed from a plate into 500 μl of SM buffer (0.1M NaCl, 5mM $MgSO_4$, 50mM Tris-HCl pH 7.5, 0.01% gelatin) with a drop of chloroform, then refrigerated at 4°C for several hours. A 100 μl fraction of this phage suspension was added to 500 μl of LE392 plating cells

(OD_{600} = 1.0) in a 50 ml Falcon™ tube, and incubated at 37°C for fifteen minutes. Ten milliliters of NZY broth was then added to the tube, which was left to shake at 37°C overnight.

The next day, two drops of chloroform was added to the tube and it was shaken at 37°C for another ten minutes. The cellular debris was then pelleted at 3000 rpm for twenty minutes. The phage-containing supernatant was removed into a 15 ml Falcon™ tube and treated with 15 µl of DNase (10 mg/ml) and 15 µl RNase (10 mg/ml). The enzymes were given thirty minutes at 37^0C to degrade all free nucleic acids in the suspension. After this, 2.5 ml of a 40% PEG/2.5M NaCl solution was added to the suspension, and the tube was stored at 4^0C for at least two hours to precipitate the phage particles. The precipitated phage was pelleted at 3000 rpm for ten minutes, and the pellet was resuspended in 0.5 ml SM buffer.

To extract the DNA from phage particles, the intact particles were cracked by first adding 5 µl 10% SDS and 5 µl 0.5M EDTA, then incubating for fifteen minutes at 68°C. Following this, the DNA was extracted first with phenol, then with 1:1 phenol:chloroform, and finally with chloroform alone. A 1/10 volume of 3M sodium acetate and an equal volume of isopropanol were added to precipitate the DNA. The DNA was then pelleted by centrifuging for ten minutes at 3000 rpm. After washing the pellet in 70% ethanol it was allowed to dry, then resuspended in 100 µl TE buffer and stored at -20^0C.

Small-Scale Plasmid DNA Preparation

Plasmid DNA 'mini-preps' were performed following a standard protocol (Sambrook, et al., 1989). A plasmid-containing colony of bacterial cells was picked from a petrie dish, dropped into 2 ml of liquid medium, and grown overnight at 37^0C in a shaker incubator. The next day, 1.5 ml of the cell culture was pipetted into a 1.5 ml Eppendorf™ tube and microcentrifuged for five minutes at 3000 rpm. The media was removed and the bacterial pellet was resuspended in 100 µl of lysis buffer (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA pH 8.0). Next, 200 µl of freshly prepared lysis solution (0.2M NaOH, 1% SDS) was added, and the mixture was gently inverted several times. Lastly, 150 µl of ice-cold precipitation solution (60 ml 5M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml water) was added, and the tube was again inverted gently several times. The tube was then left on ice for 3-5 minutes, after which it was microcentrifuged for five minutes at 13,000 rpm. The supernatant was carefully pipetted into a new 1.5 ml eppendorf tube, and an equal volume of 1:1 phenol:chloroform was added. The tube was inverted several times, then microcentrifuged for five minutes at 13,000 to separate the phases. The aqueous phase was removed to a new eppendorf tube, and two volumes of 100% ethanol was added to precipitate the plasmid DNA. The tube was again centrifuged for five minutes at 13,000 rpm to pellet the DNA. The ethanol was removed and 200 µl of 70% ethanol was added to wash the pellet. The tube was again microfuged, the ethanol removed, and the tube left open and inverted for twenty minutes to allow the remaining ethanol to

evaporate off. The dried pellet of purified plasmid DNA was then resuspended in 30-50 µl of either TE buffer or water.

Restriction Digests

Restriction digestions of plasmid and phage DNA were carried out with various enzymes under uniform conditions. Regardless of the volume of the reaction, each digestion consisted of the following: a variable amount of DNA suspended in either TE buffer or water, 1-2 units of enzyme per µg of DNA, and 1/10 volume of 10X reaction buffer. Also present in each digestion were small amounts of spermidine and RNase A. Digestion reactions were allowed to proceed at 37°C for at least two hours. For digests in which two or more restriction enzymes were used and different buffers were required, the digests were first set up with the buffer of lowest salt concentration and its corresponding enzyme(s). After allowing the digestion to proceed for one hour at 37°C, the higher salt concentration buffer and its corresponding enzyme(s) were added, and the digestion was given another two hours to proceed at 37°C.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze bacteriophage and/or plasmid DNA fragments after all restriction digestions and ligation reactions. Depending on the expected size of the DNA fragments, a gel was prepared as 0.6%

to 1% w/v agarose in 1X TAE buffer (39mM Tris, 20mM acetic acid, 10mM EDTA). This mixture was boiled in order to melt the agarose, after which several microliters of ethidium bromide from 10 mg/ml stock solution were added. The solution was poured into a gel tray, a gel comb was inserted, and the gel was allowed to harden. The comb was removed from the hardened gel and the gel tray was submersed in a reservoir filled with 1X TAE.

Several microliters of loading buffer (0.25% bromophenol blue and 40% sucrose dissolved in dH_2O) were added to each DNA sample. The samples were then each loaded into a separate lane on the gel, and a DNA size marker was loaded into another lane. Electrodes were connected to the gel reservoir and the samples were electrophoresed until independent DNA bands could be satisfactorily resolved on the gel. Gels were given variable amounts of time to run, from one hour to overnight. After electrophoresis, the gel was visualized and photographed next to a ruler on an ultraviolet transilluminator.

Isolation of DNA Fragments

In some cases, a particular fragment of digested DNA was required. To isolate this fragment, it was first resolved by gel electrophoresis. Using a razor blade, the fragment was then cut out of the gel and placed in a 1.5 ml Eppendorf™ tube. To dissolve the gel, three volumes of freshly prepared 6M sodium iodide was added, and the tube was incubated for five minutes at 55°C. Five microliters of glassmilk solution (silica suspended in dH_2O) was added to the tube, and it was

kept at 55°C for another five minutes. The tube was centrifuged for five seconds at 13,000 rpm, and the supernatant was discarded. The pellet was washed twice in 500 µl of glassmilk buffer (50mM NaCl, 10mM Tris pH 7.5, 2.5mM EDTA pH 7.5, 50% ethanol). The DNA was then eluted from the silica pellet by adding 10-15 µl of either TE or water and incubating the sample for five minutes at 55°C. After microcentrifuging the sample for thirty seconds at 13,000 rpm, the supernatant was removed and saved.

Ligation Reactions

In constructing any specialized plasmid, the gene of interest was subcloned into the vector plasmid at a specific restriction site. To accomplish this, the insert DNA was prepared so that its ends matched the restriction site at which it was to be inserted into the plasmid. The plasmid vector was linearized at that particular restriction site, then dephosphorylated for thirty minutes at 37°C in a reaction mixture containing phosphatase and phosphatase buffer. This reaction was then rinsed in 1:1 phenol:chloroform in order to remove the phosphatase. As in the mini-prep protocol, the DNA was then precipitated in 100% ethanol and resuspended in water.

Next, a ligation reaction was set up by combining the insert DNA with the linearized plasmid vector in a 2:1 insert to vector ratio, and bringing the volume to 8 µl in water. To this was added 1 µl of T4 DNA ligase and 1 µl of 10X ligation

buffer. The ligation reaction was then allowed to proceed overnight at 16°C. The next day, the ligation reaction was stopped and the DNA was again precipitated with ethanol and resuspended in water. The ligated DNA was electroporated into electrocompetent cells, which were then plated on selective media and grown overnight at 37°C. To verify the presence of the insert in the plasmid, several colonies were picked and grown overnight. The next day, plasmid minipreps were performed, followed by restriction analysis of the plasmids.

On one occasion, this method of verifying a construct did not work, and a screening approach was used. Nylon membranes were pressed onto NZY plates, and the cells were then plated directly onto the membranes and grown overnight. The next day, duplicate membranes were made and screened with a radiolabeled probe derived from the insert DNA. This proved to be quite effective in identifying the desired subclones.

Southern Blotting

In order to locate a DNA sequence of interest on an electrophoresed gel, southern analysis was used (Sambrook,et al., 1989). The gel was first soaked for fifteen minutes in 0.2M HCl, then rinsed briefly in 0.4M NaOH. The blotting apparatus was set up as follows: A reservoir was filled with 0.4M NaOH and a glass bridge was laid perpendicularly across the top of it. A piece of Whatman™ 3MM paper was laid over the glass bridge as a wick with the ends of the paper being submerged in opposite ends of the reservoir. Another piece of Whatman™ paper matching the size of the gel was placed on the middle of the bridge and the

gel was positioned face up on top of it. A piece of Hybond™-N nylon membrane (Amersham) cut to the size of the gel was placed over the gel, and another piece of gel-sized Whatman™ 3MM paper was placed on top of this. Finally, a tower of paper towels was stacked on top of the last piece of Whatman™ 3MM paper, effectively acting to draw the NaOH upward and the DNA out of the gel and onto the nylon membrane. The southern apparatus was left to run overnight. The next day, the nylon membrane was rinsed briefly in 2X SSPE. It was then ready to be washed in prehybridization buffer, and subsequently exposed to a radiolabeled probe using either the random prime or end-labeling method.

Preparing Electrocompetent Cells

Electrocompetent stocks of *E. coli* strains MC1061[p3] and DH5α were prepared as follows. Cells were grown up in 250 ml of LB broth to an OD of A_{600} = 1.0. The cell culture was then chilled in ice water for several minutes before centrifuging at 3000 rpm and 0°C for fifteen minutes. After thoroughly draining off and discarding the supernatant, the bacterial pellet was resuspended and washed five times in cold de-ionized water. After the fifth wash, the pellet was resuspended and washed once in 20 ml of 10% glycerol in cold de-ionized water. The cells were again spun down and the supernatant thoroughly drained. Cells were then resuspended by vortexing in a minimal amount of 10% glycerol (one or

two drops). Cells were then pipetted in 20 µl aliquots into pre-chilled 0.7 ml Eppendorf™ tubes and then frozen on dry ice and stored in a -80°C freezer.

Electroporation

In order to transfer plasmids into electrocompetent cells by electroporation, it was important to ensure the plasmid DNA preparations were pure and free of salts. Two to five microliters of a plasmid preparation was brought to 30 µl with 10% glycerol and then placed on ice. Electrocompetent cells were removed from a -80°C freezer and thawed on ice. A cuvette (2 mm chamber width) was also placed on ice. Ten microliters of the thawed cells was mixed with the plasmid, and the entire 40 µl mixture was pipetted into the cuvette. The electroporator was set to 2500 volts, a resistance of 400 ohms and a capacitance of 25 farads. The cuvette was then loaded into its chamber and electroporated. Immediately afterward, the cells were transferred into a tube containing 500 µl of S.O.C. (20 g/l select peptone 140, 5 g/l yeast extract, 0.5 g/l NaCl, 20mM glucose) medium. The tube was incubated at 37°C for 45 minutes in a shaker incubator before the cells were plated at different dilutions on selective media. Plates were incubated at 37°C overnight to allow transfected colonies to grow.

Library Screening

Using a 1.6-kb *Timp3* cDNA probe, the λTK1 mouse genomic library was screened to isolate genomic clones containing exon five of *Timp3*. For the primary screening, approximately $1x10^6$ phages were plated onto 28 150 mm plates, with approximately $5x10^4$ phages on each plate. After cooling the plates at 4°C, a Hybond™-N nylon membrane was pressed onto the surface of each plate in order to transfer phage nucleic acids from the plate to the membrane. Using a hypodermic needle, holes were punched through the membrane and the agar in order to establish the physical orientation of the membrane on the plate. Each membrane was carefully removed from its respective plate and allowed to soak for five minutes in a denaturing solution (0.5M NaOH, 1.5M NaCl). After this, membranes were transferred to a neutralizing solution (0.5M Tris pH 8.0, 1.5M NaCl) for another five minutes, then briefly rinsed in 2X SSPE (0.15M NaCl, 10mM NaH_2PO_4-H_2O, 1mM EDTA pH 8.0). Membranes were allowed to dry on Whatman™ 3MM paper before being stored in empty petrie dishes at 4°C.

Random Prime DNA Fragment Labeling

Before screening the membranes, they were incubated for two hours at 65°C in prehybridization buffer (5X SSPE, 0.5% SDS, 5X Denhardts [50X stock: 10 g/l Ficoll, 10 g/l polyvinylparilidone, 10 g/l BSA (Fraction V)], 250 μg/ml salmon sperm DNA). Using the random prime method, a ^{32}P radiolabelled probe

was prepared from a 1.6-kb *Timp3* cDNA fragment. Approximately 200 ng of the cDNA fragment was placed in a 1.5 ml Eppendorf™ tube and brought up to 16.5 µl volume in water. The cDNA was then boiled for two minutes, after which the tube was immediately placed on ice. To this was added 0.5 µl (3 units) of Klenow polymerase, 3 µl of 30 µCi α^{32}P-dCTP, and 5 µl of 5X random prime buffer.

Random Prime 5X Buffer:

Combine Solutions A, B, C, and D.

