DEVELOPMENT OF A PLASMID CONSTRUCT AND AN EMBRYONIC STEM CELL LINE FOR HOMOLOGOUS RECOMBINATION GENE DELETION OF MOUSE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1

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MING HU

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ABSTRACT

The biological actions of insulin-like growth factors(IGFs) are believed to be modulated by insulin-like growth factor binding proteins(IGFBPs). Of the six known IGFBPs, IGFBP-1 has been the most extensively studied *in vitro* and *in vivo* to date. However, the biological function of IGFBP-1 remains undefined. To understand better the biological role of IGFBP-1 in embryonic development and growth, I have developed a plasmid construct and generated an Embryonic Stem(ES) cell line for future use in developing transgenic mice. A targeting vector was designed to delete the second and third exon of the mouse IGFBP-1 gene. The targeting vector contains the 5' and 3' fragments of mouse IGFBP-1 genomic DNA, a bacterial neomycin resistance gene(Neo^r) and a herpes simplex virus thymidine kinase(HSV-TK) gene. Linearized plasmid DNA was introduced into ES cells by electroporation. A positive-negative selection protocol was applied to enrich for homologous recombination events. This method involves simultaneously selecting for the Neo^r gene integrated within the target DNA and against the HSV-TK gene which is placed at the end of targeting vector. After double selection, more than 300 ES cell colonies were analyzed by the polymerase chain reaction(PCR). No homologous recombinants were detected. A further 342 colonies were screened by Southern blotting. One colony showed the

predicted mutant allele of 4.5 kb. This mutated cell line will be used to develop transgenic mice carrying a disrupted IGFBP-1 gene. We believe that the homologous recombination gene deletion experiment will provide a powerful tool to study the physiological role of IGFBP-1 in development and growth.

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair(s)
°C	degree centigrade
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CTP	cytosine triphosphate
dNTP	deoxyribonucleotide triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ES cell	embryonic stem cell
FBS	fetal bovine serum
G418	geneticin
hr	hour(s)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HSV-TK	herpes simplex virus thymidine kinase
IGF	insulin - like growth factor
IGFBP	insulin - like growth factor binding protein
kb	kilobase
kDa	kilodalton
L	litre
LIF	leukemia inhibitory factor
m	mouse
min	minute

ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
M	molar
MW.	molecular weight
Neo^r	neomycin resistance gene
ng	nanogram
nM	nanomolar
O/N	overnight
PCR	polymerase chain reaction
pg	picogram
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13 acetate
r	rat
RNase	ribonuclease
SDS	sodium dodecyl sulfate
Sec	second
SSC	standard saline citrate
SV40	simian virus 40
TE	Tris-HCl/EDTA
µg	microgram
µl	microlitre

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Chapter 1. Introduction

The family of insulin-like growth factor(IGFs) and their binding proteins(IGFBPs) consists of IGF-I, IGF-II and at least six IGFBPs. IGF-I and IGF-II, which have a structural similarity to proinsulin, act as both a mitogen and a differentiation factor. Postnatally, IGF-I mediates many of the growth-promoting effects of growth hormone by binding to the IGF-I(type 1) receptor, whereas the essential growth promoting function of IGF-II is restricted to the period of embryogenesis in the mouse(Daughaday *et al.*, 1989; Schofield *et al.*, 1992; Liu *et al.*, 1993). The production of IGFs in many tissues suggests that they play autocrine/paracrine roles, although they also function as classical hormones, since they circulate in the plasma in association with IGFBPs which bind and modulate the biological action of IGF-I and IGF-II.

Insulin-like growth factor binding protein(IGFBP)-1 is one of the six structurally similar proteins. Human IGFBP-1 was first isolated from human amniotic fluid(Povoa *et al.*, 1984) and Hep G2 human hepatoma cell conditioned medium(Povoa *et al.*, 1985). The amino acid sequences of the N-terminal region of the IGFBP isolated from amniotic fluid and the Hep G2 cell line were identical. Koistinen *et al.*(1986) found that placental protein 12 (PP12), the major secreted protein of the human decidua, had IGF-binding activity and an N-

terminal sequence identical to that of amniotic fluid and Hep G2 cell-derived IGFBP. Later studies have identified other proteins from various biological fluids that are immunochemically and biochemically similar to PP12 and are most likely IGFBP-1 or another very similar IGFBP(Waites *et al.*, 1989; 1990). The complementary DNA for the Hep G2 derived IGFBP was first reported in 1988(Lee *et al.*, 1988). Identical sequences were subsequently reported for cDNAs from human placenta, liver(Brinkman *et al.*, 1988) and decidual tissue(Brewer *et al.*, 1988; Julkunen *et al.*, 1988).

1.1. Molecular characterization of IGFBP-1

Human IGFBP-1 amino acid sequence has been predicted from the cDNA sequence and confirmed by IGFBP-1 protein sequencing(Brewer *et al.*, 1988; Luthman *et al.*, 1989). Human IGFBP-1 consists of 234 amino acids and has a predicted molecular mass of 25.3 kDa. It migrates with an apparent MW of 28 kDa on non-reducing SDS-polyacrylamide gel electrophoresis(Lee *et al.*, 1988). Rat IGFBP-1 cDNA was cloned by Murphy *et al.*(1990) from rat decidual and liver cDNA libraries. The predicted protein contains 272 amino acids and a 25 residue N-terminal signal peptide. The primary amino acid sequence of human IGFBP-1 is characterized by an N-terminal cluster of 12 cysteines and a C-terminal cluster of six cysteines that are conserved in rat and bovine IGFBP-1 and may participate in ligand binding. It has been suggested

that these cysteines may be involved in intrachain disulfide linkage(Brinkman *et al.*, 1991). An Arg-Gly-Asp(RGD) sequence is located near the C-terminal of human, rat and bovine IGFBP-1(Mohn *et al.*, 1991; Murphy *et al.*, 1990; Senyers *et al.*, 1991). However, the physiological significance of the IGFBP-1 RGD sequence is currently undefined.

Human IGFBP-1 undergoes post-translational modifications such as phosphorylation which appear to be related to the affinity for IGF peptides and consequently modulate the biological action of IGFs(Waites *et al.*, 1989). Site-directed mutagenesis of Ser101 to Ala101 prevents the phosphorylation and results in decreased affinity for IGF-I(Jones *et al.*, 1991). IGFBP-1 does not contain N-linked glycosylation sites but has potential O-glycosylation sites(Julkunen *et al.*, 1988).

Human IGFBP-1 mRNA is a transcript of 1.55 kb. The 3'-untranslated region contains five ATTTA motifs that are characteristic of mRNA species with a very short half life(Lee *et al.*, 1993). The human IGFBP-1 gene has been cloned(Brinkman *et al.*, 1988; Cubbage *et al.*, 1989) and localized to human chromosomal region 7p14-p12(Ekstrand *et al.*, 1990). The gene is a single copy gene and divided into four exons with a total span of 5.2 kb(Brewer *et al.*, 1988; Brinkman *et al.*, 1988). The rat IGFBP-1 gene was isolated and sequenced in this laboratory. The structural organization and

size of the rat gene are similar to the human IGFBP-1 gene. Comparison of human and rat IGFBP-1 cDNA sequence demonstrates 67% amino acid identity(Murphy *et al.*, 1990).

The promoter of the human IGFBP-1 gene has been characterized by Suwanichkal *et al.*(1990). The region immediately 5' to the IGFBP-1 mRNA cap site is typical of a eukaryotic promoter, with a TATA sequence beginning 28 bp and a CCAAT promoter element beginning 72 bp upstream from the cap site. Their studies identified the CAATT box region as a major *cis* element involved in basal IGFBP-1 promoter activity in Hep G2 cells. The liver factor B1 is the major *trans*-acting factor which stimulates basal IGFBP-1 promoter activity in Hep G2 cells.

1.2. Regulation of IGFBP-1

Age and fasting

IGFBP-1 levels are elevated in fetal sera and are approximately 15 fold higher than maternal serum levels, which also rise during pregnancy(Drop *et al.*, 1984; Baxter *et al.*, 1987). IGFBP-1 levels fall during puberty and are inversely correlated with age. Studies have demonstrated that serum IGFBP-1 levels are increased in fasting human subjects(Busby *et al.*, 1988; Yeoh *et al.*, 1988). An increase in both serum IGFBP-1 and hepatic IGFBP-1 mRNA abundance in

fasting rats was also demonstrated by Murphy *et al*(1991). In the rat, refeeding caused a prompt decline in hepatic IGFBP-1 mRNA.

Insulin

Hall, *et al.* (1980) first reported that insulin was a regulator of PP12(IGFBP-1) levels. Insulin administration suppressed IGFBP-1 secretion by Hep G2 cells *in vitro*(Conover *et al.*, 1990). Studies with human fetal liver, decidual cells and human hepatoma cells have also demonstrated the inhibitory effects of insulin on IGFBP-1(Lewitt *et al.*, 1989; Thraikill *et al.*, 1990; Singh *et al.*, 1990). The inhibitory effects of insulin occur at the transcription level. The 5' flanking region from 103 to 509 bps is responsible for the inhibitory effect of insulin in a chloramphenicol acetyltransferase(CAT) assay(Powell *et al.*, 1991). Other studies have also shown that suppressive effects of insulin on IGFBP-1 mRNA levels are not due to a decrease in the half-life of IGFBP-1 mRNA(Powell *et al.*, 1991; Unterman *et al.*, 1991). Similar results have also been observed in *in vivo* studies. It has been reported that IGFBP-1 mRNA is increased in the liver and kidney of diabetic and ketotic rats(Ooi *et al.*, 1990; 1992; Gelato *et al.*, 1992). Further, insulin treatment rapidly decreased IGFBP-1 mRNA to the levels of non-diabetic control rats within 1 hr(Ooi *et*

al., 1992). Restoration of euglycemia is not necessary for insulin to decrease IGFBP-1 mRNA in diabetic rat liver.

Elevated IGFBP-1 levels have been found in patients with insulin dependent diabetes mellitus(IDDM). IGFBP-1 levels are negatively correlated with insulin levels in fasting subjects as well as in patients with disorders of insulin secretion, including IDDM and insulinoma(Suikkari *et al.*, 1988; Brismar *et al.*, 1988; Conover *et al.*, 1992). It has also been reported that IGFBP-1 levels are elevated in streptozotocin induced diabetes in rats(Unterman *et al.*, 1989; Graubert *et al.*, 1991). Serum IGFBP-1 levels decreased after insulin administration. however, the levels rose again if insulin treatment was discontinued(Unterman *et al.*, 1990). Yeoh *et al.*(1988) reported that the insulin-induced hypoglycemia in human subjects was correlated with elevated IGFBP-1 levels. Similar results were also reported in rats(Lewitt *et al.*, 1992) and *in vitro* studies with rat liver explants(Lewitt *et al.*, 1990), indicating that increased glucose rather than insulin might inhibit IGFBP-1 production. Lee *et al*(1993) suggested that the hypoglycemia-associated elevated IGFBP-1 levels might also be expressed by a secondary suppression of insulin secretion into the hepatic portal circulation. Recent studies demonstrated that an acute increase in glucose concentration within a physiologic range had no independent inhibitory effect on IGFBP-1 production in the presence of a nonsuppressive insulin level(Lee *et al.*, 1993). The relative

roles of insulin and glucose in IGFBP-1 regulation remain to be defined.

