TWO INVESTIGATIONS IN MOLECULAR PATHOPHYSIOLOGY

by

PAUL CHRISTOPHER ORBAN

B. Med. Sc., The University of New South Wales, 1981
M.B., B.S., The University of New South Wales, 1984
B.A., The University of Sydney, 1984

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Genetics Programme)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 1993

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature) _____

Department of MEDICAL GENETICS

The University of British Columbia
Vancouver, Canada

Date 30 AUG 1993

ABSTRACT

This thesis is in two discrete parts. The first part deals with the mechanism of auto-stimulatory growth in the pathogenesis of malignant neoplasia. Experiments were undertaken to investigate the question of whether antagonism of growth-factor activity can lead to the death of the cells of a murine model of auto-stimulatory leukemia. Although many previous workers have examined the ability of growth-factor antagonists to inhibit the growth of the cells of human leukemias and of animal leukemia models, none have documented the complete blockage of growth, and none have documented the death of such cell populations. A model of auto-stimulatory leukemia was generated by transfecting a mouse IL-2-dependent cell line with vectors designed to cause expression of IL-2 in these cells. One series of clones was derived which grew in the absence of exogenous IL-2, and produced tumours in syngeneic mice. Cells of these clones produced very small amounts of IL-2, but their growth was not completely inhibitable by antibodies to IL-2 or the IL-2 receptor. Another clone was derived, which produced no detectable IL-2, but grew independently of exogenous IL-2. The growth of cells of this clone was completely inhibited by antibody, and death of the cells resulted. The experiments described here represent the first demonstration that antibody antagonists of the growth factor can induce the death of cells that grow by auto-stimulatory mechanisms. They support the hope that cytokine antagonists may find use as therapeutic reagents in the treatment of auto-stimulatory neoplasms.

The second part of the thesis presents a new technique in gene targetting, which has broad applications in the study of gene function. Using a bacterial recombinase under the control of a tissue-specific developmentally regulated promoter, transgenic animals were derived, in which a target gene was deleted in thymocytes and their daughter cells, but no other tissues. This technique circumvents the impediment embryonic lethality may present in some gene deletion experiments, and allows questions of tissue-specifc function of genes in pathogenesis and normal development to be addressed.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab.	antibody
AML	acute myelogenous leukemia
bp	base pair(s)
cDNA	complementary DNA
CM	conditioned medium
CML	chronic myelogenous leukemia
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
ES cell	embryo stem cell
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
hGH	human growth hormone
hIL-2	human IL-2
IL	interleukin
kbp	kilo base pairs
LTR	long terminal repeat
M	molar
M.A.	medium alone (no growth factor added)
mIL-2	murine IL-2
ml	millilitre(s)
mM	millimolar
mRNA	messenger RNA
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RNA	ribonucleic acid

βGAL	beta-galactosidase.
µg	microgram(s)
µl	microliter(s)
^{o}C	degrees Celsius

Although the convention of naming genes in italics has been adhered to, in general, all transgene fragments have been named in upright capital letters in this work.

ACKNOWLEDGEMENT

I wish to express my gratitude to my supervisor, Dr. John Schrader, whose enthusiasm and breadth of knowledge were a source of inspiration, and without whose open-mindedness and generosity of spirit, project two would not have been undertaken. In addition, I am deeply indebted to Dr. Jamey Marth of the Biomedical Research Centre, who supervised the work described in the second part of the thesis, and to Daniel Chiu, who assisted with much of the work reported in the second part. I should also like to acknowledge Dr. Hermann Ziltener of the Biomedical Research Centre, who supplied several antibodies, and taught me some of the basic techniques of working with them, Dr. Ian Clark-Lewis, of the Biomedical Research Centre, who supplied synthetic growth factors, and Dr. Allen Delaney of the Biomedical Research Centre who provided computer software and hardware support for this work.

FOREWORD

Materials and methods aside, this thesis is in two parts, each dealing with a hypothesis and the experiments carried out to test it. The first part of the thesis deals with a project proposed by my supervisor. The second part deals with a project devised by myself. The link between the two is not obvious from the purely scientific point of view, but the historical path from the first to the second was quite simple. The coincidence of three factors influenced me in conceiving the second project: 1) reading a review relating to the first project (see introduction to Part 2), 2) general reading of scientific journals, and 3) the arrival of a new scientific technology at the Biomedical Research Centre (where this work was carried out). The materials and methods have been presented as a single section, since many of the techniques there described were used in both parts of the work.

During the initial stages of work on project two, it became clear that there would be considerable competition from the group which donated the reagents essential to this project. The author's efforts were therefore diverted towards completing project two as rapidly as possible.

AUTOSTIMULATORY MECHANISMS IN LEUKEMOGENESIS

INTRODUCTION

There is an abundant literature on the subject of autostimulatory tumours. Many relevant reviews have been published (e.g. Sporn and Roberts, 1985; Lang and Burgess, 1990). Rather than attempting a comprehensive review of all the relevant publications, or simply providing an additional general review, the aim of this introduction is to make clear the reason for undertaking yet another study of autostimulatory neoplasia - to identify the unanswered questions that prompted the present work.

1) BACKGROUND

In vitro, proliferation of mammalian cells depends on the presence of poly-peptide growth factors, and it is thought that the same growth factors regulate cell growth *in vivo*. More than 30 years ago, it was suggested that production of a growth factor by a cell that would respond to it might be a component of tumourigenesis (Hsu, 1961). Secretion of growth factor that could then act on the secreting cells receptors was termed "autocrine secretion" by Sporn and Todaro, 1980. In this original sense, the term "autocrine" strictly implied secretion of a factor which acted directly on the cell secreting it. Since the coining of this term, others have proposed alternative mechanisms of growth factor mediated self-support in tumours. In particular, the "paracrine" mechanism is an indirect mechanism involving secretion of a factor which induces surrounding cells to in turn secrete a factor that stimulates growth of the tumour cells (e.g. Griffin et al., 1987; Leslie et al., 1991). The first suggestions regarding autostimulatory mechanisms implied action of growth factor on receptors displayed at the cell surface, however, many workers (see below) have more recently proposed that growth factors may function from within the cell on receptors that are not exposed to the extra-cellular environment making specific antagonsim of the autostimulatory factor more difficult. Growth factor antagonists could clearly have a role in intervention in paracrine loops, however, the present work concerns the possibly more difficult problem of direct mechanisms, in which factor and receptor are present in (or on) the same cell. To allow for the possibility of intracellular function, but make clear the distinction from the paracrine mechanism of self-supporting growth factor loops, the term "autostimulation" is used here.

Tumourigenesis has long been considered to be a multi-step process (Foulds, 1958; Hunter, 1991), involving several genetic lesions in a cell that gives rise to a malignancy. Any given genetic lesion or

phenotypic trait of a tumour (such as autostimulatory growth factor production) may, however, be epiphenomenal with respect to the mechanism of tumourignesis. It is only by examining the frequency of the occurence of the trait in tumours of a given type, or by observing the effect on tumourigenesis when the trait is reversed, that one could make claims about the involvement of the trait in the tumourigenic process. The first part of this introduction will be concerned with the basis of the claim that autostimulatory mechansims are significant in tumourigenesis.

Early suggestions as to the presence of autostimulatory factors in tumourigenic cells came from Todaro and De Larco (1978). These workers found that mouse fibroblastoid cells transformed to the tumourigenic phenotype by various means, including transfer of DNA from human tumour tissue, released "transforming growth factors" into their conditioned medium. These factors would in turn stimulate growth of non-transformed cells. Since then, however, it has become apparent that the production of these two factors, termed TGF alpha and TGF beta, is not specific for tumour cells, and many cell types in normal animals produce them (Sporn and Roberts, 1985).

One of the first direct pieces of evidence that production of an autostimulatory factor could have a causal role in oncogenesis involved platelet-derived growth factor (PDGF). As its name suggests, this factor is normally derived from platelets, which release it when activated. It is therefore present in serum used for much mammalian tissue culture, many cell types in culture requiring the presence of this factor for growth. The transforming protein of the simian sarcoma virus (SSV), encoded by the oncogene v-*sis*, is very closely related to a portion of one form of PDGF, and the presence of this oncogene is essential to the tumourigenic activity of the virus (Doolittle et al., 1983; Waterfield et al., 1983). Production of PDGF-like molecules occurs in several human tumours, e.g. osteosarcomas (Graves et al., 1983; Betsholtz et al., 1984) glioma (Nister et al., 1984), and the bladder carcinoma line T24 (Bowen-Pope et al., 1984), many of which also have functional PDGF receptors. In addition some simian virus 40 (SV40) -transformed cells as well as mouse fibroblasts transformed by murine sarcoma viruses produce PDGF-like molecules, and in some of these latter cells, neutralising antibody to PDGF reduces the growth of the lines *in vitro* (Bowen-Pope et al., 1984). Additionally, a correlation was observed between the amount of PDGF produced by simian sarcoma virus (SSV) -transformed cells and their growth rate in nude mice (Huang et al., 1984). Johnsson et al. (1985) were able to demonstrate some reduction in the ability of SSV to transform fibroblasts in the presence of high concentrations of neutralising antibody to PDGF. It has since been shown that arterial smooth muscle cells isolated from normal young rats also release biologically significant amounts of these factors into their culture medium (reviewed in Sporn and Roberts, 1985). This puts into question the suggestion that the production of PDGF-like substances is always aberrant in a tumour cell, rather than a simple part of the physiology of the corresponding normal cell type.

Autocrine loops in other tumours and the tumour models discussed below may indeed be reflections of physiological events. Nevertheless, one may still propose the autocrine loop as a mechanism in the pathogenesis and maintenance of these tumours. Cells which would otherwise pass through a stage of differentiation in which autostimulation was physiological might, in the process of mutation resulting in the genesis of the tumourigenic clone, be "frozen" at this stage of differentiation. Alternatively, mature cells might lose their normal ability to down-regulate a physiological autostimulaton. T-cells, for example, when stimulated by antigen in the presence of appropriate cooperative signals, will up-regulate interleukin-2 (IL-2) receptor levels, and secrete IL-2 (reviewed in Dinarello, 1991). Various mechanisms subsequently operate to down-regulate both the IL-2 production and the level of receptor, and the loss of such mechanisms would lead to pathological autostimulation. Of course, tumour cells may well acquire alternative mechanisms of autonomous growth, irrespective of whether they have passed through an autostimulatory stage during differentiation, or lost their ability to down-regulate an autostimulatory loop. Such cells may be producing a potentially autostimulatory growth factor as an epiphenomenon.This consideration stresses the importance of showing more than the simple presence of growth factor production and of the corresponding receptor on the cells of a tumour, in order to establish the autocrine loop as essential to the tumour's viability. Studies aimed at inhibiting growth with growth factor antagonists therefore acquire greater importance in suggesting the involvement of autostimulatory mechanisms in tumourigenesis.

An example of spontaneous tumours that displayed autostimulation and susceptibility to inhibition was that of the human small-cell carcinomas of the lung that produce gastrin-releasing-peptide, the mammalian equivalent of bombesin (reported by Cuttitta et al.,1985, and others). Gastrin-releasing-peptide (GRP) is a poly-peptide hormonal factor usually produced by foregut-derived tissue, but aberrant production of hormones is a quite common feature of small-cell lung cancers, and many instances involve the production of GRP. Neutralising monoclonal antibodies to bombesin signifcantly inhibited growth of the tumours *in vitro.* When transplanted into nude (genetically immunocompromised) mice, cells of this tumour are able to proliferate and metastasise. Treatment of such mice with neutralising antibodies to GRP results in a significant, although temporary inhibition of the proliferation of these cells. The tumours eventually adapt to the presence of antibody, possibly by outgrowth of some of the cells with additional or pre-existing mutations which allow growth independently of GRP.

Several examples of the role of insulin-like growth factor-I (IGF-I), in human tumours have been reported, in which antibodies to this factor have slowed growth of the tumour cells *in vitro.* These involved neoplasms of the lung (Minuto et al., 1988) and breast (Huff et al., 1986), and osteosarcoma (Blatt et al., 1984). IGF-I was also more recently the subject of another experimental

strategy to interfere with an autostimulatory loop in a spontaneously arising tumour (Trojan et al, 1992). Cells of the transplantable rat glioma C6 were found to produce IGF-I, and the receptors for this factor. Cells of this glioma were transfected with a plasmid that produced an anti-sense IGF-I transcript and thereby caused a significant reduction of the amount of IGF-1 produced in the cells. C6 cells transfected with the anti-sense plasmid were unable to produce tumours in syngeneic rats, whereas cells transfected with empty vector were tumourigenic.Trojan et al. (1993) subsequently observed that injection of anti-sense transfected C6 cells could cause regression of existing control C6 tumours and found lymphocytic infiltration at the sites of tumour regression. This suggested that an immune response was responsible for the death of tumour cells, and that the production of IGF-I may have acted to overcome such an immune response. Since these authors make no claim as to the effect of the anti-sense plasmids on *in vitro* growth, it is at least conceivable that the IGF-I produced by these cells was acting not as a growth factor, but simply as a mechanism of avoiding the immune response. A more likely explanation of these findings might be that the growth-promoting activity of IGF-I was allowing the tumour cells to overwhelm the response. The autocrine action of IGF-1 in these tumour cells may also be an indirect reflection of physiological events - there is some evidence to suggest that central nevous system precursors of glial cells may produce IGF-1 in their normal proliferation and differentiation during fetal ontogeny (Drago et al., 1991).

Human melanomas liberate a factor, termed MGSA (for "melanoma growth stimulating activty") which stimulates the growth of normal melanocytes and melanoma cells. Indeed this factor was purified and characterised from the supernatant of a melanoma line. (Richmond et al., 1988; Richmond and Thomas, 1988). In the case of one such tumour, changes in growth rate of the cells could be coorrelated with changes in the level of messenger RNA for the factor present. (Bordoni et al., 1989).

In addition to the data reviewed above, there is a further body of evidence from which one could infer that autostimulatory mechanisms might be involved in the development of malignant neoplasms. Several oncogenes have been described that act as constitutively active receptors for growth factors. The v-*erb-B* gene, for instance, encodes a truncated and activated epidermal growth factor receptor (Downward et al.,1984), and the v-*fms* gene is also constitutively activated by virtue of a mutation that distinguishes it from its cellular counterpart c-*fms* (Sher et al., 1985), the receptor for macrophage colony-stimulating factor (CSF-1). A constitutively activating mutation also makes oncogenic another growth factor receptor, the c-*kit* gene product (Geissler et al., 1988). Constitutively activated receptor signal transducers, such as mutated *ras* gene products, are also found widely distributed amongst spontaneous tumours (reviewed in Barbacid, 1987, and Hunter, 1991). These examples support the notion that the constitutive activity of a growth factor response pathway is a common mechanism in tumourigenesis.

With the exception of the *in vivo* work of Trojan et al., no instance of autostimulation in a solid tumour has been reported in which tumour cells have died as the result of growth factor antagonism. Eradication of malignancies in man generally requires destroying tumour cells - most tumours do not display any evidence of being immunogenic, and the occurence of spontaneous regression of tumours is so rare as to merit report in a journal in every instance where documentation is sufficient. Normal cells of the hemopoietic system, however, display the unique property of being absolutely dependent on growth factors for not only proliferation, but also survival. This is true of cells *in vivo* as well as *in vitro* (Crapper et al., 1984; Savill et al., 1989; Koury and Bondurant, 1990). When the growth factor is completely removed from culture, or not available to the cells *in vivo*, such cells die. Moreover, in contrast to cells of solid tumours, malignant cells of hemopoietic origin are commonly exposed to the circulation directly, rather than occuring in masses with some cells only poorly accessible from the circulation. For these reasons, hemopoietic tumours are more likely than solid tumours to be amenable to therapy with growth factor antagonists.

2) AUTOSTIMULATION IN HEMOPOIETIC NEOPLASIA

Hemopoietic precursors as well as mature cells are relatively easy to obtain from patients and experimental animals. In the right conditions, precursors can be made to differentiate *in vitro.* As a result, much is known about the factors that support the growth, differentiation and function of hemopoietic cells. Amongst the known factors are the granulocyte-macrophage, granulocyte, and macrophage colony stimulating factors (GM-CSF, G-CSF, and M-CSF or CSF-1), erythropoietin, interleukins (ILs) 1 to 13, interferons, and steel-factor (for a recent overview, see Schrader, 1992). There is also considerable evidence for the role of autostimulatory loops both in malignancies and the normal physiology of the hemopoietic system.

The first evidence of an autostimulatory loop in the pathogenesis of a leukemia was the discovery of a spontaneously factor-independent derivative of an interleukin-3 (IL-3) -dependent mouse cell line. In contrast to cells of human or even other rodent origins, mouse cells have a marked tendency to give rise to immortal clones of cells in culture. Such cell lines, if of hemopoietic origin, generally remain dependent on hemopoietic growth factors for survival and proliferation. In this instance, culture of a large number of cells in the absence of factor permitted the emergence of a single clone which grew autonomously. Whereas the parental IL-3-dependent cells died on injection into syngeneic mice in the absence of an artificial source of IL-3, the autonomous cells gave rise to leukemia in mice without an IL-3 source. *In vitro,* the autonomous cells grew more rapidly at higher cell density, suggesting the

presence of an autostimulatory mechanism. IL-3 was detectable in the supernatant of the cells in culture, and in the serum of the leukemic mice (Schrader and Crapper, 1983). Since these observations were made, numerous examples of autocrine loops in human tumours have been reported.

Interleukin-1 (IL-1), a cytokine present in many mammalian cells appears to be responsible for autostimulation of some freshly explanted adult T-cell leukemic cells (Shirakawa et al., 1989). When cultured at sufficiently low density for the effect to be observed, such cells proliferate in response to a factor present in their own conditioned medium. (At higher densities no effect was observed suggesting that the concentration of autostimulatory factor was then saturating with respect to the proliferative capacity of the cells.) A rabbit neutralising anti-serum to IL-1 specifically inhibited this response, although it did not completely inhibit proliferation of the cells. This autostimulatory loop may reflect a physiological phenomenon, since activated T-cells produce IL-1, and respond to IL-1 in conjunction with antigenic stimulation, by upregulating IL-2 and IL-2 receptor production, in the course of an immune response.

Several laboratories have reported that IL-1 is involved in the autostimulatory growth of Epstein-Barr Virus (EBV) -transformed cells. Though not tumourigenic in healthy hosts, EBV-infected cells, present in the circulation of more than 90% of the general population over 30, may give rise to B-cell leukemias in immunocompromised patients. When removed from the circulation and placed into culture, some EBV infected cells give rise to immortal "B-lymphoblastoid" cell lines, which continue to grow apparently independently of hemopoietic growth factors. Scala et al. (1987), Wakasugi et al. (1987), and Vandenabeele et al. (1988) have reported observations on three B-lymphoblastoid lines which produced a factor that promoted their own growth (again, at lower densities). These factors had physical and biological characteristics of IL-1, and in two of the three instances, neutralising antibody to IL-1 was able to inhibit, to some extent, the growth of the cells. Bertoglio et al. (1989) studied a further 12 B-lymphoblastoid lines, and found a strict correlation between the presence of IL-1 mRNA and IL-1 receptor, consistent with an autostimulatory role of the IL-1.

Many groups have attempted to account for the apparent factor-independence of EBV-tansformed cells. Interleukin 5, (IL-5), originally described as a growth and differentiation factor produced by T-cells and acting on eosinophils and B-cells (Takatsu et al., 1988 ; Sanderson et al., 1988), has been shown to be involved in an autostimulatory loop in some lymphoblastoid lines (Paul et al., 1990; Baumann and Paul, 1992). These workers demonstrated production and high-affinity binding of IL-5 by the EBV-transformed cells, and showed that neutralising antibodies to IL-5 partially inhibited growth of these cells. Interleukin 6 (IL-6), known for its B-cell growth promting activities (reviewed in

Kishimoto et al., 1992), was used by another group in transfection of EBV-transformed lines (Scala et al., 1990). Expression of IL-6 in these cells caused a striking increase in growth rate at very low cell densities, secretion of detectable IL-6 into the culture medium, and the development of tumours in immunocompromised mice from these cells, which were not tumourigenic in the absence of IL-6 expression.

IL-6 has come to prominence within the field of autostimulatory loops, as a growth factor involved in the pathogenesis of multiple myeloma, a tumour of plasma cell origin. Kawano et al (1988) reported an IL-6-dependent autostimulatory loop in cells freshly isolated from patients. They observed production of and response to IL-6, as well as some inhibition of *in vitro* growth with neutralising antibody to IL-6. However, the presence of contaminating marrow cells in these myeloma samples could not be excluded, thus admitting of the possibilty that the IL-6 detected in these samples was not produced by the myeloma cells themselves, but by such contaminating cells. Another group, (Klein et al., 1989) found that neither of two established human myeloma lines expressing receptors for IL-6 produced biologically detectable IL-6 or IL-6 mRNA. Moreover, anti-IL-6 antibody did not effect the growth of these cells. When these workers compared bone marrow samples of patients with fulminant multiple myeloma and control marrow samples, they found that stromal cells in myeloma patients, rather than the myeloma cells themselves were secreting IL-6, suggesting a possible paracrine growth loop. These workers also showed a correlation between *in vitro* responsivenes to IL-6, and *in vivo* proliferative status in 13 patients studied (Zhang et al., 1989). However, Schwab et al, using one of the same lines (nominally) used by Klein et al., were able to show secretion and response to IL-6, as well as the presence of IL-6 message. They also showed a 70% inhibition of the growth of the cells in the presence of a neutralising antibody to IL-6. The findings of several laboratories in relation to IL-6 and multiple myeloma have led to clinical trials (e.g. Klein et al., 1991) of neutralising monoclonal antibodies in myeloma patients, with some success in inhibition of myeloma growth *in vivo.*

Although no reports have described an activating mutation in the IL-6 gene in human multiple myeloma cells, Blankenstein et al. (1990) have described the insertion of an intracisternal A particle (IAP) in the IL-6 gene of a mouse myeloma-like tumour, the plasmacytoma MPC11. An IAP is a transposable DNA sequence with some features of the retroviral long terminal repeats (LTRs), and is capable of activating genes by insertion 5' of the coding sequence. The presence of this rearrangement supported the hypothesis that activation of IL-6 production was involved in the pathogenesis of the plasmacytoma. Additionally, plasmacytosis occurs in transgenic mice expressing IL-6 specifically in B-cells, and in the presence of an immortalising mutation (such as an activation of the c-*myc* gene), these mice develop plasmacytomas (Suematsu et al., 1989; and 1992).

The human immunodeficiency virus (HIV) has also provided an example of autostimulatory growth in tumour development. The HIV *tat* gene product has a growth-promoting role in Kaposi's sarcoma cells. These tumour cells produce IL-6 and the growth-promoting activity of the *tat* product can be specifically reversed, at least in part, by anti-sense oligonucleotides to IL-6 (Miles et al., 1990).

In view of the physiological autostimulatory loop mediated by IL-2 in T-cells (mentioned above), it is not surprising that such a mechanism appears to operate in some T-cell leukemias. Indeed one of the earliest reports suggesting an autostimulatory loop in hemopoietic neoplasms (Gootenberg et al., 1981), described cells of several human cutaneous T-cell lymphoma lines producing and responding to what was then known as "T-cell growth factor". Subsequently, Duprez et al. (1985) reported the establishment of a line from a T-cell lymphoma, which secreted and responded to human IL-2. The growth of this line was strictly density-dependent, and the cells prodcued very little IL-2 (0.6 units per ml in a 25 x concentrated cell-culture supernatant). Neutralising antibody to IL-2 was able to inhibit the growth of these cells by 90%. The same workers later showed that the autostimulatory loop in these cells could be blocked by Cyclosporin A, which blocked IL-2 transcription (Dautry-Varsat et al., 1988).

Physiological production of myeloid growth factors has been demonstrated for several myeloid cell types. Macrophage/monocyte lineage cells secrete GM-CSF, CSF-1 and G-CSF in response to inflammatory stimuli, and can respond to GM-CSF and CSF-1 (reviewed in Moore, 1991). Eosinophils and neutrophils can secrete GM-CSF and IL-3 when stimulated with other cytokines (Kita et al., 1991; Moqbel et al., 1991). Mast cells can be stimulated, by cross-linking of cell-surface antibodies with antigen, to produce IL-3, GM-CSF, IL-4, and IL-5 (Burd et al, 1989; Plaut et al, 1989; Wodnar-Fillipowicz et al., 1989; Razin et al., 1991), and can respond to IL-3 and IL-4. In all these situations, some form of ligand-mediated physiological stimulation of cell-surface receptors is required to induce secretion of growth factor. Such stimuli function through intracellular pathways that share common components and some components of these pathways are activated by oncogenic mutations. Activating mutations of the N-*ras gene,* for example, are found in many acute myeloid leukemias (Bos et al., 1987). Therefore, it is conceivable that oncogenic mutations may secondarily activate the production of potentially autostimulatory growth factors, which do not participate in the survival or proliferation of the tumour cells. This highlights the importance of studies using growth factor inhibition before drawing conclusions as to the role of autostimulatory factors in the genesis of tumours in cell types that display a variety of physiological autostimulatory mechanisms.

For many years, it was thought that culture of human acute and chronic myeloid leukemic (AML and CML) cells required the addition of exogenous growth factors (reviewed in Metcalf, 1989). Culture of AML blasts at high density, however, (e.g.over 10^6 per ml) permits growth of purified leukemic blast

populations in the absence of exogenous factor. Such density-dependence is consistent with the activity of an autostimulatory mechanism in these cells. Many workers have described the production, in blast-cell enriched populations of myeloid leukemias, of factors known to stimulate the growth of the corresponding normal cells (e.g.Young and Griffin, 1986; Young et al., 1987; Young et al, 1988; Sakai et al., 1987; Oster et al, 1989; Reilly et al., 1989; Freedman et al., 1992). Several of these reports simply described this finding of growth factor production, or of growth factor message, in freshly isolated cells, without attempting to establish the requirement for autostimulation in maintenance of growth and many reports were subject to the criticism that it was possible that the growth factor detected was present not in the leukemic cells themselves, but in contaminating normal cells present in the isolates. Nevertheless, it is clear that some populations enriched for leukemic cells produce GM-CSF *in vitro*, and that neutralising antibody to GM-CSF reduces the growth rate of such cells (Young and Griffin, 1986). Similarly, there is good evidence for the presence of IL-1 in such populations, and again, *in vitro* inhibition of IL-1 activity with antibodies, or more recently, with the naturally occurring IL-1 antagonist IL-1ra has slowed growth in cultured cells (Cozzolino et al., 1989; Rodriguez-Cimadevilla et al., 1990, Bradbury et al., 1990; Rambaldi et al., 1991; Estrov et al, 1992).

With respect to IL-1, at least, there is evidence from *in situ* hybridisation studies showing that mRNA for IL-1 is present in the leukemic blasts themselves (Nakamura et al., 1990). In the case of GM-CSF, however, there is some evidence that the growth factor is produced by non-proliferating progeny of the blasts present in leukemic isolates (Murohashi et al., 1989). This would be consistent with a paracrine rather than an autocrine mechanism, in which IL-1 stimulates production of GM-CSF in such populations, and evidence for this has been reported in human AML (Delwel et al., 1989; Estrov et al., 1992), and in a spontaneous mouse myelo-monocytic leukemia (Leslie et al., 1991).

The techniques used for isolating leukemic blasts themselves may induce the production of growth factors (Kaufman et al., 1988). These blasts represent cells at various stages of differentiation of the myeloid pathway, and it has not yet been determined what features of the apparent autostimulatory and paracrine loops are physiological in cells at these various differentiation stages. Although gene rearrangements, particularly translocations, are common in leukemic cells, there are no data to suggest that any such rearrangement has directly activated a growth factor gene. The inability to detect autostimulatory factors need not necessarily imply that they are functionally absent - very small amounts may suffice to induce proliferation (reviewed in Lang and Burgess, 1990, and Thomson, 1991; and the data presented here). For these reasons, it will be important in subsequent studies of leukemic (and normal) hemopoiesis to attempt, perhaps by sensitive *in situ* polymerase chain reaction (PCR) techniques to demonstrate the presence of cytokine production in cells that are well-characterised with respect to differentation and function.

With respect to CML, there is no good evidence to suggest the involvement of autostimulatory mechanisms in the pathogenesis of the disease. The hallmark of this disease is the presence of the Philadelphia chromosome (an abnormal chromosome 22 resulting from a balanced translocation between chrmosomes 14 and 22), This results in the formation of a protein-encoding gene fusion between the *bcr* (for *b*reakpoint *c*luster *r*egion) and *abl* genes. This latter is the cellular equivalent of the oncogene found in the Abelson murine leukemia virus. Although its precise function is unclear, the c-*abl* product is a tyrosine kinase thought to be involved in pathways of signal transduction from cell surface receptors. The fusion product function in a constitutively activated manner with respect to its tyrosine kinase activity. Thus, if a growth factor pathway were involved in leukemogenesis resulting from expression of a constitutively activated c-*abl* product, the mechanism would be more likely to involve short-circuiting the growth factor response, bypassing the need for ligand to bind to receptor, rather than inducing a truly autostimulatory loop. When the *bcr/abl* product was expressed in immortal murine factor-dependent myeloid cells, these cells acquired the ability to produce IL-3, and became independent of exogenous factor (Hariharan et al., 1988). This may simply reflect the action of the activated kinase in mimicking a physiological stimulus, which would normally induce IL-3 production. Moreover, others have reported similar experiments in which no autostimulatory factor production was detected (Laneuville et al., 1991). The relevance of hemopoietic growth factor production to the pathogenesis of CML has not been established, indeed one group has reported negative evidence with respect to autostimulatory factors in CML cells (Otsuka et al., 1991).

3) ANIMAL MODELS OF AUTOSTIMULATORY HEMOPOIETIC NEOPLASIA

Much work in autostimulatory neoplasia has been carried out in animal model systems. These have the great advantage that autostimulatory loops can be constructed so that the existence and requirement of autostimulation for proliferation of cells can be ascertained. These autostimulatory loops can be manipulated *in vitro* and the effects of such manipulations can be studied in cells returned to animals.

Amongst the earliest work with animal models of autostimulatory leukemia is that of Adkins et al. (1984). These workers were investigating transformation of normal chicken myeloid cells by oncogenic retroviruses. A virus carrying a single oncogene (either v-*myb* or v-*myc*) was able to immortalise primary cells, which still required a growth factor (chicken myelomonocytic growth factor, cMGF, a distant relative of IL-6 and G-CSF) for continued proliferation *in vitro*. When super-infected with a second virus carrying a *src*-family oncogene (a constitutively activated form of a signal-transducing tyrosine kinase), the cells started to produce cMGF, and became independent of exogenous factor. The subsequent growth of such cells could be inhibited, to some extent, by

neutralising anti-serum to cMGF. Graf et al. (1986) extended these results by showing that the v-*mil* oncogene product (also involved in signal-transduction pathways) would similarly induce v-*myc* -transformed cells to produce cMGF, and would cooperate with v-*myc* in forming leukemias *in vivo*.

Somewhat related are the observations of Baumbach et al. (1987) on tumours arising after infection of mice with a retrovirus containing the c-*myc* gene. All the tumours obtained were of the monocyte-macrophage lineage, and they showed various phenotypes with respect to production of GM-CSF and CSF-1. The growth of one of these tumours was partially inhibited by neutralising antibody to CSF-1. This tumour was shown to have a CSF-1 gene rearrangement, suggesting that this mutation, resulting in the aberrant expression of CSF-1, cooperated with the *myc* gene in the genesis of the tumour. Another interesting set of experiments relating to the idea of cooperative events in tumourigenesis were those of Andrejauskas and Moroni (1989), who transfected a mouse immortal IL-3-dependent line with a vector containing an activated v-H-*ras* gene under the control of an inducible promoter. In transfected cells, induction of expression of the activated *ras* gene correlated with loss of dependence on exogenous IL-3 and with production of IL-3 by the cells, a provocative observation in the light of the role of the *ras* gene products in many signal-transduction pathways that might be involved in the physiological induction of IL-3 production in reponse to appropriate stimuli.