Solution A: 1 ml Solution O, 18 µl β-mercaptoethanol, 5 µl each of dATP, dTTP, dGTP (each dNTP was a 0.1M stock in 3mM Tris-HCl pH 7.0 and 0.2mM EDTA pH 7.0)

[Solution O: 1.25M Tris-HCl pH 8.0, 125mM $MgCl_2$]

Solution B: 2M Hepes pH 6.6 adjusted with NaOH

Solution C: Hexadeoxyribonucleotides (90 OD units/ml) in TE

Solution D: 100 mg/ml BSA

The 25 µl labeling reaction was incubated for one hour at 37°C. Using a Sephadex G-50 filtration column, the radiolabeled probe was separated from free nucleotides. The probe was then boiled for two minutes and added to the nylon membranes still soaking in prehybridization buffer.

The hybridization reaction was allowed to run overnight at 65°C with gentle rocking. On the following day, the membranes were twice washed for ten minutes at room temperature in 500 ml 2X SSPE, 0.5% SDS. Membranes were then washed for one hour at 65°C in 500 ml 0.5X SSPE, 0.5% SDS. After drying

the membranes on Whatman™ 3MM paper they were loaded onto autoradiographic cartridges, which were then loaded with film and stored overnight at –80°C. The film was developed the next day, and hybridized plaques were identified and picked from their respective plates into 1.5 ml Eppendorf™ tubes containing 500 µl SM buffer.

Secondary and tertiary screenings were necessary to ensure purification of *Timp3* phage clones. For these screenings, the same procedure was carried out as mentioned above, with the exception that less phage were plated in each subsequent stage. This made it possible to pick lone isolated clones from each plate. Once individual *Timp3* phage clones were obtained they were grown up, and their DNA was isolated and mapped using restriction enzymes.

Plate Lysates

Plate lysates were carried out by infecting 200 µl samples of plating cells with 10 µl of varying dilutions of phage. These infections were incubated at 37°C for twenty minutes to allow the phages to adsorb to the cells. After this, 4 ml of molten top agar, kept at 55°C, was added to each sample and immediately poured onto a plate containing solid medium. Plates were incubated overnight at 37^0C to allow for lysis of the cells. The next day, 3-5 ml of SM buffer was pipetted onto the plate that best exhibited a confluent lawn of plaques. This plate was then rocked slowly for at least two hours in order to draw the phage particles out of the

top agar and into the buffer solution. The plate was then set at an angle for five minutes to allow the phage-containing liquid to drain to one side of the plate. The liquid was pipetted into a tube, and 10 µl of chloroform was added to it in order to lyse any remaining cells. The cellular debris was pelleted by centrifugation, and the supernatant was removed to a fresh tube where another drop of chloroform was added. This final suspension of phage particles was then spot titered.

Spot Titering of Bacteriophage

Bacteriophage stocks were maintained at 4°C in SM buffer. Each phage stock was spot-titered as follows: A 100 µl fraction of LE392 plating cell suspension was added to 4 ml NZY top agar (7.5 g/l agar) that was first melted and cooled to 55°C. The LE392 top agar mixture was poured onto an NZY plate and allowed to solidify.

Phage dilutions were made in SM buffer, ranging from 10^{-2} to a 10^{-10} dilution. A 10 µl fraction was taken from each dilution and pipetted onto a labeled spot on the NZY-LE392 plate. The plate was incubated overnight at 37°C, and the phage titer was determined according to the spot on the plate where the number of plaques was countable.

DNA Sequencing

Using the Big Dye Kit from Perkin Elmer Applied Biosystems, sequencing was performed on phage DNA to confirm the presence of a 2-bp mutation. Having purified the DNA, 200-500 ng (in a volume of 3 µl) was placed in a 200 µl PCR tube to act as sequencing template to which was added 1 µl (3.2 pmoles) primer, 4 µl 2.5X sequencing buffer, 4 µl term ready reaction mix, and 8 µl dH_2O. The 20 µl sample was then placed in a PCR machine and run for 25 cycles (one cycle = 96°C, 30 sec; 50°C, 15 sec; 60°C, 4 min).

The thermocycled PCR mix was placed in a 1.5 ml eppendorf tube and precipitated by adding 16 µl dH_2O and 64 µl 95% ethanol. The tube was placed on ice for thirty minutes to ensure complete precipitation of the DNA. The tube was then spun for ten minutes at 13,000 rpm, and the supernatant completely removed. The invisible pellet was washed in 200 µl 70% ethanol, and centrifuged again for ten minutes. After removing the supernatant, the pellet was allowed to dry, then delivered to the sequencing facility (Faculty of Medicine, University of Calgary).

CHAPTER THREE: RESULTS

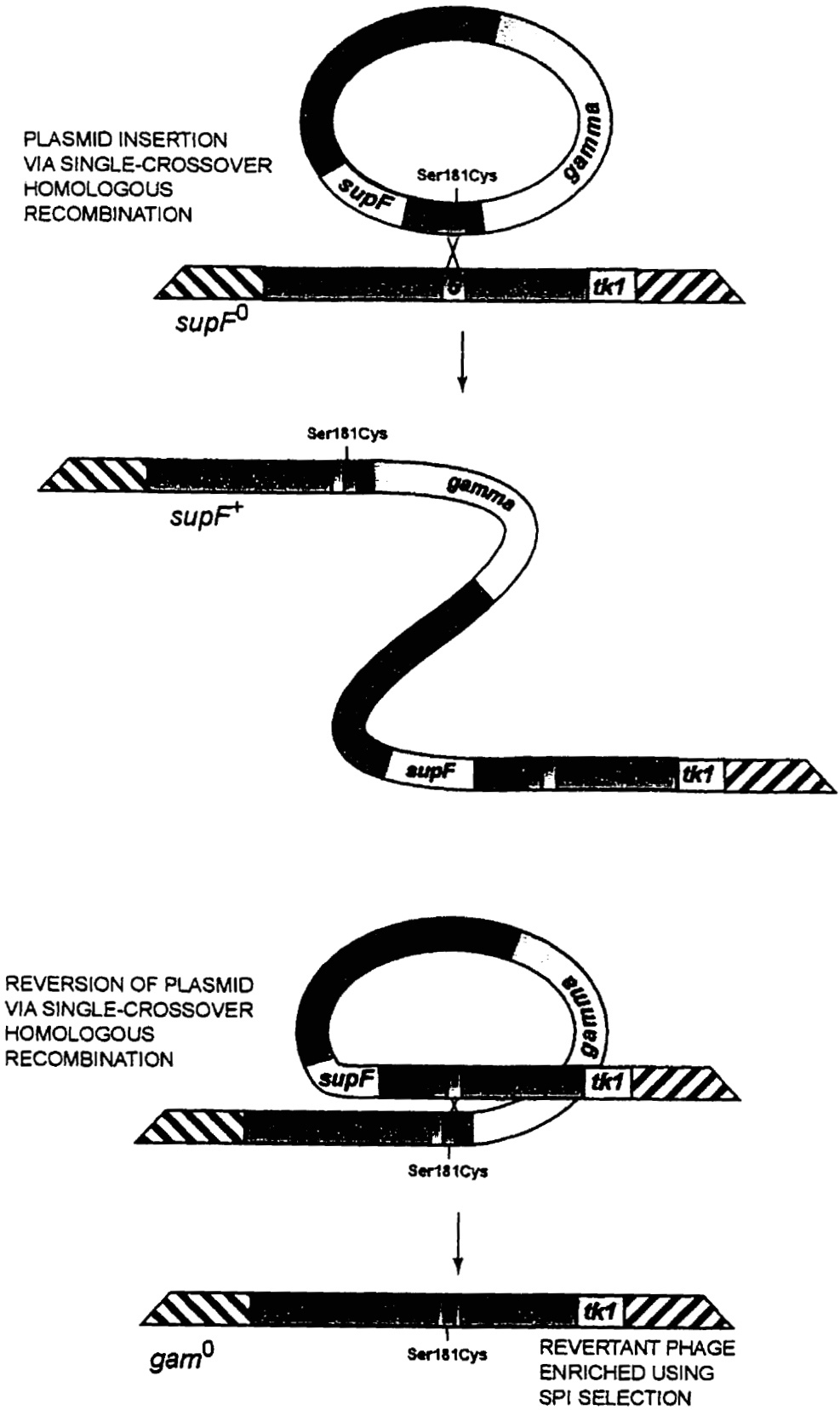
I set out to generate a novel targeting vector that could be used to deliver the Ser181Cys Sorsby fundus dystrophy mutation to its genomic locus in exon five of *Timp3* in mouse embryonic stem cells. To accomplish this goal, a novel method of *in situ* mutagenesis was employed. This method uses phage-plasmid recombination to generate targeting vectors in bacteriophage λ. The efficacy of this transplacement mutagenesis system is ensured by the exploitation of a system of positive-negative selection. In generating the completed phage targeting vector, I also took advantage of PNS in such a way as to replace the genomic sequence with only the 2-bp mutation, leaving no exogenous markers behind in the targeted locus.

Transplacement Mutagenesis

As Tsuzuki and Rancourt (1998) demonstrated, it is possible to construct TVs in phage *in situ*. By applying the principals of the hit and run method of gene targeting to TV construction in phage, I proposed that it should be possible to introduce point mutations into phage vectors *in situ* via insertional phage-plasmid recombination, a process we term transplacement mutagenesis (Unger, et al., 1999). In transplacement mutagenesis, it should be possible to insert a plasmid vector containing the SFD mutation, *gamma*, and *supF* into a specific site within the phage vector via single-crossover recombination (Figure 10). Recombinants could then be selected for on a *supF*° bacterial host. After selection conditions are relaxed and condensation allowed to take place, the phage would be passaged over

Figure 10. Transplacement mutagenesis in bacteriophage λ.

Flow diagram of novel transplacement mutagenesis using phage-plasmid recombination. The plasmid πSFDγ (red) bears only 50 bp of homology to the *Timp3* phage clone λTK-112 (blue). The homology is indicated here by a black box in the plasmid and a light blue box in the phage, representing exon 5 of *Timp3*. The 2 bp Ser181Cys mutation is also indicated within the plasmid homology. Through single crossover phage-plasmid homologous recombination, the plasmid is completely inserted into the phage clone. Phages having taken up the plasmid become *supF*^{r} are selected for on *supF*^{o} host cells. Due to the duplication of homology within the recombinant phages, relaxation of selection conditions (growth on a *supF*^{r} host) allows plasmid DNA to revert out of some of the phages. Revertant bacteriophages are selected for the absence of *gamma* using spi selection. If in the reversion event recombination occurs on the opposite side of the mutation as in the insertion event, the mutation remains behind in the phage clone, effectively replacing the wild-type sequence. This will be the case in a fraction of revertant phage clones.


PLASMID INSERTION
VIA SINGLE-CROSSOVER
HOMOLOGOUS
RECOMBINATION
Ser181Cys
supF
gamma
tk1
supF0
Ser181Cys
supF+
gamma
supF
tk1
REVERSION OF PLASMID
VIA SINGLE-CROSSOVER
HOMOLOGOUS
RECOMBINATION
gamma
supF
tk1
Ser181Cys
tk1
gam0
Ser181Cys
REVERTANT PHAGE
ENRICHED USING
SPI SELECTION

a P2 lysogenic host. Only phages that had undergone a reversion would grow, as evidence that the plasmid insert containing *gamma* had condensed out of the phage vector.

Isolation of Timp3 Phage Clone

The mouse genomic library, λTK1, was screened with a 1.6-kb probe derived from the 3' end of a *Timp3* cDNA. Three phage clones were identified and isolated. Restriction analysis of each clone was performed and compared to an existing restriction map of mouse *Timp3* (Apte, et al., 1995). Of the three bacteriophage clones, λTK-112 was chosen as the best candidate in which to construct the targeting vector. The clone insert was found to be approximately 14 kb in length, with the target locus of exon five centrally positioned within the insert sequence (Figure 11).