Growth Hormone

Previous studies in this laboratory have shown dramatic increases in hepatic IGFBP-1 mRNA levels in hypophysectomized rats with a 4 fold increase in the rate of hepatic IGFBP-1 gene transcription compared to pituitary intact rats(Senevirante *et al.*, 1990). The abundance of a 30 kDa IGF-I binding protein was elevated in both hepatic extracts and serum from hypophysectomized rats. Both long term and short term administration of GH caused a marked decrease in hepatic IGFBP-1 mRNA. A single dose of GH restored the hepatic IGFBP-1 mRNA levels and the gene transcription rates to basal levels. However, GH had no effect on IGFBP-1 expression in primary rat hepatocytes(Villafuerte *et al.*, 1992).

Earlier studies showed that serum IGFBP-1 was increased in GH-deficient patients and decreased in patients with acromegaly(Povoa *et al.*, 1984; Busby *et al.*, 1988). Holly *et al.*(1991) studied the inter-relations between GH, insulin and IGFBP-1 in patients with acromegaly. They found that the low IGFBP-1 levels were unrelated to mean GH levels but inversely related to fasting insulin levels. The short and long term effects of GH on serum IGF-I, IGFBP-1 and insulin were

investigated by Tapanainen *et al.*(1991). IGF-I and insulin levels did not change during 4 hr after GH administration, whereas IGFBP-1 levels decreased significantly. During the 11 day follow up period, IGF-I and insulin were significantly higher in GH-treated than in placebo-treated women. These results suggested that the IGFBP-1 was not completely GH independent. They concluded that a direct inhibitory effect of GH on the IGFBP-1 levels was unlikely since the high GH concentration during fasting was concomitant with high IGFBP-1 levels and low insulin levels. They proposed that an inverse relation between GH levels and the IGFBP-1 concentration was probably due to the GH-induced insulin release(Degerblad *et al.*, 1989). One study in this laboratory showed that administration of GH, but not insulin caused a decrease in elevated hepatic IGFBP-1 mRNA levels in fasted rats, suggesting that increased expression of IGFBP-1 in fasted rats might be a consequence of GH deficiency rather than insulin deficiency(Murphy *et al.*, 1991). The effects of GH on IGFBP-1 will await more investigation.

Glucocorticoid

A study from this laboratory has demonstrated that administration of dexamethasone to rats results in elevated levels of serum IGFBP-1 and a 2-10 fold increase in hepatic IGFBP-1 mRNA abundance(Luo *et al.*, 1990). Similar results have also been reported in growth retarded fetal rats(Price

et al., 1990). Multiple studies *in vitro* have found that dexamethasone has a stimulatory effect on IGFBP-1 protein and mRNA accumulation in H4IIE rat hepatoma cells(Orlowski *et al.* 1990; 1991), primary adult rat hepatocytes(Villafuerte *et al.*, 1992) and human Hep G2 hepatoma cells(Powell *et al.*, 1993). Recent studies in Hep G2 cells demonstrated that dexamethasone stimulated IGFBP-1 gene transcription and facilitated the stimulatory effects of cAMP and theophyllines on mRNA and protein levels(Powell *et al.*, 1993). The stimulatory effect of dexamethasone on IGFBP-1 is at the level of transcription by IGFBP-1 promoter *cis* elements located between 357 and 103 bp 5' to the transcription start site. In H4IIE cells, it was reported dexamethasone not only stimulated transcription, but also increased mRNA stability(Orlowski *et al.*, 1990; 1991). However, Lewitt *et al.*(1989) reported the opposite results in human fetal liver explants.

Recently, Conover *et al.*(1993) investigated the regulation of plasma IGFBP-1 by cortisol in humans. When infused with cortisol, the subjects showed a 3 fold increase in IGFBP-1 levels over the 6 hr study period as compared with saline-infused controls. These data suggested a stimulating role for glucocorticoids on IGFBP-1 production and a possible role for IGFBP-1 in glucocorticoid-induced catabolism.

Other hormones and factors

It has been reported that glucagon, octreotide, cAMP, relaxin and progesterone have stimulatory effects on IGFBP-1 *in vitro* or/and *in vivo*(Lewitt *et al.*, 1989, 1991; Jensen *et al.*, 1991; Hilding *et al.*, 1992; Ezzat *et al.*, 1991, 1992; Ren *et al.*, 1992; Conover *et al.*, 1989; Pekonen *et al.*, 1992; Thrailkill *et al.*, 1990; Bell *et al.*, 1991). Later studies have demonstrated that the effects of cAMP occur at the transcription level in Hep G2 cells(Suwanichkul *et al.*, 1993). cyclic AMP increased IGFBP-1 gene transcription, mRNA levels and protein levels. DNase protection assays showed that Hep G2 nuclear extract footprinted the region from 273 to 249 bp 5' of the cap site; this region has a central CGTCA motif common to cAMP responsive elements(CREs). Drop *et al*(1984) reported that estrogen increased serum IGFBP-1 levels in girls and androgen suppressed serum IGFBP-1 levels in boys. Another study demonstrated that hyperestrogenemia was correlated with elevated serum IGFBP-1 levels(Martikainen *et al.*, 1992). Tamoxifen, a partial antiestrogen used therapeutically in treatment of breast cancer, was also shown to increase plasma IGFBP-1 levels.(Lonning *et al.*, 1992).

Thrailkill *et al.*(1990) reported that IGF-I suppressed IGFBP-1 production in human decidual cells. The stimulatory effects of IGF-I have been observed in fetal fibroblasts(Hill *et al.*, 1989). However, IGF-I was shown to have no effect on

IGFBP-1 expression in cultured primary rat hepatocytes (Villafuerte *et al.*, 1992). The effect of IGF-I on IGFBP-1 concentrations remains to be clarified *in vivo*.

The stimulatory effects of phorbol ester, phorbol 12-myristate 13-acetate (PMA) on IGFBP-1 production were also reported in human ovarian granulosa cells(Jalkanen *et al.*, 1989). It was suggested that the protein kinase C system might be involved in regulation of IGFBP-1 production, since PMA is a PKC activator. Unterman *et al*(1992)demonstrated that PMA rapidly enhanced IGFBP-1 mRNA in rat H4IIE hepatoma cells. Similar results in human endometrial cancer cells have been observed in this laboratory(Gong *et al.*, 1992). The abundance of mRNA increased 4 times after PMA administration in human endometrial carcinoma cells.

1.3. Biological function of IGFBP-1

At present all the known actions of IGFBP-1 are related to its ability to specifically bind and modulate the action of the IGFs. The IGFBP-1 affinity for IGFs plays an important role in modifying the action of IGFs. Studies have shown that IGFBP-1 inhibits IGF binding to cell surface receptors. It is postulated that a conformational change resulting from IGFBP-1/IGF association may mask or alter the receptor binding site on the IGF molecule(Oh Y *et al.*, 1991).

In vitro, inhibitory effects of IGFBP-1 on IGFs have been shown in numerous mammalian cell culture systems(Ritvos *et al.*, 1988; Frauman *et al.*, 1989; Angervo *et al.*, 1991; Liu *et al.*, 1991). It has also been reported that IGFBP-1 inhibited basal and IGF-I stimulated increases in cartilage dry weight, thymidine incorporation into DNA, and ^{35}S incorporation into proteoglycan(Burch *et al.*, 1990). However, studies have demonstrated that IGFBP-1 may enhance IGF-I mediated actions *in vitro*. IGFBP-1 enhanced IGF-I stimulated mitogenesis in porcine aortic smooth muscle cells, chick embryo fibroblasts and human fibroblasts(Eglin *et al.*, 1987). Koistinen *et al.*(1990) demonstrated that IGFBP-1 inhibited binding of IGF-I to fetal skin fibroblasts but stimulated their DNA synthesis. In the only direct *in vivo* study published to date, infusions of human IGFBP-1 into rats

inhibit the hypoglycemic effect of an equimolar infusion of human IGF-I(Lewitt *et al.*, 1991)

The presence of different forms of IGFBP-1 may partly accout for the discrepancy of biological function. Busby *et al*(1988) obtained two forms of IGFBP-1 by using ion exchange chromatography. Amino acid sequence analysis showed that both forms had identical N-terminal sequences. However, these two forms, termed form C and form B, had marked difference in bioactivity. Form C inhibited both basal and IGF-I stimulated DNA synthesis; form B showed the stimulating effect on DNA synthesis reported by Eglin *et al.*(1987). Form B was shown to bind to the cell surface in association with [^{125}I] IGF-I, whereas form C did not bind. Some undefined post translational modifications may account for the difference in biological activities of the two forms of IGFBP-1. IGFBP-1 phosphorylation may play a role in mediating its effects. It has been reported that IGFBP-1 exists in non-phosphorylated and phosphorylated isoforms(Frost *et al.*, 1991; Jones *et al.*, 1991). Phosphorylated IGFBP-1 has much higher affinity for IGF-I than dephosphorylated form. Frost *et al.*(1991) have proposed that IGFBP-1 is secreted as a phosphoprotein and subsequently dephosphorylated *in vivo*. Non-phosphorylated IGFBP-1 was phosphorylated by multiple protein kinases *in vitro*(Frost *et al.*, 1991). These data indicated that the phosphorylation was a physiologically important post-translational modification. The difference in phosphorylation

between form B and form C has not been clarified yet. Jones reported human IGFBP-1 was phosphorylated on three serine residues(Jones *et al.*, 1993). Additional studies are required to understand the biological function of the IGFBP-1 isoforms.

1.4. Development of transgenic model

In spite of extensive studies on IGFBP-1, its exact functions are not well understood. To understand better the physiological role of IGFBP-1 in embryonic development and growth, work in this laboratory has been directed toward the development of transgenic mouse models which would provide evidence for IGFBP-1 function. Two approaches have been undertaken at this laboratory: 1. Development of IGFBP-1 overexpressing mice. 2. IGFBP-1 gene deletion by homologous recombination .

In this laboratory transgenic mouse models that over express IGFBP-1 have been developed(Murphy *et al.*, 1993). A number of plasmid constructs were developed and used to generate transgenic mice. Initially rat IGFBP-1 cDNA driven by the SV40 promoter was employed because our previous studies showed that this construct produced high levels of IGFBP-1 in mammalian cells *in vitro*. Elevated IGFBP-1 mRNA in the kidney has been observed in some of the F1 transgenic mice. No significant differences were found in serum IGFBP-1

levels, litter size, birth weight and weight gain compared to non-transgenic wild-type mice. Other constructs were made using the rat IGFBP-1 gene and the mouse metallothionein promoter(MTK1) or the mouse phosphoglycerate kinase promoter(PGK K1). Preliminary studies have shown that there is a reduction in litter size, suggesting that IGFBP-1 may have inhibitory effects on embryonic development(Murphy *et al.*, 1993). Further studies of these transgenic mice are currently underway by colleagues in this laboratory.

Gene targeting experiments provide a powerful tool for studying gene function since the experiments can target preselected genes of interest. Gene targeting means that we have the potential to generate mice of virtually any desired genotype. Recombination DNA technology is used to altered a cloned DNA sequence of a chosen locus; the modified DNA is then introduced into a pluripotent stem cell derived from a mouse embryo, and homologous recombination between the exogenous and endogenous chromosomal sequences transfers the mutation into the genome. Microinjection of the stem cells containing the modified locus into mouse blastocysts is used to generate germ-line chimaeras. Finally, interbreeding of heterozygous siblings yields animals homozygous for the desired mutation. This technology was first used to create germ-line containing targeted disruption in the β-globin gene in 1985(Smithies *et al.*). Since then it has been widely used to study specific gene biological functions(Nandi *et al.*,

1988; Stanton *et al.*, 1990; DeChiara *et al.*, 1991; Liu *et al.*, 1993). For each experiment, the particular genetic designs and appropriate protocols vary; however, the underlying concept is the same. It is based on the observation that DNAs transfected into cells in culture can recombine in a homologous fashion with their chromosomal equivalents (Capecchi *et al.*, 1990). Essentially, one needs to construct a recombinant DNA molecule wherein the mutagenic change one desires to make in the gene of interest is flanked by regions of complete homology, often of considerable length of that same gene(Thomas *et al.*, 1987). Such a recombinant molecule, upon transfection into a recipient cell, may undergoes homologous recombination in the two flanking regions such that the interior portion of the gene of interest is removed and replaced by its mutated equivalent.