Strong support was given to the notion that acquisition of an autostimulatory mechanism might be a frequent cooperative factor in the formation of tumours from otherwise immortal cells by the observations of Stocking et al. (1988). These workers produced 11 autonomous variants of a factor-dependent mouse cell line by random insertional mutagenesis with a retrovirus. Ten of the 11 clones produced one of the two growth factors to which the parental line responded (GM-CSF or IL-3), and in most cases these workers were able to demonstrate the insertion of the retrovirus in proximity to the GM-CSF or IL-3 genes, which induced production of the factors. Similarly, Duhrsen et al. (1990) observed that another mouse factor-dependent myeloid line, also responsive to both IL-3 and GM-CSF, spontaneously gave rise to tumours when injected into irradiated syngeneic mice. The cells of about one third of the tumours they isolated displayed production of either GM-CSF or IL-3, and in the majority of these cases it could be shown that this growth factor production was due to the insertion of an IAP in the proximity of the relevant gene.

When transfected with vectors that determine expression of the product of cDNAs of the relevant growth factors, mouse immortal factor-dependent cell lines are reproducibly converted to independence of exogenous growth factor. Such experiments have been performed by many workers, and in some cases, density-dependence and the ability of neutralising antibody to growth factor or receptor to inhibit growth has been examined.

Lang et al. (1985) conferred the ability to express GM-CSF on cells of a GM-CSF-dependent immortal line, and showed that the resultant cells were autonomous and tumourigenic. Although the amount of GM-CSF in the supernatant of these cells cultured at low density was too small to be able to support the growth of untransfected factor-dependent cells, the autostimulatory cells showed no density-dependence and were not inhibited by antibody to GM-CSF. These latter observations led the authors to suggest the possibility that the engagement of ligand and receptor took place intracellularly in these autostimulatory cells. (This suggestion and other evidence relating to it are dealt with in some detail in the discussion section of this portion of the thesis.) Laker et al. (1987), however, using similar expression vectors and the same cell-line, observed that GM-CSF-producing autostimulatory cells were indeed density-dependent, at least for some period following transfection. Neutralising anti-serum to GM-CSF significantly inhibited the growth of these cells, although cell death was not documented. These workers made the additional observation that such clones underwent a transition to a state in which their growth was no longer density-dependent, nor inhibitable by antibody. This transition occured more rapidly in clones initially secreting higher amounts of GM-CSF, although no explanation for this was apparent. Clearly, although an autostimulatory mechanism was involved in the generation of these cells, in the post-transition state they could not be shown to be autostimulatory, despite continued production of GM-CSF. These observations highlight the possibility of such progressions in the pathogenesis of spontaneous leukemias.

At least two groups have made IL-2 dependent models of autostimulatory growth. Taniguchi et al. (1987) using a retroviral vector and a human IL-2 cDNA, obtained clones that produced IL-2, from an IL-2-dependent cytotoxic T-cell line. These cells grew autonomously, but appeared no longer to respond to exogenous IL-2 (except for some inhibition of growth at higher concentrations of IL-2). These workers were able to achieve some inhibition of growth of such cells with a neutralising antibody to the IL-2 receptor. Cells of these clones were able to induce lymphomas in syngeneic mice. Karusayama et al., using a different vector system, and another IL-2 dependent line, obtained IL-2 producing clones that, while capable of growth without exogenous IL-2, were still responsive to exogenous IL-2. Moreover, these clones proliferated in a density-dependent manner, and their growth could be significantly, but not completely, inhibited by neutralising antibodies to IL-2 or to the IL-2 receptor. It is worth noting that this group used the mouse anti-human monoclonal antibody DMS-1 to apparently inhibit the action of mouse IL-2. The DMS-1 monoclonal antibody preparation generated by the present author (see results section) did not appear to be able to neutralise mouse IL-2 (data not shown). These authors also observed that all of the autostimulatory clones they had generated ,except the clone producing the least IL-2, were capable of inducing tumours in mice, and that the latency of tumour induction was inversely correlated with the level of IL-2 production by the clones *in vitro*.

Several groups have generated autostimulatory cells in mouse IL-3-dependent cell lines, (Hapel et al, 1986; Jirik et al., 1987; Wong et al., 1987). In these cases, autostimulatory clones were tumourigenic, but density-dependence and the effects of neutralising antibodies to IL-3 were not documented. Dunbar et al, (1989) performed an elegant set of experiments in an IL-3 dependent autostimulatory model, which are discussed later. Cells of an IL-5-dependent line spontaneously acquired the ability to produce IL-5 during culture, as a result of the activating insertion of an IAP 5' of the IL-5 gene. Proliferation of these autostimulatory cells was partially inhibited by a neutralising monoclonal antibody to IL-5 (Tohyama et al., 1990).

Some salient observations resulting from this considerable body of investigation in the field of autostimulatory hemopoietic tumourigenesis are as follows:

1. The weight of the evidence suggests that autostimulatory mechanisms are probably involved in the pathogenesis of acute myeloid leukemia, and may be involved in the development of other neoplasms.

2. In instances where attempts have been made to inhibit the growth of autostimulatory cells with antagonists of the growth factor or its receptor, investigators have either failed to see any inhibition, or obtained incomplete inhibition. In no instance has growth factor antagonism been reported to result in the death of the autostimulatory cells.

These observations have prompted the present investigation into the inhibition of autostimulatory growth with growth factor antagonists. The answer to the question of whether blocking the action of growth factor can lead to the death of the cells of an autostimulatory tumour has clear implications for the feasibility of developing growth factor antagonists as therapeutic agents in some forms of malignant neoplasia.

4) EXPERIMENTAL STRATEGY

In its most general form, the question that this project was designed to address can be stated simply as: can specific antagonism of the relevant growth factor result in the death of growth factor-dependent autostimulatory cells? Underlying this question is the hope that a positive answer might encourage the development of substances that could find clinical application in inhibiting the growth

of human tumours that have an autostimulatory component, with minimal non-specific toxicity. In choosing an experimental model, the following criteria were considered:

1) The model should be as near to clinical relevance as practicable. The cells should be of mammalian origin, preferably, and the autostimulatory cells should produce malignant tumours in animals.

2) The cells should for practical reasons be "immortal" *in vitro*, allowing repeated studies of cells of essentially homogeneous phenotype.

3) The growth factor requirements of the cells used should be well understood (in so far as it is possible to say this of any mammalian cells), and the required growth factor(s) should not be physiologically present in the circulation in concentrations that would support growth of these cells.

4) In order to facilitate the interpretation of results, the nature of the cells should be such that interference with the factor-receptor interaction to the extent of factor starvation would lead to cell death, rather than merely growth arrest, i.e. the cells should be factor-dependent rather than merely factor-responsive.

5) The proposed autostimulatory growth factor cDNA should be available, so that it would be possible, given an appropriate expression vector, to introduce this cDNA into the chosen cells and thereby render them autostimulatory. This would offer the advantage that one might be able to regulate the level of production of growth factor by using an inducible promoter, permitting one to address the question of whether varying the amount of factor produced would vary the resistance of the autostimulatory loop to antagonists.

6) The proposed autostimulatory growth factor should be one to which neutralising antagonists that function by interfering with the interaction between the factor and its cell-surface receptor would be readily available.

Of all mammalian cell types that have been studied and manipulated *in vitro*, those best conforming to criteria 1-4 are those of the hemopoietic system. Since almost all reports of putative pathological autostimulatory loops in hemopoietic neoplasias have concerned myeloid cells, the criterion of clinical relevance determined the choice of a myeloid cell line. Given the availability of cell lines, and the relative ease of manipulation of the animal in the laboratory, cells of murine origin were deemed to be

most suitable for these studies. At the time of initiating these studies, the only known specific hemopoietic growth factor antagonists were antibodies. In view of the size of these molecules, relative to most pharmacological therapeutic reagents, hemopoietic cells provided an ideal model, since they are, in general, readily accessible to substances present in the circulation.

The following subsidiary criteria were established to determine the system finally selected for study:

a) The cells should be responsive to more than one growth factor so that optimal controls for non-specific toxicity of antibody preparations could be performed. Thus, an antibody preparation which inhibited proliferation of the cells in the presence of growth factor A, could be shown to have no effect on the same cells in the presence of growth factor B at concentrations at which its inhibitory effect was specific.

b) The growth factor requirement of the cells should be such that one of the factors to which it would be responsive could be anatagonised by antibodies of a species in which one could produce tumours using the auto-stimulatory cells. In the assessment of the inhibition of tumour growth *in vivo*, one might otherwise expect significant interference with the activity of injected antibodies due to immune response to xenogeneic protein.

c) It would be desirable to select a system in which inhibitory antibodies to the receptor, as well as to the growth factor were available, as this would allow a second line of attack should antagonism by anti-factor antibody alone prove insufficient to prevent survival of autostimulatory cells.

A system was designed conforming to all these criteria, in which cells of the imortal cell line FD.C/2 were made autostimulatory using a human IL-2 cDNA. Le Gros et al. (1985) derived the IL-2 responsive clone, FD.C/2, from an IL-3-dependent murine myeloid cell line (FDC-P2, Dexter et al., 1980). The cells of this line can be grown in either IL-2 or IL-3 and human IL-2 is an effective ligand for the mouse IL-2 receptor (see, e.g., Taniguchi et al, 1986, and references therein). Hybridomas producing murine neutralising monoclonal antibodies to human IL-2 (DMS-1 and DMS-2, Smith et al., 1983), and rat neutralising monoclonal antibodies to the mouse IL-2 receptor (PC61, Lowenthal et al., 1985) were readily available. A human IL-2 (hIL-2) cDNA had been cloned and expressed, using a retroviral vector, in an immortal mouse IL-2-dependent T-cell line, rendering infected cells autostimulatory and tumourigenic (Yamada et al., 1987). Neither IL-2 nor IL-3 are present in the mouse circulation at levels sufficient to support the proliferation of physiologically responsive cells other than those in the immediate micro-vicinity of the source of these factors, except in conditions of

severe immunological stress such as hyperimmunisation or graft-versus host disease (Kuziel and Greene, 1991, Schrader, 1991). Thus, autostimulatory derivatives of FD.C/2 cells would most likely grow as tumours by an autostimulatory mechanism in mice of the strain of origin (DBA/2). Although this system would involve stimulation of myeloid cells with IL-2, which is traditionally thought of as a lymphoid-cell stimulatory factor (see e.g. Smith, 1988, Kuziel and Greene, 1991), several publications have reported data on the presence of IL-2 receptor molecules on myeloid cell lines (Le Gros et al., 1985, Koyasu et al., 1986, Le Gros et al., 1987) on monocytes (Hermann et al., 1985, Armitage et al., 1986, Rambaldi et al, 1987, Holter et al., 1987), and on the blasts of acute myeloid leukaemias (Hermann et al., 1985, Armitage et al., 1986, Yamamoto et al., 1986) by the time of commencement of this project. These data suggested at least the possibility that IL-2 might serve as an autostimulatory growth factor in some stage of the pathogenesis of myeloid leukaemias, increasing the clinical relevance of the proposed model system.

RESULTS

1. CREATION AND CHARACTERISATION OF AUTOSTIMULATORY CLONES

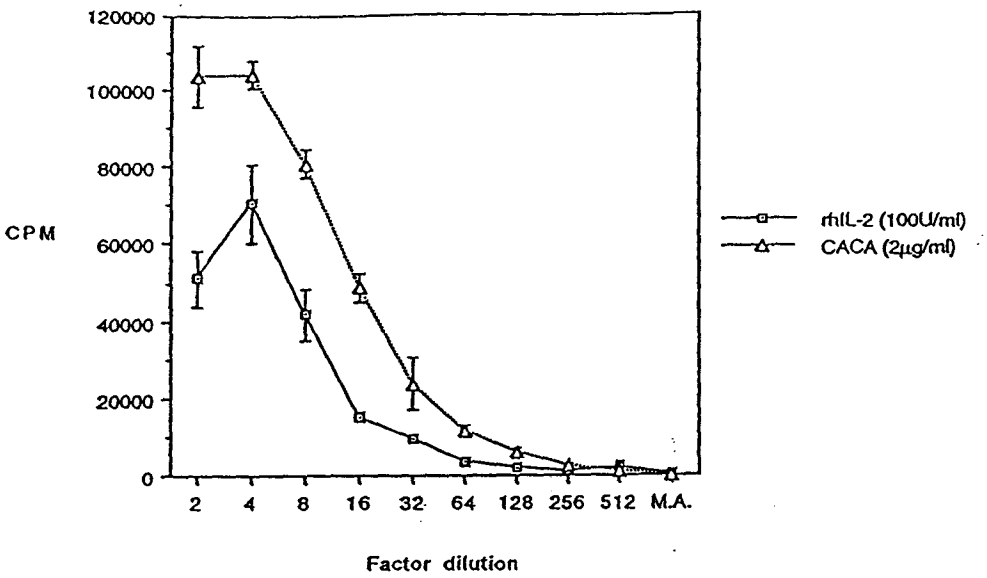
A) CHARACTERISTICS OF FD.C/2 CELLS.

i) IL-2 and IL-3 growth responses.

Prior to any attempt to create autostimulatory clones, the growth of FD.C/2 cells in response to various concentrations of IL-2 and IL-3 was determined. When cells were maintained in medium containing both IL-2 and IL-3, growth responses were as represented in figure 1.1. Ample responses to both IL-3 and IL-2 were observed, but the IL-3 response tended to be stronger than the IL-2 response, in terms of maximal thymidine incorporation. When cells were grown in IL-3 in the absence of IL-2 for 10 passages (50 days), the proliferative response to IL-2 was very poor in comparison to the response to IL-3 (fig. 1.2A). When such IL-3-passaged cells were washed free of IL-3 and replated in IL-2 alone, the cell population was observed to undergo a crisis such that there was a net decrease of viable cell numbers, with death of some 90% of the population over 2 weeks. Most of this death was apparent in the first 48 hours, followed by the outgrowth of the remaining cells. Cells that had been "converted" to IL-2-dependent growth in this manner showed similar maximal responses to IL-2 and IL-3 in ^{3}H-thymidine incorporation assays (fig. 1.2B) . Moreover such IL-2-passaged cells did not undergo a crisis when reconverted to IL-3-dependent growth, but continued to proliferate in the absence of IL-2 (but the presence of IL-3) without any evidence of cell death, or decline in proliferation rate. These results are consistent with the findings of Le Gros et al. (1985), who examined the conversion of the parent IL-3-dependent cells (FD.C/1) into FD.C/2 cells. These authors were unable to detect the p55 chain of the IL-2 receptor on the surface of the FD.C/1 cells, but after the conversion crisis, the resulting growing cells, which they designated FD.C/2, displayed "readily detectable" levels of p55 as determined by FACS analysis. Furthermore, addition of IL-3 to these FD.C/2 cells growing in the presence of IL-2 did not affect levels of cell-surface p55, whereas withdrawal of IL-2 and replacement with IL-3, while allowing continued growth, resulted in a 50% decline of cell-surface p55 within 20 hours (ibid.).

Very high concentrations of murine IL-4 permitted the survival of some FD.C/2 cells for a few days, yielding a ^{3}H-thymidine-incorporation curve as seen in figure 1.1, part B. The cells could not, however, be maintained in passage medium containing IL-4 alone.

A



B

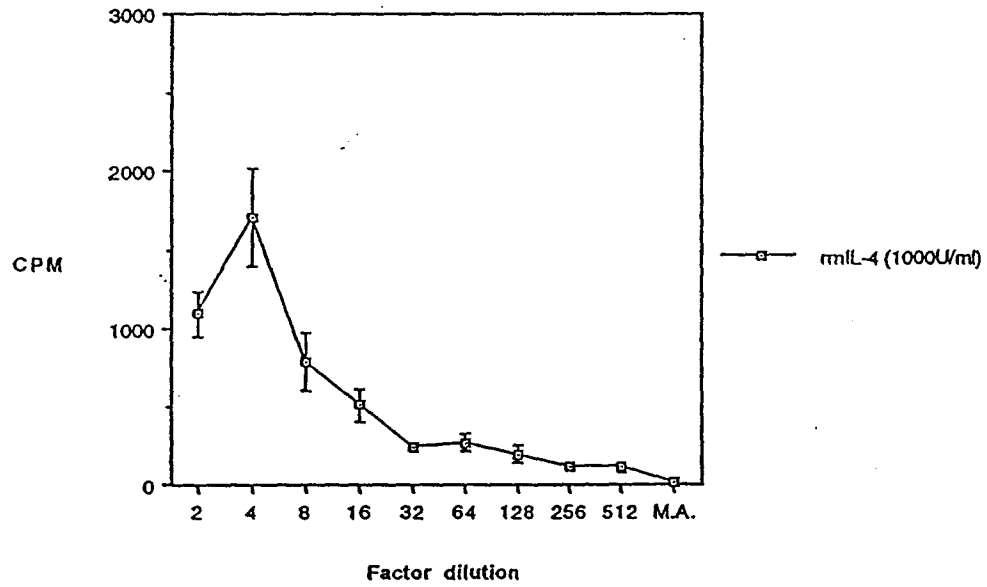
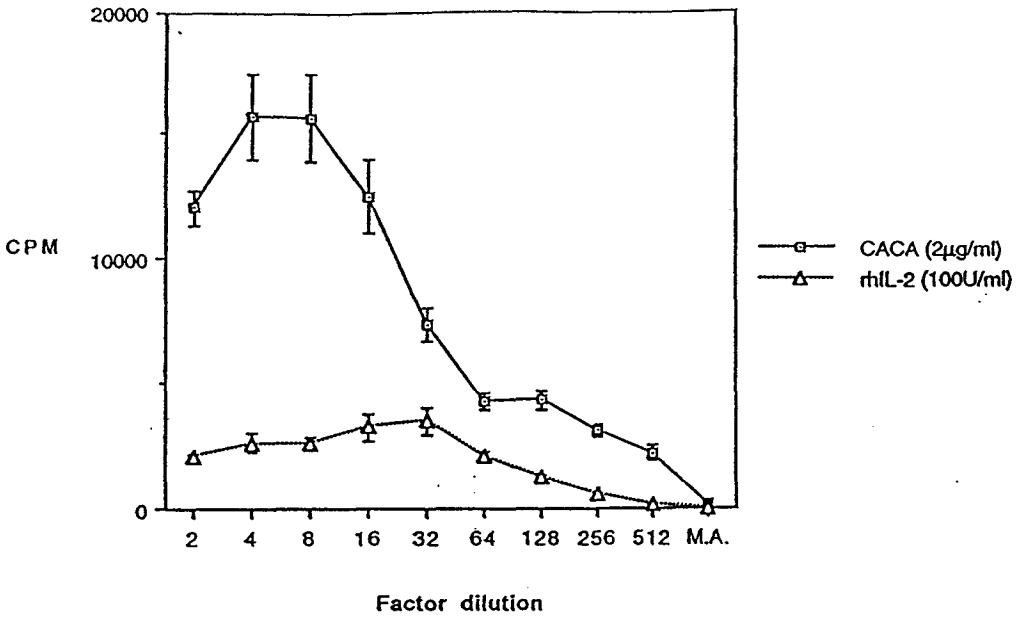


Fig. 1.1. Factor responses of FD.C/2 cells maintained in IL-2 and IL-3 A) FD.C/2 cells that had been passaged in a mixture of IL-2 and IL-3 were plated at 500 cells per 10 µl per well in Terasaki HLA microtiter plates on titrations of IL-3 (CACA - a synthetic murine IL-3 kindly provided by Dr. Ian Clark-Lewis of the Biomedical Research Centre) or IL-2 (recombinant human IL-2). After 48 hours of incubation, wells were pulsed with ^{3}H-thymidine and incubation continued a further 12 hours before harvesting. B) FD.C/2 cells that had been passaged in a mixture of IL-2 and IL-3 were assayed for proliferative response to mouse IL-4. 48 hour incubation followed by 12 hour ^{3}H-thymidine pulse. The concentrations of factor indicated in the legend represent the final concentration in the first well of the titration. The units given are as calculated from the manufacturers specifications (see Appendix 1). Error bars in this and all subsequent ^{3}H-thymidine assays show standard error of the mean of triplicates. M.A. indicates medium alone - i.e. without growth-factor.

A



B

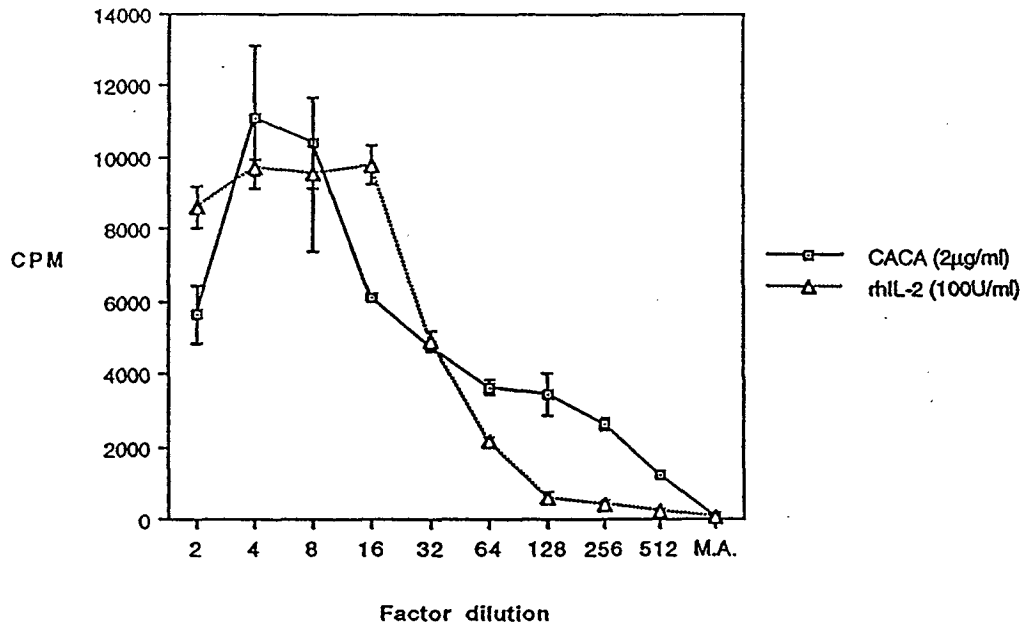


Fig. 1.2. Factor responses of FD.C/2 cells maintained in IL-2 *or* IL-3 A) FD.C/2 cells that had been passaged in IL-3 were assayed for response to IL-2 and IL-3 as in figure 1.1. After 36 hours of cultivation, wells were pulsed with ^{3}H-thymidine and incubation continued a further 6 hours before harvesting. B) FD.C/2 cells that had been passaged in IL-2 were assayed for response to IL-2 and IL-3.

In the absence of exogenous IL-2 or IL-3, FD.C/2 cells were not only unable to incorporate ^{3}H-thymidine (M.A. in figure 1.1), but died within 24 hours of withdrawal of growth factor. This conclusion was supported by phase-contrast microscopic inspection of proliferation assays such as that shown in figure 1.1, and by a separate experiment in which cells were washed free of factor-containing passage medium, and replated in 5 ml cultures in medium without factor at 10^5 cells/ml. After 24 hours, these cultures were harvested by centrifugation and the cells resuspended in 10 µl of medium. Ten µl of eosin (2 cultures) or trypan blue (2 cultures) were added and the suspensions were examined by light microscopy. In no instance were dye-excluding cells seen, confirming the absolute dependence on FD.C/2 cells for exogenous growth factor for maintenance of viability. FD.C/2 cells did not demonstrate detectable responses to GM-CSF or IL-6.

While the FD.C/2 cells grow in "suspension", i.e. do not adhere to tissue culture dishes, their density and physical characteristics are such that they will roll or otherwise accumulate under the influence of gravity at the lowest point in a culture well or plate, and proliferation from a single cell at the edge of an undisturbed culture vessel will give rise to a "carpet" of adjacent cells. These characteristics facilitated assays involving assessment of growth in 96-well culture plates, and cloning procedures, described below.

ii) Determination of G418 resistance of FD.C/2 cells.

Preliminary experiments to determine the level of G418 to be used in selection of transfectants were designed as follows: 2×10^3 cells were plated in 1 ml of medium containing IL-2 in the wells of a 24 well culture plate. G418 was added to the wells so as to achieve final concentrations ranging from 0 to 2000 µg per ml in increments of 200 µg/ml. The cells were observed daily with a phase-contrast microscope. At concentrations of 1000 µg/ml and above, there was no apparent proliferation and all cells appeared dead by 48 hours. At concentrations of 400 µg/ml and below, surviving cells were apparent at up to 4 days of culture. By day 6, however, all cells appeared dead at 400 µg/ml. On the basis of this titration, it was decided that 500 µg/ml G418 be used for subsequent selection of transfectants.

B) ISOLATION OF TRANSFECTED CLONES.

The Ψ2-3.1 cell line is a mouse fibroblastoid cell line that produces infectious but packaging-defective retrovirus, which contains hIL-2 and neomycin-resistance sequence, from the transposon Tn5 (fig.

1.3, Yamada et al., 1987). Cells infected with this virus can be selected in the neomycin analogue G418, and the expression of G418-resistance and of hIL-2 are under the control of Moloney leukemia virus LTR in such cells. Ψ2-3.1 cells were cocultivated with FD.C/2 cells in tissue-culture treated Petri dishes in conditions such that a given dish might be left for 5 days without overgrowth of either cell population. 2 x 10^5 cells of the Ψ2-3.1 line were seeded onto dishes, and allowed to adhere overnight. The following day, dishes were gently agitated by hand, and the medium was removed along with any dead or otherwise non-adherent cells. The medium was replaced with medium containing mIL-2, in which were suspended 4 x 10^5 FD.C/2 cells. After 5 days, the non-adherent cells were aspirated, washed by centrifugation twice, and plated at a density of 50 cells/ml in soft agar with medium containing mIL-2 and G418 at 500 μg/ml. Colony growth was observed over the next 10 - 14 days, and 8 individual colonies were plucked with a pulled Pasteur pipette, and plated into individual wells of a 24-well plate, each containing 1 ml of mIL-2 medium and G418 at 500μg/ml.

Cells were allowed to grow out of the agar into the liquid medium, and when the cells formed a carpet of over half the floor of the well, the contents of each well were gently resuspended and 500 μl of the contents of each well were transferred to an empty well, and 500μl of medium containing G418, but no IL-2, was added to these fresh wells. This process of weaning from exogenous IL-2 in the presence of G418, was continued until, between two and three weeks after the initial plucking of colonies, the contents of a well were resuspended and washed free of any remaining exogenous IL-2 that might have been carried over from the original plating, and replated in fresh medium containing G418, but no IL-2, at a density of 2 x 10^5 cells per ml. This high density was selected on the basis of the density tolerated by parental cells in super-saturating levels of IL-2, and in the expectation that the clones would show density-dependent growth (see Holter et al., 1987, and section D, below). Of the 8 colonies initially plucked, 5 survived this weaning process, the remaining 3 dying within the first week of weaning. The cells of these surviving populations resembled the parental FD.C/2 cells morphologically (under phase-contrast microscopy), and showed no tendency to adhere to tissue-culture plastics. These 5 clones were designated FD.C/2Ψ.1 through Ψ.5.

After this weaning process, cells were passaged in medium without IL-2 by 1:1 dilution every second or third day, following inspection of the plates. While such passages were routinely performed in the absence of G418, a succession of three passages in the presence of 500 μg/ml G418 were performed approximately once every two months, with little or no apparent cell death attributable to the presence of G418.

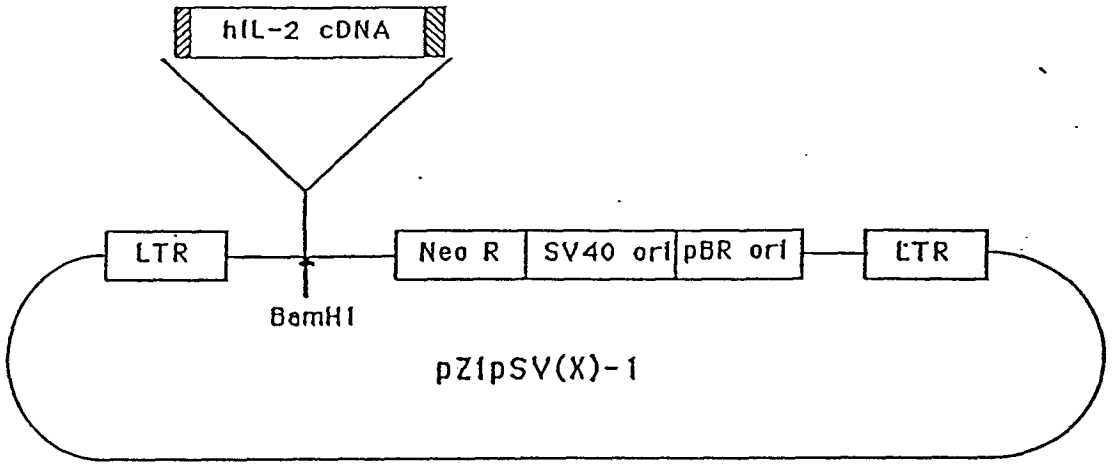


Fig. 1.3. The proviral construct used to make Ψ2-3.1 cells. LTR - Moloney Murine Leukaemia Virus LTR, NeoR - Neomycin resistance gene from Tn5, SV40 ori - origin of replication of SV40 virus, pBR ori - bacterial origin of replication.

C) GROWTH CHARACTERISTICS OF AUTOSTIMULATORY CLONES.

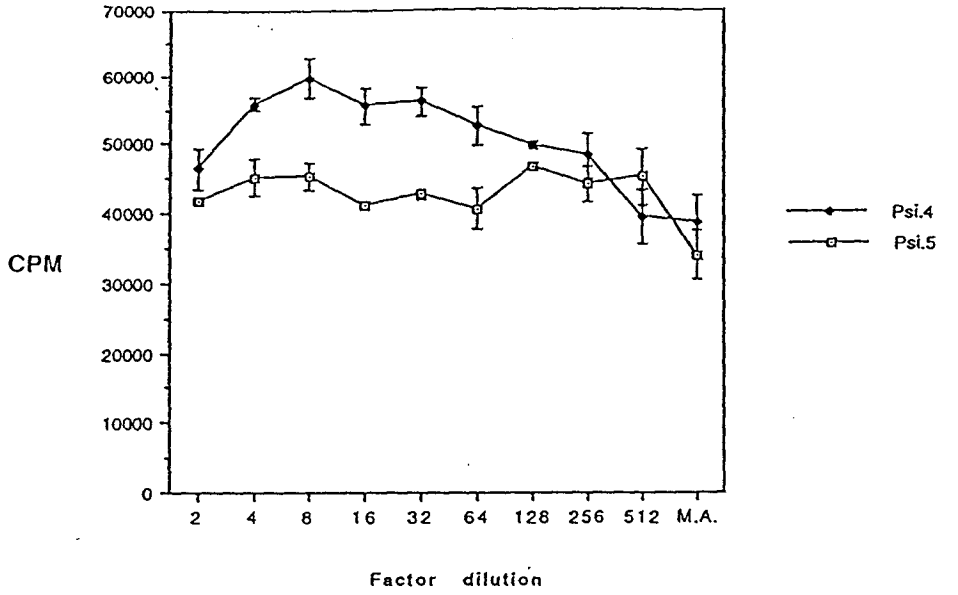
i) Confirmation of FD.C/2 derivation of FD.C/2Ψ cells - response to exogenous growth factors.