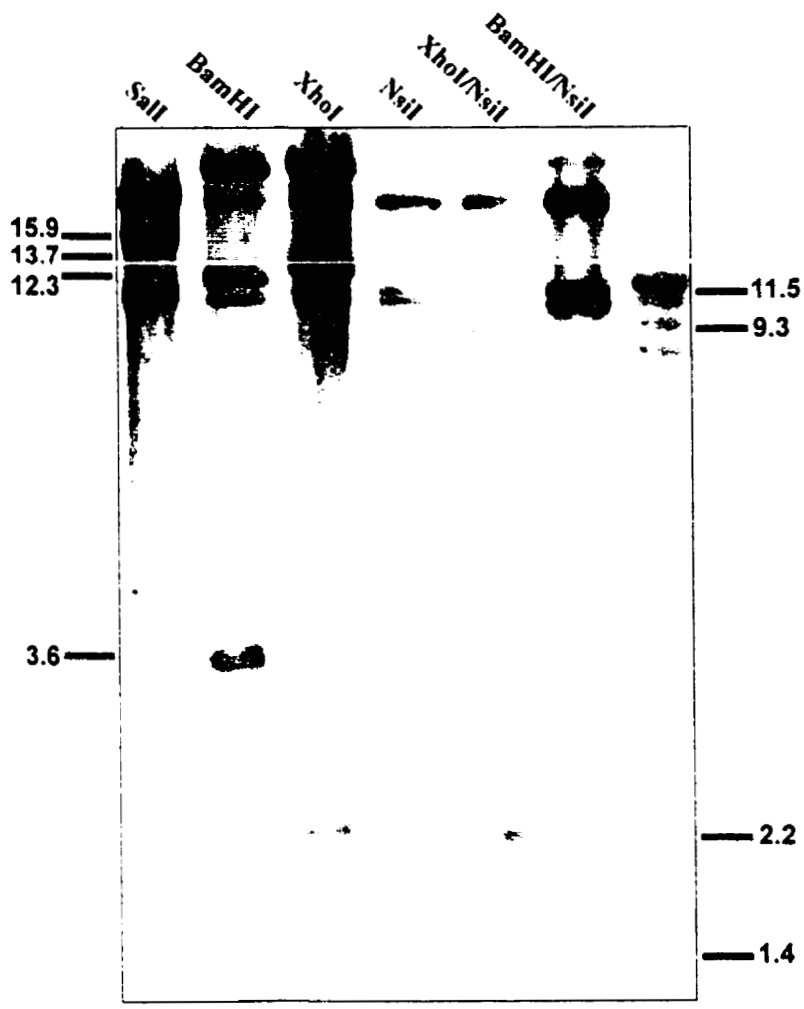
Mutagenic Oligonucleotides

Two complementary 52-base SFD oligonucleotides were prepared, which bear 50 bp of homology to a portion of exon five of the mouse *Timp3* gene. A 2-bp substitution in the center of the oligonucleotides serves to encode the Ser181Cys mutation, as well as creating a diagnostic *Nsi*I restriction site (Figure 12).

Figure 11. *Timp3* phage clone λTK-112.

A. Photograph of restriction digested λTK-112 electrophoresed on an agarose gel. A 1-kb DNA ladder (far-right lane) was used a size marker. All samples were digested with *Sal*I as well as the enzymes indicated above each lane. It should be noted that the 11-kb bacteriophage arm appears in each lane, and should not be confused with cloned insert fragments. The intact cloned insert (~15.9 kb) is present in the sample digested with *Sal*I alone. B. Schematic representation of *Timp3* bacteriophage clone λTK-112, as determined by restriction analysis. The clone includes exons 2 through 5 of *Timp3*, indicated as numbered in light blue boxes. The clone is positioned upstream of the *tk1* gene (orange box), and known restriction endonuclease sites are indicated. Phage arms are represented by cross-hatched boxes on each end of the clone insert.

A.


SalI
BamHI
XhoI
NsiI
XhoI/NsiI
BamHI/NsiI
15.9
13.7
12.3
3.6
11.5
9.3
2.2
1.4

B.

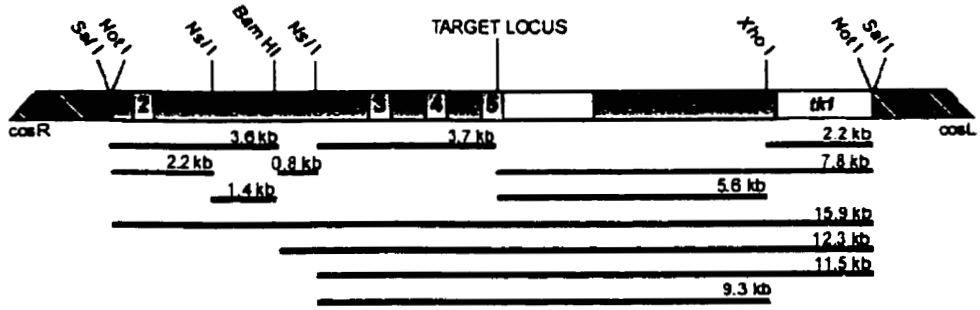

Sal I
Not I
Nsi I
Bam HI
Nsi I
TARGET LOCUS
Xho I
Not I
Sal I
2
3
4
6
tkt1
cosR
cosL
3.6 kb
3.7 kb
2.2 kb
2.2 kb
0.8 kb
7.8 kb
1.4 kb
5.6 kb
15.9 kb
12.3 kb
11.5 kb
9.3 kb

Figure 12. Sorsby fundus dystrophy mutagenic oligonucleotide.

Sequence of mutant SFD oligonucleotide. Wild type *Timp3* exon 5 encodes a serine as residue 181. The 52-bp SFD fragment made from complementary oligonucleotides emulates the Ser181Cys mutation by introducing a 2-bp substitution into the sequence. By changing the wobble base of the adjacent lysine, a novel *Nsi*I restriction endonuclease site is generated which can be used as a diagnostic indicator of the presence of the mutation after mutagenic experiments.

Wildtype *TIMP3*

172	173	174	175	176	177	178	179	180	**181**	182	183	184	185	186	187	188
Tyr	Arg	Gly	Trp	Ala	Pro	Pro	Asp	Lys	Ser	Ile	Ser	Asn	Ala	Thr	Asp	Pro
TAC	CGA	GGA	TGG	GCT	CCC	CCA	GAC	AAG	AGC	ATC	AGC	AAC	GCC	ACA	GAC	CCC

SFD Transplacement Oligo

	Tyr	Arg	Gly	Trp	Ala	Pro	Pro	Asp	Lys	Cys	Ile	Ser	Asn	Ala	Thr	Asp	Pro
5' C	TAG	TAC	CGA	GGA	TGG	GCT	CCC	CCA	GAC	AAA	TGC	ATC	AGC	AAC	GCC	ACA	GAC	CCC	T 3'

Nsi I

Plasmid Constructs

The size limitation on packageable DNA was a major consideration in choosing a plasmid vector. The plasmid had to be small because a large region of homology would be added to it, together with selectable markers. The entire construct would have to be inserted into the λ phage vector, after which the phage, with integrated plasmid, would then have to be short enough to be packaged into the phage capsid. The plasmid πAN13 was an ideal vector.

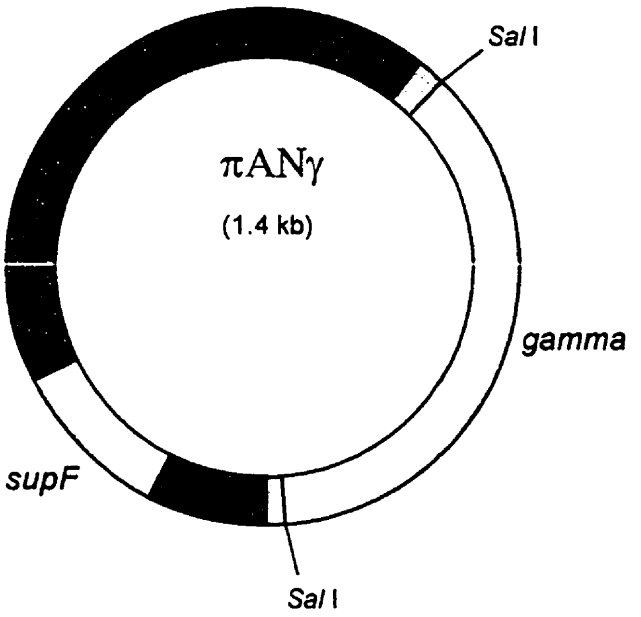
πANγ

For the purposes of this work, πAN13 provided two clear advantages. This plasmid is relatively small (895 bp) and contains the positive selectable marker *supF*. The 500-bp negative selectable marker *gamma* was inserted into the *Sal*I restriction site of πAN13 to give rise to the universal transplacement vector πANγ (Figure 13a) (Unger, et al., 1999). Because *supF* can be selected for in phage and *gamma* can be selected against in a P2 lysogenic host, πANγ should prove to be a very useful transplacement vector for the proposed thesis experiments as well as future ones.

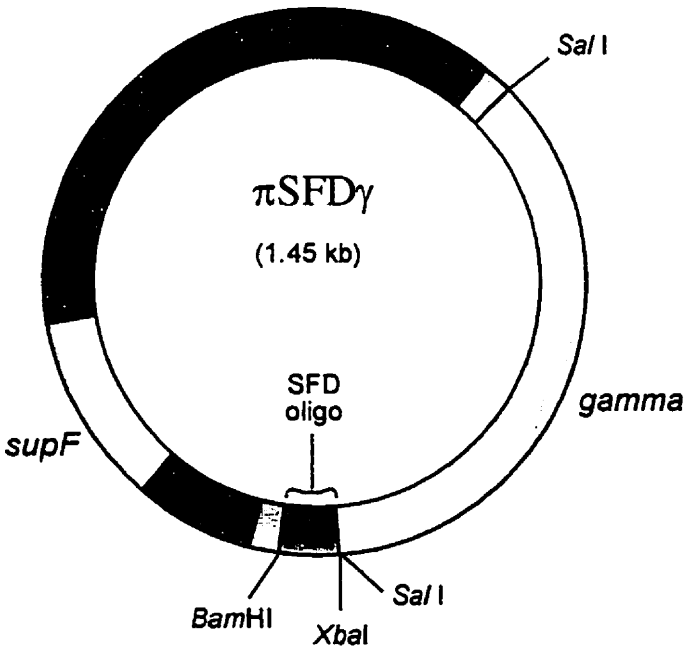
Figure 13. Plasmid constructs πANγ and πSFDγ.

Schematic representations of plasmid transplacement vectors. A. The vector πANγ is a derivative of πAN13 (Lutz, 1987). The 500-bp selectable marker λgamma (beige) was ligated into the *Sal*I restriction endonuclease site of the polycloning region (orange) of πAN13, giving rise to the 1.4 kb vector. B. In constructing 1.45 kb πSFDγ, a 52-bp mutagenic oligonucleotide (black) was ligated between the *Bam*HI and *Xba*I restriction sites in the polycloning region of πANγ.

A.


πANγ
(1.4 kb)
Sal I
gamma
supF
Sal I

B.


πSFDγ
(1.45 kb)
Sal I
SFD
oligo
gamma
supF
BamHI
XbaI
Sal I

πSFDγ

The double-stranded oligonucleotide containing the 2-bp Ser181Cys mutation and unique *Nsi*I restriction endonuclease site was inserted into the multiple cloning site of the πANγ vector using restriction enzymes *Xba*I and *Bam*HI. The completed 1.45 kb plasmid construct was named πSFDγ (Figure 13b).

Transplacement Mutagenesis

To successfully transfer the SFD mutation into λTK-112, the phage vector had to be passaged over cells carrying the mutagenic oligonucleotide. Specifically, the λTK-112 phage vector was passaged under non-selective conditions over MC1061[p3; πSFDγ] cells, and the phages were harvested the next day in SM buffer. The phage suspension was spot titered on LE392 cells, and also passaged over *supF*° LG75 cells on NZY plates (with Xgal and IPTG) to identify single-crossover *supF*$^+$ recombinant phages (blue plaques). As expected, only blue plaques appeared on the plates, and the incidence of these single-crossover recombinants was 5×10^{-3}.

One blue phage plaque was chosen and passaged over LE392 under relaxed conditions to allow the plasmid to revert out of the phage. The phages harvested from this experiment were again spot titered on LE392, then passaged over the

supF$^+$ P2 lysogen, P2392. This effectively took advantage of spi selection to select against non-reverted phages that still retained the λ *gamma* gene. The incidence of plasmid condensation in this step was 1.5 x 10^{-2}, substantially higher than the initial integrative recombination event.

To determine which, if any, of the condensatants retained the SFD mutation in place of the endogenous wildtype sequence, individual revertant phage clones were picked and grown on P2392 in small-scale liquid lysates. Restriction and southern analysis of fourteen clones revealed that all had excised the plasmid, and that five of the fourteen had retained the 2-bp SFD substitution (Figure 14). The high fidelity of this *in situ* mutagenesis technique was confirmed by sequence analysis of one of the *Nsi*I positive transplaced phage clones (Figure 15).

Completion of the SFD Targeting Vector

To complete the phage replacement TV, another plasmid containing *supF*, *Hprt*, and a large region of homology to *Timp*3 was inserted into the phage via single-crossover recombination. Because there is a limit to the length of exogenous DNA that can be packaged into λ phage, a λTK1 library has been constructed in which only one copy of *tk* is present in the phage. The size of the clones in this library has been restricted to ~12 kb. This allows for the insertion of the homologous *supF*$^+$ *Hprt*$^+$ plasmid into the phage. Recombinants were selected

Figure 14. Restriction and southern analysis of reverted λTK-112.