Homologous recombination is a rare event in eukaryotic cells. Johnson *et al.*(1989) reported homologous recombination frequencies between 1 in $5x10^7$ and 1 in $6.6x10^6$ cells transfected with targeting constructs. It used to be a problem because of lack of appropriate screening protocols to find the cells which carry the disrupted gene of interest. To identify a subset of homologous recombinants, Mansour *et al.*(1988) devised a protocol they call positive/negative selection. With this method, the positive-selectable marker, a neomycin resistance gene(Neo^r), is inserted between the flanking regions of homology. The negative-selectable marker,

herpes simplex virus thymidine kinase(HSV-TK), is placed at the end of the construct outside the region of homology. The purpose of using the Neo^r gene in the targeting construct is twofold: first, to disrupt the coding sequence of the chosen gene and second, to act as a selective marker(conferring resistance to the drug G418) for cells containing an integrated copy of the recombinant vector. The vector is designed so that when replacement of the endogenous sequence by the exogenous one occurs via homologous recombination, the HSV-TK gene will not be transferred into endogenous target. The HSV-TK gene represents a discontinuity in the incoming vector between homology and non-homology with the endogenous target sequence. Cells in which the targeting events occurred will therefore be Neo^{r+} and HSV-TK^-. On the other hand, random integration of the target vector into the recipient cell genome should result in most cases, in cells that are Neo^{r+} and HSV-TK^+. The drug ganciclovir, which kills cells expressing HSV-TK, is used to select against cells carrying the HSV-TK gene. Predictably, the homologous recombinants will be both G418- and ganciclovir-resistant. The positive/negative selection protocol makes it feasible to enrich for homologous recombination events and subsequently detect cellular clones in which the gene has been disrupted. To date the most successful protocol involves a positive/negative selection strategy in embryonic stem(ES) cells(Mansour *et al.*, 1988).

Sedivy *et al.*(1989) developed a technique that resulted in a frequency of targeted gene disruption of 1 in 10,000 cells incorporating exogenous DNA. This procedure employs a targeting vector encoding Neo^r that lacks a translation-initiation codon and thus selects for in-frame insertions within exons of active genes transcribed by RNA polymerase II.

ES cell lines are derived directly from the inner cell mass of mouse embryos. They retain the potential to participate in normal embryonic development. ES cells can be maintained in an undifferentiated state by growth on a feeder layer of an embryonic fibroblastic cell line or in the presence of leukemia inhibitory factor(LIF) in the culture medium(Evans *et al.*, 1981; Martin *et al.*, 1981). In addition, ES cells can undergo genetic manipulation *in vitro* without any apparent loss in developmental potential if the cells are maintained properly. When ES cells are introduced into a carrier embryo, they will become assimilated into the inner cell mass and take part in the formation of many tissues, thus providing a chimeric animal which may be used to develop a mouse with a defective gene of interest(Smithies et al., 1985).

Gene targeting technique is being used to study the physiological role of insulin-like growth factors(IGFs) and their binding proteins(IGFBPs). DeChiara *et al.*(1990, 1991)

disrupted one of the IGF-II alleles in the cultured mouse ES cells by gene targeting and constructed chimeric mice. Germ-line transmission of the inactivated IGF-II gene from male chimaeras yielded heterozygous progeny that were growth deficient(60% of normal birthweight). In contrast, when the disrupted IGF-II allele was transmitted maternally, the offspring were phenotypically normal. This result showed that the IGF-II gene is subject to parental imprinting; the paternal IGF-II allele is expressed, while the maternal allele is silent in most tissues. Recently, it was reported that mice homozygous for a targeted disruption of IGF-I also exhibited a growth deficiency similar in severity to that previously observed in viable IGF-II mutants(Liu *et al.*, 1993). Mice heterozygous for a targeted disruption of IGFBP-2 has also been developed. The offspring are phenotypically normal(Wood *et al.*, 1993). They proposed that other IGFBPs may compensate for IGFBP-2 function lacking in genetic mutants. These studies provide the first direct evidence for the physiological role of IGF-I, IGF-II and IGFBP-2.

My project described in this thesis was to develop a plasmid construct necessary for deletion of the IGFBP-1 gene and generate the ES cell line for future use in generating chimeric mice. Transgenic mice carrying a disrupted IGFBP-1 gene will be developed in this laboratory in the near future.

Chapter 2. Materials and Methods

2.1. Materials

Restriction enzymes and DNA modifying enzymes were supplied by Pharmacia(Canada) Inc.(Baie d'Urfe, Quebec), Gibco/BRL Life Technologies Inc.(Burlington, Ontario), Boehringer-Mannheim(Laval, Quebec) and New England Biolabs Inc.(Beverly, Massachusetts). [Alpha - ^{32}P]dCTP was purchased from ICN Biochemical Canada Ltd.(Mississauga, Ontario) and Amersham Canada Ltd.(Oakville, Ontario). Nick translation kits were obtained from Amersham Canada Ltd. DNA sequencing reagents were obtained from United States Biochemical Corp. (Cleveland, Ohio). Oligonucleotides were synthesized and obtained from DNA laboratory in Faculty of Medicine, University of Manitoba, Winnipeg. Mouse genomic DNA library(adult BALA/c male liver) was purchased from CLONTECH laboratories, Inc. (Palo Alto, CA, USA). Mouse embryonic stem(ES) cells were purchased from American Type Culture Collection(ATCC, Rockville, Maryland). Leukemia inhibitory factor(LIF) and ES cell culture reagents were obtained from Gibco BRL. *Herpes simplex* virus thymidine Kinase(HSV-TK) cassette(plasmid p^{PNT}) was kindly provided by Dr. J Rossant, Samuel Lunenfeld Research Institute, Toronto, Ontario.

2.2. Labelling of probes

The nick translation reaction(Feinberg *et al.*, 1983), catalyzed by *Escherichia coli* DNA polymerase I, was used to introduce radioactively labelled nucleotides into DNA. The nick translation reaction was carried out as described by the supplier of the kit. Usually 7 µl of 3,000 Ci/mmol [alpha-^{32}P] dCTP and 100 - 200 ng DNA were used per reaction. Radioactively labeled DNA was separated from unincorporated [alpha-^{32}P]dCTP by G100 Sephadex gel filtration chromatography.

2.3. Subcloning, small and large scale preparation of plasmid DNA

The desired DNA insert was cleaved with appropriate restriction enzyme(s) and isolated by gel electrophoresis. In order to reduce background, enzyme(s) cleaved vector DNA was treated with calf intestinal alkaline phosphatase(CIP). Although alkaline phosphatase treatment lowers the absolute efficiency of cloning the desired DNA molecule, the great reduction in background greatly enhances the isolation of the desired clone(Dugaiczyk *et al.*, 1975; Struhl *et al.*, 1985). The concentration of insert DNA(s) was relatively high in order to facilitate ligation to vectors. The molar ratio of insert to vector used in this study was 3 to 1. The amount of DNA in a 20 µl ligation mixture was 0.01 - 0.1 µg. The

products of the ligation were introduced into competent E.coli(JM109), and recombinants containing the gene for ampicillin resistance were selected directly on ampicillin containing plates.

The alkaline lysis procedure was used for the isolation of small quantities of plasmid DNA from bacterial cells (minipreps). The protocol is a modification of the methods of Birnboim and Doly(1979). The large scale method for plasmid DNA preparation was used as described by Maniatis *et al.* (1984).

2.4. ES cell culture

E14TG2a-ES cells(129/Ola mouse, pluripotent embryonic stem cells,) were cultured in Dulbecco's Modified Eagles Medium(DMEM) with 4.5 g/L glucose and 10^{-4} M 2-Mercaptoethanol, supplemented with 15% heat inactivated Fetal Bovine Serum(FBS) in a constant environment of 37^0C, 5%CO_2 and 100% humidity(Thomas *et al.*, 1987). In the past, ES cells required the presence of a feeder layer of fibroblast cells to prevent this differentiation *in vitro*. In this study, leukemia inhibitory factor (LIF) was used instead of feeder layers to inhibit spontaneous differentiation of ES cells(Evans *et al.*, 1981; Martin *et al.*, 1981). 10^{-3} units/ml LIF was used to maintain the cells in an undifferentiated state. The cultures were examined daily and passaged every 2

or 3 days. Subcultivation ratio was 1:5. ES cell differentiation was determined by staining with Giemsa (Williams *et al.*, 1988). Compact stem cell colonies could be distinguished from diffuse differentiated colonies.

2.5. Transfection and selection of ES cells

DNA was introduced into ES cells by electroporation using a gene pulser electroporator (Bio-Rad). Confluent plates of ES cells were harvested by trypsinization and resuspended in a buffer containing 20mM HEPES(pH 7.0), 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM dextrose, and 0.1mM 2-mercaptoethanol at $4x10^7$ cells per ml. The NotI linearized IGFBP1 targeting construct was added to ES cells at a final concentration of 20μg/ml just prior to electroporation. Aliquots (0.5 ml) of the cells with DNA were electroporated in a 0.4 cm cuvette of a Bio-Rad gel pulser set at 500V, 25μF(Tybulewicz *et al.*, 1991; Liu *et al.*, 1993). The cells were equally distributed into three 10 cm plates immediately after electroporation.

To identify a subset of transfected cells that might contain an homologously recombined allele, a positive/negative selection strategy(Mansour *et al.*, 1988) was used to identify cells with a disrupted mIGFBP1 allele. The positive marker we exploited was a neomycin resistance gene(Neo^r), which confers resistance to the antibiotic G418.

The negative selection marker was the *herpes simplex* virus thymidine kinase(HSV-TK) gene placed at the end of the construct outside of the region of homology. The drug ganciclovir, which kills cells expressing HSV-TK, was used to select against cells carrying the HSV-TK gene.

To allow expression of the Neo^r and HSV-TK genes, the cells were first plated after electroporation on three 10cm plates in nonselective medium and refed the next day. G418 (300μg/ml) was added 2 days after plating. For double selection (positive selection and negative selection), ganciclovir was added to 2 of 3 plates 3 days after the addition of G418. Plates were refed every 3 or 4 days. After 9 days of culture, colonies were picked. Each colony was disrupted in trypsin(30 μl of 0.25% trypsin with 0.57mM EDTA) and transferred to one well of the 96 well plates. After a further 5 days of growth, most wells were confluent and were transferred to two sets of 24 well plates. It took another 4 days of growth to become confluent. One set of cells were used for processing DNA, and the other set of the cells were kept growing on another 24 well plate after each colony was trypsinized(Liu *et al.*, 1993).

2.6. Preparation of ES cell DNA

After aspiration and washing with PBS, 500μl of lysis buffer (100mM NaCl, 100mM Tris.HCl[pH8.0], 25mM EDTA[pH8.0],

0.5% sodium dodecyl sulfate[SDS], 0.2 mg/ml proteinase K) was added to each well of the 24 well plates containing ES cells. The lysate was transferred to a 1.5ml microcentrifuge tube and incubated at 55°C overnight. Cell lysates were extracted with phenol and chloroform, and the DNA was precipitated with ethanol and recovered by centrifugation and resuspended in 50μl of TE[pH8.0]with RNase.