The morphology of the resultant cloned cells, and the process in which they were created (since non-transformed fibroblasts, such as the Ψ2-3.1 cells, are unable to grow in agar), made it unlikely that the clones were of other than FD.C/2 origin. Assays of the response of these clones to exogenous IL-2 and IL-3 were performed to confirm that these cells were FD.C/2 derived and that IL-2 and IL-3 responsiveness were maintained, despite growth in the absence of exogenous IL-2 or IL-3. Initial experiments using the same cell-density as used in bio-assays of parental FD.C/2 cells (500 cells in 10μl per well of a Terasaki microtiter tray), while revealing some apparent response to IL-2 and IL-3, showed that such response might be largely concealed by the degree of proliferation evident in the absence of added factors (fig. 1.4). The bio-assays were repeated at lower cell-densities (200 cells/10μl/well) , and over a broader range of IL-2 and IL-3 doses to increase the likelihood of covering the range of concentrations at which an effect would be apparent. The results showed that all 5 lines were responsive to exogenous IL-2 and IL-3 (fig. 1.5). A comparison with IL-2-passaged parental FD.C/2 cells of the responsiveness to IL-2 and IL-3 was undertaken. The results of this comparison in one clone are shown in figure 1.6. Interestingly, while responses to exogenous IL-3 were qualitatively similar, the shape of the IL-2 response curve of the FD.C/2Ψ.1 cells was very different from that of the parental FD.C/2 cells - significantly less IL-2 being needed to achieve maximal growth stimulation of the Ψ.1 cells. Figures 1.4, 1.5 and 1.6 show that the FD.C/2Ψ cells are capable of incorporating ^{3}H-thymidine in the absence of any exogenous factor.

ii) Density dependence of FD.C/2Ψ clones.

Although proliferation continued in the absence of exogenous IL-2 for many passages (over 100 for some of the clones), a few dead cells (up to 5% of the total cell number) were often observed at the time of passage. When populations were depleted of the majority of dead cells, by Ficoll gradient centrifugation, it was possible to maintain a higher level of viability (> 98% viable cells, as judged by eosin uptake) by passaging daily on a 2:1 dilution basis (2 volumes of existing culture to 1 volume of fresh medium). Parental cells maintained in IL-2 (or IL-3, or IL-3 + IL-2) could be maintained at > 99.5% viability when cell density was kept low enough to keep cells in log-phase growth (below approximately 5×10^5 cells/ml). Allowing cells of autostimulatory clones to fall to lower densities than those produced by the above passage conditions (i.e. to below approximately 1×10^4 cells per ml), resulted in decreased viability of these cell populations.

A



B

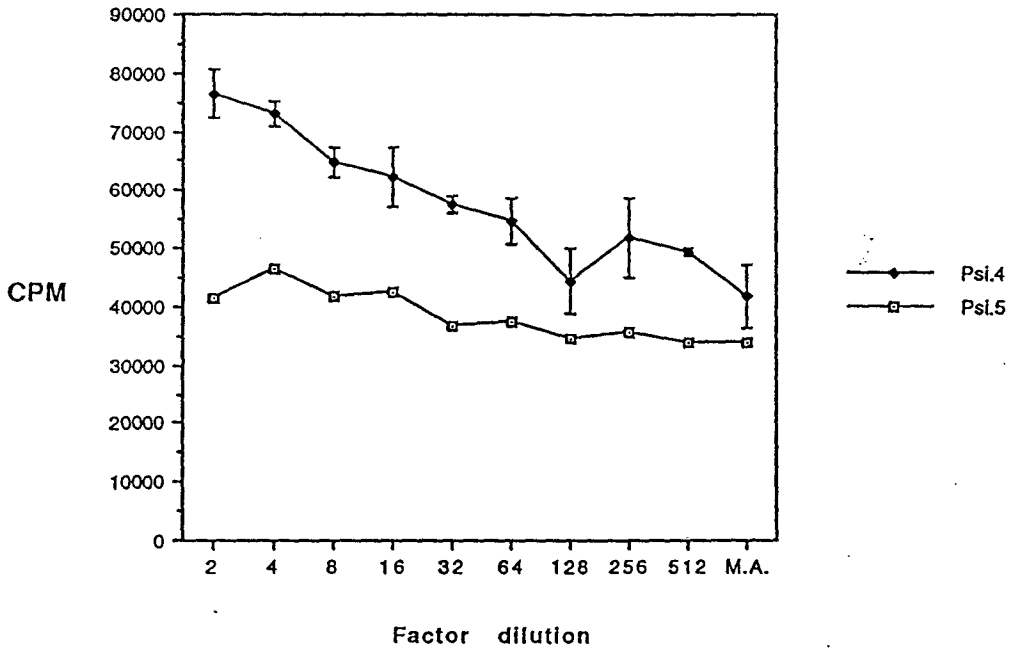
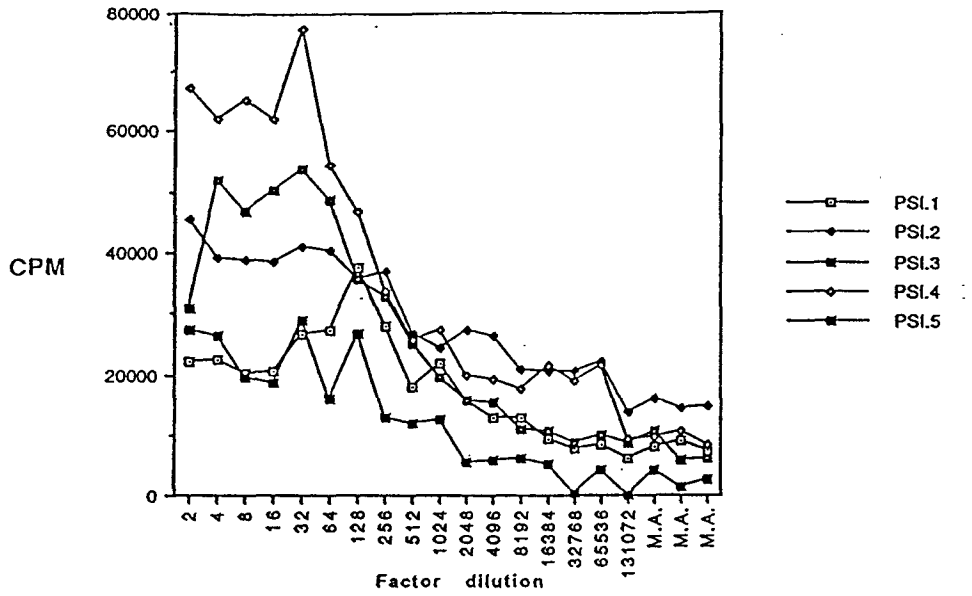


Fig. 1.4. FD.C/2Ψ cells showed some response to IL-2 and IL-3 Cells of two of the FD.C/2Ψ clones (Psi.4 and Psi.5) were assayed for responsiveness to exogenous IL-2 (A) and IL-3 (B). 500 cells per well. 48 hours incubation followed by 12 hour ^{3}H-thymidine pulse. The growth-factors were used in the form of supernatants and the highest concentrations were above the level required to give optimal responses in the FD.C/2 line (and other IL-2 responsive cells). In this and subsequent figures where units of activity are not given, growth-factor has been applied from sources without previous calculation of the units of activity, since interpretations of the results shown rely on internal comparisons (see Appendix 1).

A



B

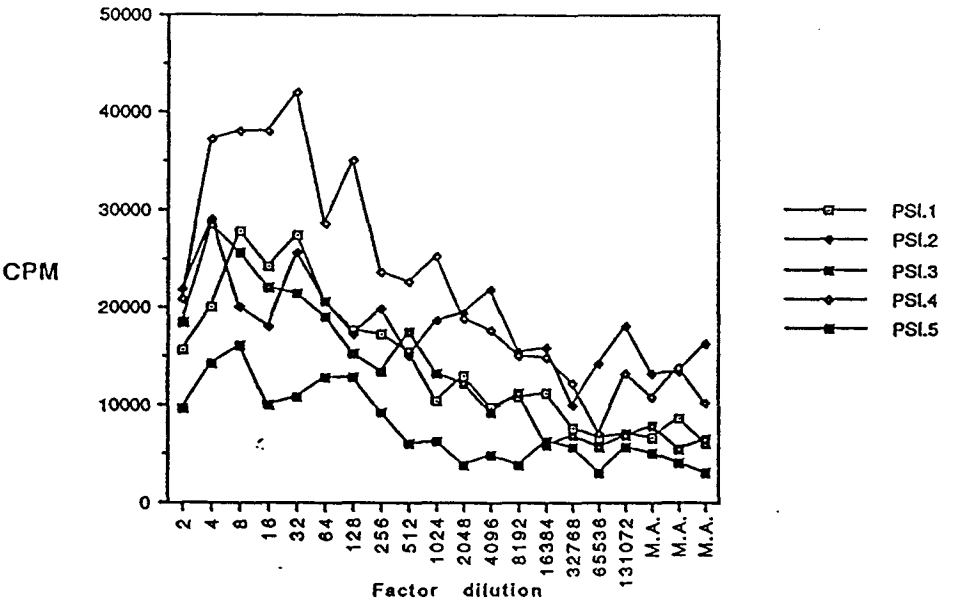
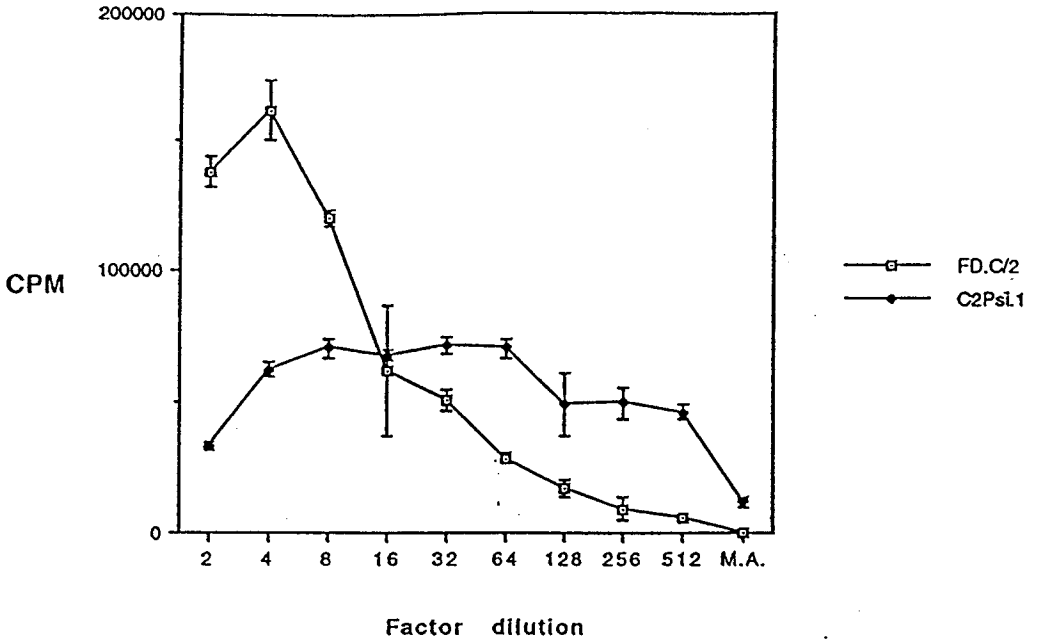


Fig. 1.5. Response of FD.C/2Ψ cells to IL-2 and IL-3 was more obvious at lower initial cell density
Cells of the FD.C/2Ψ lines were assayed for responsivenes to exogenous IL-2 (A) and IL-3 (B). 200 cells per well. 48 hours incubation followed by 12 hour ^{3}H-thymidine pulse. The C/2Ψ clones are referred to as simply "Psi.1", etc., in the legened.

A



B

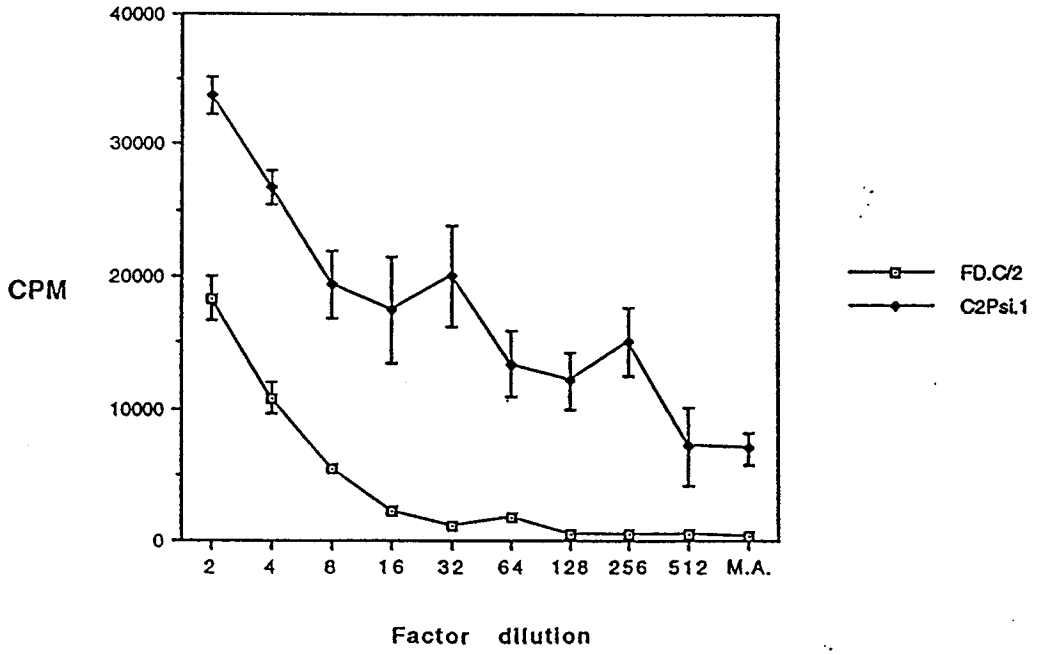


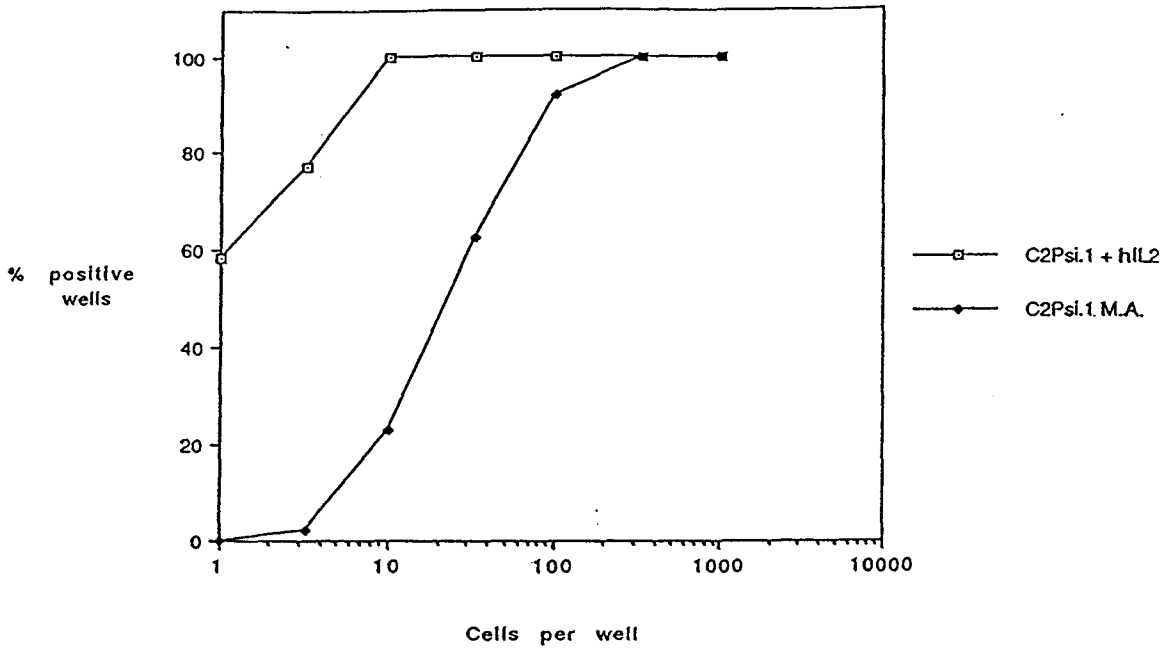
Fig. 1.6. Comparison of responses of FD.C/2Ψ.1 cells with those of the parental FD.C/2 cells A) IL-2 responses. B) IL-3 responses. Both cell lines were plated at 500 cells per well. 48 hour incubation plus 8 hour ^{3}H-thymidine pulse. In the legend the letter "Ψ" is replaced by "Psi".

This apparent density-dependence of FD.C/2Ψ cells was confirmed by the following experiment. Passaged cells were washed by centrifugation and divided into medium alone or medium containing exogenous IL-2, at various cell densities. The cells were then plated out into the wells of 96-well flat-bottomed tissue-culture plates in 150µl of medium per well, and cultured for 6 weeks. Cultures were inspected once a week, and wells scored for viable cells by phase-contrast microscopy. At higher densities, viability of wells' contents was not determined by presence of live cells at the end of the 6 week culture period, since in many cases cells in such wells had overgrown the culture medium, but by the presence of obvious proliferation at earlier time points. The results of these assays for 2 of the FD.C/2Ψ clones are shown in figure 1.7. Although the clones differed in their ability to support their own growth at different densities in these conditions, they all displayed a density-dependence that was abrogated by the presence of exogenous IL-2. In the presence of exogenous IL-2, the curves of percent growth-positive wells versus cell number plated per well resembled the curve constructed to assess the plating efficiency of the parental FD.C/2 cells in exogenous IL-2 (fig. 1.8).

iii) Production of growth factors by FD.C/2Ψ cells.

The bio-assays used to assess production of cytokines by FD.C/2Ψ cells involve adding 5 µl of assay sample to 5 µl of target cell suspension, so the addition of spent medium (such as that harvested from the FD.C/2Ψ clones) to a final concentration of 50% of the contents of the first well in the proliferation assay titration, could be expected to mask the effect of small amounts of growth factor activity, due to the relative depletion of essential nutrients in the medium of this first well. Earlier observations of the passaging of the FD.C/2Ψ cells (see section ii above) suggested that they might produce only small amounts of IL-2 activity, and this was confirmed by preliminary experiments in which no IL-2 bioactivity was detected in the unconcentrated supernatants of some clones. For these reasons, assays of factors produced by cells were routinely carried out on 10-fold concentrated culture supernatants. Cells were harvested from passage, washed 4 times in medium without growth factor, resuspended at 2×10^5 cells/ml, and plated in 10 ml cultures in the absence of exogenous IL-2 or IL-3. After 3 days, the medium was harvested and concentrated 10-fold by Amicon ultrafiltration using a filter with a molecular weight cut-off of 5000 M_r prior to performance of the bio-assays.

As shown in figure 1.9, FD.C/2Ψ cells liberated a small but detectable amount of IL-2 into their culture medium. These assays were performed on the T-cell line HT-2, which responds to IL-2 and IL-4 but to no other known cytokine (Hermann et al., 1985, and see Appendix 1). This cell line has been widely used to assay IL-2 activity, and was found to be somewhat more sensitive to IL-2 than the FD.C/2 cells themselves (see Appendix 1).



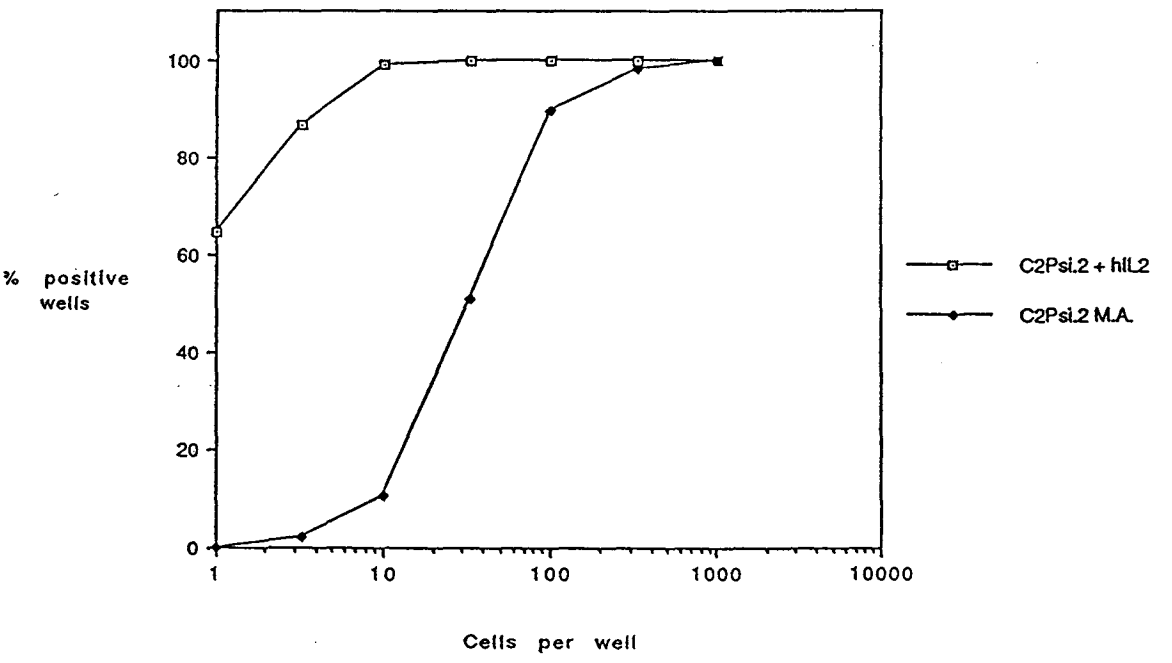


Fig. 1.7. Density-dependence curves for 2 FD.C/2Ψ clones.

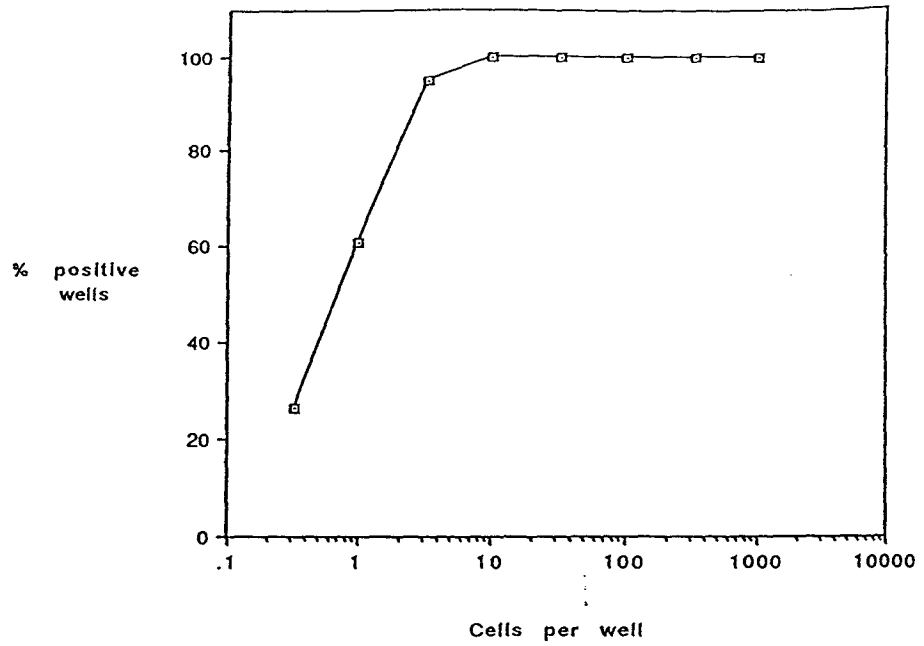
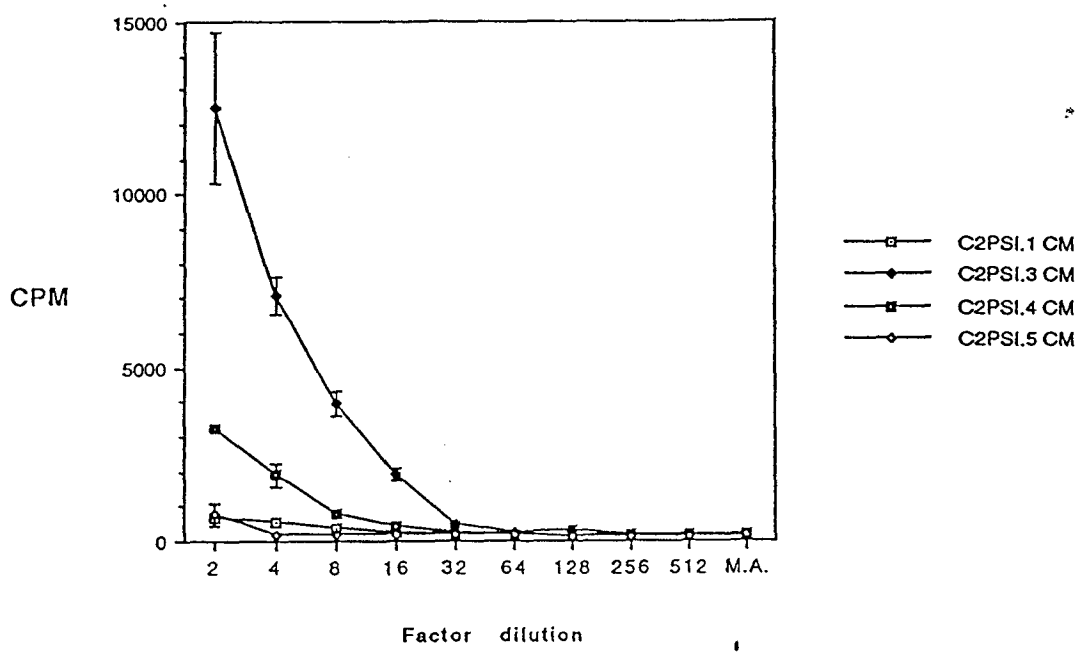


Fig. 1.8. Plating efficiency of FD.C/2 cells Plating efficiency was determined in the presence of saturating amounts of IL-2 as described in the text. The data shown for 0.33 cells per well and one cell per well represent the averages of per cent positive wells in 4 separate plates (384 wells) in each case.

A



B

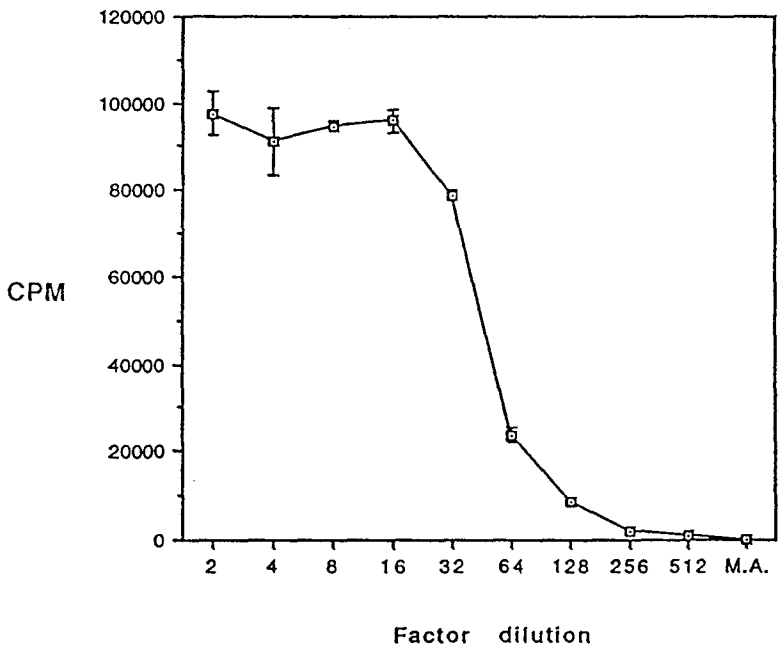


Fig. 1.9. IL-2 activity in the supernatants of FD.C/2Ψ cells A) 10-fold concentrated conditioned media (CM) of FD.C/2Ψ cells were assayed on HT-2 cells in the presence of the neutralising anti-mIL-4 antibody, 11B11. 1000 cells per well; 36 hour incubation followed by 12 hour ^{3}H-thymidine pulse. B) Assay of responsiveness of HT-2 cells to IL-2, performed in parallel with the assays shown in part A. In the legend the letter "Ψ" is replaced by "Psi".

To exclude the possibility that the result seen was due to the presence of IL-4 in the supernatants, the assay was performed in the presence of a specifically inhibitory concentration of anti-mIL-4 monoclonal antibody 11B11 (see Appendix 2). The stimulation of HT-2 cells by these supernatants was no greater in the absence of this antibody and the signals seen on HT-2 cells were completely abolished by specifically inhibitory amounts of the anti-hIL-2 antibody DMS-1 (Smith et al., 1983, and Appendix 2), suggesting that the FD.C/2Ψ cells did not liberate any IL-4 into their culture supernatants. The supernatants of FD.C/2Ψ cells were also able to stimulate the growth of parental FD.C/2 cells, as shown in figure1.10.

The retroviral construct which transferred the hIL-2 cDNA into the FD.C/2Ψ cells, was so designed that the production of hIL-2 and the neomycin-resistance enzyme would occur as a result of alternate splicing of a single mRNA transcribed from the same (5' LTR) promoter. As the factors controlling this splicing are not well understood, and in particular, were not controllable or predictable within the FD.C/2Ψ cells, it was expected that there might be variation with time, and between individual cells of clones, as to the amount of hIL-2 produced. Additionally, temporary differences in IL-2 receptor and IL-2 consumption levels would affect the amount of IL-2 detectable in supernatants. These predictions were confirmed in experiments in which supernatants were again collected from three of the cell lines some six months after the initial collections, similarly concentrated, and again subjected to assay on HT-2 cells in the presence of 11B11 antibody (fig. 1.11). While the amounts of IL-2 activity were again small for all three clones, the relative levels of supernatant IL-2 activity produced by the three clones were found to be different from those observed in the initial assessments.

Since the parental FD.C/2 cells were known to respond to IL-3, the (first set of) FD.C/2Ψ culture supernatants were assayed for the presence of IL-3. In this instance, the culture supernatants were assayed on R6X cells (Schrader et al., 1983, see Appendix 1). A representative result is shown in figure 1.12. The apparent presence of IL-3 activity in the supernatant is confirmed by the abolition of the signal by a specifically inhibitory concentration of a rabbit anti-mIL-3 anti-serum, Rab7 (kindly donated by Dr. Hermann Ziltener, Biomedical Research Centre, see Appendix 2). The supernatants were also assayed on the FDC-P1 cell line (Dexter et al., 1980, see Appendix 1) which is known to respond to mIL-3 and to GM-CSF. Surprisingly, the activity from the supernatant detected with FDC-P1 cells was only partly abolished by Rab7 antibody, suggesting that the supernatant might also contain GM-CSF activity (fig.1.13). Accordingly, specifically inhibitory concentrations of a rabbit anti-mGM-CSF anti-serum, Rab39 (kindly donated by Dr. Hermann Ziltener, see Appendix 2), were also able to partially abolish the signal, confirming the presence of GM-CSF in the supernatant (fig. 1.14).

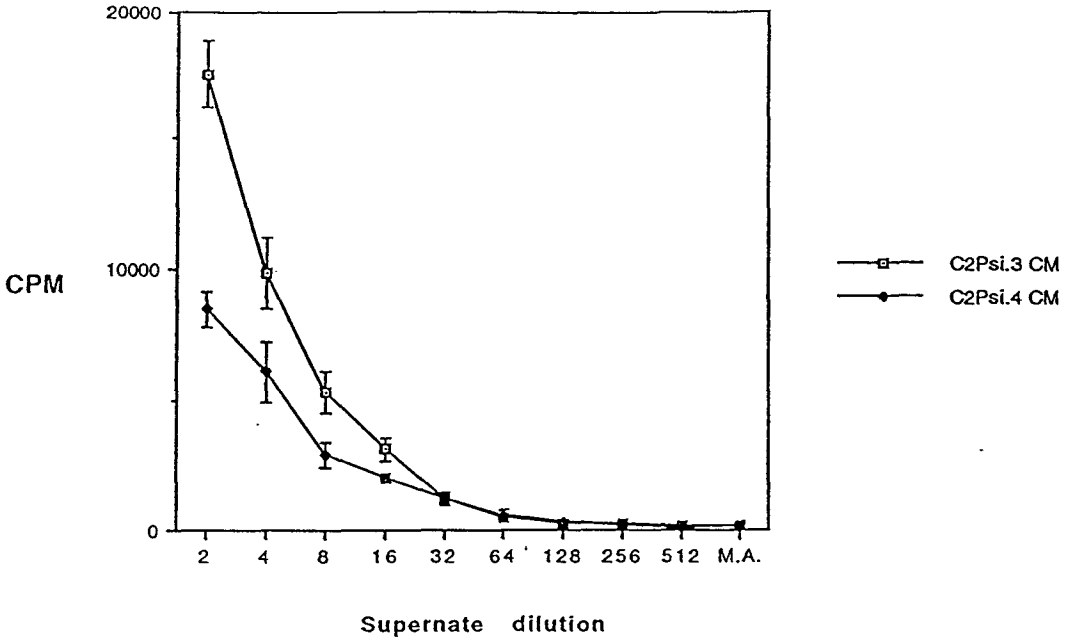


Fig. 1.10. Response of FD.C/2 cells to FD.C/2Ψ cell supernatants. 10-fold concentrated conditioned media of 2 FD.C/2Ψ clones were assayed on FD.C/2 cells. 500 cells per well. 48 hour incubation plus 12 hour ^{3}H-thymidine pulse.