Identification of SFD mutation within phage clones after transplacement. After performing the insertion and reversion steps of transplacement mutagenesis on λTK-112, fourteen revertant phage clones were analyzed for the presence of a 2-bp SFD mutation that creates a unique *NsiI* restriction endonuclease site. The DNA from each clone was cut with *NsiI*, then electrophoresed on an agarose gel. The agarose gel displayed here shows eight of the fourteen λTK-112 phage clones and a 1-kb DNA ladder used as a marker. Two distinctive banding patterns are evident: an extra band of 3.7 kb appears in lanes 2, 6, and 8. Southern analysis was performed using a single-stranded SFD oligonucleotide probe. The resulting autoradiographic image identifies the same unique 3.7 kb bands in lanes 2, 6, and 8, indicating the presence of an additional *NsiI* restriction site in these clones.

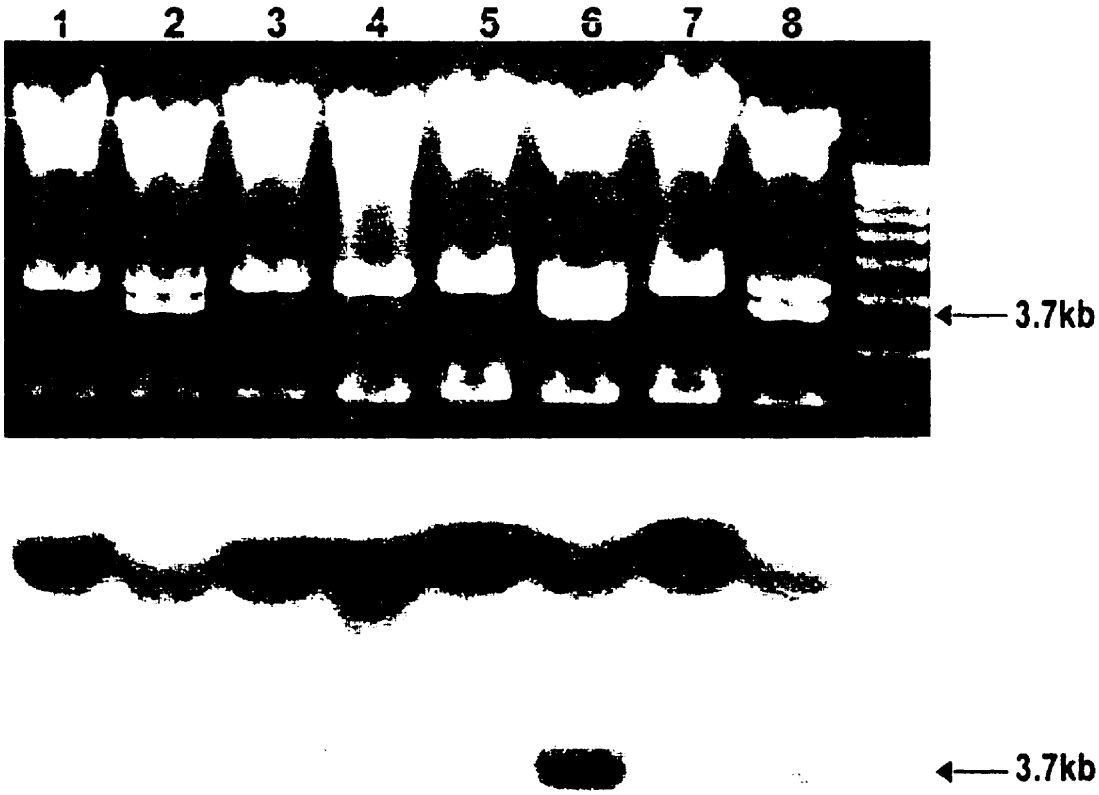
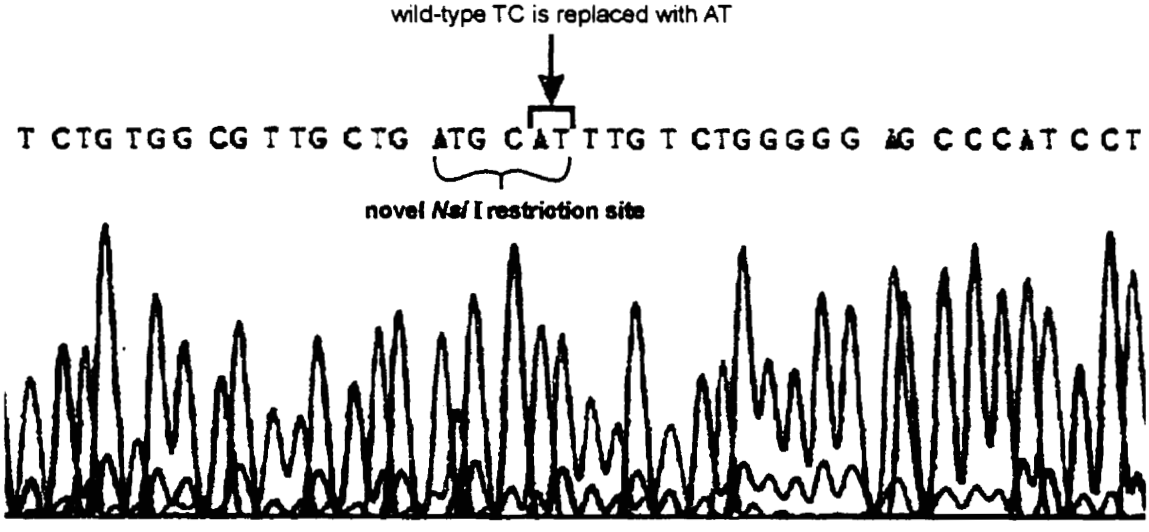

1
2
3
4
5
6
7
8
3.7kb
3.7kb

Figure 15. Sequence analysis of λTK-112 after transplacement.

Sequencing analysis of reverted λTK-112 clone. An extra band appeared in several reverted λTK-112 phage clones that had been cut with *NsiI*. To confirm that this extra band was a result of successful transplacement mutagenesis, sequencing analysis was done on one of the clones. As indicated here, a 2-bp substitution is present, and serves both to emulate the SFD Ser181Cys mutation and to create a unique *NsiI* restriction site.


wild-type TC is replaced with AT
T CTG TGG CG T TG CTG ATG CAT T TG T CTGGGGG AG CCCAT CCT
novel NsiI restriction site

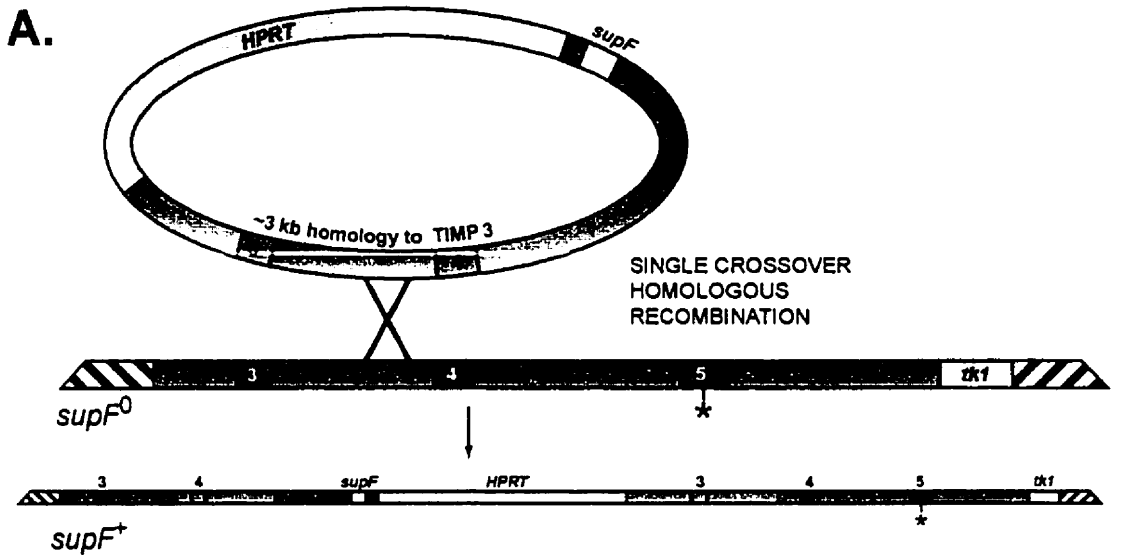
for on a *supF*° bacterial host. The completed TV will be used in a novel gene targeting strategy called replacement-condensation (RC).

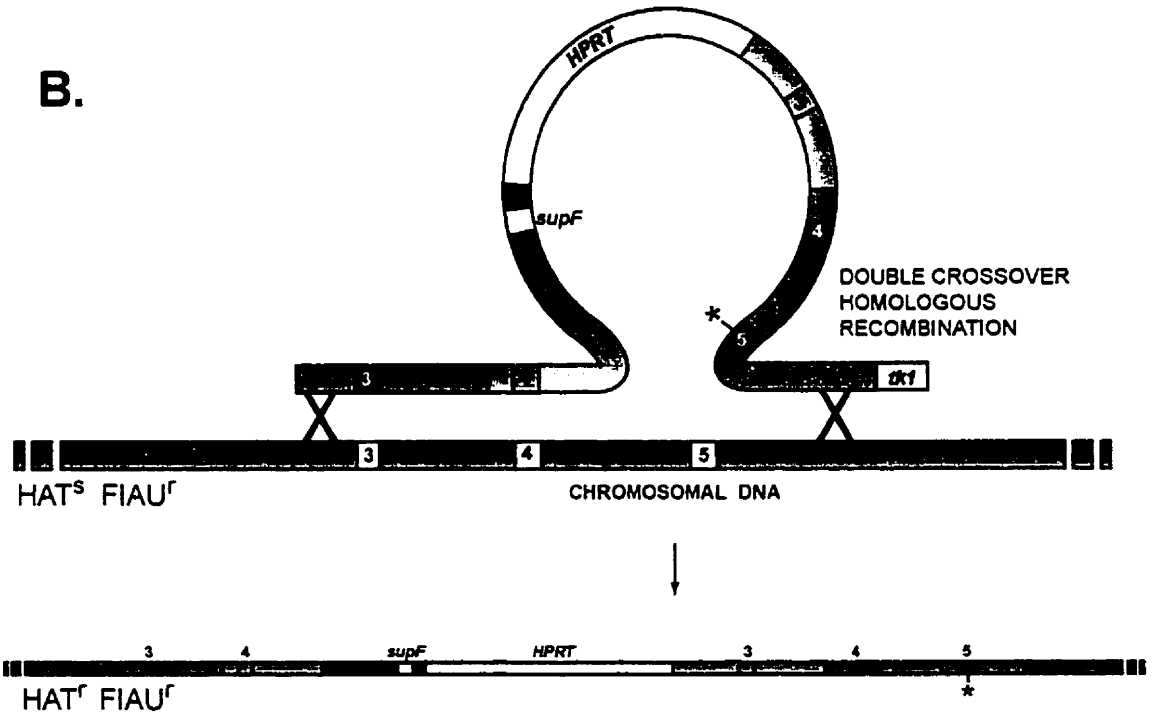
Replacement-Condensation Gene Targeting

The purpose of constructing such an unconventionally configured TV is to apply a novel gene targeting approach we term replacement-condensation to target the subtle SFD mutation to mouse ES cells. RC is a novel approach which applies the principals of the hit-and-run technique to replacement gene targeting. Using phage-plasmid recombination, replacement TVs are constructed which contain a large duplication of homology to the targeted locus. This duplication is separated by plasmid-derived DNA containing positive and negative selectable markers. The duplicated region of homology is flanked by *tk* genes. After transfecting cells with this replacement vector, homologous double-crossover recombination occurs at the target locus while random integration of the TV takes place at a much higher frequency. Cells are then grown in media that selects only for homologous recombination events (Figure 16). In other words, *tk* is selected against while the positive marker is selected for. Selection conditions are then relaxed so that a reversion may occur within the duplication of homology. As in the hit-and-run approach, the plasmid DNA and one copy of the duplicated region recircularize and revert out via intrachromosomal recombination. During this 'condensation' reaction, both negative and positive selectable markers are also removed from the

Figure 16. Replacement step in RC gene targeting.

Flow diagram of replacement gene targeting step for novel replacement-condensation (RC) strategy. A. The RC targeting vector in phage is generated via homologous phage-plasmid recombination. In a single crossover event, a plasmid containing *supF*, *Hprt*, and a large region of homology is inserted into the mutagenic phage. This insertion results in a large duplication of homology (represented here as duplications of exons 1 and 2 in green boxes) that is separated by plasmid DNA (red) and selectable markers (beige). Phage arms are represented here by cross-hatched boxes. A subtle mutation in the phage is indicated by an asterisk downstream of the duplicated region. The completed vector is then selected for on a *supF*° host. B. The genomic sequence (black) is targeted via homologous double crossover recombination. This event is selected for on a medium containing both HAT and FIAU. HAT selects for the presence of *Hprt* while FIAU selects against *tk*.