2.7. Southern blot hybridization

After digesting the DNA samples with appropriate restriction enzyme(s), DNA was separated by electrophoresis in a 1% agarose gel with DNA size markers, and photographed. The gel was then washed in 1.5M NaCl and 0.5M NaOH for 20 min to denature the DNA and neutralized in 0.5M Tris and 3.0M NaCl[pH 7.0] for 40 min. DNA was then transferred to nitrocellulose filters(Nitro Plus) and the blots were baked for 2 hr at 80°C or at 60°C overnight. Nitrocellulose filters were prehybridized for at least 2 hr. at 42°C in a solution containing 50% formamide, 20mM NaH_2PO_4[pH 7.0], 4xSSC(1x=0.15M NaCl, 0.015M sodium citrate), 2mM EDTA, 4x Denhardt' solution(1x=0.02% BSA, ficoll, and polyvinylpyrrolidine), 0.1% SDS and 100μg/ml sonicated, denatured salmon sperm DNA. Hybridization was performed at 42°C overnight in the same solution with the addition of the DNA probes. After hybridization, blots were washed initially at room temperature with 2xSSC and 0.1% SDS for 15-30 min and finally

washed for 20 min at 65^{0}C in 0.1xSSC and 0.1% SDS. Autoradiography was performed by exposing Kodak X-Omat AR film to the nitrocellulose filters in the presence of an enhancing screen up to 7 days(Southern *et al.*, 1975).

2.8. DNA sequencing

The DNA sequence was generated using United States Biochemical Corp.(USB) sequencing kit with Sequenase version 2.0. This procedure is based on the chain termination DNA sequencing method(Sanger *et al.*, 1977). It includes annealing template and primer, labelling reaction and termination reactions. T7 primer was used in this study. Four separate reactions, each with a different ddNTP give complete sequence information. The labelled chains of various length were visualized by autoradiography after separation by electrophoresis in a 6% acrylamide gel.

2.9. Oligo primer design and polymerase chain reaction(PCR)

To search for and select oligonucleotides for polymerase chain reaction(PCR) from a sequence file, primer analysis software(OLIGO™ version 4.0 for Macintosh, published by National Biosciences Inc., Plymouth, MN.) was used. The PCR cycling protocol was for 1 min at 94^{0}C, 1 min at 60^{0}C and 3 min at 72^{0}C for 35 cycles. Each PCR reaction containing 50

pmol of each primer, 0.2mM dNTPs, 2.0mM $MgCl_2$, 20mM Tris-HCl(pH8.4), and 50mM KCl and 1.0 unit of Taq polymerase (Gibco/BRL) in a final volume of 50μl.

Chapter 3. Results

To obtain a targeted disruption of the IGFBP-1 gene, we designed a targeting vector that would delete part of the IGFBP-1 gene, corresponding to the second and third exons of the rat IGFBP-1 gene. In this study, the fragments of IGFBP-1 genomic DNA, transcriptionally competent cassettes of the bacterial neomycin-resistance gene(Neo^r) and the *herpes simplex* virus thymidine kinase gene(HSV-TK), both under the control of phosphoglycerate kinase(PGK) promoter, were used to construct the targeting vector. The positive-negative selection protocol was applied to enrich for homologous recombination events by simultaneously selecting for a Neo^r within the homologous DNA and against HSV-TK gene placed at the end of the targeting vector.

3.1. Identification and subcloning of the mouse equivalents of the rat IGFBP-1 5' and 3' fragments

Rat IGFBP-1 and mouse IGFBP-1 gene were previously cloned in this laboratory. Rat IGFBP-1 contains 4 exons and spans 5 kb (Figure 1A). Preliminary data suggested that mIGFBP-1 is very similar(Figure 1B). Two bacteriophage lambda mouse genomic clones(mouse genomic DNA library, adult BALB/c male liver), DB11b and DB2, were available in this laboratory and contained the 5' region and 3' region of the mIGFBP-1 gene respectively.

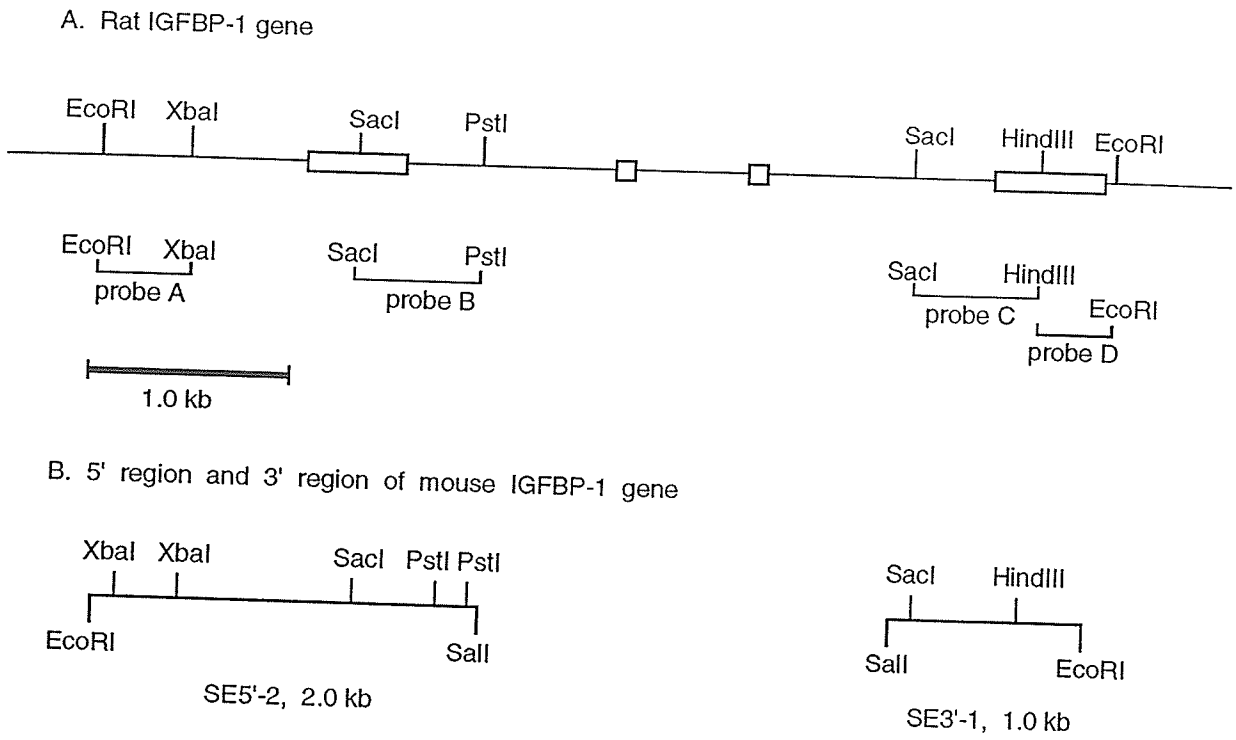


Figure 1. Restriction enzyme map of the rat IGFBP1 gene(A) and the two mouse IGFBP-1 genomic fragments, SE5'-2 and SE3'-1 (B, See Figure 6). Rat IGFBP-1 contains 4 exons. Preliminary data available in this laboratory suggested that the mouse genomic IGFBP-1 gene has a similar restriction map. Rat IGFBP-1 probes(A, B, C and D) used to determine the orientation of SE5'-2 and SE3'-1 are shown.

In order to make a plasmid construct useful for homologous recombination gene deletion of mIGFBP-1, 5' and 3' IGFBP-1 genomic DNA fragments first had to be identified and subcloned from the bacteriophage into a plasmid vector. Different fragments of rat IGFBP-1 gene were used as probes to identify the mouse equivalents. DNA from bacteriophage lambda clones, DB11b and DB2, were doubly digested with a variety of restriction enzymes. DNA was separated by electrophoresis in a 1% agarose gel, transferred to nitrocellulose filters, and then analyzed by Southern blot hybridization with the SacI-PstI fragment(probe B) containing the first exon and the SacI-EcoRI fragment(probe C+D) containing the fourth exon of rat IGFBP-1. A 2.0 kb SalI-EcoRI fragment from DB11b which hybridized with the SacI-PstI fragment(probe B) was chosen and subcloned into pBluescript SK(Figure 2, 4, 5, 6). This plasmid, pSE5'-2, was used to make the 5' fragment of the targeting construct. A 1.0 kb SalI-EcoRI fragment from DB2 which hybridized with the SacI-EcoRI fragment(probe C D) was chosen and subcloned(Figure 3, 4, 5, 6). This plasmid, pSE3'-1, was used to make the 3' fragment of the targeting construct. For confirmation, a Southern blot containing these two fragments were hybridized with a mIGFBP-1 cDNA previously isolated in this laboratory(Figure 5).

Figure 2: Southern blot analysis of bacteriophage lambda clone, DB11b. DNA from DB11b was doubly digested by a variety of restriction enzymes, as indicated above, separated by electrophoresis in a 1% agarose gel, and then analyzed by Southern blot hybridization with the SacI-PstI fragment(probe B, see figure 1). A 2.0 kb SalI-EcoRI fragment indicated by arrow was chosen and subsequently subcloned into pBluescript SK(+). This pSE5'-2 was used to make the 5' fragment of the replacement construct.

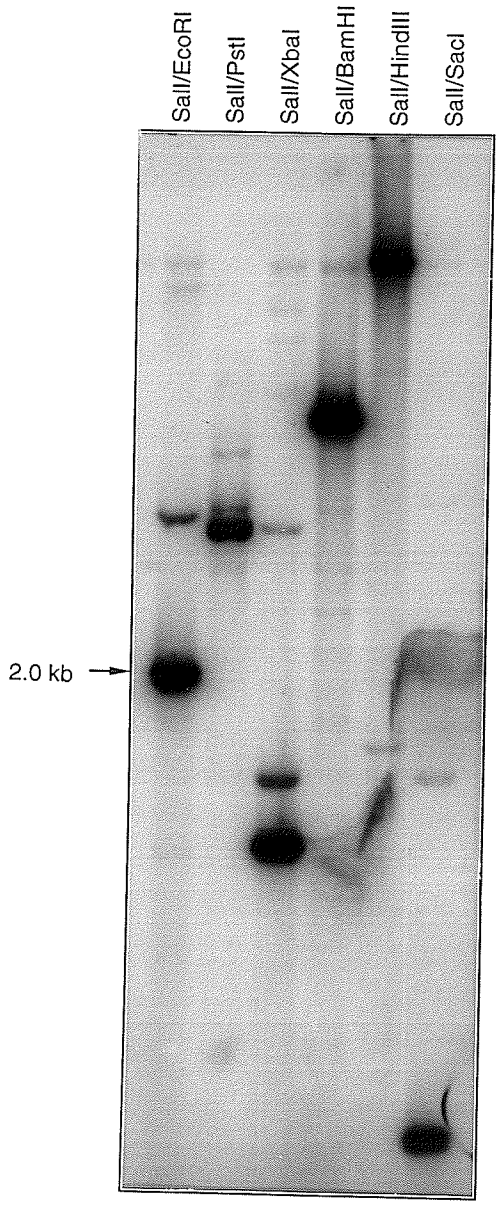

SalI/EcoRI
SalI/PstI
SalI/XbaI
SalI/BamHI
SalI/HindIII
SalI/SacI
2.0 kb

Figure 3: Southern blot analysis of bacteriophage lambda clone, DB2. DNA from DB2 was doubly digested by a variety of restriction enzymes, as indicated above, separated by electrophoresis in a 1% agarose gel, and then analyzed by Southern blot hybridization with the SacI-EcoRI fragment containing the fourth exon of rat IGFBP-1(probe C+D, see Figure 1). A 1.0 kb SalI-EcoRI fragment indicated by arrow was chosen and subsequently subcloned into pBluescript SK(+). This pSE3'-1 was used to make the 3' fragment of the replacement construct.