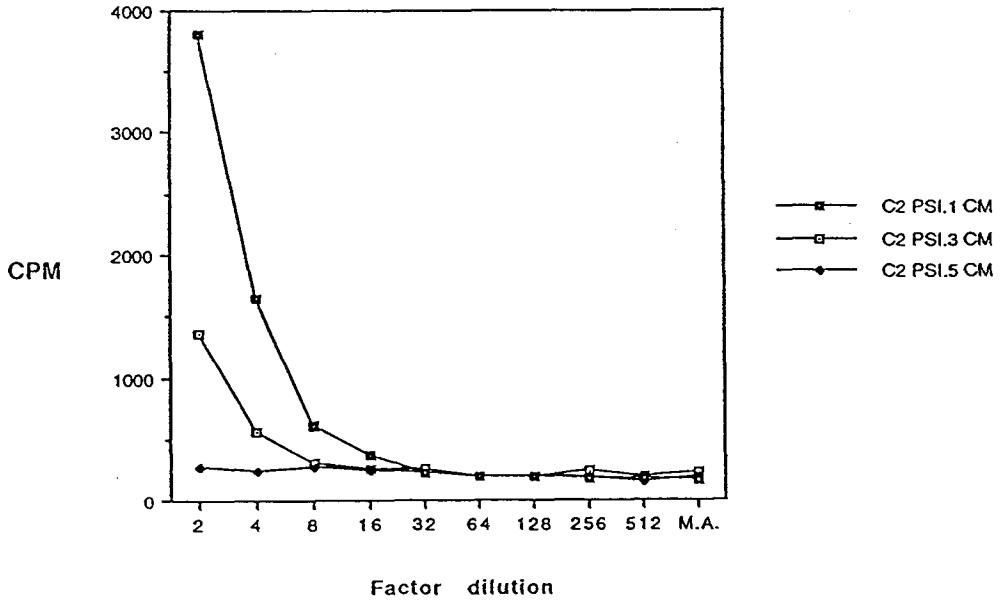


Fig. 1.11. Re-assay of FD.C/2Ψ supernatants 10-fold concentrated conditioned media of FD.C/2Ψ cells, from a separate collection to those used in figures 1.9 and 1.10, were again assayed on HT-2 cells in the presence of the neutralising anti-mIL-4 antibody, 11B11. 1000 cells per well; 36 hour incubation followed by 8 hour ^{3}H-thymidine pulse.

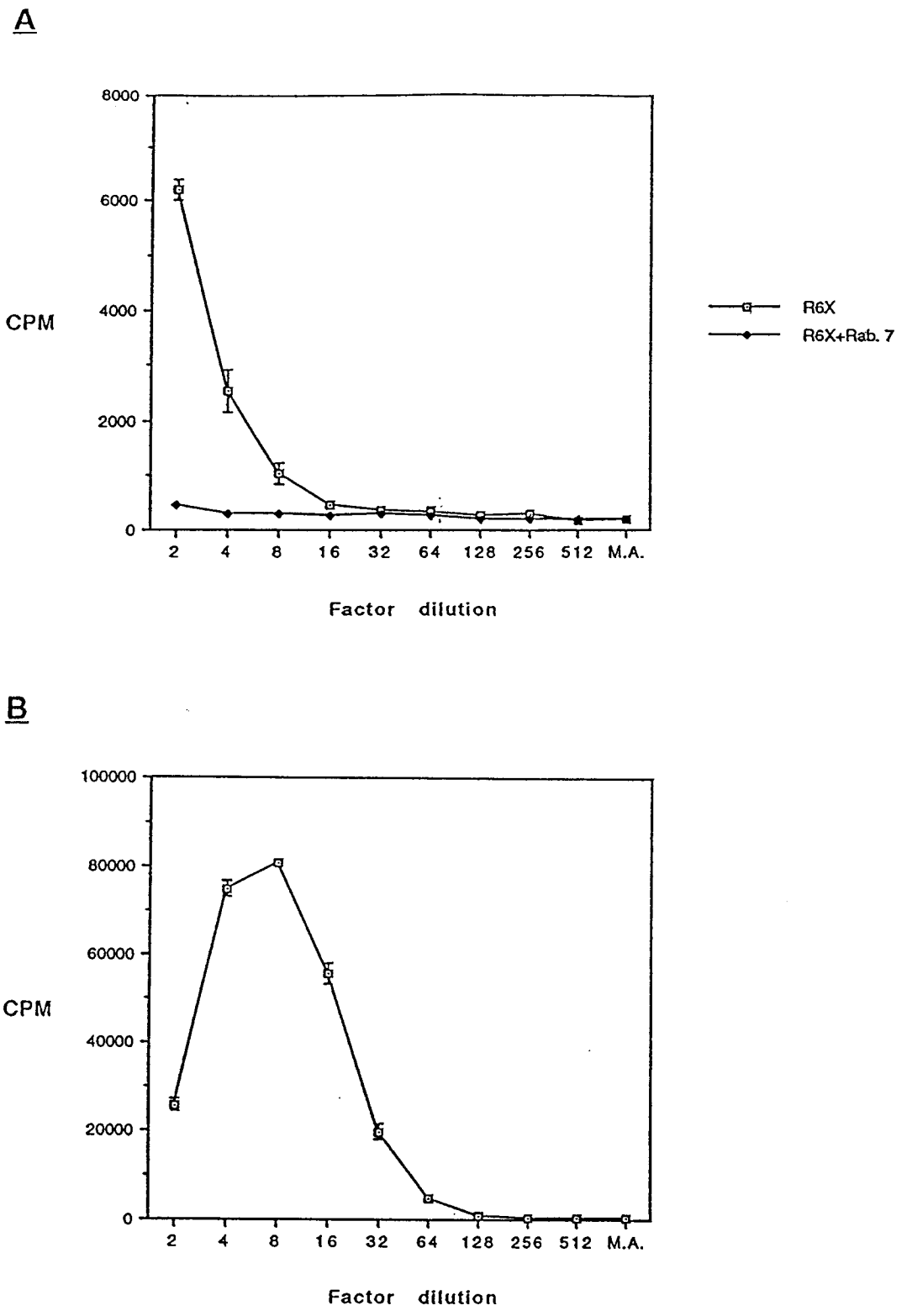
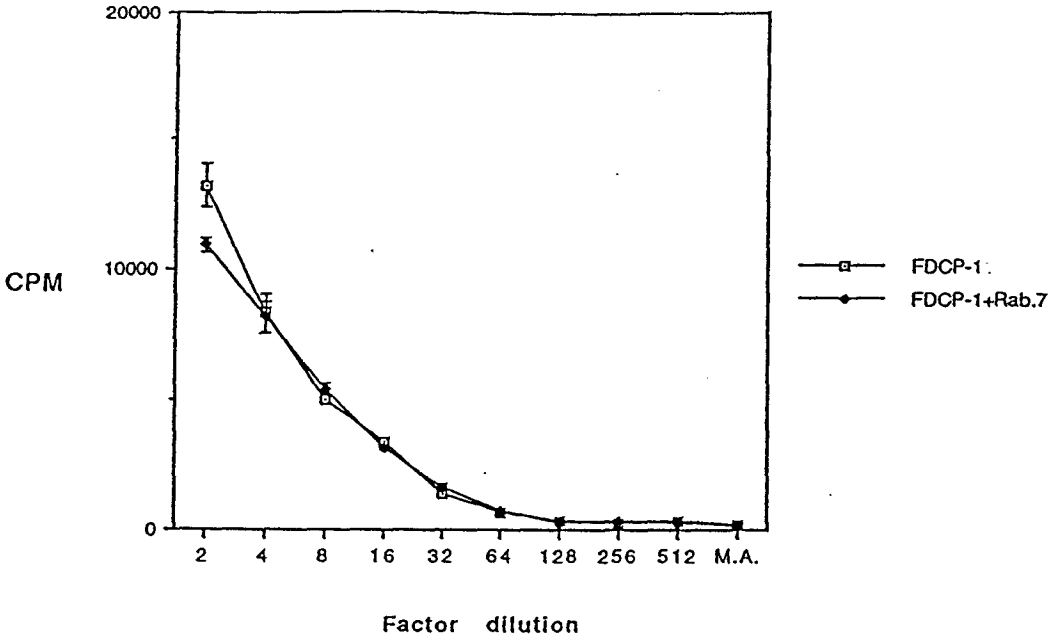


Fig. 1.12. IL-3 activity in the supernatants of FD.C/2Ψ cells A) 10-fold concentrated conditioned medium of FD.C/2Ψ.1 cells was assayed on R6X cells alone or in the presence of the neutralising anti-mIL-3 antibody, Rab7. 500 cells per well. 60 hour incubation followed by 12 hour ^{3}H-thymidine pulse. B) Assay of responsiveness of R6X cells to IL-3, performed in parallel to assays shown in part A.

A



B

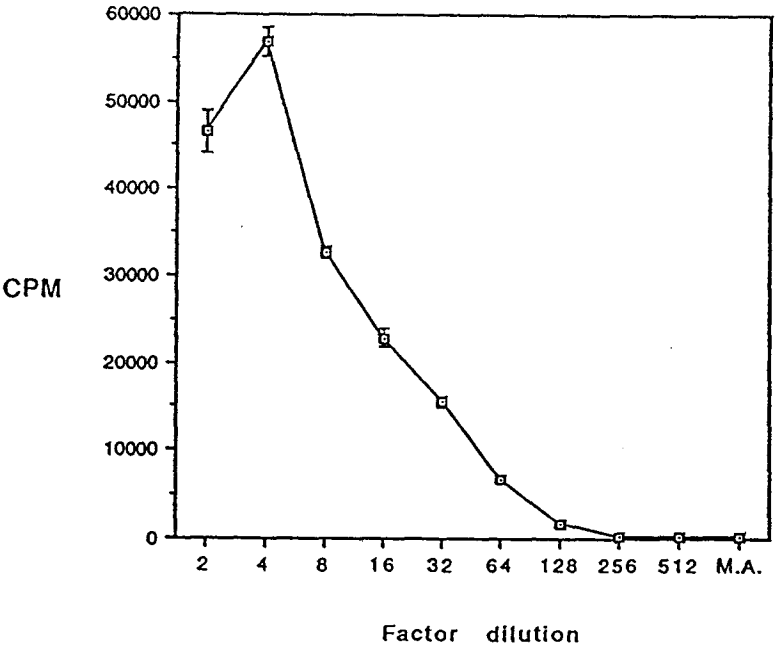
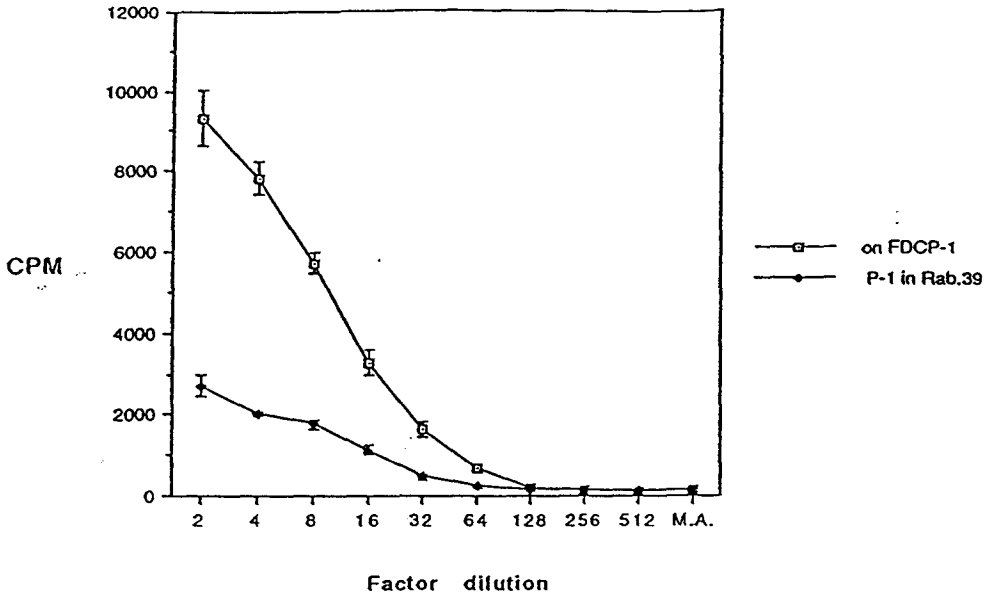


Fig. 1.13. IL-3-mediated response of FDC-P1 cells to FD.C/2Ψ.1 supernatant A) 10-fold concentrated conditioned medium of FD.C/2Ψ.1 cells was assayed on FDC-P1 cells alone or in the presence of the neutralising anti-mIL-3 antibody, Rab7. 500 cells per well. 60 hour incubation followed by 12 hour ^{3}H-thymidine pulse. B) Assay of responsiveness of FDC-P1 cells to IL-3, performed in parallel to assays shown in part A.

A



B

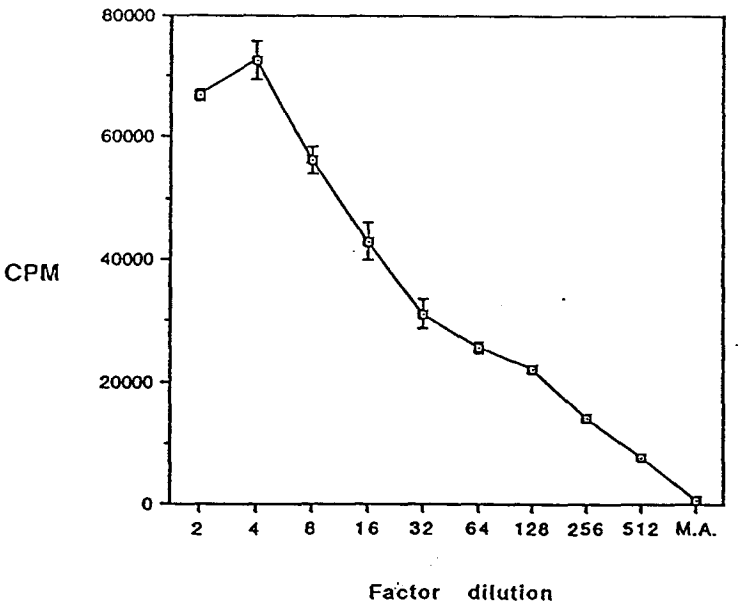


Fig. 1.14. GM-CSF-mediated response of FDC-P1 cells to FD.C/2Ψ.1 supernatant A) 10-fold concentrated conditioned medium of FD.C/2Ψ.1 cells was assayed on FDC-P1 cells alone or in the presence of the neutralising anti-mGM-CSF antibody, Rab39. 500 cells per well. 48 hour incubation followed by 12 hour ^{3}H-thymidine pulse. B) Assay of responsiveness of FDC-P1 cells to GM-CSF, performed in parallel to assays shown in part A.

Purified antibodies from these rabbit anti-sera were not available at the time of these assays, and FDC-P1 cells were found to be slightly stimulated by rabbit serum, (Dr. Hermann Ziltener, personal communication, and see figure 1.46). The inhibitory effect of Rab39 anti-serum in particular was partially masked by this effect. This observation may explain the failure of a combination of Rab7 and Rab39 to completely remove the signal seen on FDC-P1 cells as shown in figure 1.15).

As a result of growing interest in IL-6 amongst workers in the interleukin field at the time of these studies, the FD.C/2Ψ supernatants were assayed on the IL-6-responsive line, 41E5, derived by Dr. Hermann Ziltener at the Biomedical Research Centre (see Appendix 1). The supernatants were found (fig. 1.16) to contain an activity that stimulated growth of these cells and was completely removed by specifically inhibitory concentrations of the anti-mIL6 monoclonal antibody 6B4 (Vink et al., 1988, see Appendix 2). Exogenous sources of IL-6 activity did not, however, stimulate growth of either the parental FD.C/2 cells or the FD.C/2Ψ cells.

The supernatant of the parental FD.C/2 cells revealed the identical profile of growth-factor activities, with the exception of IL-2, which was not detected in 25-fold concentrated supernatant of FD.C/2 cells grown in IL-3 (fig. 1.17). The production of IL-3 by FD.C/2 cells is consistent with the detection of traces of IL-3 mRNA in these cells (Dr. J.D. Watson, personal communication).

iv) FD.C/2Ψ cells are functionally free of infectious retrovirus.

Although retroviral infection methods of gene transfer are designed to avoid this outcome, there is a recognised incidence of recombination between endogenous and introduced retrovirus in the cell lines used to generate the infectious particles, leading to the presence of infectious retrovirus in the ultimate target cells. Since the presence of infectious retrovirus in FD.C/2Ψ cells might well interfere with assessment of tumours resulting from *in vivo* growth of these cells, an assay to determine the presence of infectious retrovirus was conducted.

FD.C/2Ψ cells and Ψ2-3.1 cells were plated into fresh medium at 2×10^5 cells/ml, and allowed to grow for 3 days. The supernatants were then harvested and subjected to centrifugation and 0.2 μm filtration to remove cells. These supernatants were then applied to cultures of growing 3T3 cells (Aaronson and Todaro, 1968) as follows: culture medium was removed from the adherent 3T3 cells by gentle aspiration, and replaced with a 1:1 mixture of filtered supernatant and fresh medium, containing 6 μg/ml of polybrene. This was repeated every second day for 6 days. The 3T3 cells were then harvested by trypsinisation, and replated in the presence of fresh medium containing G418 at a

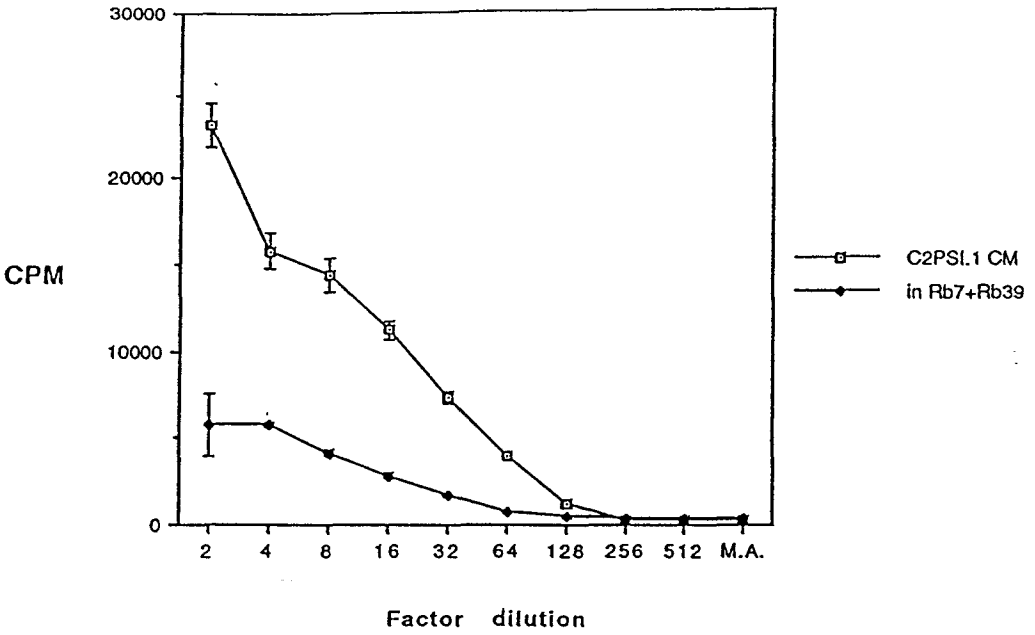
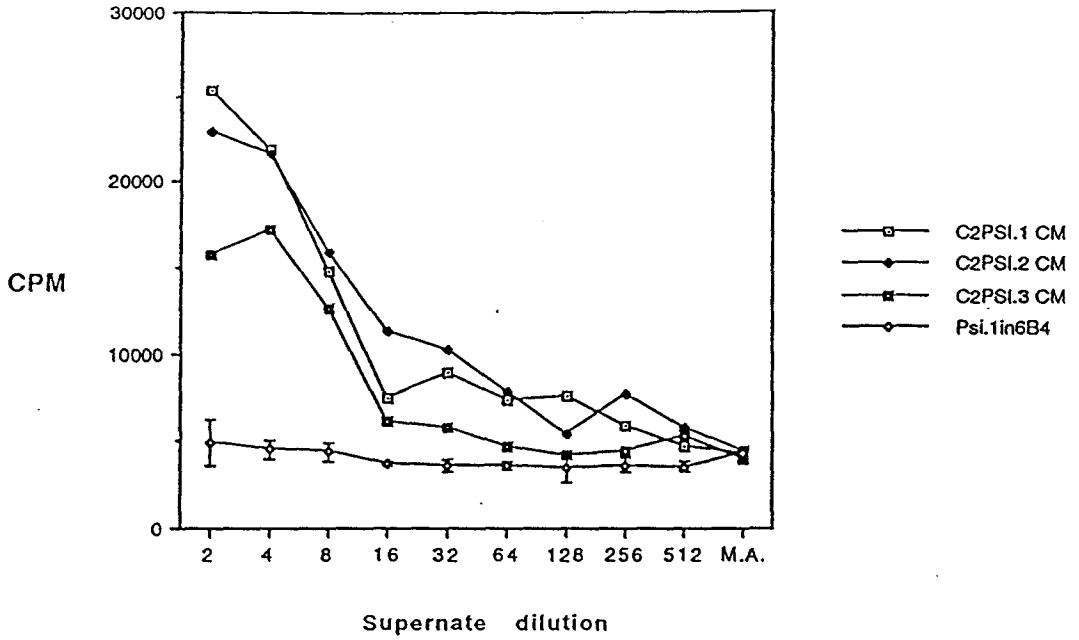


Fig. 1.15. Rabbit antibodies to IL-3 and GM-CSF partly abolish the effect of FD.C/2Ψ.1 supernatant on FDC-P1 cells 10-fold concentrated conditioned medium of FD.C/2Ψ.1 cells was assayed on FDC-P1 cells alone or in the presence of both antibody Rab7and Rab39. 500 cells per well. 60 hour incubation followed by 12 hour ^{3}H-thymidine pulse.

A



B

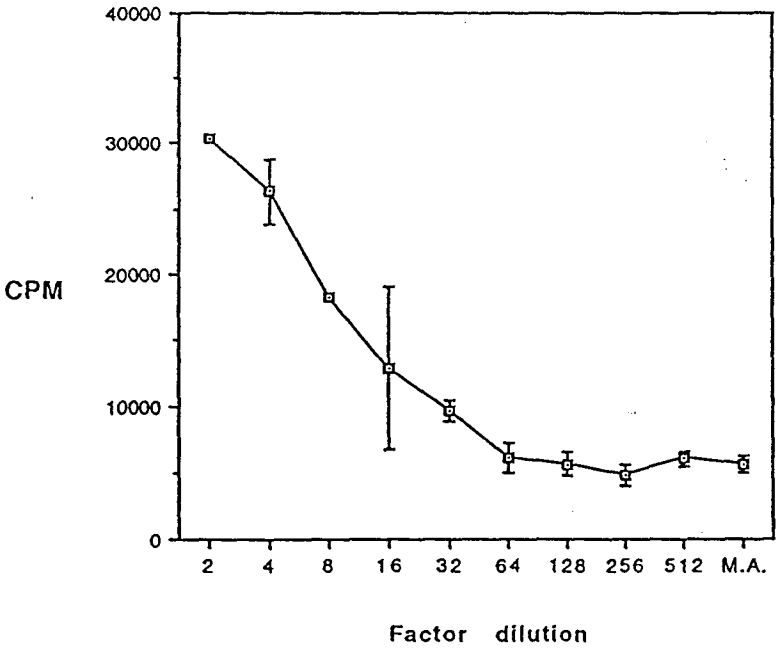


Fig. 1.16. IL-6 activity in the supernatant of FD.C/2Ψ.1 cells A) 10-fold concentrated conditioned medium of FD.C/2Ψ.1 cells was assayed on 41E5 cells alone or in the presence of the neutralising anti-mIL-6 antibody, 6B4. B) Assay of responsiveness of 41E5 cells to mIL-6, performed in parallel to assays shown in part A.

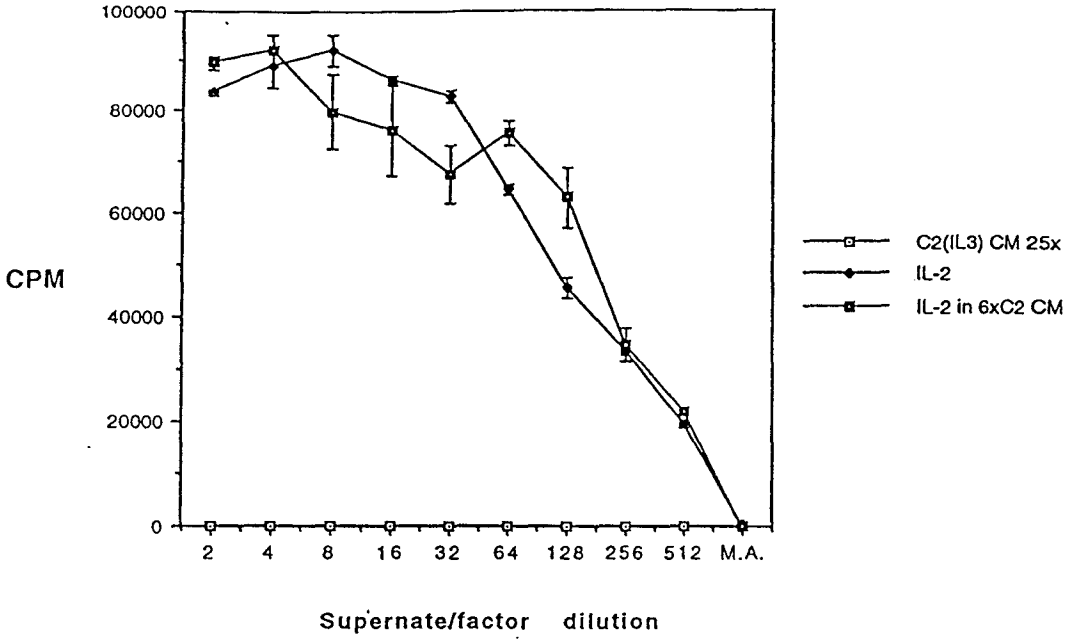


Fig. 1.17. Supernatant of FD.C/2 cells contains no detectable IL-2 activity 25-fold concentrated conditioned medium of FD.C/2 cells maintained in IL-3 was assayed on HT-2 cells. Also shown are a titration of IL-2 on the cells, alone, or in the presence of a 1/4 dilution of the 25 x supernatant. 1000 cells per well. 24 hour incubation followed by 8 hour ^{3}H-thymidine pulse.

concentration (600 µg/ml active) that had previously been determined to be lethal to 3T3 cells. 12 of 12 separate 3T3 cultures that had been exposed to Ψ2-3.1 cell supernatants contained cells that were able to continue growing in G418, whereas none of 12 3T3 cultures that had been exposed to FD.C/2Ψ cell supernatants contained such cells.

FD.C/2Ψ cell supernatants did not inhibit transfer of G418 resistance to 3T3 cells as 6 additional 3T3 cultures which had been exposed to a 1:1 mixture of FD.C/2Ψ and Ψ2-3.1 supernatants also contained G418-resistant cells. These results, while not completely ruling out the possibility, suggest that it was unlikely that FD.C/2Ψ cells produced infectious retrovirus. Any such virus would have lost the ability to transfer G418 resistance, and G418 resistance or the presence of the intact G418-resistance gene could be assessed in cells recovered from any tumours that might arise following *in vivo* transfer of FD.C/2Ψ cells.

v) *In vivo* growth of FD.C/2Ψ cells.

To assess the ability of FD.C/2Ψ cells to form malignant tumours, 6 to 8-week old mice of the DBA/2 strain (from which the FD.C/2 cells were derived) were inoculated with cells of each of the 5 tumours. In each case, 5×10^5 cells were injected subcutaneously into each of two mice and a third received the same number of cells intraperitoneally. A similar set of three mice received cells of the parental FD.C/2 line. All 15 mice that had received inocula of FD.C/2Ψ cells developed subcutaneous tumours or ascites, though the latency of the tumours (i.e. the time between inoculation and sacrifice of the animals due to signs of tumour growth) varied from 6 to 10 weeks. The latency periods did not show any correlation with the amount of FD.C/2 - stimulatory activity in the supernatant of the cells of each clone in culture. In contrast to mice that received inocula of the FD.C/2Ψ clones, mice that received FD.C/2 inocula remained healthy for at least 14 months after inoculation.

2) ANTIBODY-MEDIATED INHIBITION OF GROWTH OF FD.C/2Ψ CELLS.

A) PREPARATION OF ANTIBODIES.

The data presented show that the supernatants of growing FD.C/2Ψ cells contained IL-2, mIL-3, mGM-CSF, and mIL-6, but no mIL-4. The parental FD.C/2 cells and their FD.C/2Ψ derivatives responded to IL-2, IL-3, and IL-4, but not to GM-CSF nor IL-6. Only anti-IL-2 (and anti-IL-2 receptor) and anti-IL-3 antibodies were tested, therefore, for their ability to inhibit growth of FD.C/2Ψ cells. The

IL-2 antagonist antibodies used were DMS1 and DMS2 (Smith et al., 1983), mouse antibodies to human IL-2, and PC61 (Lowenthal et al,. 1985), a rat antibody to the p55 chain of the mouse IL-2 receptor. The anti-IL-3 antibody, Rab7, was used as serum (see above and Appendix 2)

After purification of anti-IL-2 antibodies (see Appendix 2), all preparations were tested for specificity on parental FD.C/2 cells grown in either IL-2 or IL-3. Examples of these specificity assays are shown in figures 1.18 and 1.19. These titrations allowed the identification of concentrations of antibody which displayed specific inhibition of IL-2 or IL-3 activity. In preliminary experiments it was observed that maximal inhibitory activity was obtained by four days of assay, with no further cell death apparent thereafter. Inhibition experiments were therefore carried out as 4-day assays. It was claimed in the original description of the antibodies DMS1 and DMS2 (Smith et al., 1983) that they exhibited a synergistic antagonism of IL-2. Consequently, these antibodies were used as a cocktail in all these experiments. From figure 1.18, it will be seen that the anti-receptor antibody PC61 showed a much greater "window" of specific versus non-specific inhibitory activity than did the anti-hIL-2 antibody cocktail. Indeed, a combination of the DMS cocktail with PC61 was no more potent in terms of specific antagonism of IL-2 activity than was PC61 alone, possibly as a result of the combination of greater affinity of PC61 for the IL-2 receptor than that of the DMS antibodies for hIL-2, with relatively higher non-specific cytotoxicity apparent in the DMS preparation.

B) INHIBITION OF AUTOSTIMULATORY GROWTH OF FD.C/2Ψ CELLS BY ANTIBODIES.

i) Antibody antagonists of IL-2 significantly inhibit autostimulatory growth.