A.
HPRT
supF
~3 kb homology to TIMP 3
SINGLE CROSSOVER
HOMOLOGOUS
RECOMBINATION
3
4
5
tk1
supF0
3
4
supF
HPRT
3
4
5
tk1
supF+


B.
HPRT
supF
4
5
DOUBLE CROSSOVER
HOMOLOGOUS
RECOMBINATION
3
tk1
3
4
5
HATS FIAUr
CHROMOSOMAL DNA
3
4
supF
HPRT
3
4
5
HATr FIAUr

chromosomal DNA (Figure 17). Successful condensatants can then be selected for by the absence of the negative selectable marker.

πHPRT

The next construct I made also took advantage of the attributes of πAN13. The negative selectable marker *tk* is to be used to select against random integration of the targeting vector, but an additional negative selectable marker must be used for the purposes of this novel gene targeting system. The *Hprt* gene is ideal for this purpose because, due to its ability to function as both a positive and a negative selectable marker, it negates the need for a separate positive marker. To utilize the benefits of *Hprt*, the *Hprt* minigene along with its *pgk* promoter was inserted into the *Eco*RI restriction site of πAN13 to create another universal vector, πHPRT (Figure 18a).

πHPRT-TIMP112

A 4-kb fragment of the λTK-112 phage clone upstream of the target locus was cloned into pGEM®-7Zf between the *Bam*HI and *Kpn*I restriction sites. A 3-kb portion of this *Timp3* fragment was isolated after digesting the plasmid with *Xba*I, then ligated into the *Xba*I restriction site in πHPRT. The final 6.6-kb construct was named πHPRT-TIMP112 (Figure 18b). The purpose of this

Figure 17. Condensation in RC gene targeting.

Flow diagram of the condensation step in replacement-condensation gene targeting. Due to the large duplication of homology (indicated as exons 1 and 2 in green boxes) present in the chromosome after the replacement gene targeting event, intrachromosomal recombination between the duplicated regions is able to occur when selection conditions are relaxed. This results in a large portion of the original phage targeting vector condensing out of the chromosome, taking one copy of the duplicated region with it, as well as both selectable markers (orange). Because the mutation (indicated here by an asterisk) is not part of the duplicated sequence, it is invariably left behind in the genome. Cells having undergone condensation of the targeting vector are selected against the presence of *Hprt* in 6-thioguanine liquid medium.

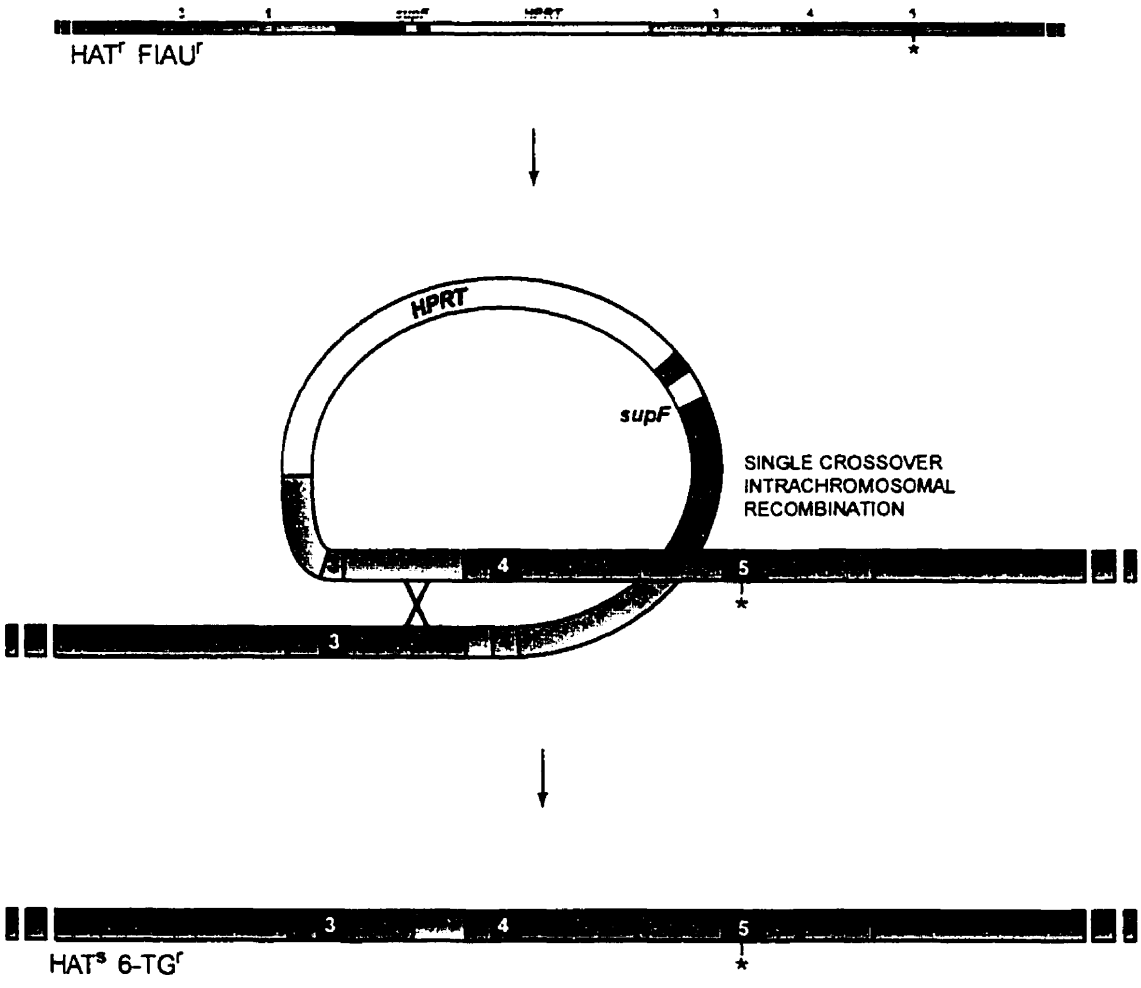
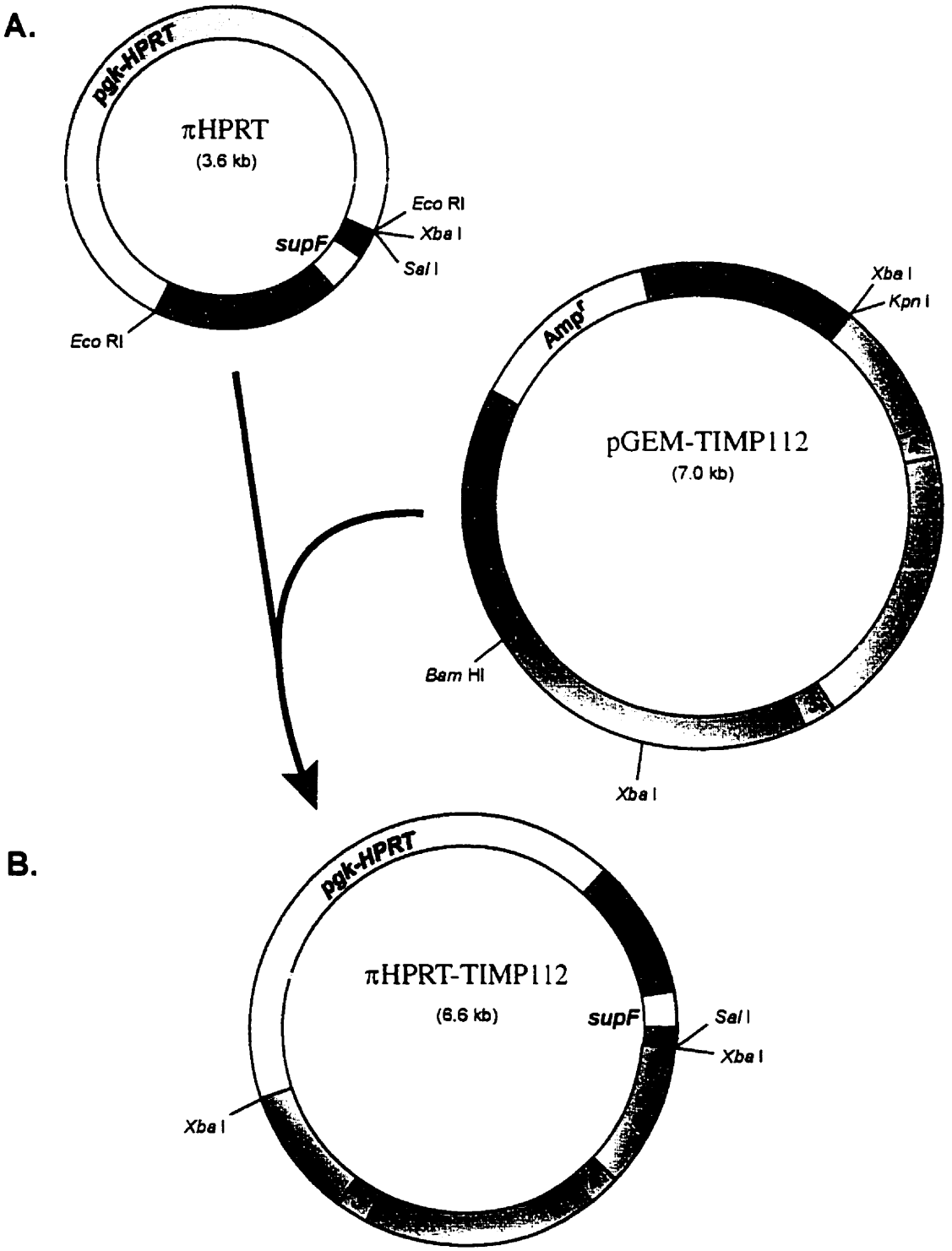

HAT^r FIAU^r
HPRT
supF
SINGLE CROSSOVER
INTRACHROMOSOMAL
RECOMBINATION
4
5
3
3
4
5
HAT^s 6-TG^r

Figure 18. Plasmid vectors πHPRT and πHPRT-TIMP112.

Schematic representation of replacement-condensation plasmid vectors. A. An *Hprt* minigene with *pgk* promoter (Magin, et al., 1992) was ligated into the *Eco*RI restriction endonuclease site of πAN13 (Lutz, 1987) to yield the 3.6-kb plasmid vector πHPRT. Another plasmid, pGEM-TIMP112, was constructed by ligating a 4-kb fragment of *Timp3* phage clone λTK-112 (indicated here in light blue) between the *Kpn*I and *Bam*HI restriction endonuclease sites in the polylinker region of pGEM®-7Zf (Promega). The subcloned region of *Timp3* includes exons 3 and 4, as indicated in green boxes. B. A 3-kb portion of *Timp3* in pGEM-TIMP112 was excised with *Xba*I, then ligated into the *Xba*I restriction endonuclease site in πHPRT. The resulting construct, πHPRT-TIMP112, is 6.6 kb and contains the selectable markers *Hprt* and *supF*, as well as 3 kb of homology to genomic *Timp3*.


A.
pgk-HPRT
πHPRT
(3.6 kb)
supF
Eco RI
Xba I
Sal I
Eco RI
Xba I
Kpn I
Ampr
pGEM-TIMP112
(7.0 kb)
Bam HI
Xba I
B.
pgk-HPRT
πHPRT-TIMP112
(6.6 kb)
supF
Sal I
Xba I
Xba I

construct was to introduce both *Hprt* and the 3-kb region of *Timp3* homology into the TV by phage-plasmid recombination.

Completing the Targeting Vector

Having successfully delivered the SFD mutation into the phage vector, the next step was to insert the full πHPRT-Timp112 plasmid into the vector just upstream of the mutation. This was accomplished using the same technique as previously mentioned. Using *supF* as a positive selectable marker, λTK-SFD was passaged over MC1061[p3; πHPRT-Timp112] cells. The single-crossover event then was allowed to occur between a point in the phage DNA and the 3-kb region of *Timp3* homology within the plasmid. The resulting recombinant phage vector contained a large duplication of homology separated by the plasmid sequence and *Hprt* minigene (Figure 19), all upstream of the mutation. This completed TV was called λSFDTV. The purpose of this uniquely designed vector is to target endogenous *Timp3* using a novel approach we term replacement-condensation (RC) gene targeting.

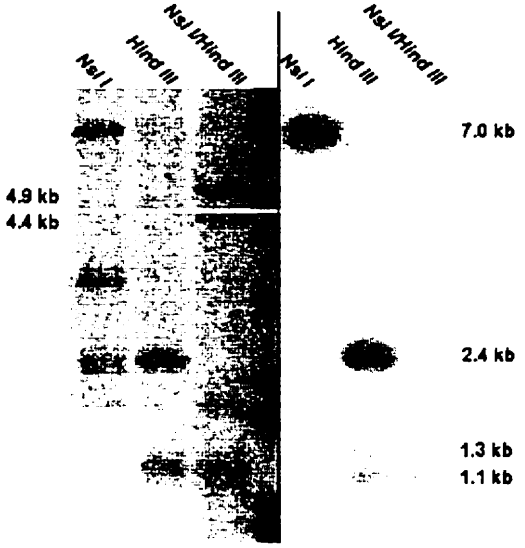
Generating a Flanking Probe

Once cells have been successfully targeted and the plasmid allowed to condense out of the chromosomal DNA, it is necessary to confirm the integrity of the chromosomal sequence and to ensure that the only alteration in the sequence is

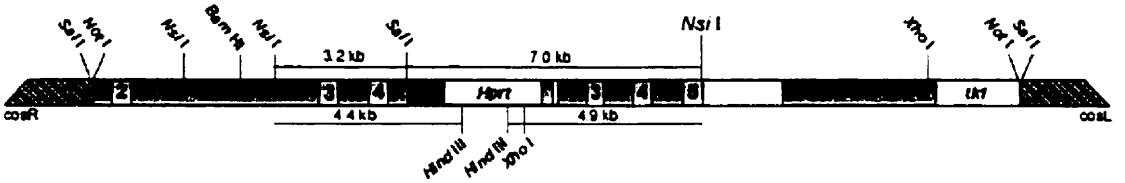
Figure 19. RC targeting vector λSFDTV.