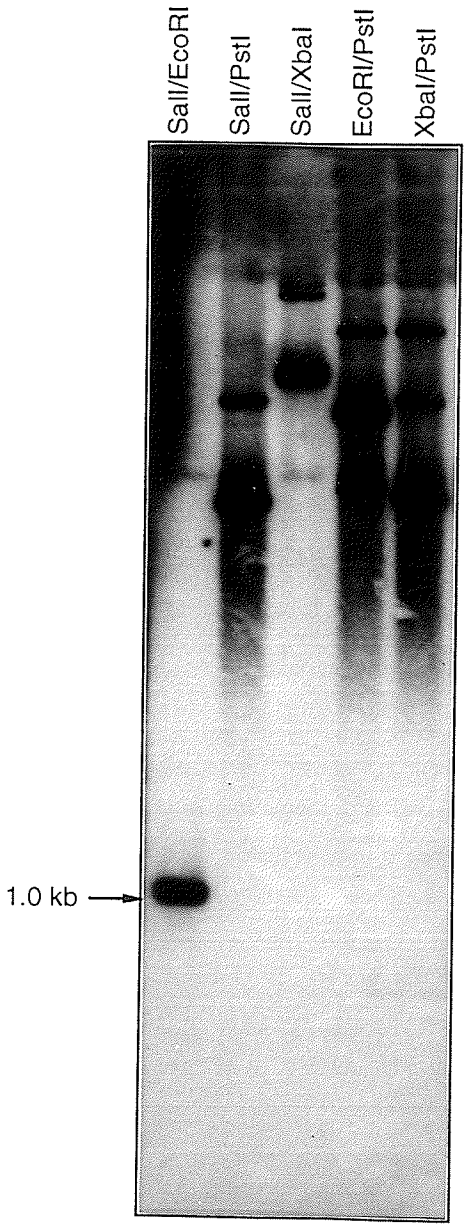
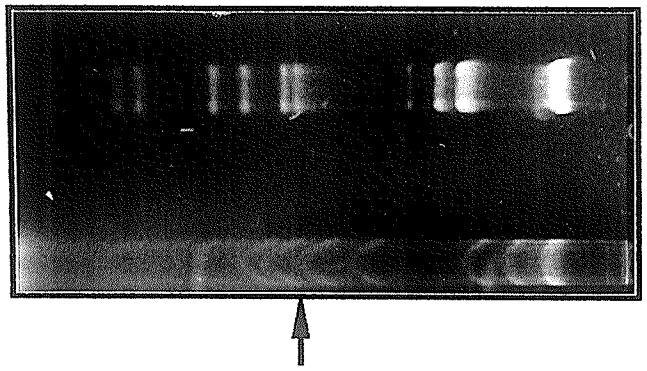
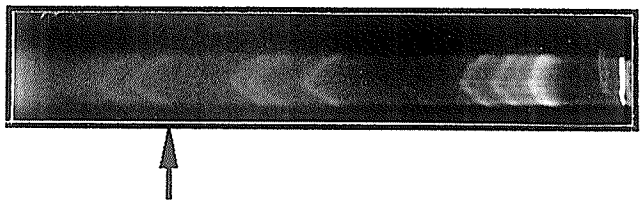

SalI/EcoRI
SalI/PstI
SalI/XbaI
EcoRI/PstI
XbaI/PstI
1.0 kb

Figure 4: Isolation of inserts from bacteriophage lambda clones, DB11b and DB2. DNA from DB11b and DB2 were doubly digested by SalI and EcoRI, separated by electrophoresis in a 0.6% low melting point(LMP) agarose gel. The 2.0 kb band indicated by arrow from DB11B and the 1.0 kb band indicated by arrow from DB2 were cut out and subcloned into pBluescript SK. For comfirmation, a Southern blot containing these two fragments were hybridized with a mIGFBP-1 cDNA previously isolated in this laboratory(see Figure 5)



Lambda HindIII/EcoRI

DB11b, SalI/EcoRI



DB2, SalI/EcoRI

Figure 5: Subcloning of 5' fragment and 3' fragment of mouse IGFBP-1 gene. Plasmid DNA was prepared from ampicillin resistant colonies. The DNA was then digested with SalI and EcoRI and separated by electrophoresis in a 1% agarose gel. Lane 1-7 shows plasmid DNA from the ligation of the 2.0 kb fragment of DB11b(see figure 4). Lane 8-9 shows plasmid DNA from the ligation of the 1.0 kb fragment of DB2(see figure 4). For confirmation, the gel was transferred to nitrocellulose membrane and hybridized with mouse IGFBP-1 cDNA. Panel A shows ethidium bromide stained gel. Panel B shows Southern blot hybridization.

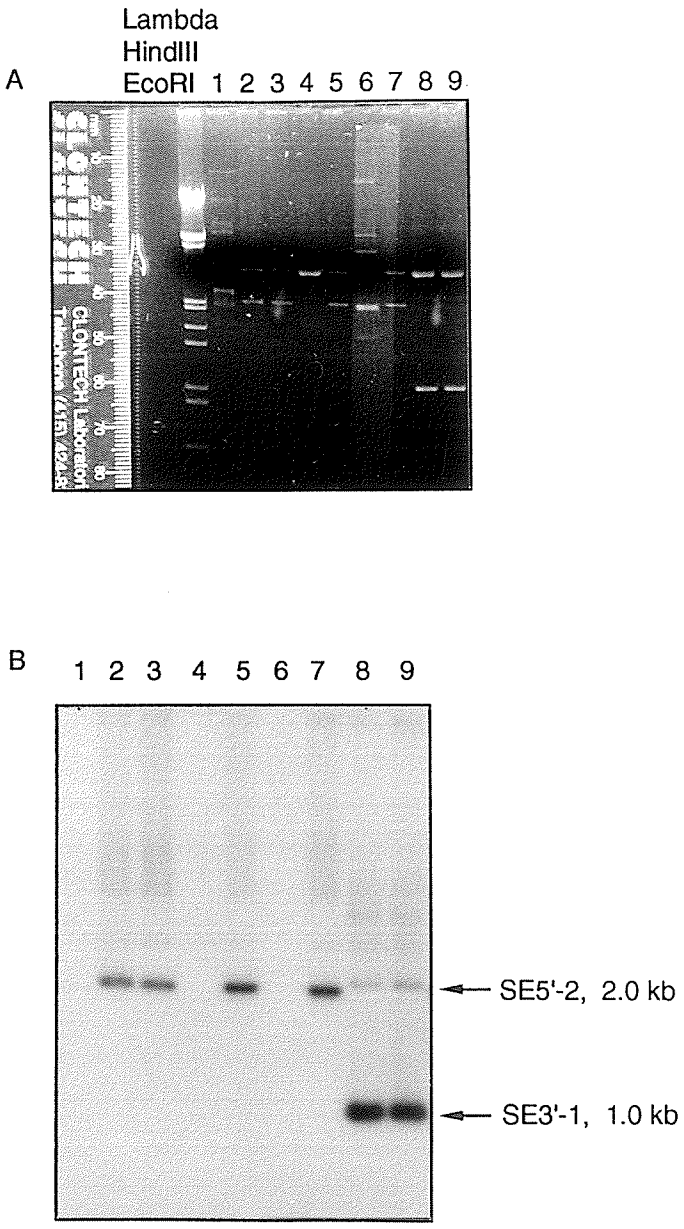
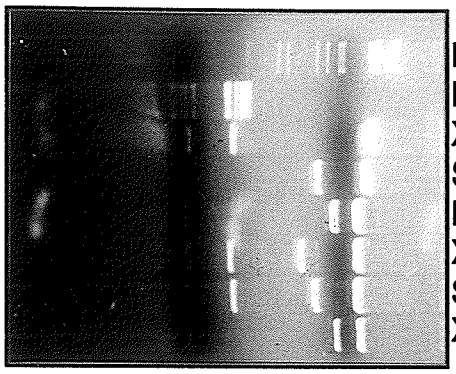
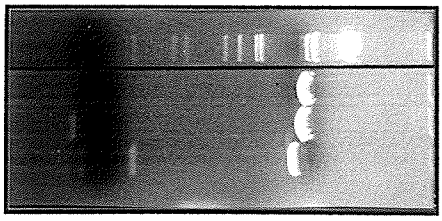

A
Lambda
HindIII
EcoRI 1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
SE5'-2, 2.0 kb
SE3'-1, 1.0 kb

Figure 6: Restriction enzyme analysis of pSE5'-2(panel A) and pSE3'-1(panel B) Plasmids were digested with the restriction enzymes as indicated above. DNA was separated by electrophoresis in a 1% agarose gel.

A


Lambda H E
PBR 322
XbaI
SacI
PstI
XbaI/PstI
SacI/PstI
XhoI/PstI

B

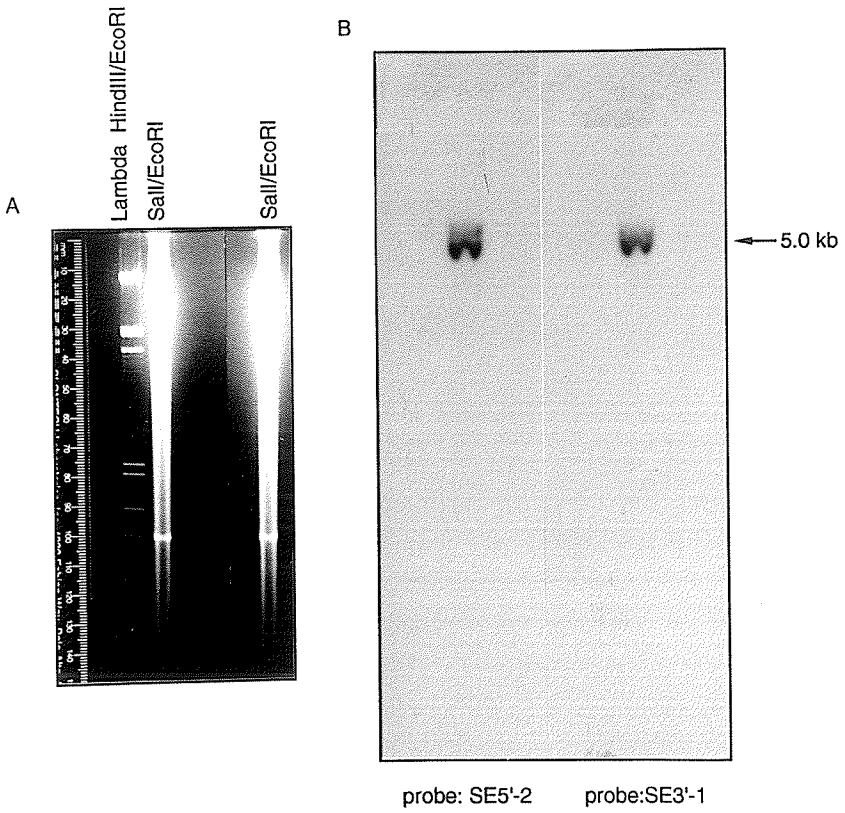

Lambda HindIII/EcoRI
HindIII
HindIII/EcoRI
HindIII/SacI

pSE5'-2 and pSE3'-1 inserts were used as probes to hybridize with genomic DNA digested with EcoRI/SalI in order to determine whether there were highly repetitive sequences contained within these genomic DNA fragments. No repeat sequences were found in these fragment(Figure 7).