Cells of FD.C/2Ψ lines were plated at 250 cells per well in the presence of maximal specifically-inhibitory concentrations of either the DMS cocktail or the PC61 antibody. In these conditions, it was possible to significantly inhibit the growth of all clones, and all antibody-containing wells contained numerous dead cells. (This phase-contrast microscopic appearance was substantiated by uptake of trypan blue). Nevertheless, visual observation, as well as thymidine-incorporation data, revealed that all antibody-containing wells contained live cells. The IL-2 antagonism exhibited by the antibodies could be overcome by exogenous IL-2 and circumvented by exogenous IL-3, confirming that these clones were not susceptible to a non-specific toxic effect of the antibodies. Since FD.C/2Ψ cells had been found to liberate small amounts of IL-3, and displayed proliferative responses to IL-3, it was postulated that this residual cell growth may have been due to the presence of this IL-3. The amount of IL-3 in question appeared from the FD.C/2Ψ cell supernatants to be very small in comparison with that used for assaying the inhibitory specificity of the anti-IL-3 anti-serum.

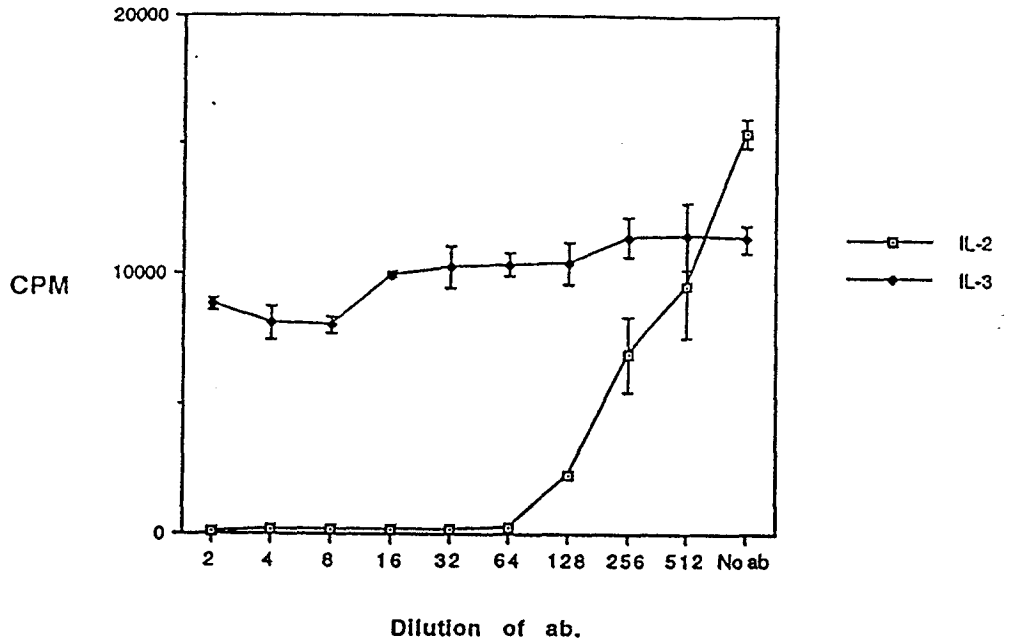


Fig. 1.18. Specificity assay for the preparation of PC61 antibodies used in these studies The preparation of purified PC61 antibody was titrated onto FD.C/2 cells passaged in a mixture of IL-2 and IL-3, growing in approximately one unit of IL-2 or one unit of IL-3, as indicated. 500 cells per well. 48 hour incubation followed by 8 hour ^{3}H-thymidine pulse. The highest concentration of antibody in the titration was approximately 50 µg/ml (see Appendix 2).

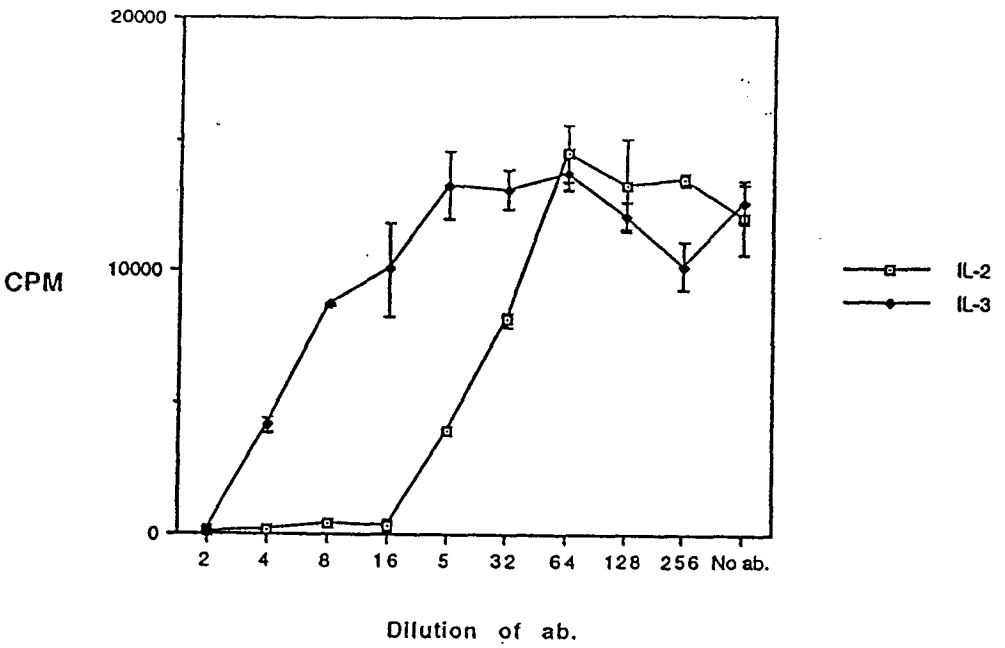


Fig. 1.19. Specificity assay for the preparation of DMS antibodies used in these studies The preparation of purified DMS antibody was titrated onto FD.C/2 cells passaged in a mixture of IL-2 and IL-3, growing in approximately one unit of IL-2 or one unit of IL-3, as indicated. 500 cells per well. 48 hour incubation followed by 8 hour ^{3}H-thymidine pulse. The highest concentration of the antibody preparation used was approximately 200µg/ml (see Appendix 2).

Accordingly, a concentration of Rab7 was selected which, while able to inhibit a unit of IL-3 activity (see Appendices 1 and 2), showed no stimulatory activity on FD.C/2 cells, and the anti-IL-2 antibody experiments were repeated in the presence of this concentration of Rab7 anti-serum. The results were simlilar to those obtained with the DMS and PC61 antibodies alone - all wells contained live cells after 4 days, demonstrating that IL-3 secreted by the cells did not account for their continued growth. Thymidine-incorporation data from the clone showing the greatest susceptibility to inhibition are shown in figures 1.20 and 1.21.

ii) Surviving cells are not mutants with respect to IL-2-dependence.

The incomplete inhibition by antibodies suggested the possibility that mutations may have arisen within the FD.C/2Ψ clones, rendering mutant cells independent of autostimulatory IL-2. To test this possibility, surviving cells from antibody inhibition experiments of each clone were harvested from antibody-containing wells, and cultivated in the presence of saturating concentrations of IL-2 until they had reached sufficient density to wash them free of exogenous IL-2 (10 to 14 days), and returned to passage in medium alone. After 2 passages in the absence of exogenous IL-2, the antibody inhibition experiment was repeated, and yielded qualitatively similar results to those initially observed. In each case, most but not all cells died, and all antibody-containing wells contained viable cells. This result demonstrated that the antibody-surviving cells had not undergone a mutation that rendered them insusceptible to the effects of IL-2 antagonist antibodies.

C) AUTOSTIMULATORY FD.C/2Ψ CELLS HAVE A MARKED ADVANTAGE OVER FD.C/2 CELLS IN ACCESS TO GROWTH FACTORS.

Although significant inhibition of growth of FD.C/2Ψ cells by antibodies had been demonstrated, the failure to bring about the death of all cells was surprising in view of the paucity of IL-2 liberated by these cells, as determined by the collection of conditioned medium (see section 1,C, iii, above), in comparison to the inhibitory capacity of the antibody preparations. Autostimulatory cells will consume a portion of the factor they synthesise, so the amount of factor found in collections of conditioned medium is a poor reflection of the ongoing synthesis of factor by such cells. In order to better assess the amount of IL-2 (and IL-3) produced by autostimulatory cells, a strategy was designed to determine the abililty of FD.C/2Ψ cells to support the growth of the parental FD.C/2 cells under conditions of co-culture. Under these conditions, the FD.C/2 cells would have "immediate" access to growth factor present in the wells in which it was being synthesised by FD.C/2Ψ cells.

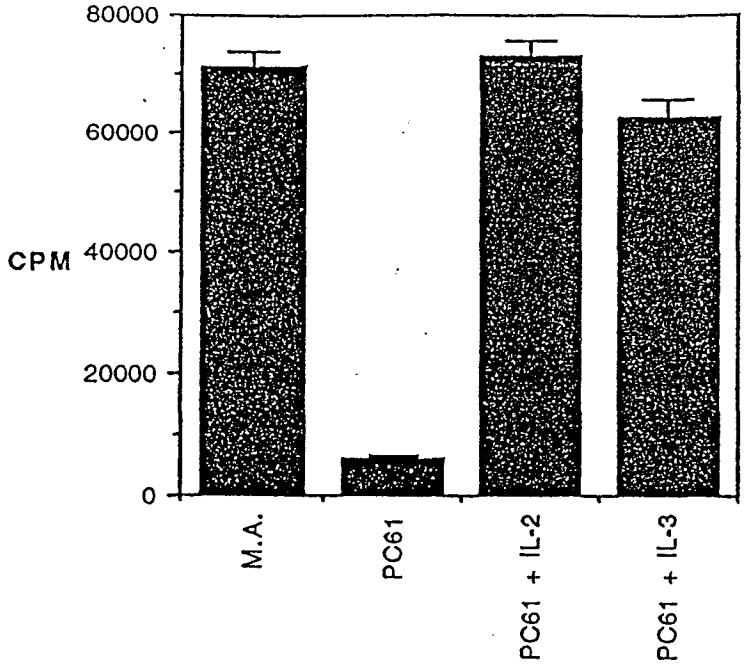


Fig. 1.20. PC61 antibody significantly and specifically inhibits the growth of FD.C/2Ψ cells FD.C/2Ψ.5 cells were assayed alone, in the presence of neutralising anti-mIL2-receptor antibody, PC61 (approximately 5 μg/ml), or in the presence of antibody and either IL-2 or IL-3. 250 cells per well. 96 hour incubation followed by 16 hour ^{3}H-thymidine pulse.

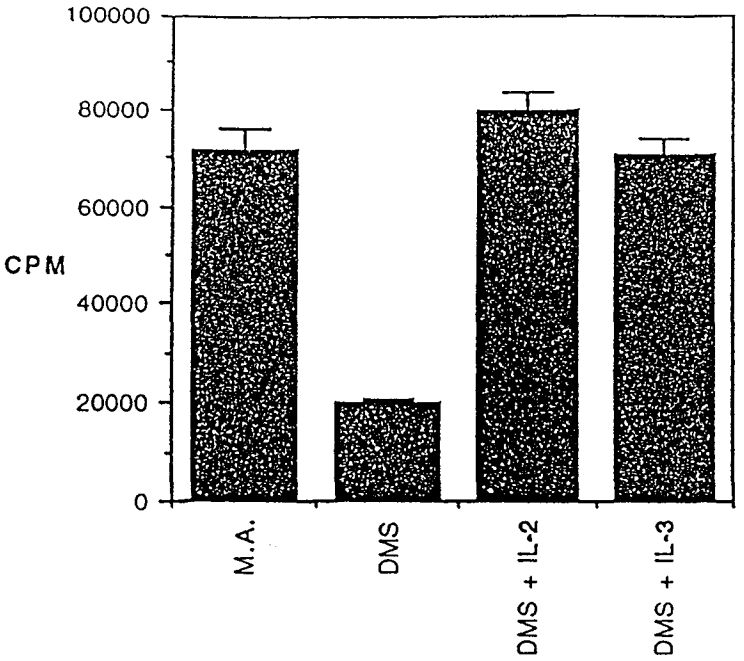


Fig. 1.21. DMS antibody significantly and specifically inhibits the growth of FD.C/2Ψ cells Growth of FD.C/2Ψ.5 cells was assayed alone, in the presence of neutralising anti-mIL2 antibodies, DMS1 and DMS2, (approximately 40 μg/ml)or in the presence of antibodies and either IL-2 or IL-3. 250 cells per well. 96 hour incubation followed by 16 hour ^{3}H-thymidine pulse.

i) Co-culture with populations distinguished by fluorescence.

Cells of the parental FD.C/2 line were stained with the fluorescent dye PKH2 so as to be able to distinguish them from unmarked FD.C/2Ψ cells during the experiment. PKH2 has similar excitation and emission characteristics to fluorescein. This dye was chosen because it was claimed by the supplier to be a) evenly partitioned amongst daughter cells of a stained cell, b) not susceptible to leaking from viable cells, and c) not taken up by cells in tissue culture conditions. Staining of cells with the dye required brief incubation with a supplied (proprietary) staining solution, in the absence of serum or culture medium, at room temperature. The staining solution was found to be toxic to cells, but after preliminary experiments in which duration of the staining period was varied between 4 and 10 minutes, stained cells were derived whose response to IL-2 was similar to that of unstained FD.C/2 cells (fig. 1.22). One x 10^5 stained FD.C/2 cells were co-cultivated with proliferating 3T3 fibroblasts (initially approximately 10% confluent in a 100mm tissue culture dish) in the presence or absence of IL-2 for 6 days. At the end of this period the dish was gently agitated by hand, and non-adherent cells were harvested, pelleted by centrifugation, and subjected to fluorescence microscopy. When IL-2 had been present in the culture, 98% of the non-adherent cell population were fluorescent, the remaining 2% consisting of dead cells and cells with fibroblast morphology. The adherent population was harvested by trypsinisation, and contained no detectable fluorescent cells (i.e. less than one in 1600). When IL-2 had been omitted from the culture medium, no viable fluorescent cells were found in either adherent or non-adherent populations. These results substantiated the manufacturers claims, and suggested that PKH2 cells would be suitable for the proposed co-culture assays.

A preliminary experiment was conducted in which 250 cells of the line FD.C/2Ψ.1 were co-cultivated with PKH2-stained washed FD.C/2 cells, in the presence or absence of saturating amounts of IL-2. Wells were examined after 4 days, and as expected, wells plated with IL-2 contained numerous fluorescent cells. In wells to which no IL-2 had been added, however, no fluorescent cells could be seen, suggesting that so little IL-2 was liberated by the FD.C/2Ψ.1 cells, that they would not support the growth of FD.C/2 cells. The experiment was repeated but scored after 3 days of co-culture with similar results. Thymidine-incorporation assays were performed on cultures established in parallel with this 3 day assay (fig. 1.23).

These experiments were unsatisfactory on several counts. Firstly, it was noted during these experiments that fluorescence was much more difficult to judge in Terasaki wells than on a microscope slide, a potential source of error particularly since cells that would survive 4 days would be likely to be daughters or grand-daughters of those originally stained, and therefore would fluoresce with lesser intensity. Secondly, it was impossible to be sure that transfer of the contents of a well to a slide would

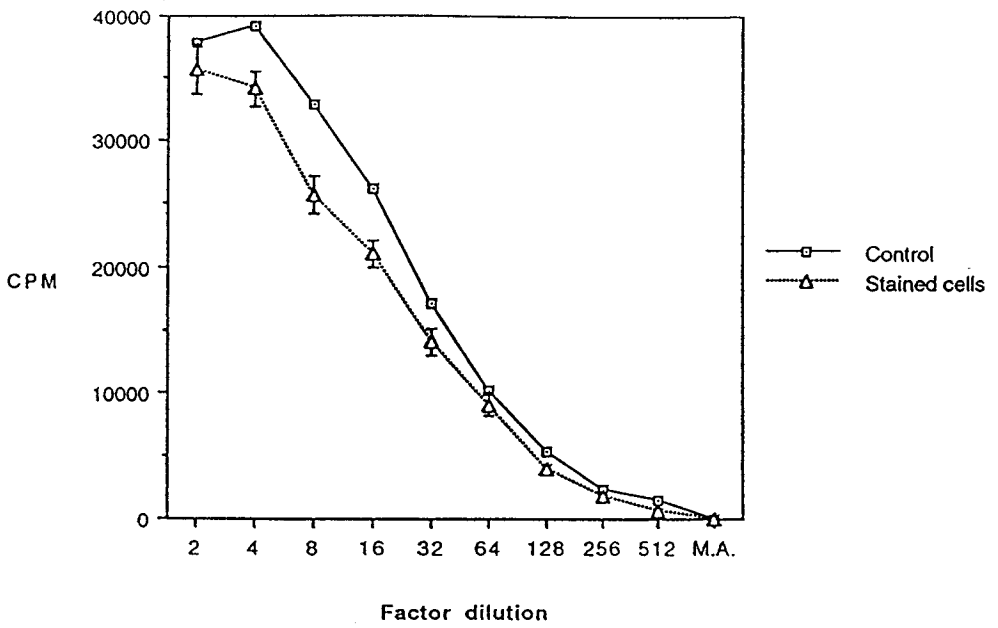


Fig. 1.22. Growth-factor response of stained FD.C/2 cells is similar to that of unstained cells Assay of responsiveness of PKH2-stained FD.C/2 cells to IL-2, in comparison with unstained cells. 500 cells per well. 36 hour incubation followed by 12 hour ^{3}H-thymidine pulse.

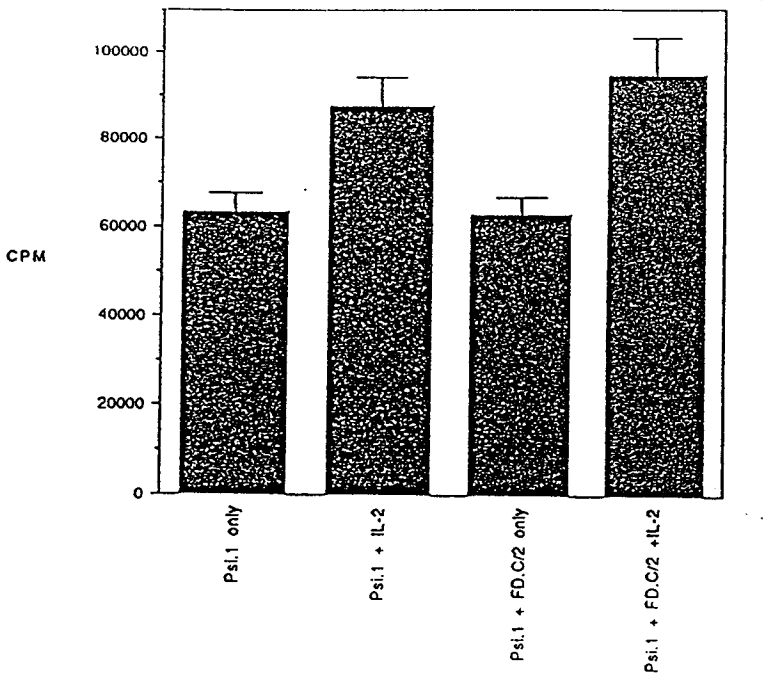


Fig. 1.23. Assessment of the ability of FD.C/2Ψ.1 cells to support the growth of FD.C/2 cells FD.C/2Ψ.1 cells were plated at 250 cells per well, either alone or with 50 FD.C/2 cells. The cells were plated either in medium alone, or with a saturating concentration of IL-2, as indicated. (Psi.1 = FD.C/2Ψ.1 cells). 3 day assay followed by16 hour ^{3}H-thymidine pulse.

be complete, and that no fluorescent cells would be missed. Thirdly, PKH2-stained cells were a finite resource, in that staining of a population would eventually diminish with time in passage, and staining of another population would be necessary prior to each set of experiments.

ii) Co-culture with cells distinguished by hygromycin resistance.

As an alternative to the use of dye, therefore, a population of FD.C/2 cells was marked by transfection with a vector that rendered them resistant to the antibiotic hygromycin B. The plasmid used is shown in figure 1.24. Linearisation and electroporation of the plasmid into FD.C/2 cells yielded a clone (FD.C/2Hyg) of cells that were resistant to 600 μg/ml of hygromycin B, whereas untransfected cells were killed by 500 μg/ml in 8 days. Like the parental FD.C/2 cells, these cells showed vigorous proliferative responses to IL-2 and IL-3 (fig. 1.25). Two clones of FD.C/2Ψ cells (FD.C/2Ψ.1 and FD.C/2Ψ.2) were assayed for resistance to hygromycin B, and were found to be killed by 500 μg/ml. Conversely, FD.C/2Hyg cells were killed by 500 μg/ml G418.

Co-culture experiments were conducted as follows: Each well of a Terasaki microtiter plate was seeded with 50 cells of the FD.C/2Hyg line in 5 μl of medium. To 18 wells 500 cells of the FD.C/2Ψ.1 line were added, to 18 wells, 150 cells, and to 18 wells 50 cells of the FD.C/2Ψ 1 line, in each case in 5 μl of medium. To the remaining 6 wells, as a positive control, 50 FD.C/2Ψ.1 cells were added in 5 μl of medium supplemented with twice the amount of IL-2 used to passage IL-2 dependent FD.C/2 cells. This resulted in assays in which "stimulator" (FD.C/2Ψ.1) cells and "responder" (FD.C/2 cells) were present at 10:1, 3:1, and 1:1 ratios, at cell numbers at which it was known that consumption of factors other than IL-2 would not limit growth. After 3 days of co-culture, to each well was added 10 μl of medium containing twice the passage-medium concentration of IL-2 and 1.2 mg/ml of hygromycin B. Incubation was allowed to proceed for a further 8 days, when 5 μl of medium was carefully removed from each well without disturbing the cells, and replaced with 5μl of ^{3}H-thymidine for a further 16 hours of incubation. This manoeuvre was necessary as the size of the wells prohibits harvesting volumes greater than 20 μl. As a "negative" control for each assay, another Terasaki was set up with identical conditions except that the FD.C/2Hyg were suspended in medium containing G418 at 1000 μg/ml. The final G418 concentration in the wells during the first 3 days of assay was thus 500 μg/ml - sufficient to kill the FD.C/2Hyg cells, but to allow growth of the FD.C/2Ψ cells. The medium added to these wells after 3 days, containing IL-2 and hygromycin, also contained 500 μg/ml of G418.

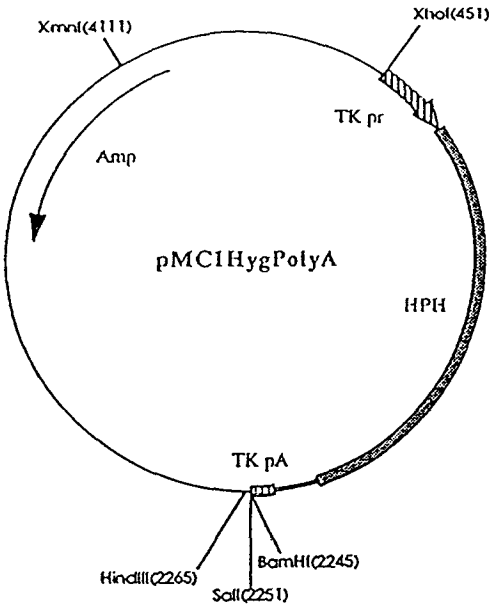


Fig. 1.24. The vector used to confer hygromycin resistance upon FD.C/2Hyg cells This vector was constructed by replacing the MluI - RsrII NeoR fragment of pMC1NeoPolyA with a HindIII - BglII fagment of pSV2Hyg containing the hygromycin phophotransferase coding sequence. Both original plasmids were kindly donated by Dr. Jamey D. Marth of the Biomedical Research Centre. TK pr - Herpes simplex virus thymidine kinase promoter; HPH - hygromycin phophotransferase coding sequence; TK pA - Herpes simplex virus thymidine kinase poly-A addition signal; Amp - Ampicillin resistance gene. The plasmid was linearised at the XmnI site prior to electroporation.

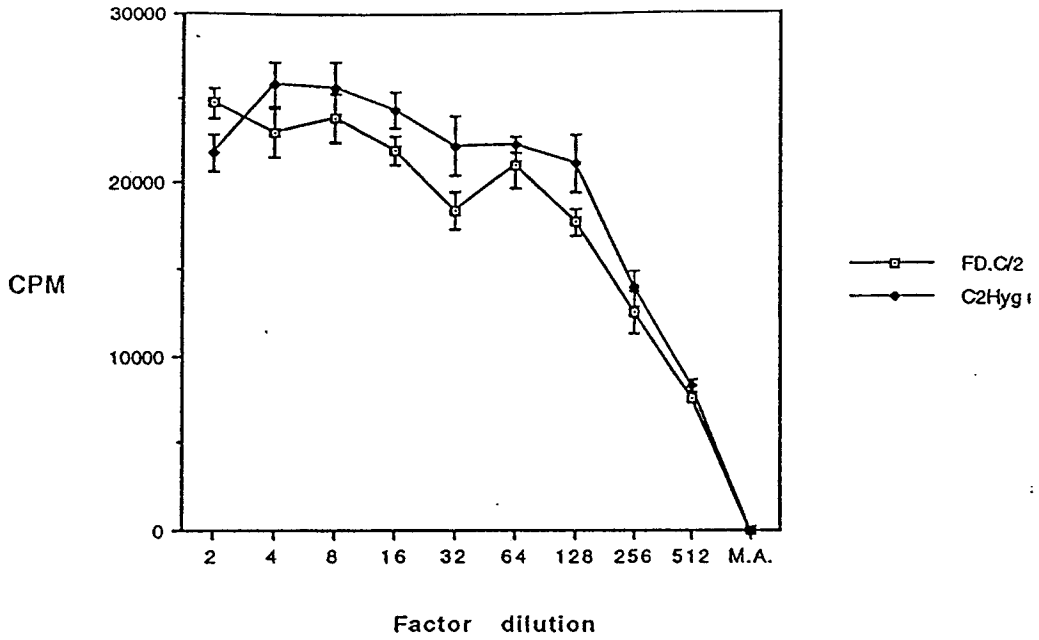
This assay was performed with FD.C/2Ψ.1 and FD.C/2Ψ.2 cells as "stimulator" populations. The results are shown in figure 1.26. In the assays set up with G418 (and hygromycin), no viable cells were apparent by inspection at the end of the assay period. Assays without G418, revealed that both FD.C/2Ψ.1 and FD.C/2Ψ.2 cells were capable of supporting the growth, or survival, of FD.C/2Hyg cells, but only at the highest (10:1) ratio used. The presence of exogenous IL-2 during the first three days of the assay resulted in the expected proliferation of hygromycin-resistant cells.

The remaining 3 clones of FD.C/2Ψ cells (.3, .4, and .5) were also subjected to this assay. Two of these remaining 3 clones (.4 and .5) were capable of supporting growth of hygromycin-resistant cells, but only at the 10:1 ratio of "stimulators" to "responders", as judged by visual inspection of wells at the end of the hygromycin selection period. The FD.C/2Ψ.3 clone was not capable of supporting FD.C/2Hyg cells in these conditions.

D) AUTOSTIMULATORY FD.C/2Ψ CELLS ARE MARKEDLY MORE RESISTANT TO GROWTH INHIBITION BY IL-2-INHIBITORY ANTIBODIES THAN ARE FD.C/2 CELLS.

Since co-culture conditions had been established in which FD.C/2Hyg cells were supported by FD.C/2Ψ cells, assays were designed to address the question of whether in these conditions, surviving FD.C/2Hyg cells were resistant to IL-2-antagonist antibodies. Assays were performed in which 500 FD.C/2Ψ cells and 50 FD.C/2Hyg cells were co-cultured in the presence or absence of specifically inhibitory concentrations of DMS or PC61 antibodies. Each well of 4 Terasaki plates was seeded with 50 FD.C/2Hyg cells in 5 µl of medium. Cells of the FD.C/2Ψ.1 and FD.C/2Ψ.2 clones were suspended in medium alone or in medium containing specifically inhibitory concentrations of either the DMS or PC61 antibodies. 5 µl of medium containing 500 cells of either the FD.C/2Ψ.1 clone (2 plates) or the FD.C/2Ψ.2 clone (2 plates) was added to the Terasaki wells, so that in each plate, 18 wells contained FD.C/2Ψ cells and FD.C/2Hyg cells in medium alone, 18 contained cells in medium with DMS antibody, and 18 contained cells in medium with PC61 antibody. After 3 days of co-culture, 10 µl of medium containing a saturating concentration of IL-2 and 1.2 mg/ml hygromycin was added to each well of two plates (one containing FD.C/2Ψ.1 cells and the other containing FD.C/2Ψ.2 cells as "stimulators"), and 10 µl of medium containing IL-2 and 1 mg/ml G418 to each well of the other two plates. Incubation was allowed to proceed as previously, for 8 days in the case of hygromycin-containing plates, but only 4 days in the case of G418 plates, since this was known to be sufficient to kill all G418 susceptible cells at this concentration of G418.

A



B

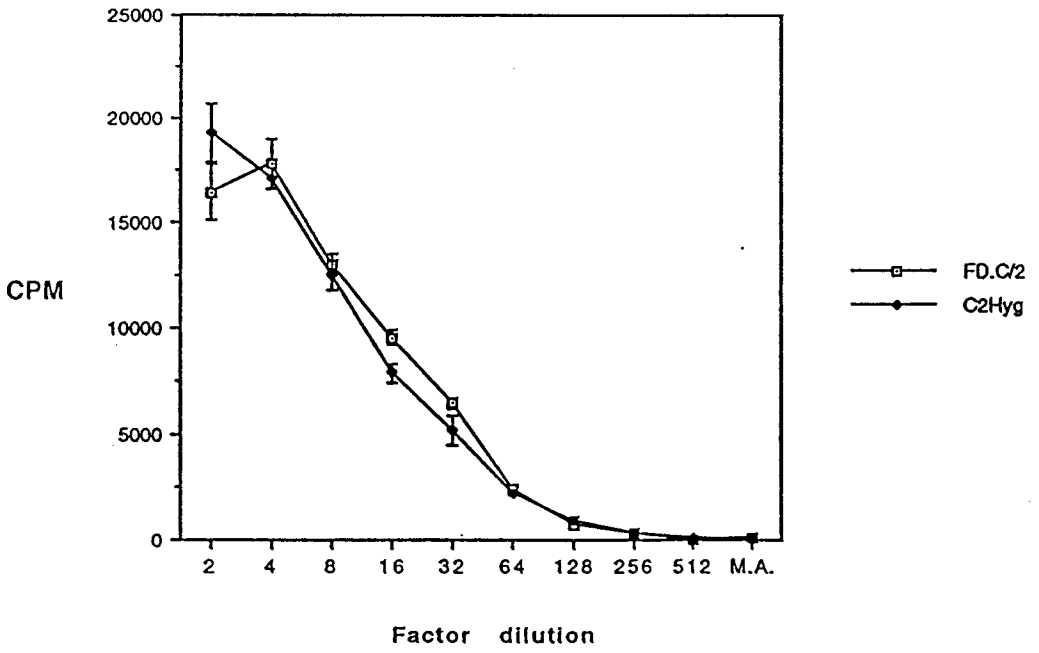


Fig. 1.25. Growth-factor responsiveness of FD.C/2Hyg cells is similar to that of FD.C/2 cells A) IL-2 responses. B) IL-3 responses. 500 cells per well. 36 hour incubation followed by 12 hour ^{3}H-thymidine pulse.

The results of these assays are illustrated in figure 1.27. For both the FD.C/2Ψ.1 and FD.C/2Ψ.2 clones, all antibody-resistant cells were shown to be G418-resistant, but hygromycin-sensitive, the profile expected of the autostimulatory FD.C/2Ψ cells. These data demonstrate that the survival of FD.C/2Hyg cells resulting from the presence of FD.C/2Ψ cells was completely abrogated by IL-2-inhibitory antibodies in conditions in which the growth of the FD.C/2Ψ cells was only partially inhibited.

E) THE IL-2 RECEPTORS ON FD.C/2Ψ CELLS ARE UNLIKELY TO ACCOUNT FOR THE GROWTH ADVANTAGE OF THESE CELLS.