Restriction analysis of λSFDTV. Using phage-plasmid homologous recombination, the plasmid vector πHPRT-TIMP112 was inserted into a λTK-112 clone bearing the Ser181Cys mutation. A. The positioning of the plasmid elements was confirmed by restriction and southern analysis. All samples shown were cut with restriction endonuclease *Sal*I in addition to the enzymes indicated above each lane. A 1-kb DNA ladder was used as a marker. A 1.1 kb *Hind*III fragment of the *Hprt* minigene was used as a probe for southern analysis. A 7.0 kb *Nsi*I/*Sal*I fragment is clearly labeled by the probe. In the sample cut with *Hind*III/*Sal*I, the 2.4 kb, 1.3 kb, and 1.1 kb fragments are also labeled in varying degrees. The sample cut with all three enzymes shows definite bands of 4.9 kb and 4.4 kb, but only 1.3 kb and 1.1 kb bands are labeled. B. A schematic representation of the completed RC targeting vector λTKSFD shows that *pgk-Hprt* is oriented backwards in the clone. However, this will not affect the expression of the selectable marker. *Pgk* here is represented by a purple box.

A.


Nsi I
Hind III
Nsi I/Hind III
Nsi I
Hind III
Nsi I/Hind III
7.0 kb
4.9 kb
4.4 kb
2.4 kb
1.3 kb
1.1 kb

B.


Sal I
Not I
Nsi I
BamHI
Nsi I
Sal I
Nsi I
Xho I
Not I
Sal I
3.2 kb
7.0 kb
cosR
4.4 kb
4.9 kb
cosL
Hprt
tk1
Hind III
Hind III
Xho I

the designed mutation. For this purpose, it is necessary to construct a flanking probe beforehand that is homologous to a region just outside of the homology incorporated into the TV. I encountered great difficulty in generating a flanking probe for λSFDTV. I intended to generate the flanking probe from one of the other two *Timp3* phage clones that had been isolated from the library; however, my attempts at mapping these clones were unsuccessful.

Re-Screening the Library

I decided to screen the library once again for more *Timp3* clones, hoping that one of them could be used to generate a flanking probe. I isolated three more clones. One of the clones, λTK-TU14, encompassed the same region as λTK-112 minus one or two kilobases on each end. I revised my strategy at this point and decided to use the newly isolated clone as the TV and generate the flanking probe from λTK-112. An added advantage of using the shorter clone as a TV is that it should be more packageable into bacteriophage after the extra homology and *Hprt* are added to it.

Repeating the Transplacement Step

I realized that I would did not have enough time left to repeat the entire transplacement experiments using the newly isolated clone λTK-TU14. Bearing that in mind, I decided to repeat only the first transplacement step in order to

compare the phage-plasmid recombination frequencies of λTK-TU14 to that of λTK-112.

The frequency of insertion of πSFDγ into the λTK-TU14 was lower than in the original experiment. In this round of transplacement, the frequency of targeted insertion was only 10^{-4}, whereas in the original experiment, the frequency was 5 x 10^{-3}. This difference in frequency of insertion might have been due to differences in the plasmid to phage ratios.

Two recombinant phage plaques were picked and taken through the relaxation and condensation steps. The frequencies of plasmid reversion from these phages were 2 x 10^{-3} and 10^{-3}. This, too, was significantly lower than the reversion frequency observed in λTK-112. One notable similarity exists between the λTK-112 and λTK-TU14 experiments, however. In both experiments the incidence of reversion observed was about an order of magnitude higher than that of the insertion event.

CHAPTER FOUR: DISCUSSION

Overview

I have demonstrated a novel method of *in situ* mutagenesis in bacteriophage λ (Unger, et al., 1999). Exploiting the principles of naturally occurring homologous recombination, a subtle mutation within an oligonucleotide is transferred to a target site in bacteriophage λ using transplacement. In this procedure the oligonucleotide that was subcloned into a recombination plasmid bears homology to the target sequence within the phage clone. Homologous recombination between phage and plasmid results in the insertion of the entire plasmid. Due to the duplication of homology resulting from incorporation of the oligonucleotide into the phage clone, reversion of the plasmid out of the phage DNA is possible. When this condensation reaction occurs, the entire plasmid is removed along with one of the duplicated regions of homology. The second recombination event occurs at a higher frequency than the initial insertion event. This suggests that recombination from the same homology sequence occurs more frequently in *cis* than in *trans*, even though the alignment of *cis* homology sequences in condensation requires a significant conformational change of the linear plasmid DNA. Of course, the ratio of plasmid to phage DNA is also a factor that must be considered in the insertion event, because recombination in *trans* is reliant on random interactions between the two molecules. Previous work has shown that the frequency of phage-plasmid recombination can be increased using higher copy number plasmids (Lutz, et al., 1987).

After the condensation event, a significant fraction of the reverted phages retain the mutation as the wildtype sequence is removed with the plasmid. Here, I have demonstrated the efficacy of this procedure with as little as 50 bp of homology between targeted bacteriophage and mutagenic plasmid.

The fidelity of oligonucleotide synthesis and phage-plasmid recombination are both very high; however, in synthesizing the mutagenic oligonucleotides with a 5' overhang in this situation, the chances of generating the correct sequence were increased because oligonucleotides are synthesized from 3' to 5'. This essentially obviates the DNA sequencing step that is standardly required as part of all *in vitro* mutagenesis procedures. In this occasion, however, a mutated phage clone was sequenced only to confirm the validity of this novel methodology. This sequencing step could also have been done in the plasmid before transplacement was carried out.

I have shown that a desired subtle mutation can be easily screened for if it has been designed with a unique restriction site. In cases where it is not possible to engineer such a diagnostic site, other methods are available to detect the presence of the mutation. By using small oligonucleotides as probes, mutant clones could be detected by differential hybridization under stringent conditions. That is, a short probe containing the mutation would bind more strongly to mutant phage clones, whereas the degree of hybridization to wildtype clones would be noticeably lower. Alternatively, competitive oligonucleotide priming (COP) may be applied to detect clones in which a mutation as subtle as a single base pair has

been incorporated (Gibbs, et al., 1989). In this method a universal downstream primer and two small differently labeled primers, one wildtype and one carrying the mutation, are used in PCR. The two competitive primers compete for the same binding site on the clonal template DNA. The nature of the phage clones are then distinguished by their differential affinity for binding the primers.

I have shown that transplacement mutagenesis simplifies the manipulation of large DNA constructs. Where traditional *in vitro* methods of mutagenesis involve the removal and replacement of smaller regions of DNA following mutagenesis, transplacement mutagenesis allows modifications to be transferred to specific sites *in situ* without the use of restriction enzymes or polymerase strategies. These large mutated constructs are then maintained stably in bacteriophage.

Relying largely on the strengths of positive-negative selection, recombinant clones in which the mutagenic plasmid has been incorporated are easily identified and enriched for, as are recombinant clones that follow the excisional step. It should be noted that there are several other possible phage vectors that can be utilized for transplacement mutagenesis, being equipped with the essential attributes for effective PNS. The prerequisite genetic characteristics for these vectors are rap^+, gam^-, and $supF^\circ$. In addition, there must be no homology between the phage and plasmid. Other characteristics, such as amber mutations on essential genes, are useful but not essential. Potential phage vectors for use in transplacement mutagenesis include Charon4A (A^{am}, B^{am}, S^{am}, gam^-), Charon21A

(W^{am}, E^{am}, *gam*$^-$), and EMBL3,4 (A^{am}, B^{am}, *gam*$^-$). Even though none of these vectors are *rap*$^+$, *rap* can be supplied in *trans* in each case.

Although transplacement mutagenesis was shown to be effective using phage-plasmid recombination, there is also potential for this system to be used with other template combinations, such as plasmid-plasmid, phage-phage, and plasmid-cosmid (Crouse and McEwan 1988; Tripodi, et al., 1990). In each of these cases, a suitable PNS system may be developed to exploit the specific attributes and genetic requirements of the given mutagenic vector and targeted clone. For instance, in the system developed by Tripodi et al. (1990), a mutagenic plasmid carrying the ampicillin resistance gene and a lac operator sequence is incorporated into a cosmid clone carrying kanamycin resistance. After transducing the packaged plasmid-cosmid cointegrates into bacterial cells, they are selected for on a solid medium containing both ampicillin and kanamycin. Reversion of the plasmid from the cosmid is later identified by white colonies, as the lac operator is removed from the cointegrate.

Applications of Transplacement Mutagenesis

The utility of transplacement mutagenesis has just begun to be explored in the Rancourt lab. One application in which we have tested this technique is in screening genomic libraries (Woltjen, et al., 2000). Conventional library screening methods employ the use of a labelled RNA or DNA probe to hybridize to and identify phage clones immobilized on a filter (Lutz, et al., 1987). Using

similar principles to those applied to constructing TVs via transplacement mutagenesis, a less expensive method was developed to screen phage libraries accurately without the need for labelled probes. Originally developed in 1983 (Seed 1983), the recombination screening method was recently modified and shown to be effective (Woltjen, et al., 2000). In the more recent version of the method, we termed retro-recombination screening (RRS), an oligonucleotide homologous to the gene of interest is first constructed in πANγ. The library is then passaged over the plasmid in a *supF*° host. Insertional homologous recombination occurs between the phage clones of interest and the plasmid, creating a tandem duplication of homology separated by plasmid DNA and markers. Recombinants are selected for on a *supF*° host, then selection conditions are relaxed to allow for reversion of the plasmid and markers. Reversion will only occur at duplicated regions, thus no random integrants will undergo reversion. Clones having undergone reversion can then be selected for by the absence of *gamma* during spi selection.

This new higher throughput approach to library screening makes possible the rapid construction of TVs in phage, because it combines the construction phase of TVs with the initial library screening process, effectively killing two birds with one stone. As well, we are currently investigating the possibility of introducing mutations into bacteriophage clones during the library screening step. Though the fidelity of library screening could be expected to decrease with the introduction of a mutation into the homologous region on the plasmid probe, increasing the size of

homology within the plasmid should be enough to sufficiently override this problem. One such experiment is currently underway in the Rancourt lab and involves the generation of a targeting vector in which the gene of interest is knocked out by the introduction of a *neo* cassette into one of its exons. To achieve this, a region of homology to the specific exon would first need to be synthesized and subcloned into πANγ. To complete the mutagenic probe, the *neo* cassette would then be inserted to interrupt the homologous sequence. Following the RRS protocol, isolated clones having undergone reversion of the plasmid probe could then be screened for the presence of *neo*.

Both transplacement mutagenesis and RRS can be performed within a matter of days under optimal conditions. However, preliminary data suggests that this timeframe may be shortened even further by foregoing the passaging of the recombinant phage over LE392 host cells (relaxed selection conditions). It has been observed in the Rancourt lab (unpublished data) that excision of integrated plasmid from recombinant phage clones can occur during the passage of the clones over the selective P2393 host, albeit at a much lower frequency than if they are first passaged over LE392.

Although transplacement mutagenesis was used in this work to introduce a 2-bp substitution into a bacteriophage clone, the technique is by no means limited in its application. Transplacement mutagenesis could prove useful, not only for delivering subtle mutations into large clones, but also for introducing other types of mutations into DNA and cDNA clones. Such mutations could include deletions

of small or large sequences of DNA, changes to splice donor and acceptor sites, and alterations to promoters. As well, new promoters or enhancers could be introduced into a clone using this method. Larger DNA manipulations could also be possible using transplacement, such as the attachment of reporter genes and even the generation of fusion proteins. Transplacement mutagenesis could also be effective in deleting introns in a genomic DNA clone, simply by passaging the phage clones over host cells that carry a cDNA clone of the same gene in πANγ.

For the purposes of this project, transplacement mutagenesis was shown to be a valuable tool for generating targeting vectors in phage, a relatively recent strategy in gene targeting. This technique should have applications far beyond the realm of gene targeting, however. For instance, the fields of structural biology and protein engineering, in particular, could exploit the benefits of this system by using a single-phage expression construct and several mutagenic plasmids to achieve high throughput saturation mutagenesis of a large protein coding sequence. Similarly, this method has potential for DNA manipulations involving artificial chromosomes in bacteria and yeast. Whatever the field of research, transplacement mutagenesis should prove advantageous in any situation in which rapid, high fidelity manipulation of large genomic or cDNA sequences is desired.