3.2. Determination of the orientation of the mouse fragments

The appropriate rat IGFBP-1 probes were used to determine the orientation of mouse fragments. The pSE5'-2(Figure 1B, 6) was digested with SacI, XbaI and PstI. The rat IGFBP-1 SacI-PstI fragment(probe B) and EcoRI-XbaI fragment(probe A) hybridized with the PstI fragment and the XbaI fragment of SE5'-2, repectively(data not shown). This indicated that the EcoRI site of SE5'-2 was at the 5' end and the SalI site of SE5'-2 was at the 3' end. For the SE3'-1 fragment(Figure 1B, 6), DNA was digested with SacI, EcoRI/HindIII, SacI/HindIII. The rat IGFBP-1 SacI-HindIII fragment(probe C) and HindIII-EcoRI fragment(probe D) hybridized with SacI-HindIII fragment and the HindIII-EcoRI fragment of SE3'-1, respectively(data not shown). This indicated that the EcoRI site of SE3'-1 was at the 3' end and the SalI site was at the 5' end.

Figure 7: Determination of genomic repeat sequences. pSE5'-2 and pSE3'-1 inserts were used as probes to hybridize with the mouse genomic DNA digested with SalI and EcoRI in order to examine whether there is a highly repetitive sequence in genomic DNA. Panel A shows the genomic DNA digested with SalI and EcoRI. Panel B shows the Southern blot hybridization. Note the SalI site at the 3' end of pSE5'-2 and 5' end of pSE3'-1 originally came from the bacteriophage polylinker. The 5.0 kb fragments which hybridized with SE5'-2 and SE3'-1 probes represent an EcoRI-EcoRI fragment(see Figure 1A).This EcoRI-EcoRI fragment probably contains entire mouse IGFBP-1 gene.


A
Lambda HindIII/EcoRI
SalI/EcoRI
SalI/EcoRI
B
5.0 kb
probe: SE5'-2
probe:SE3'-1

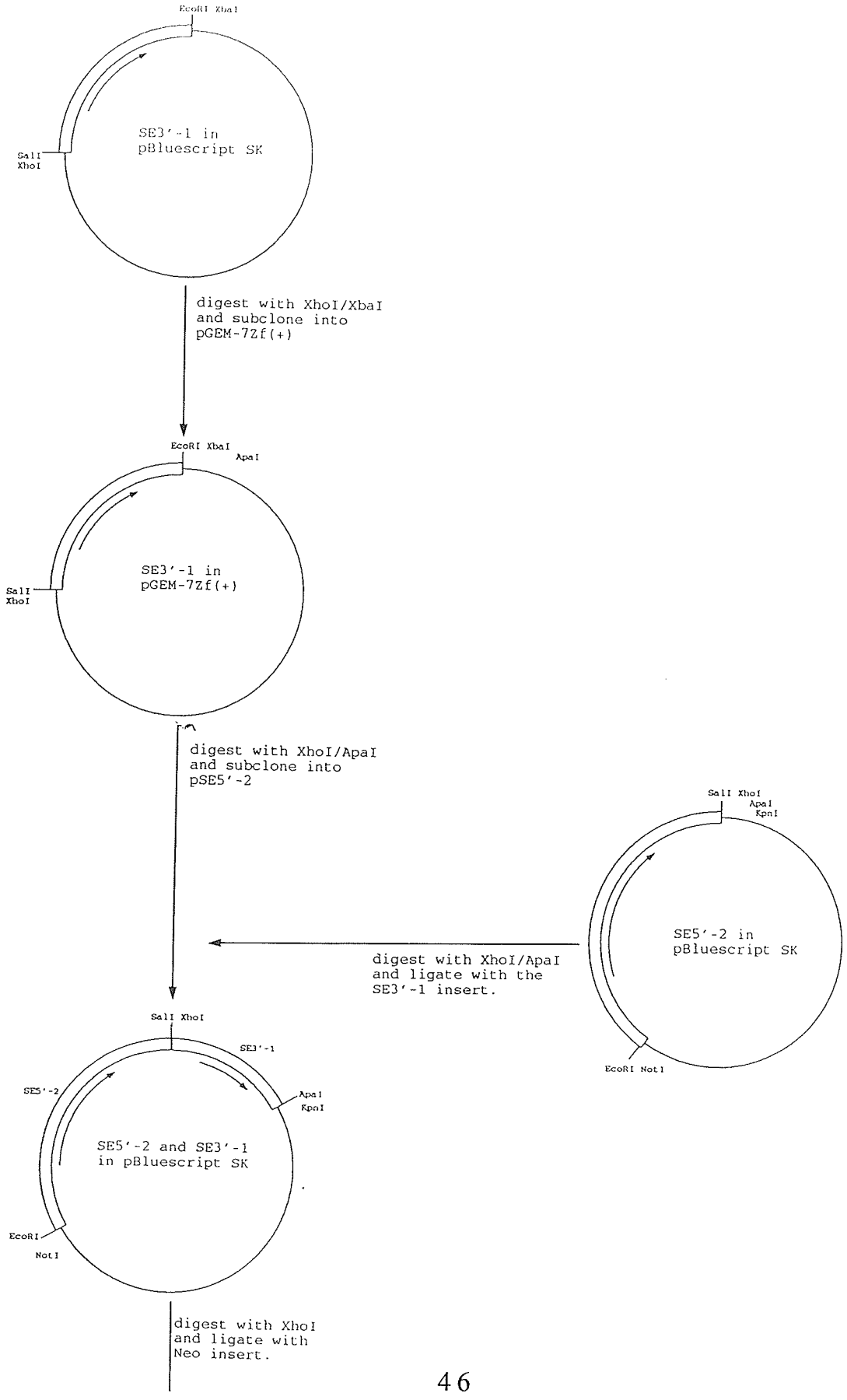
3.3. Construction of the targeting vector

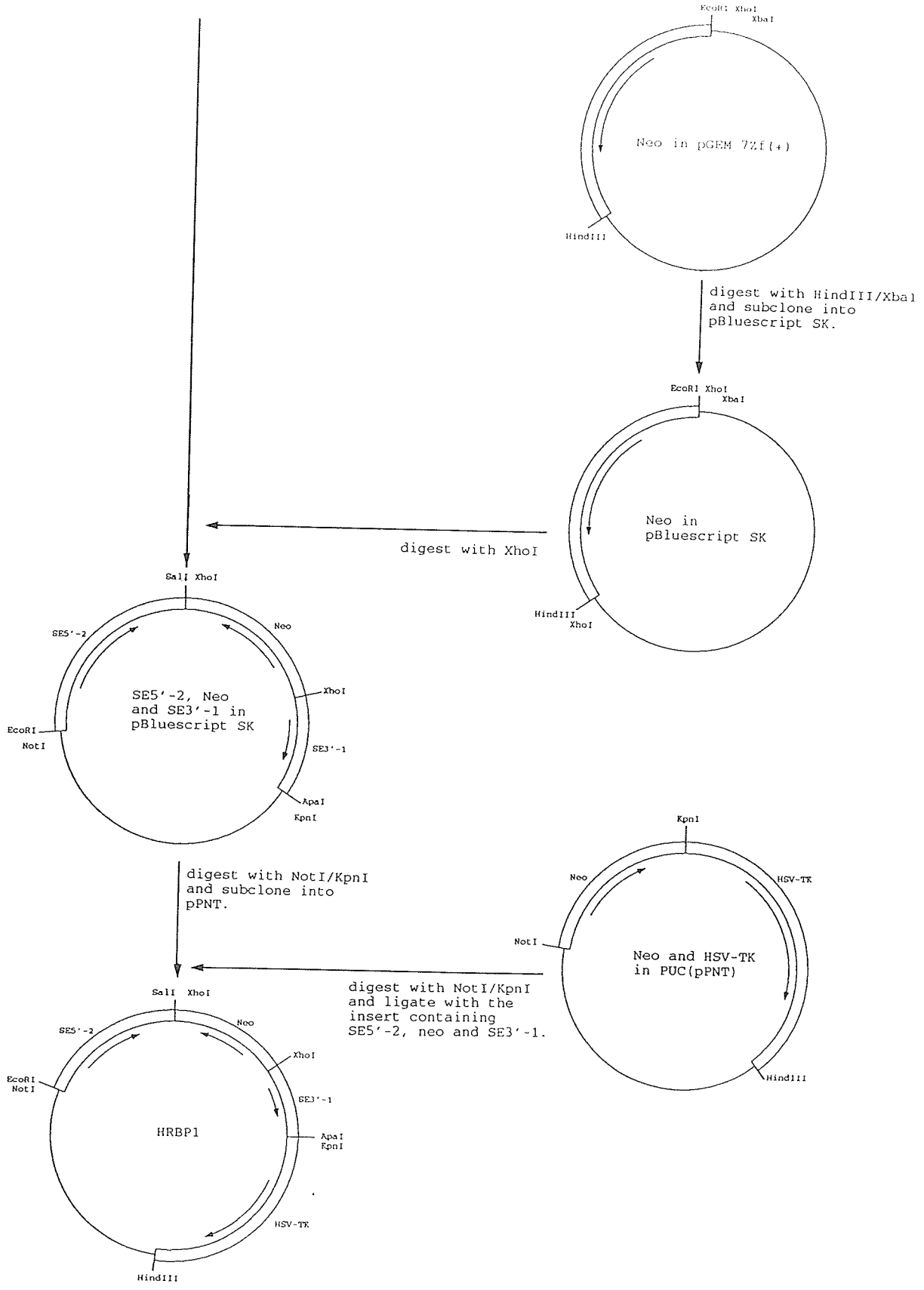
Several approaches were undertaken in order to make the construct. Only the successful approach is described in detail(Figure 8). The final product, HRBP1, consists of 5' and 3' mouse IGFBP-1 genomic fragments, Neo^r cassette and HSV-TK cassette (figure 9). To ensure the disruption of the IGFBP-1 gene, Neo^r gene was subcloned in the opposite direction of the 5' and 3' fragment of mouse IGFBP-1 genomic DNA and HSV-TK gene.

3.4. Gene targeting and ES cell DNA analysis

The targeting construct, HRBP1, was linearized at a unique NotI site and transfected on four separate occasions into ES cells by electroporation. One third of every electroporation was selected only with G418 and the remainder doubly selected with G418 and ganciclovir. Table 1 shows the results of these four experiments. The enrichment afforded by ganciclovir selection, defined as the number of G418 colonies divided by the number of G418 and ganciclovir colonies, ranged from 16.4 to 30.0.

Figure 8. Procedure for making the targeting construct, HRBP1. The final product, HRBP1, consists of 5' and 3' mouse IGFBP-1 genomic fragments, Neo^r cassette and HSV-TK cassette.


EcoRI XbaI
SE3'-1 in
pBluescript SK
SalI
XhoI
digest with XhoI/XbaI
and subclone into
pGEM-7Zf(+)
EcoRI XbaI
ApaI
SE3'-1 in
pGEM-7Zf(+)
SalI
XhoI
digest with XhoI/ApaI
and subclone into
pSE5'-2
SalI XhoI
ApaI
KpnI
SE5'-2 in
pBluescript SK
EcoRI NotI
digest with XhoI/ApaI
and ligate with the
SE3'-1 insert.
SalI XhoI
SE3'-1
SE5'-2
ApaI
KpnI
SE5'-2 and SE3'-1
in pBluescript SK
EcoRI
NotI
digest with XhoI
and ligate with
Neo insert.



Final product(HRBP1) containing SE5'-2, Neo, SE3'-1 and HSV-TK. The Direction of transcription are shown.