Several possibilities were proposed to account for the significant advantage of these autostimulatory cells over the parental cells with respect to access to IL-2 stimulation of growth. A) Some alteration in the IL-2 receptors resulted in a higher affinity for IL-2. B) Autostimulatory cells were capable of receiving IL-2 "signals" from IL-2 *within* the cells (see introduction). C) An IL-2 signal was delivered to autostimulatory cells at the cell surface, as is the case with cells dependent on exogenous IL-2, but features of the cell surface micro-environment (for instance, the glycocalyx) might result in the retention of secreted IL-2 in a manner which impeded liberation of the IL-2 from the cell surface and gave the secreting cell more immediate access to this IL-2 than a neighbouring non-producer.

The original report describing the creation and use of PC61 antibody (Lowenthal et al., 1985) showed that although PC61 could effectively block high affinity binding of IL-2 to cells, IL-2 was unable to block the binding of PC61. It was thus apparent that PC61 might be used to assess cell-surface levels of the IL-2 receptor p55 chain, even in the presence of IL-2. Consequently, fluorescent cell-sorting analysis of FD.C/2 and FD.C/2Ψ cells for expression of the p55 chain of the mIL-2 receptor was undertaken to exclude differences in receptor number. [These data were collected with the assistance of Dan Zecchini at the UBC Acute Care Unit FACS facility]. Cells were initially washed several times as for a proliferation assay, then resuspended in buffer alone or in buffer containing PC61 antibody at approximately 200 µg/ml. After incubation on ice and washing, the samples were incubated with a fluoresceinated sheep anti-rat antibody, incubated on ice again, washed, and subjected to analysis. After gating out dead cells on the basis of forward scatter, results of secondary antibody ("background") staining, and PC61 plus secondary staining, were collected for each cell line. As seen from the results shown in figure 1.28, no significant difference in the percentage of cells judged positive for PC61 staining was found between any of the FD.C/2Ψ cells and the FD.C/2 cells grown in IL-2. Indeed, the profiles of these cells were superimposable. The FD.C/2 cells that were grown in IL-3 displayed a distinctly lower level of PC61 staining than those grown in IL-3, an observation consistent with those of Le Gros et al. (1985).

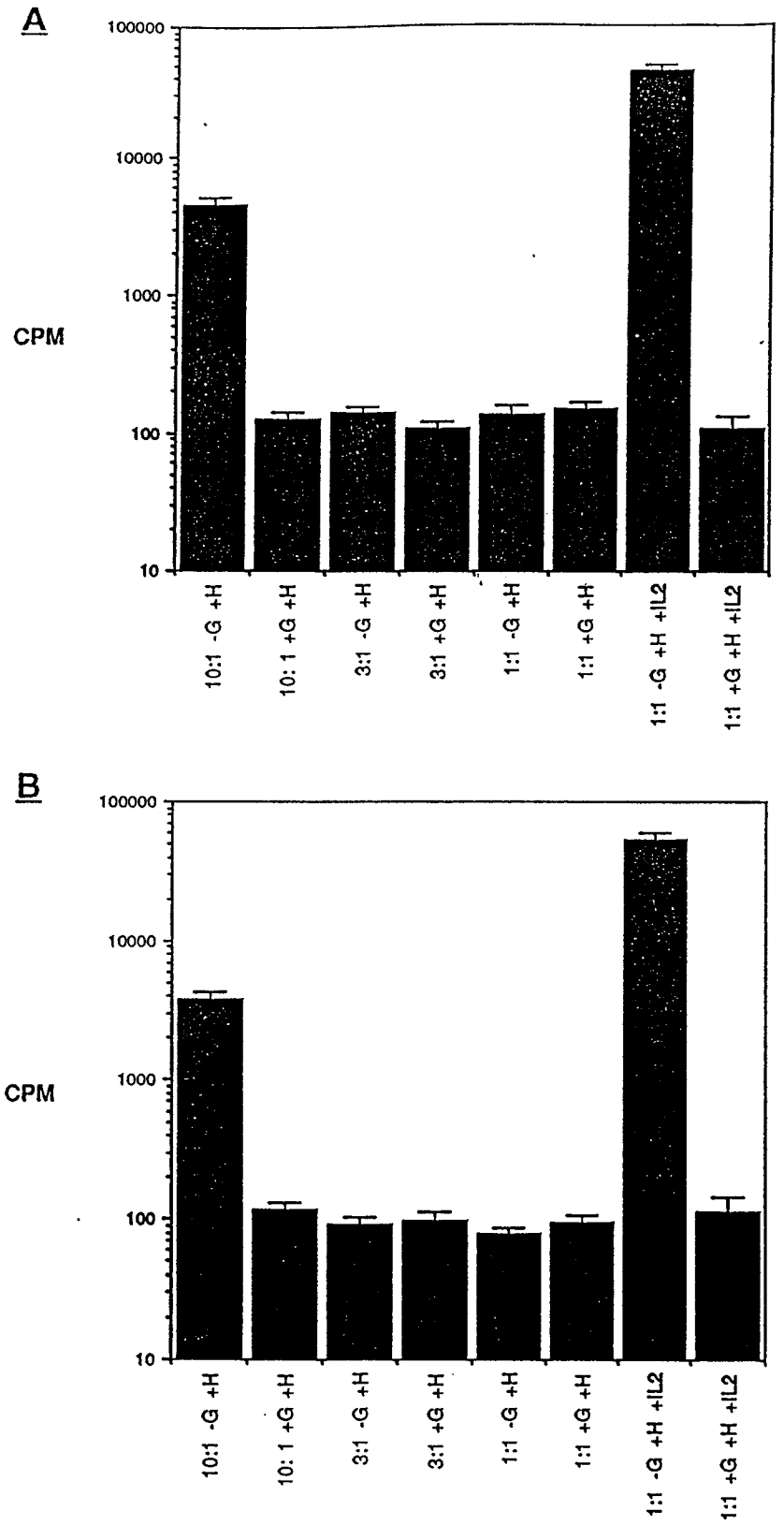


Fig. 1.26. FD.C/2Ψ cells support the survival of FD.C/2Hyg cells at high stimulator:responder ratios
A) C2Ψ.1 cells as stimulators. B) C2Ψ.2 cells as stimulators. Ratios are indicated. +G- assays with G418, +H - assays with hygromycin (see text). Note logarithmic scale of ^{3}H-thymidine incorporation. Results of less than 200 counts correspond to the absence of viable cells.

This analysis excluded the possibility of significant differences in IL-2 receptor distribution (or, at least, p55 chain distribution) accounting for the growth advantage of FD.C/2Ψ cells. At the time of these studies, no antibody to the murine IL-2-receptor beta (p70) chain was available, and the gamma chain had not been identified. Since there was no selective pressure toward mutation of IL-2 receptors in the cloning of the five FD.C/2Ψ clones, it was felt that undertaking radio-labelled IL-2 binding studies was not warranted.

3) AUTOSTIMULATORY GROWTH *CAN* BE COMPLETELY INHIBITED BY GROWTH FACTOR INHIBITORY ANTIBODIES.

A) CREATION OF A CONDITIONALLY AUTOSTIMULATORY CLONE.

The questions of intracellular stimulation by IL-2, and of cell-surface micro-environment favouring the autostimulatory cell were deemed to be beyond the scope of these studies. In order to definitively answer the question of whether antibodies could completely inhibit autostimulatory growth, however, it was necessary to derive a cell in which the amount of IL-2 produced could be regulated. A higher antibody:IL-2 ratio might overcome the advantage of auto-production of factor, providing that a significant portion of the functional factor-receptor interaction took place at the cell surface. Moloney retroviral LTR's (such as that driving production of the IL-2 message in the vectors used to generate FD.C/2Ψ cells) are generally regarded as "strong" promoters in hemopoietic cells (Keating et al., 1990) and, despite the paucity of IL-2 detectable in the supernatants of the FD.C/2Ψ autostimulatory cells, it was possible that these cells were producing more factor than could be antagonised successfully by the antibodies.

i) Single-copy integrating vector

A vector was constructed (pPO1LhIL2, fig. 1.29, A) in which the production of IL-2 message would be regulated by the metallothionein promoter. In conjunction with this inducible promoter, the sequence encoding hIL-2 was altered to include the full-length cDNA, including several ATTTA sequences in the 3' untranslated region (figure 1.29, B). These sequences were known to be involved in rapid turnover of cytokine messages in hemopoietic cells (Lindstein et al, 1989, and references therein), and their inclusion should therefore reduce IL-2 production. Linearisation of this vector and electroporation into cells, followed by selection for stable transfectants in G418, would be likely to result in cells bearing single-copy or low-copy-number genomic integrations (Potter, 1988).

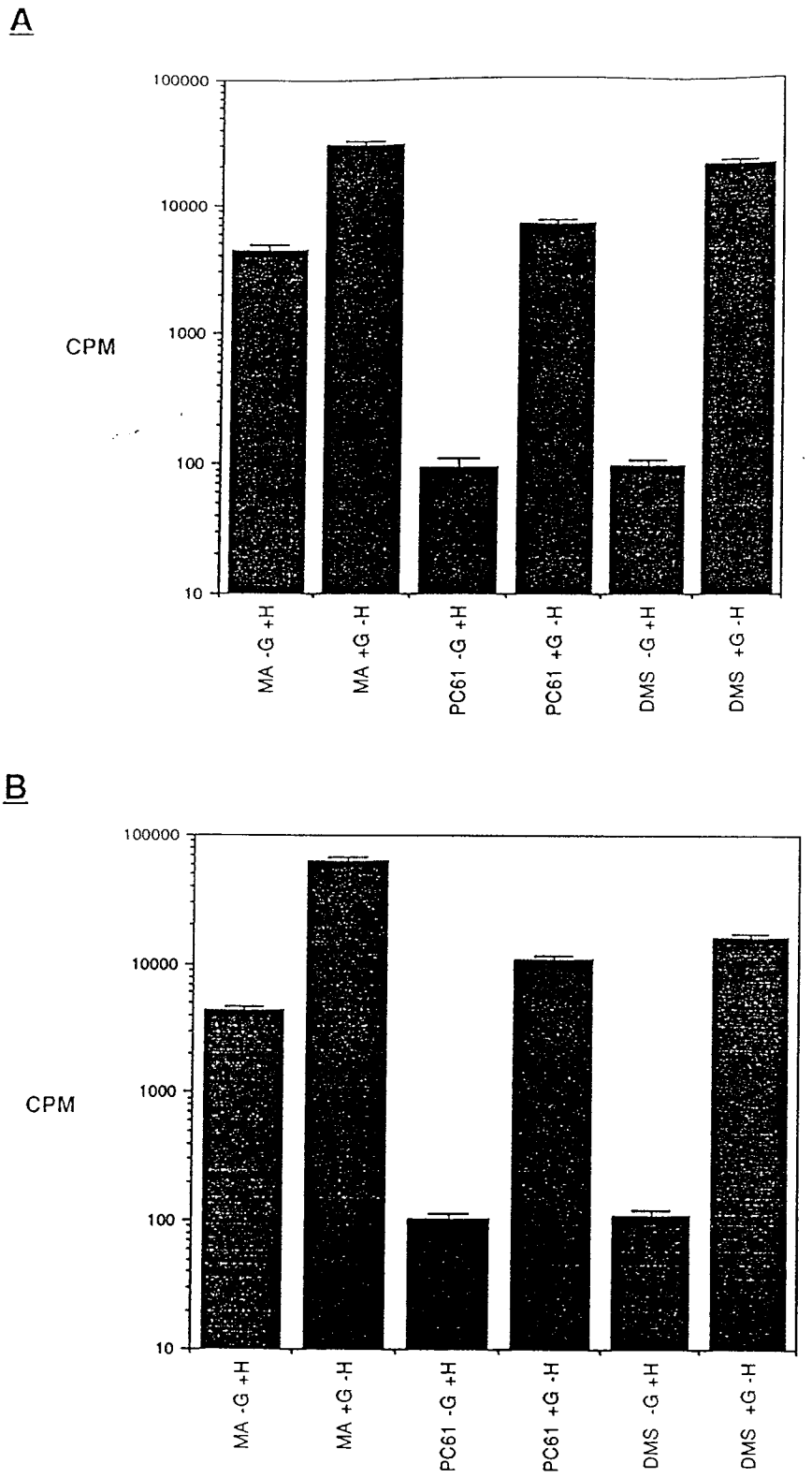


Fig. 1.27. FD.C/2Ψ cells have a dramatic advantage over FD.C/2 cells in antibody resistance A) C2Ψ.1 cells as stimulators. B) C2Ψ.2 cells as stimulators. Ratio is 10:1 throughout. +G- assays with G418, +H - assays with hygromycin (see text). Note logarithmic scale of ^{3}H-thymidine incorporation. Results of less than 200 counts correspond to the absence of viable cells.

CELL	% POSITIVE
FD.C/2 (IL-2)	95.3
FD.C/2Ψ.1	95.4
FD.C/2Ψ.2	94.8
FD.C/2Ψ.3	93.0
FD.C/2Ψ.4	96.8
FD.C/2Ψ.5	95.2
FD.C/2 (IL-3)	70.0

Fig. 1.28. FACS analyses of IL-2-receptor p55 on FD.C/2 and FD.C/2Ψ cells Percentage of p55-positive cells of various clones, as determined by FACS analysis with antibody PC61 and fluoresceinated anti-rat antibody.

Such cells would, it was hoped, display low levels of basal hIL-2 production, perhaps insufficient to maintain autostimulatory growth, and could be induced to produce more IL-2 (perhaps barely sufficient to support autostimulatory growth) with zinc or cadmium.

While the vector was in preparation, FD.C/2Hyg cells were assayed for their sensitivity to zinc chloride toxicity in vitro. It was found (figure 1.30) that FD.C/2Hyg cells would tolerate up to 300 micromolar zinc chloride without significant decrease of viability as judged by visual inspection and thymidine incorporation in the presence of sub-saturating concentrations of IL-2.

Two separate electroporations were performed on FD.C/2 cells with the pPO1LhIL2 vector. Electroporation was carried out at a capacitance of 25 μF, and voltage of 1000 V. Forty-eight hours after electroporation, viable cells were counted, and cells were distributed into the wells of 96 well plates in the presence of 500 μg/ml G418 and IL-2, at a density of 5000 viable cells per well. This density was selected on the basis of the reported frequency of stable transfectants following transfection of myeloid cells (Keating et al., 1990), so that any wells containing G418-resistant cells would be likely to contain derivatives of single cells, i.e. to contain truly clonal populations. From 8 96-well plates, 17 G418-resistant clones were obtained in the presence of exogenous IL-2. Weaning into zinc was attempted by first adding zinc chloride to a concentration of 300 micromolar (final volume 200 μl) to the wells in which G418-resistant cells were apparent, and 48 hours later replacing half the medium with medium containing G418, and zinc chloride, but lacking IL-2. Subsequently, this replacement of medium was repeated every 48 hours. When the wells became sufficiently populated to suggest expansion was in order, cells were transferred to 24 well plate wells and 200 μl medium added every 24 to 48 hours as prompted by visual inspection. When the volume of a well had reached approximately 1 ml, the regime of medium replacement was again commenced. After 3 weeks, however, it was apparent that none of the 17 clones were capable of survival in the absence of exogenous IL-2.

ii) Multi-copy episomal vector.

It was felt that the protocol of weaning to growth in the absence of exogenous IL-2 had been sufficiently gentle to warrant the conclusion that clones derived with the pPO1LhIL2 vector were unlikely to be capable of producing sufficient hIL-2 to support their own survival. Two alternatives were considered for increasing the levels of IL-2 produced while retaining some control in virtue of the inducible promoter: a) truncation of the IL-2 cDNA, removing the ATTTA elements in the 3' untranslated region, leading, presumably, to a more stable message; and b) retention of the full-

length cDNA, and utilisation of an episomally replicating vector so that the derived cells would contain several copies of the vector rather than the single integrated copy often found in stable transfectants created by electroporation. The Biomedical Research Centre had acquired such a vector, pBMGneo, and cDNA-containing derivatives of it had been shown to produce inducible secretion of various cytokines in mouse cell lines (Karusayama and Melchers, 1988). By virtue of the fragment of the bovine papilloma virus within the vector, derivatives replicate episomally, avoiding integration-position effects that result in variability of transcription from genomically integrated vectors. Although the mechanisms controlling replication of such vectors are still not fully understood, once several copies are within the nucleus of a cell, each daughter cell was believed at the time of these studies to inherit this same number of copies (Roberts and Weintraub, 1988, but see Ravnan et al., 1992 for a dissenting view).

The hIL-2 cDNA, including 3' ATTTA sequences, was cloned into the pBMGneo vector and the resultant plasmid, pBMGneoLhIL2 (fig. 1.31) was electroporated, without prior linearisation, into FD.C/2 cells. On the basis of previous experience, capacitance was set at 25μF, as before, but voltage was reduced to 750 V, and the resultant cell death was of the order of 60% (as judged by viability at 24 hours), rather than the approximately 90% previously observed. Cells were plated at 1 x 10^4 viable cells per well rather than 5 x 10^3, since it was expected that this circular vector would be several times less efficient than the linearised vector in the production of stable G418 resistance.

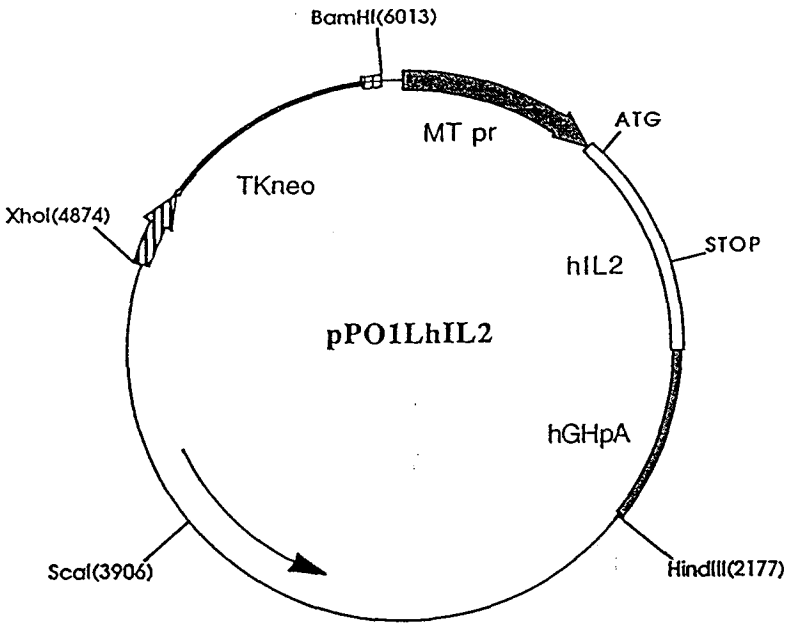
Three such electroporations were performed, resulting in 22 clones of G418-resistant cells. Following the weaning procedure outlined above, one clone, C2pBLhIL2, was able to continue proliferating in 300 micromolar zinc, without exogenous IL-2.

B) GROWTH AND FACTOR PRODUCTION OF C2pBLhIL2 CELLS.

i) Growth of C2pBLhIL-2 is zinc-dependent.

C2pBLhIL-2 cells were characterised with respect to their response to zinc concentration by carrying out a bio-assay in Terasaki wells as usual, but with a zinc chloride titration instead of cytokine. The cells in medium alone wells were all dead (as judged by visual inspection) after 94 hours, at which point ^{3}H-thymidine was added and incubation allowed to proceed a further 24 hours before harvesting. The assay was performed with cells at two different initial concentrations. The results, shown in figure 1.32, show that growth at both cell densities was strictly dependent on the presence of zinc, and correlated with the concentration of zinc present. A similar titration assay showed the growth of parental FD.C/2

A



B

ATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCCTGCCACAATGTACAGGATGCAACT
CCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACA
AAGAAAACACAGCTACAACTGGAGCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTA
ATAATTACAAGAATCCCAAACTCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGC
CACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAAT
TTAGCTCAAAGCAAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAG
TTCTGGAACTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACAGCAACCAT
TGTAGAATTTCTGAACAGATGGATTACCTTTTGTCAAAGCATCATCTCAACACTAACTTGATAA
TTAAGTGCTTCCCACTTAAAACATATCAGGCCTTCTATTTATTTAAATATTTAAATTTTATATT
TATTGTTGAATGTATGGTTTGCTACCTATTGTAACTATTATTCTTAATCTTAAAACTATAAATA
TGGATCTTTTATGATTCTTTTTGTAAGCCCTAGGGGCTCTAAAATGGTTTCACTTATTTATCCC
AAAATATTTATTATTATGTTGAATGTTAAATATAGTATCTATGTAGATTGGTTAGTAAAACTAT
TTAATAAATTTGATAAATATAAAAAAAAAAAACAAAAAAAAAA

Fig. 1.29. The integrating expression vector, pPO1LhIL2, used to attempt to make auto-stimulatory derivatives of FD.C/2 cells A) The vector. MT pr - metallothionein promoter; hGHpA - human growth hormone poly A addition signal; hIL2 - human IL-2 cDNA; NeoR - neomycin resistance cassette.
B) The hIL-2 cDNA in the vector. The initiation and termination codons are highlighted andthe 3' ATTTA sequences underlined.

cells was not supported by any concentration of zinc here assayed (as judged by visual inspection of wells).The relatively low levels of thymidine incorporation, in comparison to parental FD.C/2 cells growing in exogenous factor and to FD.C/2Ψ cells growing under autostimulatory conditions, suggested that the autostimulatory growth of C2pBLhIL2 cells in zinc was significantly slower than that of these other FD.C/2 lines. This impression was borne out during subsequent passage of these cells.

To maintain the C2pBLhIL2 clone in a zinc-dependent state, passaging of the line was carried out by 1:1 dilution into fresh medium containing 300 micromolar zinc chloride every 48 - 72 hours following visual inspection. Whereas the autostimulatory cells of the FD.C/2Ψ series could be maintained in passage at over 95% viability in passage without difficulty, cells of the C2pBLhIL2 clone were typically maintained at levels of approximately 85% viability by this regime. Moreover, if the density of viable cells was allowed to fall below approximately 1 x 10^5 per ml, the viability of the passages deteriorated. If the C2pBLhIL2 passage was first depleted of dead cells by Ficoll gradient centrifugation, and then replated at 1 x 10^5 cells/ml, it was possible to maintain populations at approximately 95% viability by 2:1 dilution in medium containing zinc chloride every 48 hours.

Although it was possible to maintain C2pBLhIL2 cells at higher viability when maintained at higher density in zinc, it was conceivable that some of the cell death was due to the loss of a given cell's ability to maintain IL-2 production, resulting from loss of the episomal plasmid. Consistent with this possibility was the observation that when C2pBLhIL2 cells were passaged for 6 weeks in exogenous IL-2 as well as zinc, and then exposed to G418 at 500 µg/ml for 5 days, 8% of the cells died, presumably because such cells had lost the episome. When the corresponding experiment had been carried out with FD.C/2Ψ.1 cells (albeit in the absence of zinc chloride), less than 1% of cells had died after exposure to G418.

Significantly, although only 8% of the C2pBLhIL2 cells died on exposure to G418, when a second population that had been expanded in exogenous IL-2 (and zinc) was washed free of exogenous IL-2 and replated at 1 x 10^5 cells/ml in medium containing zinc but no IL-2 (and no G418), approximately one third of the cells died over the next three days. This population could only be returned to normal autostimulatory growth after Ficoll gradient centrifugation and further replating at high density in zinc chloride. These results prompted suspicion that the C2pBLhIL2 clone might not have been truly clonal in nature (despite arising from one of 22 G418-resistant wells, of 768 wells seeded for the G418 selection). However, subsequent repetition of the experiment produced similar results, an observation not consistent with the suggestion that the C2pBLhIL2 population may have been derived from two clones of cells, a G418 resistant population, and a G418 sensitive population initially

maintained in G418 by leakage of the phosphotransferase from resistant cells (Bayever, 1990). Additionally, clones from other cell lines transfected with pBMGneo-derived constructs at the Biomedical Research Centre showed similar rates of loss of G418 resistance in the absence of continuous G418 selection pressure (Helen Merkens, personal communication). Given the unlikelihood of the two clone explanation, these data suggest that it may be necessary for the C2pBLhIL2 cells to maintain more copies of the pBMGneoLhIL2 plasmid to support optimal zinc-dependent growth than to display G418 resistance.

ii) Density dependence of C2pBLhIL2 cells.

An experiment to assess the density-dependence of C2pBLhIL-2 cells was performed, identical in design to that carried out with FD.C/2Ψ cells, except that the C2pBLhIL-2 cells were grown in 300 micromolar zinc (rather than simply medium alone) with or without IL-2. The results demonstrated that C2pBLhIL-2 cells showed a more acute sensitivity to density than did FD.C/2Ψ cells, but that this density-dependence was similarly abrogated by the addition of exogenous IL-2 (fig. 1.33).

iii) IL-2 production by C2pBLhIL2 cells is not detectable in 20 x supernatant.

Ten mls of C2pBLhIL2 conditioned medium were obtained by carefully expanding cells with daily addition of approximately 1/3 the volume in the dish of medium containing zinc chloride (but no IL-2 or IL-3). This supernatant was concentrated 20-fold as before and assayed on the HT-2 cell line for the presence of IL-2 as previously. No IL-2 activity was detected in this assay (fig. 1.34). To check whether this cell line might be liberating an inhibitor of HT-2 proliferation, which might have masked any possible IL-2 activity, a standard IL-2 titration was performed in the presence of a 1/4 dilution of the 20-fold concentrated C2pBLhIL2 supernatant (ibid.). No inhibitory activity was demonstrable under these conditions.

The 20 x supernatant was dialysed against 100 volumes of medium free of zinc, and then assayed on C2pBLhIL2 cells in the absence of zinc chloride. The dialysed supernatant did not support thymidine incorporation of these cells, and no viable cells were seen in the wells. When assayed on IL-3-dependent R6X cells, the 20 x supernatant showed a trace of stimulatory activity, which could be completely abolished by Rab 7 anti-mIL-3 antibody, demonstrating that, like other FD.C/2-derived cells, C2pBLhIL2 cells elaborate a small amount of IL-3 (fig. 1.35).

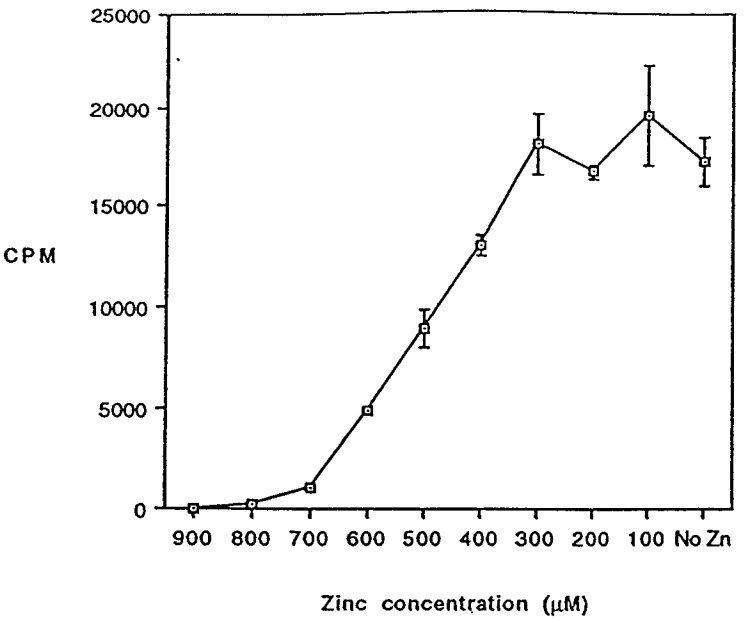


Fig. 1.30. Effect of zinc concentration on growth of FD.C/2Hyg cells Assay of proliferative response of FD.C/2Hyg cells in the presence of zinc chloride. 500 cells per well. 36 hour incubation followed by 12 hour ^{3}H-thymidine pulse.

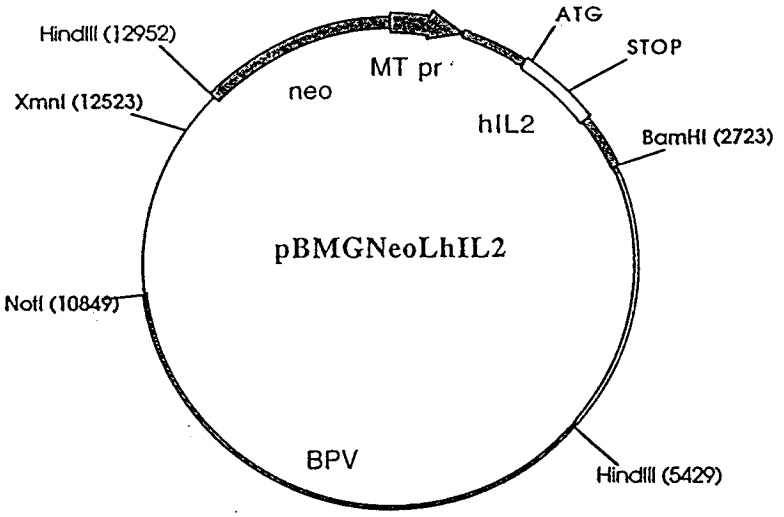


Fig. 1.31. The episomally replicating expression vector, pBMGNeoLhIL2, used to make auto-stimulatory derivatives of FD.C/2 cells neo - Neomycin resistance cassette, MT - mouse metallothionein I promoter, hIL-2 - human IL-2 cDNA. The shaded areas on either side of the cDNA represent splicing and polyA addition regions from the rabbit *ß-globin* gene. BPV - the "69%" fragment of the bovine papilloma virus genome that confers episomal replication.

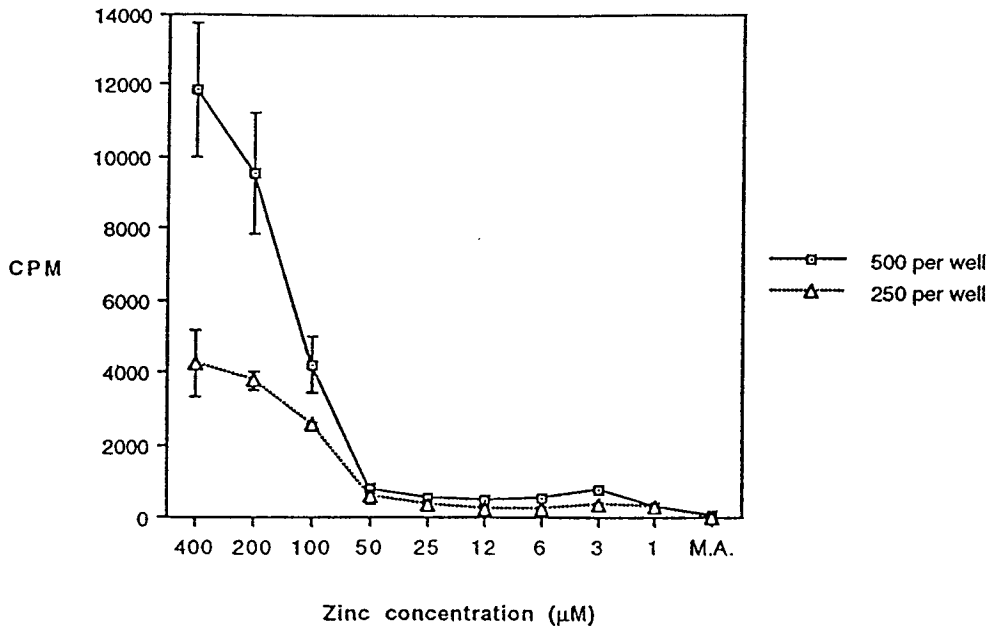


Fig. 1.32. Zinc-dependence of FD.C/2pBLhIL2 cells Assays were performed using cells at two different initial densities, as shown. 96 hour incubation followed by 16 hour ^{3}H-thymidine pulse.

iv) C2pBLhIL2 cells are unable to support parental FD.C/2 cells in co-culture conditions.