Considerations of RC Gene Targeting

The λSFDTV targeting vector constructed in this study was designed to enable novel replacement-condensation gene targeting. That is, targeted double-

crossover homologous recombination would result in the phage TV replacing a region encompassing several kilobases of endogenous *Timp3*, including the locus encoding Serine-181. Targeted cells would be identified by *Hprt*-conferred HAT resistance. Selection conditions would then be relaxed to allow the πHPRT-TIMP112 plasmid to condense out of the chromosome, and these cells would be identified by resistance to 6-TG indicating their lack of *Hprt* activity. A flanking probe would then be used to confirm the presence of the subtle mutation via genomic southern analysis.

The possibility exists that during the replacement step in RC gene targeting, the mutatagenic region of the TV may not be incorporated into the genome because both crossover events occur upstream of it. It is also possible that while the mutated sequence misses its mark, *Hprt* could still be incorporated into the genome with the rest of the TV. Cells having undergone these replacement events would then be selected for in HAT medium as if they had been successfully targeted. The proximity of *Hprt* to the mutation would be a factor in controlling the frequency of false positives, because the smaller the distance between the marker and the mutation, the lower the chance that recombination will occur between them. As well, the availability of a probe flanking the targeted genomic sequence ensures that successfully targeted cells can be distinguished from false positives by genomic southern analysis.

Until now, a targeted mutation in which only a couple of bases are deliberately substituted and no markers are left behind has seemed to be an

unrealistic feat. Therefore, the RC gene targeting system described in this thesis could prove to be very valuable in the area of transgenics.

Although one of the original goals of my project was to carry out and examine the efficiency of replacement-condensation gene targeting, the problems I encountered in generating a flanking probe prevented me from reaching this point. As a result, the technique remains theoretical and untested. Due to the absence of a reliable map of *Timp3*, I was unable to isolate a DNA fragment that I could confidently use as a flanking probe. To solve this problem, a fragment at one end of the original clone λTK-112 could be removed from the clone and used as a flanking probe. Alternatively, I could construct a new TV in a shorter *Timp3* clone I have isolated. In this case, I would also use a fragment from one end of λTK-112 as a flanking probe.

Importance of SFD Mice

The targeting of mouse ES cells using this vector would be beneficial, not only in proving the validity of RC gene targeting, but also in leading to the generation of a new murine model for SFD. The value of such a model has been mentioned here, but cannot be understated. Gene therapy strategies are rapidly being developed to treat genetically based diseases, so animal models are crucial for the advancement of these technologies to a point where they can be confidently used in a clinical setting. The fact that the histopathology of SFD so closely

resembles that of AMD contributes to the urgency in developing SFD mouse models.

References

Anand-Apte, B., M.S. Pepper, E. Voest, R. Montesano, B. Olsen, G. Murphy, S.S. Apte, and B. Zetter. (1997). Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3. Invest Ophthalmol Vis Sci **38**: 817-23.

Apte, S.S., M.G. Mattei, and B.R. Olsen. (1994). Cloning of the cDNA encoding human tissue inhibitor of metalloproteinases-3 (TIMP-3) and mapping of the TIMP3 gene to chromosome 22. Genomics **19**: 86-90.

Apte, S.S., B.R. Olsen, and G. Murphy. (1995). The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem **270**: 14313-8.

Araki, K., M. Araki, and K. Yamamura. (1997). Targeted integration of DNA using mutant lox sites in embryonic stem cells. Nucleic Acids Res **25**: 868-72.

Arbones, M.L., H.A. Austin, D.J. Capon, and G. Greenburg. (1994). Gene targeting in normal somatic cells: inactivation of the interferon-gamma receptor in myoblasts. Nat Genet **6**: 90-7.

Baribault, H., and R. Kemler. (1989). Embryonic stem cell culture and gene targeting in transgenic mice. Mol Biol Med **6**: 481-92.

Birkedal-Hansen, H. (1995). Proteolytic remodeling of extracellular matrix. Curr Opin Cell Biol **7**: 728-35.

Bok, D. (1985). Retinal photoreceptor-pigment epithelium interactions. Friedenwald lecture. Invest Ophthalmol Vis Sci **26**: 1659-94.

Bradley, A., M. Evans, M.H. Kaufman, and E. Robertson. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature **309**: 255-6.

Bressler, N.M., L.A. Frost, S.B. Bressler, R.P. Murphy, and S.L. Fine. (1988). Natural course of poorly defined choroidal neovascularization associated with macular degeneration. Arch Ophthalmol **106**: 1537-42.

Capecchi, M.R. (1989). Altering the genome by homologous recombination. Science **244**: 1288-92.

Capon, M.R., J. Marshall, J.I. Krafft, R.A. Alexander, P.S. Hiscott, and A.C. Bird. (1989). Sorsby's fundus dystrophy. A light and electron microscopic study. Ophthalmology **96**: 1769-77.

Carrero-Valenzuela, R.D., M.L. Klein, R.G. Weleber, W.H. Murphey, and M. Litt. (1996). Sorsby fundus dystrophy. A family with the Ser181Cys mutation of the tissue inhibitor of metalloproteinases 3. Arch Ophthalmol **114**: 737-8.

Copp, A.J. (1995). Death before birth: clues from gene knockouts and mutations. Trends Genet **11**: 87-93.

Cremers, F.P., D.J. van de Pol, M. van Driel, A.I. den Hollander, F.J. van Haren, N.V. Knoers, N. Tijmes, A.A. Bergen, K. Rohrschneider, A. Blankenagel, A.J. Pinckers, A.F. Deutman, and C.B. Hoyng. (1998). Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt's disease gene ABCR. Hum Mol Genet **7**: 355-62.

Crouse, G.F., R.N. McEwan. (1988). Gene manipulation by homologous recombination in Escherichia coli. DNA **7**: 729-34.

Curcio, C.A., N.E. Medeiros, and C.L. Millican. (1996). Photoreceptor loss in age-related macular degeneration. Invest Ophthalmol Vis Sci **37**: 1236-49.

D'Armiento, J., S.S. Dalal, Y. Okada, R.A. Berg, and K. Chada. (1992). Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. Cell **71**: 955-61.

Dean, W., L. Bowden, A. Aitchison, J. Klose, T. Moore, J.J. Meneses, W. Reik, and R. Feil. (1998). Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. Development **125**: 2273-82.

Della, N.G., P.A. Campochiaro, and D.J. Zack. (1996). Localization of TIMP-3 mRNA expression to the retinal pigment epithelium. Invest Ophthalmol Vis Sci **37**: 1921-4.

Deng, C. and M.R. Capecchi. (1992). Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. Mol Cell Biol **12**: 3365-71.

Denhardt, D.T., B. Feng, D.R. Edwards, E.T. Cocuzzi, and U.M. Malyankar. (1993). Tissue inhibitor of metalloproteinases (TIMP, aka EPA): structure, control of expression and biological functions. Pharmacol Ther **59**: 329-41.

Doetschman, T., N. Maeda, and O. Smithies. (1988). Targeted mutation of the Hprt gene in mouse embryonic stem cells. Proc Natl Acad Sci U S A **85**: 8583-7.

Evans, M.J. and M.H. Kaufman. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature **292**: 154-6.

Fariss, R.N., S.S. Apte, B.R. Olsen, K. Iwata, and A.H. Milam. (1997). Tissue inhibitor of metalloproteinases-3 is a component of Bruch's membrane of the eye. Am J Pathol **150**: 323-8.

Felbor, U., H. Stohr, T. Amann, U. Schonherr, E. Apfelstedt-Sylla, and B.H. Weber. (1996). A second independent Tyr168Cys mutation in the tissue inhibitor of metalloproteinases-3 (TIMP3) in Sorsby's fundus dystrophy. J Med Genet **33**: 233-6.

Felbor, U., H. Stohr, T. Amann, U. Schonherr, and B.H. Weber. (1995). A novel Ser156Cys mutation in the tissue inhibitor of metalloproteinases-3 (TIMP3) in Sorsby's fundus dystrophy with unusual clinical features. Hum Mol Genet **4**: 2415-6.

Felbor, U., E.A. Suvanto, H.R. Forsius, A.W. Eriksson, and B.H. Weber. (1997). Autosomal recessive Sorsby fundus dystrophy revisited: molecular evidence for dominant inheritance. Am J Hum Genet **60**: 57-62.

Gibbs, R.A., P.N. Nguyen, C.T. Caskey. (1989). Detection of single DNA base differences by competitive oligonucleotide priming. Nucleic Acids Res **17**: 2437-48.

Glaser, B.M., P.A. Campochiaro, J.L. Davis Jr, and M. Sato. (1985). Retinal pigment

epithelial cells release an inhibitor of neovascularization. Arch Ophthalmol **103**: 1870-5.

Goldberg, G.I., S.M. Frisch, C. He, S.M. Wilhelm, R. Reich, and I.E. Collier. (1990). Secreted proteases. Regulation of their activity and their possible role in metastasis. Ann N Y Acad Sci **580**: 375-84.

Gossler, A., T. Doetschman, R. Korn, E. Serfling, and R. Kemler. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc Natl Acad Sci U S A **83**: 9065-9.

Green, W.R. and C. Enger. (1993). Age-related macular degeneration histopathologic studies. The 1992 Lorenz E. Zimmerman Lecture. Ophthalmology **100**: 1519-35.

Greene, J., M. Wang, Y.E. Liu, L.A. Raymond, C. Rosen, and Y.E. Shi. (1996). Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J Biol Chem **271**: 30375-80.

Gregory, C.Y., S. Wijesuriya, K. Evans, M. Jay, A.C. Bird, and S.S. Bhattacharya. (1995). Linkage refinement localises Sorsby fundus dystrophy between markers D22S275 and D22S278. J Med Genet **32**: 240-1.

Guillemot, F., A. Nagy, A. Auerbach, J. Rossant, and A.L. Joyner. (1994). Essential role of Mash-2 in extraembryonic development. Nature **371**: 333-6.

Guyer, D.R., A.P. Schachat, S. Vitale, J.A. Markowitz, H. Braine, P.J. Burke, J.E. Karp, and M. Graham. (1989). Leukemic retinopathy. Relationship between fundus lesions and hematologic parameters at diagnosis. Ophthalmology **96**: 860-4.

Hageman, G.S. and D.E. Kelly. (1983). Cytoskeletal support and anchorage of the choriocapillaris. Invest Ophthalmol Vis Sci **24(Suppl.)**: 141.

Hasty, P. and Bradley, A. (1993). Gene targeting vectors for mammalian cells. In *Gene Targeting* (ed. A.L. Joyner), Oxford University Press, New York.

Hasty, P., M. Crist, M. Grompe, and A. Bradley. (1994). Efficiency of insertion versus replacement vector targeting varies at different chromosomal loci. Mol Cell Biol **14**: 8385-90.

Hasty, P., R. Ramirez-Solis, R. Krumlauf, and A. Bradley. (1991b). Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. Nature **350**: 243-6.

Hasty, P., J. Rivera-Perez, C. Chang, and A. Bradley. (1991a). Target frequency and

integration pattern for insertion and replacement vectors in embryonic stem cells. Mol Cell Biol **11**: 4509-17.

Henney, A.M., P.R. Wakeley, M.J. Davies, K. Foster, R. Hembry, G. Murphy, and S. Humphries. (1991). Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. Proc Natl Acad Sci U S A **88**: 8154-8.

Hewitt, A.T. and R. Adler. (1989). The retina pigment epithelium and interphotoreceptor matrix: structure and specialized functions. In *Retina* (ed. S.J. Ryan), Mosby, St. Louis.

Hewitt, A.T., K. Nakazawa, and D.A. Newsome. (1989). Analysis of newly synthesized Bruch's membrane proteoglycans. Invest Ophthalmol Vis Sci **30**: 478-86.

Hewitt, A.T. and D.A. Newsome. (1985). Altered synthesis of Bruch's membrane proteoglycans associated with dominant retinitis pigmentosa. Curr Eye Res **4**: 169-74.

Hicks, N.J., R.V. Ward, and J.J. Reynolds. (1984). A fibrosarcoma model derived from mouse embryo cells: growth properties and secretion of collagenase and metalloproteinase inhibitor (TIMP) by tumour cell lines. Int J Cancer **33**: 835-44.

Hogan, B., R. Beddington, F. Costantini, and E. Lacy. (1994) Manipulating the Mouse Embyo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Hogan, M.J. and J. Alvarado. (1967). Studies on the human macula. IV. Aging changes in Bruch's membrane. Arch Ophthalmol **77**: 410-20.

Hooper, M., K. Hardy, A. Handyside, S. Hunter, and M. Monk. (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature **326**: 292-5.

Ikawa, M., K. Kominami, Y. Yoshimura, K. Tanaka, Y. Nishimune, and M. Okabe. (1995). A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). FEBS Lett **375**: 125-8.