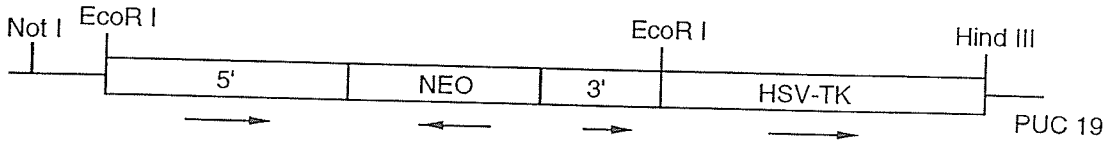


Figure 9. Structure of the targeting construct. Neo gene goes in the opposite direction of 5' fragment, 3' fragment and HSV-TK gene

Table 1. Results of Screens for Homologous Recombinants at the IGFBP-1 gene locus

Experiment	No.of cells surviving Electroporation	No.of G418 Colonies	No.of G418 and GANC Colonies	Enrichment	No. of Recombinants	Method of Analysis
1	$8X10^6$	6,200	350	17.7	0/180*	PCR
2	$1.0X10^7$	12,000	400	30.0	0/140*	PCR
3	$1.2X10^7$	8,000	300	26.6	0/158*	Southern blot
4	$1.1X10^7$	9,000	550	16.4	1/184*	Southern blot

* the number of colonies screened

Both PCR and Southern blot hybridization were used to analyze ES cell DNA. For PCR, a pair of primers were designed as described in the previous section after nucleotide sequence of a region of the IGFBP-1 locus downstream to the 3' EcoRI site present on the targeting vector was generated(Figure 10). PCR assays for IGFBP-1 targeting were performed with the primer 5'-CATAGCCGAATAGCCTCTCCA-3', which is located at a distance of 550bp 3' from the beginning of the neomycin resistance gene, and a downstream primer 5'-CAAAAGCAAACAACCCAGTGA-3'(Figure 12). To obtain this downstream primer, it was necessary to sequence a region of the IGFBP-1 locus downstream of the 3' EcoRI site(Figure 14A). To optimize the PCR conditions, another construct was developed as positive control(Figure 11, 12). Then PCR conditions were optimized using the positive control construct as template. A PCR product of correct size for the distance between the primers(1.9kb) was visualized by ethidium bromide staining if the template(positive control construct) amount was more than 1.0ng per reaction, and visualized after Southern blotting if the template(positive control construct) was more than 100pg per reaction(Figure 13).

More than 300 ES cell colonies from experiments 1 and 2(table 1) were analyzed by PCR. 2μg of each ES cell colony DNA was used as template. No homologous recombinants were

EcoRI site

+1 AATTCCAGCT TTATCTTTGT TCCCTCTGTC CGTTTGTCTT CCCCATCACC

+51 TGACTTCTGG TCTGTGCTTT GGGGAAAGAT GGTGCTTGTT TGCTTACAGA

+101 AGATCTTGTC TGACACTCTG TGATTCCATA GCCCCATTCC CAAGCAGTAT

+151 TAGGCCTCTG CCTGGGCCAG ACTAGAGATC CCCACCACCT TGGCCCATCT

+201 CCCTTTGACC TTCCAGGACC CCCAGCCTCT CTTTCCTTGA CCACTCCTCA

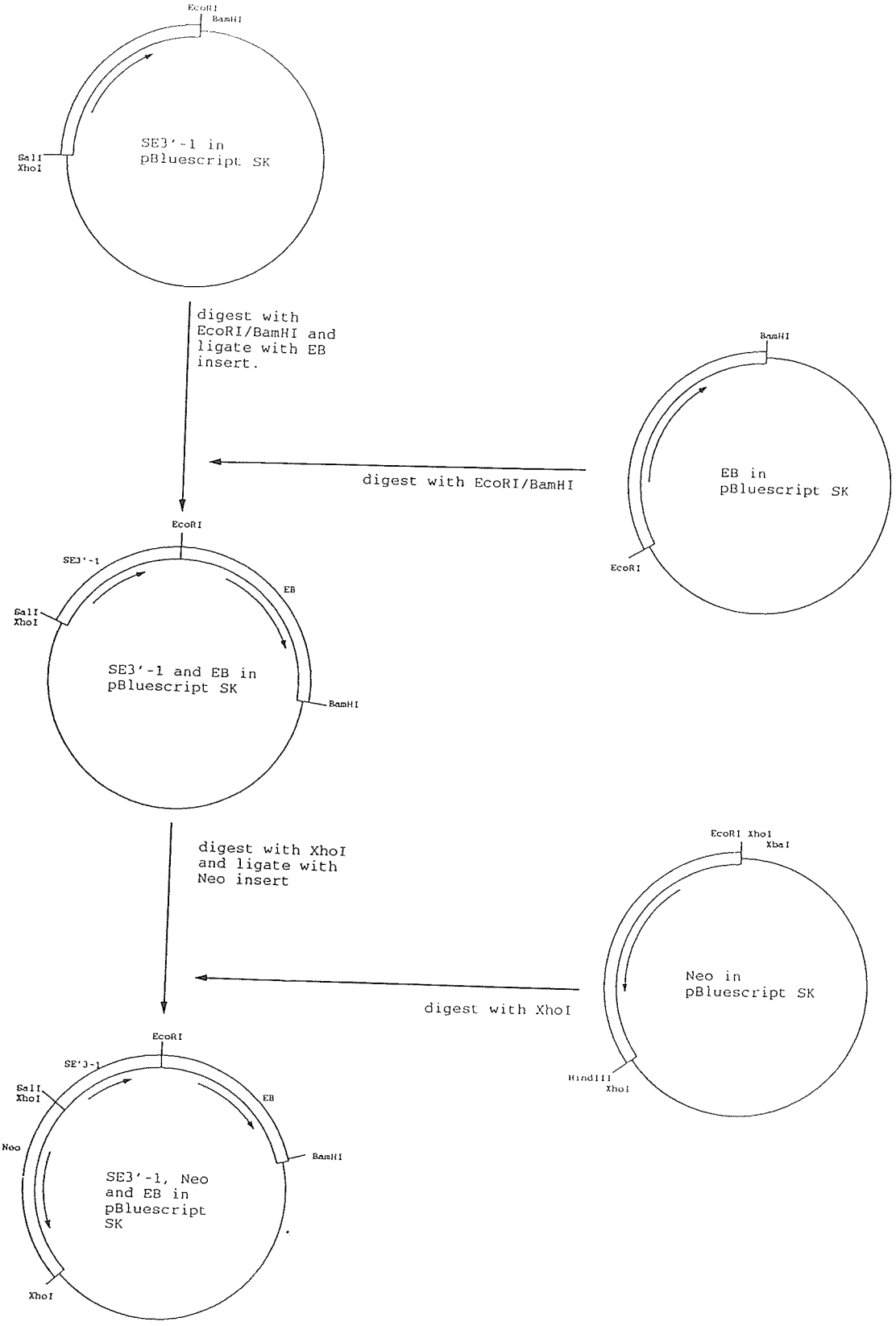
+251 ACTGCTCCAG CTGATGTCGC GTGTATCCGG GGAGCAGTCA CTGGGTTGTT

+301 TGCTTTTGAG ACAGGGTCTG ACTCTGTAGC CAGGCTGATG TAGAGTACAG

+351 CAGTCCT

Figure 10. Nucleotide sequence of the DNA fragment of mouse IGFBP-1 locus downstream to the 3' EcoRI site(See Figure 14). A, C, G and T represent deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine respectively.

Figure 11. Procedure for making the PCR positive control. PCR conditions were optimized using this positive control construct as a template.



The construct for PCR positive control

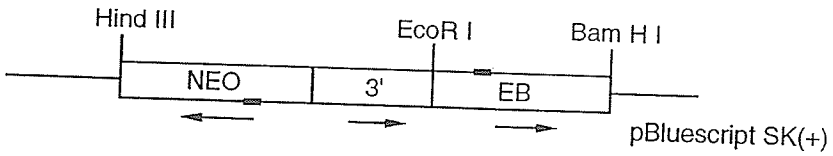


Figure 12. Construct structure for positive control. EB is a 1.5 kb genomic fragment downstream to the 3' EcoRI site present on the targeting vector. It was used as probe E. The location of Primers used in this study are shown as thick bars. Neo gene goes in the opposite direction of 3' fragment and EB fragment.

detected. The possible reasons for failing to detect homologous recombination will be discussed in the chapter 4.

For Southern blotting, probe E, an EcoRI and BamHI fragment downstream to the 3' EcoRI site present on the targeting vector was used. As a further confirmation, probe F, the Neo^r gene, was also used (Figure 14A).

A total of 342 colonies were analyzed by Southern blotting for the presence of a gene targeting event. Because the Neo^r gene contains a BamHI site, its presence introduces a new BamHI site into the IGFBP-1 gene, resulting in the production of a smaller BamHI fragment. Hybridization of BamHI digested DNA from an individual colony with probe A was predicted to show a 10 kb fragment from the original wild-type allele and a 4.5 kb fragment in a correctly targeted allele. Of the 342 colonies screened, one showed the predicted mutant fragment of 4.5 kb(Figure 15).

To test whether ES cells were differentiated or not after the screening, ES cells were stained with Giemsa, most of the ES cells retained the stem cell phenotype in the presence of LIF at concentration of 1,000 $units^{-1}$. ES cells differentiate spontaneously after withdrawal of LIF for a week(data not shown).

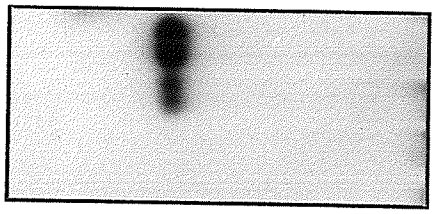
Figure 13: Optimization of PCR conditions. PCR condition was optimized with the positive control construct mixed with 4 μg genomic DNA as template before testing the samples. A PCR product of correct size (1.9 kb) was visualized by ethidium bromide staining if the template(positive control construct) amount was more than 1 ng(panel A), and visualized after Southern blotting if the template was more than 100 pg(panel B).