As a preliminary to comparing the susceptibility of C2pBLhIL2 and FD.C/2 cells to antibody in IL-2 supplied by C2pBLhIL2 cells, co-cultures with hygromycin-resistant FD.C/2Hyg cells were established in 300 micormolar zinc, at ratios of 20:1, 10:1 and 1:1. The resultant numbers per well of C2pBLhIL-2 cells were 1000, 500, and 50 respectively (corresponding to 1 x 10^5, 5 x 10^4, and 5 x 10^3 cells/ml). After 3 days of co-culture, no viable cells were visible in cells of the 1:1 ratio wells, presumably because the density of C2pBLhIL2 cells was too low at the outset to maintain their autostimulatory growth. In some of the 10:1 ratio wells, and all of the 20:1 ratio wells, however, many apparently viable wells were present. These wells were exposed to hygromycin and exogneous IL-2 as before, but after 8 days of further incubation, no viable cells were visible in any of the wells (visual impression was confirmed with trypan blue staining). Under these conditions, C2pBLhIL2 cells were unable to support the growth of IL-2 dependent parental cells.

v) C2pBLhIL-2 cells *in vivo.*

Two weeks before commencing injection of C2pBLhIL2 cells into DBA/2 mice to assess their ability to form tumours, the drinking water of the animals was supplemented to a concentration of 100 millimolar with zinc phosphate. This concentration was chosen as the highest used in experiments in which induction of metallothionein promoters had been undertaken in transgenic animals (Palmiter et al., 1983 ; Habener et al., 1989; Morahan et al., 1989; Shanahan et al., 1989,). This supplementation was continued throughout the experiment, with drinking water changed approximately every 7 days. Animals were injected subcutanously (3) or intraperitoneally (3) with 5 x 10^5 cells. After 10 weeks, all these animals appeared healthy, and showed no signs of tumour development. The experiment was therefore repeated with inocula of 2 x 10^6 cells per injection. These animals similarly showed no subsequent signs of tumour development, and remained healthy for at least 4 months following injection of C2pBLhIL2 cells.

C) Antibody-mediated antagonism of IL-2 completely inhibits growth of C2pBLhIL2 cells

Antibody assays were performed in Terasaki wells, with C2pBLhIL2 cells plated at either 1000 (one plate) or 500 (one plate) cells per well. In each plate, 12 wells contained 300 micromolar zinc, 12 contained zinc and antibody (either DMS or PC61), 12 contained zinc, PC61 and a saturating concentration of exogenous IL-2, and 12 contained zinc, PC61 and a saturating concentration of IL-3.

After 4 days, no apparent viable cells were present in either plate in wells containing only zinc and antibody. Wells containing exogenous factor in the assay set up at 1000 cells per well, showed apparent overgrowth of cells, so only the 500 cell per well assay was pulsed with ^{3}H-thymidine. Incubation was allowed to proceed a further 8 hours before harvesting the assay. The results, seen in figure 1.36, show that thymidine incorporation of C2pBLhIL2 cells was completely abrogated by both antibodies, and the cells could be "rescued" from this effect by saturating amounts of IL-2 and IL-3 added at the initiation of the assay.

This antibody-inhibition assay was repeated, and wells containing antibody (without exogenous IL-2 or IL-3) were either stained with trypan blue after 4 days, or treated by addition of exogenous IL-2 or IL-3 in medium after 4 days, and further incubated. Neither trypan blue nor this "late rescue" attempt revealed any viable cells, confirming that antibody not only abrogated thymidine incorporation but resulted in death of C2pBLhIL2 cells.

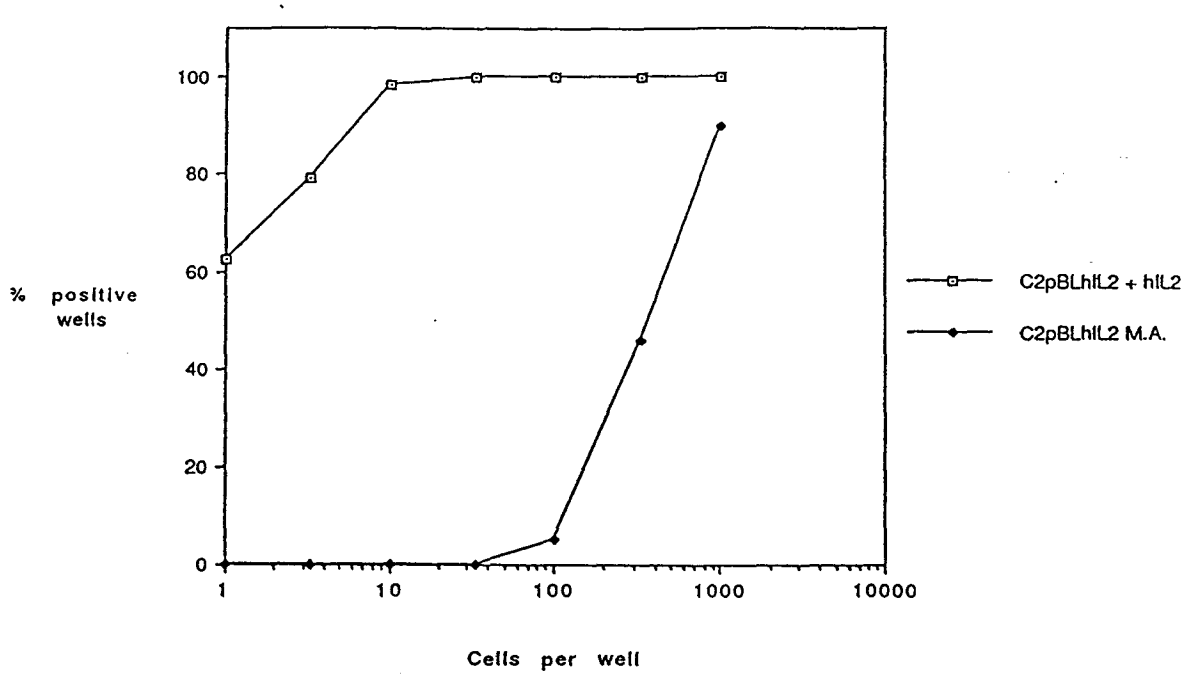


Fig. 1.33. Density-dependence of FD.C/2pBLhIL2 cells

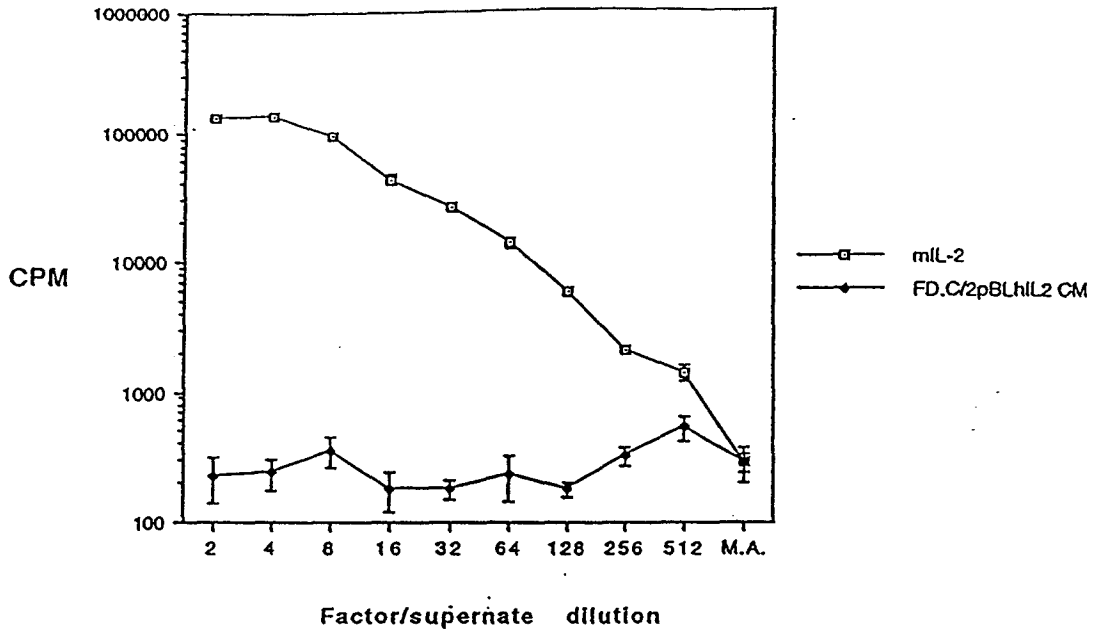


Fig. 1.34. IL-2 activity is not detectable in the supernatant of FD.C/2pBLhIL2 cells 20-fold concentrated conditioned medium of FD.C/2pBLhIL2 cells was assayed on HT-2 cells in the presence of the neutralising anti-mL4 antibody, 11B11. Also shown is an IL-2 response curve in the presence of a 1/4 dilution of the conditioned medium.1000 cells per well; 36 hour incubation followed by 12 hour ^{3}H-thymidine pulse.

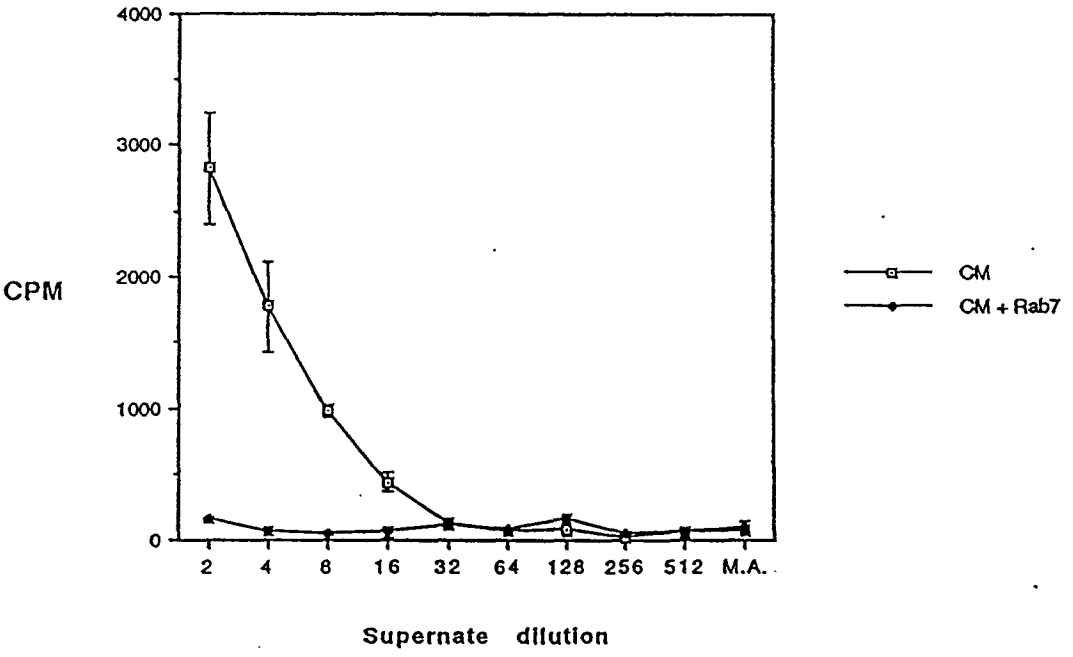


Fig. 1.35. IL-3 activity in the supernatant of FD.C/2pBLhIL2 cells 20-fold concentrated conditioned medium of FD.C/2pBLhIL2 cells was assayed on R6X cells alone, or in the presence of the neutralising anti-mIL-3 antibody, Rab7. 36 hour incubation plus 8 hour ^{3}H-thymidine pulse.

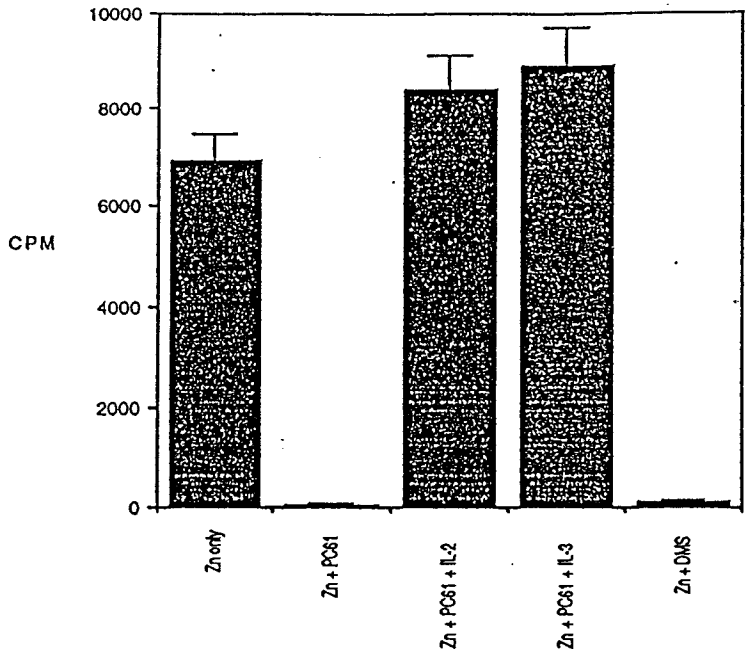


Fig. 1.36. Antibody antagonists of IL-2 can completely inhibit the growth of FD.C/2pBLhIL2 cells
Growth of FD.C/2pBLhIL2 cells was assayed in 300 micromolar zinc alone, in the presence of neutralising anti-mIL2-receptor antibody, PC61 (approximately 5 µg/ml) or in the presence of PC61 antibody and either IL-2 or IL-3, or in the presence of neutralising anti-hIL-2 DMS antibody preparation (approximately 40 µg/ml). 500 cells per well. 96 hour incubation followed by 12 hour ^{3}H-thymidine pulse.

DISCUSSION

The experiments described here represent the first demonstration that antibody antagonists of the growth factor can induce the death of cells that grow by autostimulatory mechanisms. They support the hope that cytokine antagonists may find use as therapeutic reagents in the treatment of autostimulatory neoplasms. Such therapy would be particularly applicable to neoplasms, such as leukemia, in which autostimulatory mechanisms may be necessary for the survival (as well as growth) of the abnormal cells.

These experiments also demonstrated that autostimulatory mechanisms require the production of surprisingly small amounts of growth factor by the tumour cells. Significantly, tumours could be caused by conferring upon immortal factor-dependent cells the ability to produce amounts of growth factor so small that they were almost undetectable. This finding implies that it may not be easy to determine that an autostimulatory mechanism is critical in the genesis of a given tumour, because there may be no detectable level of growth factor produced. The suggestion that it may be just those autostimulatory cells that produce the lowest amounts of growth factor that are most susceptible to therapy based on growth factor antagonists makes it more important to identify autostimulatory mechanisms involving very low levels of growth factor.

A more detailed analysis of these conclusions and the data on which they are based follows.

A) Autostimulatory cells have a marked advantage in terms of access to growth factor; very little growth factor may suffice for autostimulation.

To obtain cells producing various levels of autostimulatory growth factor, two types of vector, retroviral and episomal, were used to transfect FD.C/2 cells. Using a retroviral vector, in which the LTR served as the promoter for IL-2 production, a series of clones of autostimulatory cells were derived. These clones all retained responsiveness to IL-2 and IL-3, and exhibited density-dependence for growth in the absence of exogenous factor (a common characteristic of autostimulatory cells see introduction). Four of these 5 clones liberated detectable levels of IL-2 into their culture supernatants, but the 10-fold concentrated supernatant of the fifth contained no detectable IL-2. However, all 5 were capable of producing tumours in syngeneic animals. Cells of 4 of the 5 clones were able to support the growth of neighbouring parental factor-dependent cells in co-culture conditions, but only at high "stimulator to responder" ratios (10:1).

Using the episomal vector, a single autostimulatory clone was obtained. In this case the production of IL-2 was dependent upon a zinc-responsive metallothionein promoter. The IL-2 cDNA in this vector included 3' untranslated sequences which are known to mediate rapid degradation of cytokine message in hemopoietic cells. The features of this vector allowed isolation of a clone whose growth was dependent on the addition of zinc to the medium. This clone displayed a more marked density-dependence than the retrovirally derived clones, but its density-dependence was similarly abrogated by exogenous IL-2. Despite autostimulatory proliferation (in the presence of zinc), cells of this clone did not liberate levels of IL-2 activity detectable in 20-fold concentrated supernatant, nor could they support neighbouring factor-dependent cells in co-culture conditions. Additionally, thymidine incorporation assay and observation of passages of these cells suggested that even in optimal zinc-dependent growth conditions, these cells grew more slowly than the retrovirally derived autostimulatory cells. To date it has not been possible to obtain tumours in syngeneic animals from this clone, despite supplementation of drinking water of mice with zinc phosphate, and inocula containing 4 times as many cells as those used to obtain tumours from retrovirally derived autostimulatory lines.

B) Growth factor antagonist antibodies can bring about the death of autostimulatory cells.

All 5 retrovirally derived clones were susceptible to a significant degree of death due to antibody antagonism of IL-2. None of these clones could be completely destroyed by such treatment, not even that in which IL-2 production was undetectable. Cells surviving such treatment showed no apparent genetic resistance to antibody-induced death, as repeating the experiment with the daughters of surviving cells produced similarly incomplete death of the population. Such autostimulatory cells were markedly resistant to antibody-mediated antagonism of IL-2 in comparison with their factor-dependent neighbours, which all died when co-cultures included antibody. This advantage was not due to alteration in the levels of IL-2 receptor at the cell surface as judged by FACS analysis of distribution of the p55 chain of the receptor.

In contrast, treatment of the zinc-dependent autostimulatory cells with anti-hIL-2 or anti-mIL-2 receptor antibodies left no viable cells. This result was unlikely to be due to susceptibility of the zinc-dependent cells to non-specific toxicity of the antibody peparation as the anti-IL-2 effect could be abrogated by exogenous IL-2 or IL-3. These cells produced so little IL-2 that IL-2 activity was not detectable in 20-fold concentrated supernatant of this clone, and they were unable to support the growth of IL-2 dependent parental cells in co-culture assays.

The results presented here document for the first time the complete antibody-mediated inhibition of autostimulatory growth, and cell death. In what way do the results presented here, differ from other models in which growth was only partially inhibited?

Several other laboratories have shown incomplete blockage of autostimulatory growth with antibody (see introduction), although most have not presented the careful controls for specificity of antibody activity presented here. One straight-forward explanation presents itself as a reason for the failure to achieve complete inhibition in the models studied by other workers: in their autostimulatory model systems the antibody used was inadequate to overcome the autostimulation. To state the problem more mathematically: the product of the amount of autostimulatory factor produced, the amount of receptor present, and the affinity of the interaction between growth factor and receptor exceeded the product of the amount of antibody and its affinity for receptor or growth factor. Either their cells made too much factor to inhibit with their antibodies, or the "inhibitory" antibody they used was not of sufficient potency, as a result of its affinity being too low, or of its target epitope being in a region of the molecule that results in poor competition for the growth factor-receptor interaction. Given the present experiments, in which autostimulatory growth was completely inhibitable, any instance of failure to inhibit is open to the argument that the failure was due to the combination of insufficient concentration of antibody at the critical site and inadequate affinity of the antibody-target interaction in comparison to that of the growth factor-receptor interaction. Whereas the dissociation constants for higher affinity antibody-antigen interactions are, at best, of the order of 10^{-9}M (Goding, 1986), that of the high-affinity IL-2-receptor interaction, typical of cytokine-receptor affinities, is 10^{-11}M (Waldmann, 1991).

This explanation, however, conceals a more subtle but important possible reason for the difference between models. It has long been known that the degree of proliferation induced in response to IL-2 correlates relatively well with the proportion of cell-surface receptors occupied, in contrast to GM-CSF and IL-3, for instance. These latter cytokines display their whole range of proliferative stimulus, from barely detectable to maximal proliferation, while only varying the percentage of cell-surface receptors occupied (in equilibrium assays) from zero to less than 10%, in naturally occuring factor-responsive cells (reviewed in Kuziel and Greene, 1991, and Schrader, 1991). IL-2, on the other hand, must occupy a much higher proportion of available cell-surface receptors in order to produce maximal proliferative response. The calculated affinities for receptors of GM-CSF, IL-3 and IL-2 are all roughly similar, however, with dissociation constants of the order of 10^{-11} to 10^{-12} molar (Garland, 1991, Kuziel and Greene, 1991; Schrader, 1991). An autostimulatory mechanism involving IL-2, therefore, might be intrinsically more susceptible to inhibition than models involving GM-CSF or IL-3, since inhibition of IL-2 requires the blockade of a smaller proportion of factor-receptor interactions to

diminish resultant proliferation, than is the case for GM-CSF and IL-3. Thus, in the case of IL-2, a cell that had been growing optimally in a condition of 25% receptor occupancy might die in the presence of an antibody capable of reducing receptor occupancy by 90%, since 2.5% receptor occupancy would be insufficient to maintain growth. In the case of IL-3, on the other hand, such an antibody would not interfere with growth, since 2.5% receptor occupancy would be adequate to maintain IL-3 dependent growth.

2 QUESTIONS RAISED

1) Why is autostimulatory growth so difficult to reverse with antibody?

Although this study demonstrated that complete inhibition of autostimulatory growth was in fact possible, it also showed that a cell population which was making very little of an autostimulatory factor as judged by the amount detectable in culture supernatants, was nevertheless resistant to inhibition of autostimulatory growth by antibody. This was despite the fact that the antibody preparations were capable of blocking the effects of much more factor when applied exogenously. Other workers who have made similar observations have speculated that functional growth factor-receptor interactions were occuring within the cell. The fact that it *was* possible to isolate an autostimulatory clone which is susceptible to antibody-mediated growth inhibition, does not imply that intracellular interactions between factor and receptor could not in other circumstances be responsible for growth. All that can be concluded in this respect is that intracellular ligand-receptor interactions, if they did occur in this clone, were insufficient to maintain the viability of the cells. To state the matter more formally - the null hypothesis disproven by these studies is *NOT*: "Intracellular growth factor-receptor interactions occur and are capable of leading to growth of autostimulatory cells", but rather: "Autostimulation of growth and survival cannot be blocked by growth factor antagonists".

Ligand-receptor interaction in autostimulatory cells might conceivably occur in several compartments that are inaccessible to antibody - within the endoplasmic reticulum or the Golgi apparatus, for example. For each compartment, the question arises "Can signal transduction take place here?" The simplest hypothesis would be that further transduction of signal to the nucleus could only be initiated in the environment immediately below the cell membrane, because other components of the early steps of the cytokine signal transduction cascades initiated by ligand-receptor interaction, such as ras (Barbacid, 1987), and the src-like kinases (Resh, 1990), are firmly associated with the plasma membrane.

Is the question of whether intracellular interactions between growth factor and receptor are functional answerable? How might one go about addressing this question experimentally? An approach taken by Dunbar et al. (1989) involved the creation of a model in which, it was hoped, the IL-3 would be retained within the endoplasmic reticulum (ER). The IL-3 cDNA was so altered as to encode a carboxy-terminal sequence (lysine-aspartic acid-glutamine-leucine) which had been shown to be able to cause retention of various proteins in the ER. This cDNA was expressed with a retrovirus in an IL-3-dependent cell, as before, and autostimulatory clones were derived. However, expression of the altered IL-3 cDNA was controlled by the viral LTR, and this "strong" promoter activity resulted in apparent overloading of the ER retention signal, so that IL-3 activity was detected in the supernatant of these clones. To address this problem, the retroviral construct was altered so as to contain an internal (and relatively weak) SV40 promoter which would govern expression of the IL-3 cDNA. Using this system, the authors obtained 5 clones, of which 4 secreted no detectable IL-3, and the fifth a very low level. Neutralising antibody to IL-3 did not inhibit growth of these clones. The authors concluded that functional IL-3-receptor interaction could take place within the ER.

Although these experiments were elegant in themselves, there is a flaw in the reasoning between the data provided by these experiments and the conclusion drawn by the authors. It is quite possible that enough IL-3 escapes from the ER to occupy receptors at the cell surface and thereby promote proliferation of cells, but that this IL-3 is bound or consumed in the interaction so that it is not detectable by examination of supernatant or even of membrane lysates. This explanation would still allow for the meeting of factor and receptor within the cell, even within the ER, but would rely on the leakiness of the IL-3 retention system, and/or the effect of the un-retained receptor in "dragging" bound IL-3 to the cell surface. There is no evidence, either from the literature, or presented by these authors, that functional IL-3-receptor interaction requires levels of IL-3 that must be detectable by the most sensitive bioassay methods. The evidence from the present work would suggest just the opposite - that functional cytokine-receptor interaction can take place when cytokine is present at levels below the limits of detection.

A next step in the refinement of such experiments would be to create a model in which receptor gene is mutated so as to cause retention of the receptor molecules within intracellular compartments. Given the multi-subunit nature of many of the better-characterised cytokine receptors, however, this task would be daunting at the least, and only worth the undertaking if it were clear that results could be obtained that would not be subject to objections similar to those raised above. An alternative refinement of such experiments might be to engineer cells to express a neutralising antibody intracellularly in the compartments where factor-receptor interaction might occur. This experiment, however, would only be informative if the result were positive and autostimulation was in fact inhibited,

since many explanations for a failure to inhibit could be invoked (e.g. the folding of the antibody molecule within the cell is such as to reduce its affinity; the antibody's function is impeded by the physiological milieu of the compartment of the cell in which the important interaction is taking place; not enough antibody is being produced to saturate the receptor or factor produced in the cell). Even if a positive result were obtained, the onus would be on the experimenter to prove that the antibody had remained confined to the relevant compartment, in order that the experiment should conclusively establish that this was indeed the site of effective ligand-receptor interaction. Data relating to intracellular function of antibodies are currently being accumulated in several laboratories (Werge et al., 1990; Biocca et al., 1990) and experiments involving intracellular antibodies as inhibitors of cytokine signalling pathways are in progress at the Biomedical Research Centre.

Another system in which workers have seriously grappled with the question of intracellular activation of cytokine receptors has involved platelet-derived growth factor (PDGF). Unlike the known chains of the IL-2, GM-CSF and IL-3 receptors, this receptor has protein tyrosine kinase activity, and is known to auto-phosphorylate on binding ligand. Keating and Williams (1988) created an autostimulatory model in fibroblastoid NRK cells engineered to express the PDGF-like product of the v-*sis* oncogene. Using metabolic labelling of these cells, these workers showed that the majority of the PDGF receptor in the autostimulatory cells, in contrast with the parental cells, was incompletely processed with respect to the normal sequence of glycosylation events that occur prior to the appearance of the mature receptor at the cell surface. Moreover, the majority of the receptor was insensitive to trypsin treatment of the cells, suggesting its intracellular localisation. This trypsin-insensitive receptor was phosphorylated on tyrosine, suggesting that it had interacted with ligand. The poly-anionic compound suramin, which inhibits the interaction of PDGF with its cell-surface receptor, failed to inhibit the phosphorylation of the receptor in the autostimulatory cells. As the authors themselves admit, however, they could not exclude the possibility that a small amount of receptor-ligand complex was present at the cell-surface of their autostimulatory cells, and it is possible that these molecules could be responsible for the actual proliferation of the cells. Since the PDGF receptor is known to auto-phosphorylate on contact with ligand at the cell surface, the presence of tyrosine-phosphorylated PDGF receptor within the cell, although suggesting that it may have met its ligand there, does not imply that effective further signal transduction took place other than from the vicinity of the cell surface. Since the src family kinases with which the PDGF receptor associates to transmit a proliferative signal (Kypta et al., 1990) are localised at the inner plasma membrane, it seems likely that phosphorylated receptor must have been present in this region, in the compartment of the cell (i.e. outside the trans-Golgi) where these kinases are found.

The results of Hannink and Donoghue (1988) seem to support this conclusion. Using a heat-shock inducible promoter, these workers created a model allowing them to study the effects of a pulse of the v-*sis* product produced within fibroblasts, on phosphorylation of the PDGF receptor and the downstream induction of c-*fos*. They showed that monensin, which inhibited the transport of the sis product through the trans-Golgi, inhibited the induction of c-*fos* without inhibiting phosphorylation of the (incompletely processed) PDGF receptor, suggesting that although ligand-receptor interaction and activation of the receptor kinase had taken place, further signal transduction was blocked. In this model, suramin also inhibited c-*fos* induction suggesting, according to the authors, that receptor-ligand interaction needed to take place at the cell surface to result in effective signal transduction. This last conclusion, however, is invalid since suramin can enter and function within cells (Huang and Huang, 1988, and references therein). In fact, in the hands of Hannink and Donoghue, suramin appeared to have inhibited phosphorylation of the incompletely processed form of the PDGF receptor, as well as of the mature form. Skeptics might also argue that monensin could well have effects on the cell other than the simple inhibition of transport between Golgi and plasma membrane, making these results difficult to interpret. Huang and Huang (1988), on the other hand, using similar metabolic labelling experiments to those of Keating and Williams, concluded that signalling from the sis-product- PDGF complex did indeed take place from within the cell. They went on (Lokeshwar et al., 1990) to carry out similar detailed analyses of the biosynthesis and turnover of the sis product in autostimulatory cells to support their conclusions. However, these conclusions are based on observations of intracellular turnover, a very indirect and probably error-prone measure of the whereabouts of active ligand-receptor complexes and are thus open to similar objections to those raised against Keating and Williams (above).

Cytokines such as IL-3 may activate mitogenesis at extremely low concentrations (perhaps as few as 20 molecules per cell will suffice). It would seem impossible therefore, given current technology, and the current level of understanding of the signal transduction process, to definitively answer the question of whether the cytokine signal transduction cascade can be initiated from within cells in regions or compartments other than the "physiological" area near the cell surface. As a result, experiments currently performable, aiming to prove that one could never inhibit an autostimulatory cell from the outside because of an internal autostimulation mechanism, are bound to fall short of their mark. The only informative experiments in these models are those in which inhibition has been successful.

2) Why did zinc-dependent autostimulatory cells fail to give rise to tumours in syngeneic animals?

One explanation for this failure is that it is impossible to create concentrations of zinc in the vicinity of the injected cells sufficiently high to allow autostimulatory growth to occur in these particular cells, without poisoning the mice. Redesigning the IL-2 cDNA vector to incorporate an inducible promoter which can be more vigorously up-regulated than the mouse metallothionein promoter in pBMGNeo might overcome this problem. More recently developed expression vectors contain inducible promoters with a significantly increased dynamic range in comparison with that of the unmodified metallothionein promoters (e.g. Hu & Davidson, 1990; Labow et al., 1990).

A less likely explanation involves the possibility that the zinc concentrations achieved in the micro-environment of injected cells was higher than these cells would tolerate. This issue might be resolved by obtaining measurerments of zinc levels in the tissues of control mice, and mice receiving zinc supplementation.

A further alternative explanation of the failure to produce tumours involves the possibility of an immune response to the injected cells. It might be expected that a cell line generates several proteins or other antigens (e.g. carbohydrate determinants) that are foreign to the animal from which it was originally derived, since immortal cell lines are known to accumulate multiple abnormalities at the DNA level. Providing they carry the machinery for antigen presentation, including appropriate major histocompatibility complex molecules, it is quite likely that injection of such cells into an immunocompetent animal will result in a competition between the ability of the injected cells to proliferate and the ability of the immune system to destroy the cells. In the case of the zinc-dependent autostimulatory cells, foreign proteins (in addition to hIL-2 and neomycin phosphotranseferase also present in the retrovirally derived cells) could be predicted to be present. viz - those encoded by the bovine papilloma virus portion of the vector in these cells. It has recently been demonstrated that expression of IL-2 in an otherwise tumourigenic cell line renders the cells susceptible to immune-mediated destruction *in vivo* (Fearon et al., 1990).

To go some way towards investigating the role of rejection mechanisms in the failure of these zinc-dependent cells to produce a tumour, one might inject the cells into mice that have high levels of circulating IL-2. This could be achieved by injection or the introduction into the peritoneal cavity of a mini-pump which would continuously release IL-2. In the absence of any form of rejection mechanism , tumours should form, and it should be possible to recover from them cells that are still G418-resistant, and ideally that could be weaned into zinc-dependent growth *in vitro* (although in the absence of a selective pressure, it is conceivable that all the cells might lose the episome carrying the sequences

encoding G418 resistance and zinc-dependent IL-2 production). As distinct from the investigation of whether immune rejection of the tumour occurs, the problem might be avoided by repeating the experiment in nude mice. Indeed, it might be suggested that this experiment would more closely mimic some human leukemic conditions than the syngeneic mouse experiments, since many leukemic patients display significant compromise of immune function (albeit, generally, in the more advanced stages of disease).