Isashiki, Y., Y. Tabata, K. Kamimura, and N. Ohba. (1999). Sorsby's fundus dystrophy in two Japanese families with unusual clinical features. Jpn J Ophthalmol **43**: 472-80.

Ishibashi, T., R. Patterson, Y. Ohnishi, H. Inomata, and S.J. Ryan. (1986). Formation of drusen in the human eye. Am J Ophthalmol **101**: 342-53.

Jacobson, S.G., A.V. Cideciyan, G. Regunath, F.J. Rodriguez, K. Vandenburgh, V.C.

Sheffield, and E.M. Stone. (1995). Night blindness in Sorsby's fundus dystrophy reversed by vitamin A. Nat Genet **11**: 27-32.

Jaenisch, R. (1976). Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. Proc Natl Acad Sci U S A **73**: 1260-4.

Jomary, C., M.J. Neal, K. Iwata, and S.E. Jones. (1997). Localization of tissue inhibitor of metalloproteinases-3 in neurodegenerative retinal disease. Neuroreport **8**: 2169-72.

Jones, S.E., C. Jomary, and M.J. Neal. (1994). Expression of TIMP3 mRNA is elevated in retinas affected by simplex retinitis pigmentosa. FEBS Lett **352**: 171-4.

Karwatowski, W.S., T.E. Jeffries, V.C. Duance, J. Albon, A.J. Bailey, and D.L. Easty. (1995). Preparation of Bruch's membrane and analysis of the age-related changes in the structural collagens. Br J Ophthalmol **79**: 944-52.

Kass-Eisler, A., E. Falck-Pedersen, D.H. Elfenbein, M. Alvira, P.M. Buttrick, and L.A. Leinwand. (1994). The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. Gene Ther **1**: 395-402.

Khokha, R., P. Waterhouse, S. Yagel, P.K. Lala, C.M. Overall, G. Norton, and D.T. Denhardt. (1989). Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science **243**: 947-50.

Kuehn, M.R., A. Bradley, E.J. Robertson, and M.J. Evans. (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature **326**: 295-8.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci U S A **82** : 488-92.

Langton, K.P., M.D. Barker, and N. McKie. (1998). Localization of the functional domains of human tissue inhibitor of metalloproteinases-3 and the effects of a Sorsby's fundus dystrophy mutation. J Biol Chem **273**: 16778-81.

Langton, K.P., N. McKie, A. Curtis, J.A. Goodship, P.M. Bond, M.D. Barker, and M. Clarke. (2000). A Novel TIMP-3 Mutation Reveals a Common Molecular Phenotype in Sorsby's Fundus Dystrophy. (in press)

Leco, K.J., D.R. Edwards, and G.A. Schultz. (1996). Tissue inhibitor of metalloproteinases-3 is the major metalloproteinase inhibitor in the decidualizing murine uterus. Mol Reprod Dev **45**: 458-65.

Leco, K.J., R. Khokha, N. Pavloff, S.P. Hawkes, and D.R. Edwards. (1994). Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. J Biol Chem **269**: 9352-60.

Lutz, C.T., W.C. Hollifield, B. Seed, J.M. Davie, and H.V. Huang. (1987). Syrinx 2A: an improved lambda phage vector designed for screening DNA libraries by recombination in vivo. Proc Natl Acad Sci U S A **84**: 4379-83.

Lyda, W., N. Eriksen, and N. Krishna. (1957). Studies of Bruch's membrane. Flow and permeability studies in a Bruch's membrane-choroid preparation. Am J Ophthalmol **44**: 362-370.

Magin, T.M., C. McEwan, M. Milne, A.M. Pow, J. Selfridge, and D.W. Melton. (1992). A position- and orientation-dependent element in the first intron is required for expression of the mouse hprt gene in embryonic stem cells. Gene **122**: 289-96.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A **78**: 7634-8.

Matrisian, L.M. (1990). Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet **6**: 121-5.

Matrisian, L.M. (1992). The matrix-degrading metalloproteinases. Bioessays **14**: 455-63.

Melton, D.W., D.S. Konecki, J. Brennand, and C.T. Caskey. (1984). Structure, expression, and mutation of the hypoxanthine phosphoribosyltransferase gene. Proc Natl Acad Sci U S A **81**: 2147-51.

Melton, D.W., C. McEwan, A.B. McKie, and A.M. Reid. (1986). Expression of the mouse HPRT gene: deletional analysis of the promoter region of an X-chromosome linked housekeeping gene. Cell **44**: 319-28.

Mignatti, P., E. Robbins, and D.B. Rifkin. (1986). Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell **47**: 487-98.

Milani, S., H. Herbst, D. Schuppan, C. Grappone, G. Pellegrini, M. Pinzani, A. Casini, A. Calabro, G. Ciancio, F. Stefanini and others. (1994). Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. Am J Pathol **144**: 528-37.

Ng, P. and M.D. Baker. (1999). The molecular basis of multiple vector insertion by gene targeting in mammalian cells. Genetics **151**: 1143-55.

Okubo, A., R.H. Rosa Jr, C.V. Bunce, R.A. Alexander, J.T. Fan, A.C. Bird, and P.J.

Luthert. (1999). The relationships of age changes in retinal pigment epithelium and Bruch's membrane. Invest Ophthalmol Vis Sci **40**: 443-9.

Pelletier, J.P., F. Mineau, M.P. Faure, and J. Martel-Pelletier. (1990). Imbalance between the mechanisms of activation and inhibition of metalloproteases in the early lesions of experimental osteoarthritis. Arthritis Rheum **33**: 1466-76.

Polkinghorne, P.J., M.R. Capon, T. Berninger, A.L. Lyness, K. Sehmi, and A.C. Bird. (1989). Sorsby's fundus dystrophy. A clinical study. Ophthalmology **96**: 1763-8.

Potter, H., L. Weir, and P. Leder. (1984). Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc Natl Acad Sci U S A **81**: 7161-5.

Robertson, E., A. Bradley, M. Kuehn, and M. Evans. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature **323**: 445-8.

Sambrook, J., E.F. Fritsch, and T. Maniatis. (1989) Molecular Cloning: A Laboratory Manual, Ed. Ford, N., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sarks, S.H., J.J. Arnold, M.C. Killingsworth, and J.P. Sarks. (1999). Early drusen formation in the normal and aging eye and their relation to age related maculopathy: a clinicopathological study. Br J Ophthalmol **83**: 358-68.

Sauer, B. and N. Henderson. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A **85**: 5166-70.

Schoonjans, L., G.M. Albright, J.L. Li, D. Collen, and R.W. Moreadith. (1996). Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts. Mol Reprod Dev **45**: 439-43.

Schultz, R.M., S. Silberman, B. Persky, A.S. Bajkowski, and D.F. Carmichael. (1988). Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res **48**: 5539-45.

Seed, B. (1983). Purification of genomic sequences from bacteriophage libraries by recombination and selection in vivo. Nucleic Acids Res **11**: 2427-45.

Smith, M.R., H. Kung, S.K. Durum, N.H. Colburn, and Y. Sun. (1997). TIMP-3 induces cell death by stabilizing TNF-alpha receptors on the surface of human colon carcinoma cells. Cytokine **9**: 770-80.

Smithies, O., R.G. Gregg, S.S. Boggs, M.A. Koralewski, and R.S. Kucherlapati. (1985). Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature **317**: 230-4.

Sorsby, A.e.al. (1949). A fundus dystrophy with unusual features. Br. J. Ophthalmol. 67-97.

Steinmetz, R.L., P.C. Polkinghorne, F.W. Fitzke, C.M. Kemp, and A.C. Bird. (1992). Abnormal dark adaptation and rhodopsin kinetics in Sorsby's fundus dystrophy. Invest Ophthalmol Vis Sci **33**: 1633-6.

Sternberg, N. and D. Hamilton. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J Mol Biol **150**: 467-86.

Szybalska, E.H. and W. Szybalska. (1962). DNA-mediated heritable transformation of a biochemical trait. Proc Natl Acad Sci USA **48**: 2026-2034.

Tabata, Y., Y. Isashiki, K. Kamimura, K. Nakao, and N. Ohba. (1998). A novel splice site mutation in the tissue inhibitor of the metalloproteinases-3 gene in Sorsby's fundus dystrophy with unusual clinical features. Hum Genet **103**: 179-82.

te Riele, H., E.R. Maandag, and A. Berns. (1992). Highly efficient gene targeting in

embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc Natl Acad Sci U S A **89**: 5128-32.

Thomas, K.R. and M.R. Capecchi. (1986). Introduction of homologous DNA sequences into mammalian cells induces mutations in the cognate gene. Nature **324**: 34-8.

Thomas, K.R. and M.R. Capecchi. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell **51**: 503-12.

Thomas, K.R., C. Deng, and M.R. Capecchi. (1992). High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. Mol Cell Biol **12**: 2919-23.

Thompson, S., A.R. Clarke, A.M. Pow, M.L. Hooper, and D.W. Melton. (1989). Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell **56**: 313-21.

Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones. (1998). Embryonic stem cell lines derived from human blastocysts. Science **282**: 1145-7.

Thomson, J.A., J. Kalishman, T.G. Golos, M. Durning, C.P. Harris, R.A. Becker, and J.P.

Hearn. (1995). Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A **92**: 7844-8.

Tripodi, M., S. Perfumo, R. Ali, L. Amicone, C. Abbott, and R. Cortese. (1990). Generation of large mutation in large genomic fragments by homologous recombination: description of the technique and examples of its use. Nucleic Acids Res **18**: 6247-51.

Tsuzuki, T. and D.E. Rancourt. (1998). Embryonic stem cell gene targeting using bacteriophage lambda vectors generated by phage-plasmid recombination. Nucleic Acids Res **26**: 988-93.

Unger, M.W., S.Y. Liu, and D.E. Rancourt. (1999). Transplacement mutagenesis: a novel in situ mutagenesis system using phage-plasmid recombination. Nucleic Acids Res **27**: 1480-4.

Ward, R.V., R.M. Hembry, J.J. Reynolds, and G. Murphy. (1991). The purification of tissue inhibitor of metalloproteinases-2 from its 72 kDa progelatinase complex. Demonstration of the biochemical similarities of tissue inhibitor of metalloproteinases-2 and tissue inhibitor of metalloproteinases-1. Biochem J **278**: 179-87.

Wassarman, P. and M. DePamphilis. (1993) Guide to Techniques in Mouse

Development, Academic Press.

Weber, B.H., G. Vogt, R.C. Pruett, H. Stohr, and U. Felbor. (1994). Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) in patients with Sorsby's fundus dystrophy. Nat Genet **8**: 352-6.

Wheeler, M.B. (1994). Development and validation of swine embryonic stem cells: a review. Reprod Fertil Dev **6**: 563-8.

Williamson, R.A., F.A. Marston, S. Angal, P. Koklitis, M. Panico, H.R. Morris, A.F. Carne, B.J. Smith, T.J. Harris, and R.B. Freedman. (1990). Disulphide bond assignment in human tissue inhibitor of metalloproteinases (TIMP). Biochem J **268**: 267-74.

Winston, F., F. Chumley, and G.R. Fink. (1983). Eviction and transplacement of mutant genes in yeast. Methods Enzymol **101**: 211-28.

Woltjen, K., G. Bain, and D.E. Rancourt. (2000). Retro-recombination screening of a mouse embryonic stem cell genomic library. Nucleic Acids Res **28**: E41.

Yeow, K.M., N.S. Kishnani, M. Hutton, S.P. Hawkes, G. Murphy, and D.R. Edwards.

2000. Sorsby's fundus dystrophy Tissue Inhibitor of Metalloproteinases-3 (TIMP-3) mutants have unimpaired gelatinase inhibitory activities and extracellular matrix association, and confer increased cell adhesion. In *Matrix Metalloproteinase Protocols* (ed. I.M. Clark), Humana Press, Totowa, New Jersey.

Yoo, S.H. and A.P. Adamis. (1998). Retinal manifestations of aging. Int Ophthalmol Clin **38**: 95-101.

Zack, D.J., M. Dean, R.S. Molday, J. Nathans, T.M. Redmond, E.M. Stone, A. Swaroop, D. Valle, and B.H. Weber. (1999). What can we learn about age-related macular degeneration from other retinal diseases? Mol Vis **5**: 30.

Zimmer, A. and P. Gruss. (1989). Production of chimaeric mice containing embryonic stem (ES) cells carrying a homoeobox Hox 1.1 allele mutated by homologous recombination. Nature **338**: 150-3.

APPENDIX A: Publications Arising from this Thesis

Unger, M.W., S.Y. Liu, and D.E. Rancourt. (1999). Transplacement mutagenesis: a novel in situ mutagenesis system using phage-plasmid recombination. Nucleic Acids Res **27**: 1480-4.

Woltjen, K., M.W.T. Unger, and D.E. Rancourt. Transplacement mutagenesis: a recombination based in situ mutagenesis protocol. Methods in Molecular Biology. (in preparation)