A

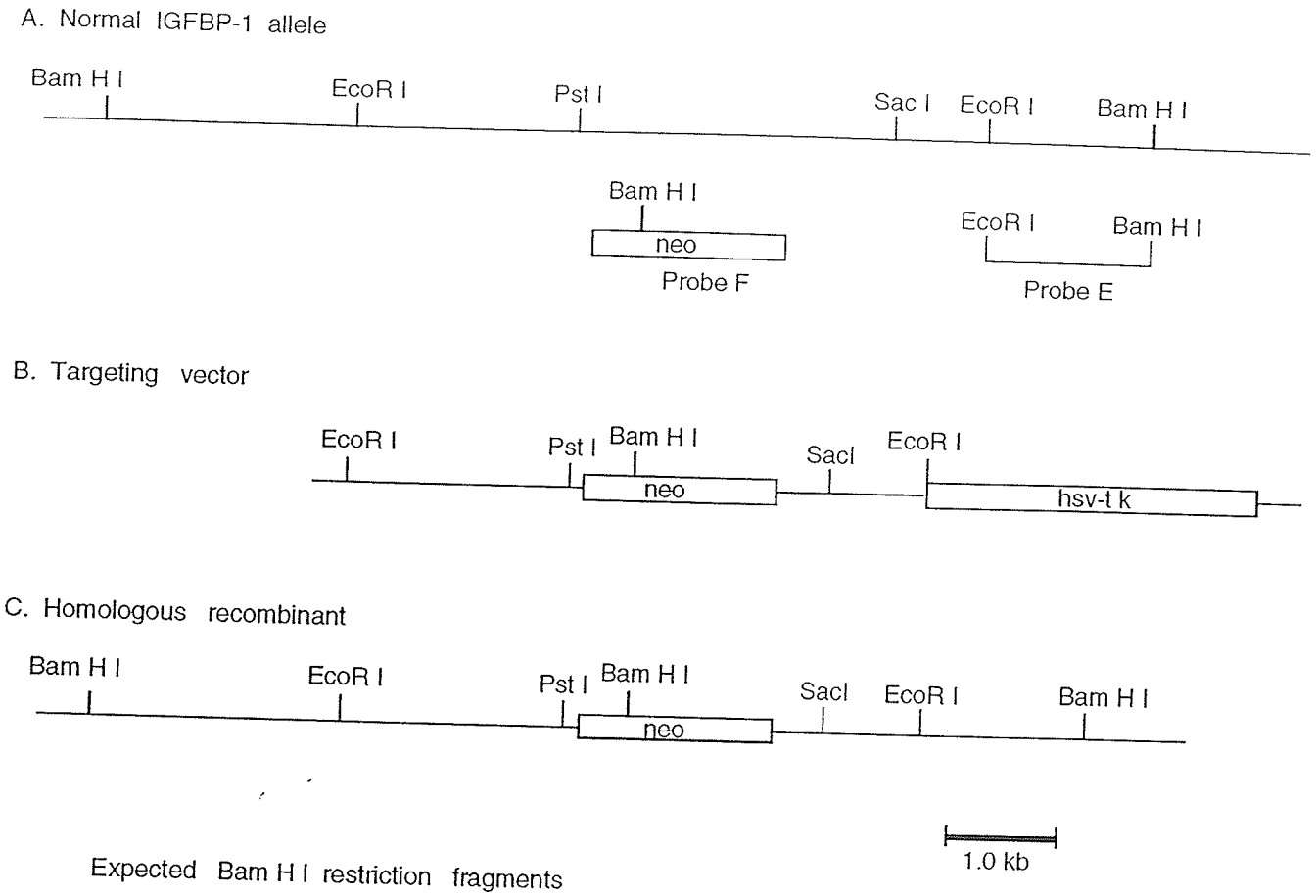
Lambda HindIII/EcoRI

100 ng
10 ng
1 ng

B



1,000 pg
100 pg
10 pg

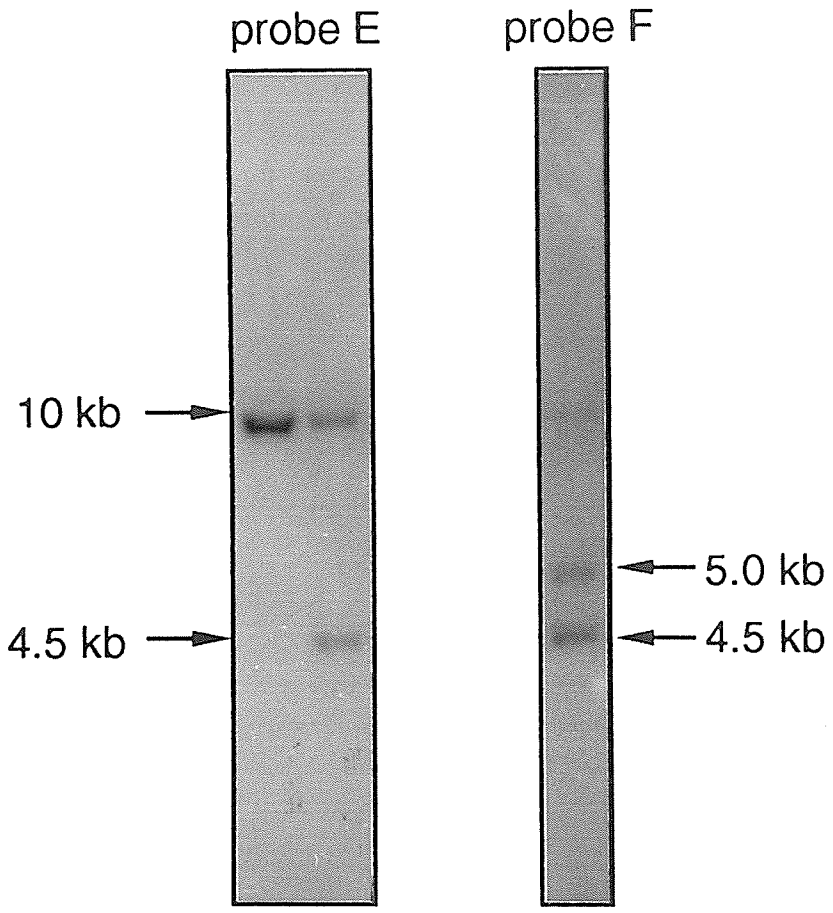


Expected Bam H I restriction fragments

	Probe E	Probe F
Endogenous	10kb	---
Recombinant	4.5kb,10kb	4.5kb, 5.0kb

Figure 14. Disruption of the IGFBP1 gene in mouse embryonic stem cells. A, Restriction map of the mouse IGFBP-1 gene. A 10 kb genomic DNA fragment containing IGFBP-1 gene is illustrated. B, Structure of targeting vector, HRBP1. A neo cassette was inserted to replace part of IGFBP1 gene and used as a marker for positive selection. A HSV-TK cassette was placed at the end of the construct for negative selection. C, the predicted structure of a homologous recombinant. The probes used for identification are shown.

Figure 15: Southern blot analysis of DNA of an ES cell colony demonstrating homologous recombination. The left panel shows the initial screening by Southern blot hybridization. DNA was digested with BamHI and analyzed with probe E(see Figure 14). The DNA sample on the right lane of the left panel demonstrates the predicted mutant allele of 4.5 kb and a reduction in the wild type allele of 10 kb. The sample of the left represents a wild type ES DNA. For confirmation, probe F consisting of the neomycin resistance gene was used to rehybridize the DNA. The right panel shows the two predicted fragments of 4.5 kb and 5.0 kb. The faint band at 10 kb region was most likely due to incomplete stripping of the bolt.


probe E
probe F
10 kb
4.5 kb
5.0 kb
4.5 kb

Chapter 4. Discussion

I have developed a targeting construct for the mouse IGFBP-1 and generated an ES cell line which will be used in the development of transgenic mice with a deficiency for IGFBP-1. This study is based on the fact that ES cells can be cultured and manipulated *in vitro* and still retain the developmental potential to form chimeric mice when injected into blastocysts.

In most previous studies, ES cells required the presence of a feeder layer of fibroblast cells to inhibit their differentiation *in vitro*. In this study, leukemia inhibitory factor(LIF) was used instead of feeder layer to prevent the differentiation of ES cells. LIF is a 20 kDa protein which originally used to induce differentiation in M1 myeloid leukemic cells. It was first reported that LIF might inhibit the differentiation of ES cell in 1981(Evans *et al.*, 1981; Martin *et al.*, 1981). Williams *et al.*(1988) have demonstrated that ES cells maintained in the medium containing LIF are capable of giving rise to germ line chimeric mice. *In vitro*, at concentrations of LIF of 1,000-5,000 units ml^{-1}, more than 95% of colonies showed the stem cell phenotype of compact colonies, which consist of small cells with a large nuclear to cytoplasmic ratio. This stem cell phenotype of ES cell was verified with the EMCA-7 antibody, which recognizes a stem cell surface specific antigen. ES cells cultured in the

medium containing the LIF express the stem cell specific antigen, whereas in the absence of LIF, less than 1% of ES cells do so(Williams *et al.*, 1988). ES cells were maintained in the presence of LIF at concentration of 1,000 units ml^{-1} for a prolonged period of time during this study. The results of this study demonstrated that ES cells could be maintained in an undifferentiated state in the presence of LIF in the medium, and could be cultured and passaged as normal cell lines.

The PCR technique was used to screen for homologous recombination events during the first part of this study. No homologous recombinants were detected in targeting experiments 1 and 2. It is possible that all the 320 colonies picked after the double selection were not homologous recombinants. However, when the PCR positive control construct was used as a template, no PCR product of the right size(1.9 kb) could be visualized, even by Southern blotting, if the control template was present at 100 pg per reaction. No PCR product of right size was visualized either when PCR was performed by using the mutant ES cell DNA as a template. The large size(1.9 kb) of the DNA fragment might account for the low sensitivity. In addition, the PCR condition optimized with a positive control construct alone might not have been suitable for amplifying the sequence in the presence of genomic DNA. The low sensitivity encountered in detecting a targeted gene by PCR and possible explanations were

described by Kim *et al.*(1991) and Nitschke *et al.*(1993). A PCR fragment of >1.0 kb is often difficult to amplify reproducibly, even various primers and $MgCl_2$ concentrations were tried. To date PCR has not been used widely to detect a targeted gene. This is probably due to the difficulty in optimizing the PCR conditions. Although PCR detection is faster, the choice of PCR as a detection method could also influence the design of the targeting vector because it would limit the size of at least one of flanking genomic fragments. While Southern blot hybridization is much more time-consuming, it would appear from the literature still to be the main method for screening of homologous recombinants at present(DeChiara *et al.*, 1991; Hasty *et al.*, 1993).

The homologous recombination experiment is based on the observation that DNA transfected into cells can homologously recombine with their endogenous chromosomal equivalents(Smithies *et al.*, 1985). The frequency of such homologous recombination events is extremely low so that it is essential to employ a sensitive screening methods to detect the cells in which homologous recombination events occurred. The positive-negative selection protocol makes it possible to screen homologous recombinants from among over 10^7 cells. In the targeting experiment 3 and 4, $1.1x10^7$ and $1.2x10^7$ cells were electroporated and doubly selected with G418 and ganciclovir. The frequency of homologous recombination events was 0.3% after double selection in this

study. It has been reported in the literature that the frequency of homologous recombinants of colonies with both G418 and Ganciclovir resistance is between 0.1% to 10%(Tybulewicz *et al.*, 1991; Mansour *et al.*, 1988). In this study, ES cells and genomic DNA library were derived from different mouse strains. This could account for the low frequencies since using DNA from the same mouse strain increases recombination frequencies because of greater homology. The small size of the 5' and 3' fragments of targeting vector probably have an impact on recombination frequencies, because the greater the length of the sequence homology, the greater the recombination frequencies. Thus, the distances flanking the mutational insertion in the targeting vector should be maximized(Mansour *et al.*, 1988).

In the process of double selection, the major problem is the growth of false positive colonies from the ganciclovir selection. The electroporated ES cell DNA was analyzed by Southern blotting hybridization with the HSV-TK probe at the beginning of this study. Most of the ES cells contained the TK gene. The reason that ganciclovir did not kill these cells was possibly that the TK gene was not expressed in these cells for unknown reasons or that the concentration of ganciclovir was not sufficient to kill these cells. To get a better selection, the addition of ganciclovir to transfected cells was delayed to prevent killing of cells which expressed its TK from non-integrated plasmid. The concentration of

ganciclovir was also increased from 2 nM to 10 nM. The effects of those modification on selection remain to be further investigated.

Gene deletion by homologous recombination in eukaryotic cells was first used to create germ-line containing targeted disruption in the β-globin gene in 1985(Smithies *et al.*). Since then it has been widely used to study specific gene biological functions and has turned out to be one of the most exciting areas of gene research(Nandi *et al.*, 1988; Stanton *et al.*, 1990; DeChiara *et al.*, 1991; Liu *et al.*, 1993). As mentioned in a previous section, this technology is also being used to study the pysiological role of IGF and IGFBP family(Dechiara *et al.*, 1990; 1991; Liu *et al.*, 1993; Wood *et al.*, 1993). In this thesis I have developed the targeting construct and generated an ES cell line carrying a disrupted IGFBP-1 gene. The next stage of this project is to transfer the our genetically modified ES cell line into the mouse germ line. Undifferentiated ES cells will be injected into mouse blastocysts in which they participate in the formation of all tissues, including reproductive germ tissue. These mice can then be breed as to develop a strain of mice which is deleted for this gene. The ability to make transgenic mice with modified ES cells will make it possible to study the effects of a deficiency for IGFBP-1 *in vivo*. Colleagues in this laboratory are also examining transgenic mice which overexpress IGFBP-1. These two approaches should provide

insights into the physiological role of the IGFBP-1 in embryonic development and growth.

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