3) The future of therapeutic approaches to autostimulatory neoplasia

i) Is anti-sense RNA a viable alternative as therapy for autostimulatory tumours?

Although the stated aim of these studies was to investigate the tumour-inhibitory potential of growth factor antagonists rather than antagonists of growth factor *production,* mention should be made of anti-sense inhibition of protein synthesis, given the popularity of this approach in inhibiting intracellular events. There are many reports of the successful application of anti-sense techniques in the literature, including at least one dealing with anti-sense inhibition of IL-2-dependent autostimulatory growth (Harrel-Bellan et al., 1988). Given the difficulty of publishing negative data, however, it is unclear in what proportion of attempts the anti-sense approach is successful. The presence of double-stranded RNA within a cell (which is thought to be necessary for anti-sense function) is known to lead to specific cellular responses including, for example, interferon production in many cell types (reviewed in De Maeyer and De Maeyer-Guignard, 1991). These cellular responses might well be related to the formation of double-stranded RNA by some viruses, since interferon production, at least, is characteristic of the cellular response to virus. Interferon is known to have a variety of effects on cell function including effects on proliferation, varying according to the cell type (ibid.). In terms of experiments in inhibition of autostimulatory growth, then, it is hard to imagine what molecule to use as a control target for these "side-effects" of anti-sense - a molecule would have to be chosen whose production could be inhibited without affecting cellular function, so that one could demonstrate that it is the inhibition of production of autostimulatory factor rather than the mere presence of double-stranded RNA within the cell that is causing the inhibition of growth. One possible way to set up such a control would be to first of all introduce into the cells a vector producing an RNA species encoding a protein product not usually found in that cell type, and which would be predicted to have no effect on cell function (or none relevant to the experiment). Anti-sense inhibition of this RNA could then be used as a control. The argument might be presented, however, that any "side-effects" of the anti-sense mechanism (e.g. inhibition of cellular proliferation by interferon-related mechanisms), might be regarded as therapeutic effects, (for instance, in the case of inhibition of

autostimulatory growth), and that experimental controls, are unneeded. Indeed, this seems to have been the attitude of many workers who have published experiments using anti-sense, since no such publication, to the knowledge of this author, has included controls of the type described above. What is commonly seen instead is the use of a sense oligo-nucleotide, as "control" for the anti-sense when synthetic oligo-nucleotides are used for experiments *in vitro.*

A major problem with anti-sense as a therapeutic tool lies in the difficulty of delivering sufficient anti-sense to cells *in vivo.* Whereas an antibody or other growth factor-antagonist could be delivered to a patient (or experimental animal) parenterally, and may function at the cell surface, the anti-sense approach would require delivery of the source of the anti-sense to the nucleus of the target cell. The simplest currently available mechanism for effecting such delivery involves production of anti-sense message by a virus which is capable of infecting the target cells *in vivo.* Narrowing the range of infected targets would then be a serious concern for many autostimulatory factors, since one would not want to create a situation in which *no* cell of the patient could ever make, for example IL-2, which is essential to the ability of a T lymphocyte to mount a normal immune response.

ii) Better cytokine antagonists.

The numerous autostimulatory models in which partial inhibition of growth was achieved with antibody, and the present data, showing that, in favourable circumstances, death of autostimulatory cells is achievable, inspire the hope that more potent antagonists will lead to therapy for autostimulatory neoplasia. Of reagents known to be capable of binding to the various cytokine receptors, those with the highest affinity are the cytokines themselves. The search for potent antagonists has resulted in the recent discovery of several cytokine analogs which are much more potent antagonists than any antibodies currently available. These antagonists are closely related to the corresponding cytokines. In the case of human growth hormone (Fuh et al., 1992), the most potent antagonist described differs from the wild-type hormone in four amino acids. An antagonist of human IL-4 has been described (Kruse et al., 1992) which has an affinity for the IL-4 receptor of the same order of magnitude as that of wild-type IL-4, and differs from it in only one amino acid. A similar antagonist of human IL-2 has been produced (J.W. Schrader, personal communication). Such antagonists may resolve problems of the relationship between cytokine structure and function, as well as allow advances in the therapy of various cytokine-related disorders, including autostimulatory neoplasia.

BIBLIOGRAPHY

Aaranson, S.A., & Todaro, G.T. (1968) *J.Cell. Physiol.* 72, pp. 141-148.

Adkins, B., Leutz, A., & Graf, T. (1984) Autocrine growth induced by *src*-related genes in transformed chicken myeloid cells. *Cell* 39, pp. 439-445.

Andrejauskas, E., & Moroni, C. (1989) Reversible abrogation of IL-3 dependence by an inducible H-*ras* oncogene. *EMBO J.* 8, pp. 2575-2581.

Armitage, R.J., Lai, A.P., Roberts, P.J., & Cawley, J.C. (1986) Certain myeloid cells possess receptors for interleukin-2. *Brit. J. Haematol.* 64, pp. 799-807.

Barbacid, M. (1987) *ras* genes *Ann. Rev. Biochem.* 56, pp. 779-827.

Baumann, M.A., & Paul, C.C. (1992) Interleukin-5 is an autocrine growth factor for Epstein-Barr virus-transformed B lymphocytes. *Blood* 79, pp. 1763-1767.

Baumbach, W.R., Stanley, E.R., & Cole, M.D. (1987). Induction of vlonal monocyte-macrophage tumours in vivo by a mouse c-*myc* retrovirus: rearrangement of the CSF-1 gene as a secondary transforming event. *Mol.Cell.Biol.* 7, pp. 664-671.

Bayever, E. (1990) Gene transfer into hematopoietic cells. (Correspondence) *Blood* 75, p. 1587.

Bertoglio, J., Dosda, J., Stancou, R., Wollman, E., & Fradelizi, D. (1989) Expression and regulation of interleukin-1 mRNA and interleukin-1 receptors in human B-cell lines. *J. Mol. Cell. Immunol.* 4, pp. 139-148.

Betsholtz, C., Westermark, B., Ek, B., & Heldin, C.-H. (1984) Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line: implications for autocrine receptor activation. *Cell* 39, pp. 447-457.

Biocca, S., Neuberger, M.S., & Cattaneo, A. (1990) Expression and targetting of intracellular antibodies in mammalian cells. *EMBO J.* 9, pp. 101-108.

Blankenstein, T., Qin, Z., Li, W., & Diamantstaien, T. (1990). DNA rearrangement and constitutive expression of the interleukin 6 gene in a mouse plasmacytoma. *J. Exp. Med.* 171, pp. 965-970.

Bordoni, R., Thomas, H.G., & Richmond, A. (1989) Growth factor modulation of melanoma growth stimulatory activity mRNA expression correlates with cell growth. *J. Cell. Biochem.* 39, pp. 421-428.

Bos, J.L., de Vries, M.U., van der Eb, A.J., et al. (1987) Mutations in N-*ras* predominate in acute myeloid leukemia. *Blood* 69, p. 1237.

Bowen-Pope, D.F., Vogel, A., & Ross, R. (1984) Production of platelet-derived growth factor-like molecules and reduced expression of platelet-derived growth factor receptors accompany transformation by a wide spectrum of agents.*Proc. Natl. Acad. Sci. USA* 81, pp. 2396-2400.

Browder, T.M., Abrams, J.S., Wong, P.M.C., & Nienhuis, A.W. (1989) Mechanism of autocrine stimulation in hematopoietic cells producing interleukin-3 after retrovirus-mediated gene transfer. *Mol. Cell Biol.* 9, pp. 204-213.

Burd, P.R., Rogers, H.W., Gordon, J.R., & Dorf, M. (1989) Interleukin-3 dependent and independent mast cells stimulated with IgE and antigen express multiple cytokines. *J. Exp. Med.* 170, p. 245.

Cozzolino, F., Rubartelli, A., Aldinucci, D., Sitia, R., Torcia, M., Shaw, A., & Di Guglielmo, R. (1989). Interleukin 1 as an autocrine growth factor for acute myeloid leukemia cells. *Proc. Natl. Acad. Sci. USA* 86, pp. 2369-2373.

Crapper, R.M., Thomas, W.R., & Schrader, J.W. (1984) *In vivo* transfer of persisting (P) cells: further evidence for their identity with T-dependent mast cells. *J. Immunol.* 133, pp. 2174-2179.

Cuttitta, F., Carney, D.N., Mulshine, J., Moody, T.W., Fedorko, J., Fischler, A., & Minna, J.D. (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature* 316, pp.823-826.

Dautry-Varsat, A., Hemar, A., Cornet, V., & Duprez, V. (1988) Autocrine growth of a human T-cell line is inhibited by cyclosporin A. *Blood* 72, pp. 588-592.

Delwel, R., van Buitenen, C., Salem, M., Bot, F., Gillis, S., Kaushansky, K., Altrock, B., & Lowenberg, B. (1989) Interleukin-1 stimulates proliferation of acute myeloblastic leukemia cells by induction of granulocyte-macrophage colony-stimulating factor release. *Blood* 74, pp. 586-593.

De Maeyer E., & De Maeyer-Guignard, J. (1991) Interferons *in* The Cytokine Handbook (A. Thomson ed., Academic Press, London) pp. 83-102.

Dexter, T.M., Garland, J., Scott, D., Scolnick, E., & Metcalf, D. (1980) Growth of factor-dependent hemopoietic prescursor cell lines. *J. Exp. Med.* 152, pp.1036-

Dinarello, C.A. (1991) Interleukin-1 and interleukin-1 antagonism. *Blood* 77, pp. 1627-1652.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., & Antoniades, H.N. (1983) Simian sarcoma virus oncogene v-*sis* is derived from the gene (or genes) encoding a platelet derived growth factor.*Science* 221, pp. 275-277.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., & Waterfield, M. (1984) Close similarity of epidermal growth factor receptor and v-*erb-B* oncogene protein sequences. *Nature* 301, pp. 521-527.

Drago, J., Murphy, M., Campbell, S.M., Harvey, R.P., & Bartlett, P. (1991) Fibroblast growth factor-mediated production of central nervous system precursors depends on endogenous production of insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA* 88, pp. 2199-2203.

Duhrsen, U., Stahl, J., & Gough, N.M. (1990) *In vivo* transformatin of factor-dependent hemopoietic cells: role of intracisternal A particle transposition for growth factor gene activation. *EMBO J.* 9, pp. 1087-1096.

Dunbar, C.E., Browder, T.M., Abrams, J.S., & Nienhuis, A.W. (1989) COOH-terminal-modified interleukin-3 is retained intracellularly and stimulates autocrine growth. *Science* 245, pp. 1493-1496.

Duprez, V., Lenoir, G., & Dautry-Varsat, A. (1985) Autocrine growth stimulation of a human T-cell lymphoma line by interleukin 2. *Proc. Natl. Acad. Sci. USA* 82, pp. 6932-6936.

Estrov, Z., Kurzrock, R., Estey, E., Wetzler, M., Ferrajoli, A., Harris, D., Blake, M., Gutterman, J.U., & Talpaz, M. (1992). Inhibition of acute myelogenous leukemia blast proliferation by interleukin-1 (IL-1) receptor antagonist and soluble interleukin-1 receptors. *Blood* 79, pp. 1938-1945.

Foulds, L. (1958) The natural history of cancer. *J. Chronic Dis.* 8, pp. 2-37.

Freedman, M.H., Cohen, A., Grunberger, T., Bunin, N., et al., (1992) Central role of tumour necrosis factor, GM-CSF, and interleukin 1 in the pathogenesis of juvenile chronic myelogenous leukemia. *Brit. J. Haematol* 80, pp. 40-48.

Fuh, G. Cunningham, B.C., Fukunaga, R., Nagata, S., Goeddel, D.V., & Wells, J.A. (1992) Rational design of potent antagonists to the human growth hormone receptor. *Science* 256, pp. 1677-1680.

Goding, J.W. (1986) Monoclonal antibodies: principles and practice. (Second edition, Academic Press, London).

Gootenberg, J.E., Ruscetti, F.W., Mier, J.W., Gazdar, A., & Gallo, R.C. (1981) Human cutaneous T cell lymphoma and leukemia cell lines produce and respond to T cell growth factor. *J.Exp.Med.* 154, pp. 1403-1419.

Graf., T. Weizsacker, F.v., Griesen, S., Coll, J., Stehelin, et al. (1986) v-*mil* induces growth and enhanced tumourigenicity in v-*myc*-transformed avian macrophages. *Cell* 45, pp. 357-364.

Graves, D.T., Owen, A.J., & Antoniades, H.N. Evidence that a human osteosarcoma cell line which secretes a mitogen similar to platelet-derived growth factor requires growth factors present in platelet-poor plasma. *Cancer Res.* 43, pp. 83-87

Griffin, J.D., Rambaldi, A., Vellenga, E., Young, D.C., Ostapovicz, D., & Cannistra, S.A. (1987) Constitutive secretion of interleukin-1 by acute myeloblastic leukemia cells in vitro induces endothelial cells to secrete colony-stimulating factors. *Blood* 70, pp. 1218-1221.

Habener, J.F., Cwikel, B.J., Hermann, H. et al. (1989) Metallothinein-vasopressin fusion gene expression in transgenic mice. Nephrogenic diabetes insipidus and brain transcripts localised to magnocellular neurons. *J. Biol. Chem.* 264, pp. 18844-18852.

Hannink, M. & Donoghue, D.J. (1988) Autocrine stimulation by the v-*sis* gene product requires a ligand-receptor interaction at the cell surface. *J. Cell Biol* 107, pp. 287-298.

Hapel, A.J., Vande Woude, G., Campbell, H.D., Young, I.G., & Robins, T. (1986) Generation of an autocrine leukemia using a retroviral expression vector carrying the IL-3 gene. *Lymphokine Res.* 5, pp. 249-254.

Hariharan, I.K., Adams, J., & Cory, S. (1988) *bcr-abl* oncogene renders myeloid cell line factor independent: potential autocrine mechansim in chronic myeloid leukemia. *Oncogene Res.* 3, 378-99.

Hermann, F., Cannistra, S.A., Levine, H., & Griffin, J.D. (1985) Expression of interleukin 2 receptors and binding of interleukin 2 by gamma interferon-induced human leukemic and normal monocytic cells. *J. Exp. Med.* 162, pp. 1111-1116.

Holter, W., Goldman, C.K., Casabo, L., Nelson, D.L., Greene, W.C., & Waldmann, H. (1987) Expression of functional IL2 receptors by lipopolysaccharide and interferon-gamma stimulated human monocytes. *J. Immunol.* 138, pp. 2917-2922.

Holter, W., Grunow, R., Stockinger, H., & Knapp, W. (1986) Recombinant interferon-gamma induces interleukin 2 receptors on human peripheral blood monocytes. *J. Immunol.* 136, pp. 2171-2175.

Hsu, T.C. (1961) Chromosomal evolution in cell populations. *Int. Rev. Cytol.* 12, pp. 69-161.

Hu, M. C.-T., & Davidson, N. (1990) A combination of the *lac* operator-repressor system with positive induction by glucocorticoid and metal ions provides a high-level-inducible gene expression system based on the human metallothionein-IIA promoter. *Mol. Cell. Biol.* 10, pp. 6141-6151.

Huang. J.S., Huang, S.S., & Deuel, T.F. (1984) Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. *Cell* 39, pp. 79-87.

Huang, S.S., & Huang, J.S. (1988). Rapid turnover of the platelet-derived growth factor receptor in *sis*-transformed cells and reversal by suramin. *J. Biol. Chem.* 263, pp. 12608-12618.

Hunter, T. (1991) Cooperation between oncogenes. *Cell* 64, pp. 249-270.

Jirik, F., Burstein, S.A., Treger, L., & Sorge, J.A. (1987) Transfection of a factor-dependent cell line with the murine interleukin-3 (IL-3) cDNA results in autonomous growth and tumourigenesis. *Leuk. Res.* 11, pp. 1127-1134.

Johnsson, A., Betsholtz, C., Heldin, C.-H., & Westermark, B. (1985). Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus. *Nature* 317, pp.438-440.
Karusayama, H., & Melchers, F. (1988) Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2,3,4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18, pp. 97-104.

Karusayama, H., Tohyama, N., & Tada, T. (1989) Autocrine growth and tumourigenicity of interleukin 2-dependent helper T-cells transfected with IL-2 gene. *J.Exp.Med.* 169, pp. 13-25.

Kaufman, D.C., Baer,M., Gao, X.Z., Wang, Z., & Preisler, H.D. (1988) Enhanced expression of the granulocyte-macrophage colony stimulating factor in acute myelocytic leukemia cells following in vitro blast cell enrichment. *Blood* 72, pp. 1329-1332.

Kawano, M., Hirano, T., Matsuda, T. et al. (1988) Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature* 332, pp. 83-85.

Keating, A., Horsfall, W., Hawley, R.G., & Toneguzzo, F. (1990) Effects of different promoters on expression of genes introduced into hematopoietic and marrow stromal cells by electroporation. *Exp. Hematol.* 18, pp. 99-102.

Keating, M.T., & Williams, L.T. (1988) Autocrine stimulation of intracellular PDGF receptors in v-*sis*-transformed cells. *Science* 239, pp. 914-916.

Kita, H., Ohnishi, T., Okubo, Y., et al. (1991) Granulocyte/macrophage colony stimulating factor amd interleukin-3 release from human peripheral blood eosinophils and neutrophils. *J. Exp. Med.* 174, p. 745.

Klein, B., Wijdenes, J., Zhang, X.-G., et al. (1991). Murine anti-interleukin-6 monoclonal antibody therapy for a patient with plasma cell leukemia. *Blood* 78, pp. 1198-1204.

Klein, B., Zhang, X.-G., Jourdan et al. (1989) Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. *Blood* 73, pp. 517-526.

Koury, M.J., & Bondurant, M.C. (1990) Erythropoietin retards DNA breakdown and prevents programmed cell death in erythroid progenitor cells. *Science* 248, pp. 278-381.

Koyasu, S., Yodoi, J., Nikaido, T., Tagaya, Y., Taniguchi, Y., Honjo, T., & Yahara, I. (1986) Expression of interleukin 2 receptors on interleukin 3-dependent cell lines. *J. Immunol.* 136, pp. 984-987.

Kruse, N., Tony, H.-P., & Sebald, W. (1992) Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement. *EMBO J.* 11, pp. 3237-3244.

Kuziel, W.A., & Greene, W.C. (1991) Interleukin-2 *in* The Cytokine Handbook (A. Thomson ed., Academic Press, London) pp. 83-102.

Kypta, R.M., Goldberg,Y., Ulug, E.T., & Courteidge, S.A. (1990) Association between the PDGF receptor and members of the *src* family of tyrosine kinases. *Cell* 62, pp. 481-492.

Labow, M.A., Baim, S.B., Shenk, T., & Levine, A.J. (1990) Conversion of the *lac* repressor into an allosterically regulated transcriptional activator for mammalian cells. *Mol. Cell. Biol.* 10, pp. 3343-335x.

Laker, C., Stocking, C., Bergholz, U., Hess, N., De Lamarter, J.F., & Ostertag, W. (1987) Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and autonomous growth are distinct but interdependent steps in the oncogenic pathway. *Proc. Natl. Acad. Sci. USA* 84, pp. 8458-8462.

Laneuville, P., Heisterkamp, N., & Groffen, J. (1991) Expression of the chronic myelogenous leukemia-associated p210*bcr/abl* oncoprotein in a murine IL-3 dependent myeloid cell line. *Oncogene Res.* 6, p. 275.

Lang., R.A., & Burgess., A.W. (1990) Autocrine growth factors and tumourigenic transformation. *Immunol. Today* 11, pp.244-249.

Lang, R.A., Metcalf, D., Gough, N.M., Dunn, A.R., & Gonda, T.J. (1985). Expression of a hemopoietic growth factor cDNA in a factor-dependent cell line results in autonomous growth and tumourigenicity. *Cell* 43, pp. 531-542.

Le Gros, G. S., Gillis, S., & Watson, J.D. (1985) Induction of IL2 responsiveness in a murine IL3-dependent cell line. *J. Immunol.* 135, pp. 4009-

Le Gros, J.E., Jenkins, D.R., Prestidge, R.L., & Watson, J.D. (1987) Expression of genes in cloned murine cell lines that can be maintained in both interleukin-2 and interleukin-3-dependent growth states. *Immunol. Cell Biol.* 65, pp. 57-69.

Leslie, K.B., Ziltener, H.J., & Schrader, J.W. (1991) The role of IL-1 and GM-CSF in the paracrine stimulation of an *in vivo* -derived myeloid leukemia.*Blood* 78, pp. 1301-1310.

Lindstein, T., June, C.H., Ledbetter, J.A., Stella, G., & Thompson C.B. (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* 244, pp. 339-343.

Lokeshwar, V.B., Huang, S.S., & Huang, J.S. (1990) Intracellular turnover, novel secretion, and mitogenically active intracellular froms of v-*sis* gene product in simian sarcoma virus-transformed cells. *J. Biol. Chem.* 265, pp. 1665-1675.

Lowenthal, J.W., Corthesy, P., Tougne, C., Lees, R., MacDonald, H.R., & Nabholz, M. (1985) High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. *J. Immunol.* 135, pp. 3988-3994.

Maggiano, N., Colotta, F., Castellino, F., Ricci, R., Valitutti, S., Larocca, L.M., & Musiani, P. (1990) Interleukin-2 receptor expression in human mast cells and basophils. *Int. Arch. Allergy Appl. Immunol.* 91, pp. 8-14.

Metcalf, D. (1989) The roles of stem cell self-renewal and autocrine growth factor production in the biology of myeloid leukemia. *Cancer Res.* 49, pp. 2305-2311.

Miles, S.A., Rezai, A.R., Salazar-Gonzalez, A.F., et al. (1990) AIDS Kaposi sarcoma-derived cells produce and respond to inerleukin 6. *Proc. Natl. Acad. Sci.USA* 87, pp. 4068-4072.

Moore, M.A.S. (1991) Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood* 78, pp.1-19.

Moore, M.A.S., Spitzer, G., Williams, N., Metcalf, D., & Buckley, J. (1974) Agar culture studies in 127 cases of untreated myeloid leukemia: the prognostic value of reclassifiaction of leukemia according to in vitro growth characteristics. *Blood,* 44, p. 1.

Moqbel, R., Hamid, Q., Ying, S., et al. (1991) Expression of mRNA and immunoreactivity for the granulocyte macrophage colony stimulant factor in activated human eosinophils. *J. Exp. Med.* 174, p. 749.

Morahan, G., Brennand, P.E., Bhathal, P.S., et al. (1989) Expression in transgenic mice of class I histocompatability antigens controlled by the metallothionein promoter. *Proc. Natl. Acad. Sci. USA* 81, pp. 2396-2400.

Murohashi, I., Tohda, S., Suzuki, T., Nagata, K., Yamashita, Y., & Nara, N. (1989) Autocrine growth mechanisms of the progenitors of blast cells in acute myeloblastic leukemia. *Blood* 74, pp. 35-41.

Nakamura, M., Kanakura, Y., Furukawa, Y., Ernst, T.J., & Griffin, J.D. (1990) Demonstration of IL-1ß transcripts in acute myeloblastic leukemia by in situ hybridization. *Leukemia* 4, p. 466.

Nister, M., Heldin, C.-H., Wasteson, A., & Westermark, B. (1984) A glioma-derived analog to platelet-derived growth factor: demonstration of receptor competing activity and immunological cross reactivity. *Proc. Natl. Acad. Sci. USA* 81, pp. 926-930.

Ohara, J. & Paul, W. (1985) Production of a monoclonal antibody to and characterization of B-cell stimulatory factor-1. *Nature* 315, pp.333-335.

Oster, W., Cicco, N.A., Klein, H., Hirano, T., Kishimoto, T., Lindemann, A., Mertelsmann, R.H., & Herrmann, F. (1989) Participation of the cytokines, interleukin 6, tumour necrosis factor-alpha, and interleukin 1-beta secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. *J.Clin.Invest.* 84, pp. 451-457.

Otsuka, T., Eaves, C.J., Humphries, R.K., Hogge, D.E., & Eaves, A.C. (1991) Lack of evidence for abnormal autocrine or paracrine mechanisms underlying the uncontrolled proliferation of primitive chronic myeloid leukemia cells. *Leukemia* 5, pp. 861-868.

Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E., & Brinster, R.L. (1983) Metallothionein-human GH fusion genes stimulate growth of mice. *Science* 222, p. 809-814.

Paul, C.C., Keller, J.R., Armpriester, J.M., Baumann, M.A. (1990) Epstein-Barr transformed B-lymphocytes produce interleukin-5. *Blood* 75, p. 1400.

Plaut, M., Pierce, J.H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P., & Paul , W.E. (1989) Mast cell lines produce lymphokines in response to crosslinkage of FcERI and calcium ionophores. *Nature* 339, p. 64.

Potter, H. (1988) Electroporation in biology: methods, applications, and instrumentation. *Anal. Biochem.* 174, pp. 361-373.

Rambaldi, A., Torcia, M., Bettoni, S., Vannier, E., Barbui, T., Shaw, A.R., Dinarello, C.A., & Cozzolino, F. (1991) Modulation of cell proliferation and cytokine production in acute myeloblastic leukemia by interleukin-1 receptor antagonist and lack of its expression by leukemic cells. *Blood 78*, pp. 3248-3253.

Rambaldi, A., Young, D.C., Hermann, F., Cannistra, S.A., & Griffin, J.D. (1987) Interferon gamma induces expression of the interleukin 2 receptor gene in human monocytes. *Eur. J. Immunol.* 17, pp. 153-156.

Ravnan, J.-B., Glibert, D., Ten Hagen, K.G., & Cohen S.N. (1992) Random-choice replication of extrachromosomal bovine papillomavirus (BPV) molecules in heterogeneous, clonally derived BPV-infected cell lines. *J. Virol.* 66, 6946-6952.

Razin, E., Leslie, K.B., & Schrader, J.W. (1991) Connective tissue mast cells in contact with fibroblasts express IL-3 mRNA: analysis of single cells by polymerase chain reaction. *J.I mmunol.*146, p. 981.

Reilly, I.A.G., Kozlowski, R., & Russell, N.H. (1989) Heterogeneous mechanisms of autocrine growth of AML blasts. *Brit. J. Haematol.* 72, pp.363-369.

Resh, M.D. (1990) Membrane interactions of pp60$^{v\text{-}src}$: a model for myristylated tyrosine protein kinases. *Oncogene* 5, pp. 1437-1444.

Rettenmeier, C.W., Jackowski, S., Rock, C.O., Roussel, M.F., & Sherr, C.J. (1987) Transformation by the v-*fms* oncogene product: an analog of the CSF-1 receptor. *J. Cell. Biochem.* 33, pp109-115.

Richmond, A., Balentien, E., Thomas, H.G., Flaggs, G., Barton, D.E., Spless, J., Bordoni, R., Francke, U., & Derynck, R. (1988) Molecular characterization and chromosomal mapping of melanom growth stimulating activity, a growth factor structurally related to beta-thromboglobulin. *EMBO J.* 7, pp. 2025-2033.

Richmond, A., & Thomas, H.G. (1988) Melanoma growth stimulatory activity: isolation from human melanoma tumours and charcterization of tissue distribution. *J. Cell. Biochem.* 36, pp. 185-198.

Roberts, J.M., & Weintraub, H. (1988) Cis-acting negative control of DNA replication in eukaryotic cells. *Cell* 52, pp. 397-404.

Rodriguez-Cimadevilla, J.C., Beauchemin, V., Villeneuve, L, Letendre, F., Shaw, A, & Hoang, T. (1990) Coordinate secretion of interleukin-1ß and granulocyte-macrophage colony-stimulating factor by the blast cells of acute myeloblastic leukemia: role of interleukin-1 as an endogenous inducer. *Blood* 76, p. 1481.

Sakai, K., Hattori, T., Matsuoka, M., Asou, N., Yamamoto, S., Sagawa, K., & Takatsuki, K. (1987) Autocrine stimulation of interlekin 1ß in acute myelogenous leukemia cells. *J.Exp.Med.* 166, pp. 1597-1602.

Sanderson, C.J., Campbell, H.D., & Young, I.G. (1988) Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. *Immunol. Rev.* 102, p. 29.

Savill, J., Wylie, A.H., Henson, J.E.et al. (1989) Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* 83, pp. 865-875.

Scala, G., Morrone, G. Tamburrini, M., et al. (1987) Autocrine growth function of human interleukin 1 molecules on ROHA-9, an EBV transformed human B cell line. *J.Immunol.* 138, pp. 2527-2534.

Scala, G., Quinto, I., Ruocco, M.R., et al. (1990) Expression of an exogenous interleukin 6 gene in human Epstein-Barr virus cells confers growth advantage and in vivo tumourigenicity. *J. Exp. Med.* 172, pp. 61-68.

Schrader, J.W., Clark-lewis, I., Crapper, R.M., & Wong, G.W (1983) P-cell stimulating factor: characterization, action on multiple lineages of bone marrow-derived cells and role in oncogenesis. *Immunol. Rev.* 76, pp. 79-104.

Schrader, J.W., & Crapper, R.M. (1983) Autogenous production of hemopoietic growth factor "P cell stimulating factor" as a mechanism for transformation of bone marrow-derived cells. *Proc. Natl. Acad. Sci. USA* 80, pp. 6892-6896.

Schrader, J.W. (1991) Interleukin-3 *in* The Cytokine Handbook (A. Thomson ed., Academic Press, London) pp. 103-118.

Schrader, J.W. (1992) Biological effects of myeloid growth factors. *In* Balliere's Clinical Haemtaology, Vol.5 No.3, (B.I.Lord & T.M.Dexter, eds. Balliere Tindall, London, 1992).

Schwab, G., Siegall, C.B., Aarden, L.A., Neckers, L.M., & Nordan, R.P. (1991) Characterization of an interleukin-6-mediated autocrine loop in the human multiple myeloma cell line, U266. *Blood,* 77, pp. 587-593.

Shanahan, C.M., Rigby, N.W., Murray, J.D., Marshall, J.T., Townrow, C.A., Nancarrow, C.D., & Ward, K. (1989) Regulation of a sheep metallothionein 1a-sheep growth hormone fusion gene in transgenic mice. *Mol. Cell. Biol.* 9, pp. 5473-5479. 9, pp. 5473-5479.

Sherr, C.J., Rettenmeier, C.W., Sacca, R., Roussel, M.F., Look A.T., & Stanley, E.R. (1985) The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 41, pp. 665-676.

Shirakawa, F., Tanaka, Y., Oda, S., Eto, S., & Yamashita, U. (1989) Autocrine stimulation of interleukin 1-alpha in the growth of adult human T-cell leukemia cells. *Cancer Res.* 49, pp. 1143-1147.

Smith, K.A., Favata, M.F., & Oroszlan, S. (1983) Production of monoclonal antibodies to human interleukin 2: strategy and tactics. *J. Immunol.* 131, pp. 1808-1815.

Sporn, M.B., & Roberts, A.B. (1985) Autocrine growth factors and cancer. *Nature* 313, pp. 745-747.

Stocking, C., Loliger, C., Kawai, M., Suciu, S., Gough, N., & Ostertag, W. (1988) Identification of genes involved in growth autonomy of hematopoietic cells by analysis of factor-independent mutants. *Cell* 53, pp. 869-879.

Suematsu, S., Matsuda, T., Aozasa, K. et al. (1989) IgG1 plasmacytosis in interleukin 6 transgenic mice. *Proc. Natl. Acad. Sci. USA* 86, pp. 7547-7551.

Suematsu, S., Matsusaka, T., Matsuda, T., et al. (1992) Generation of plasmacytomas with the chromosomal translocation t (12;15) in interleukin 6 transgenic mice. *Proc. Natl. Acad. Sci. USA* 89, pp. 232-235.

Takatsu, K., Tominaga, A., Harada, N., et al. (1988) T cell replacing factor (TRF)/interleukin 5 (IL-5): molecular and functional properties. *Immunol.Rev.* 102, p. 107.

Taniguchi, T., Matsui, H., Fujita, T., Hatakeyama M., Kashima, N., Fuse, A., Hamuro, J., Nishi-Takaoka, C. & Yamada, G. (1986) Molecular analysis of the interleukin-2 system. *Immunol. Rev.* 92, pp. 121-133.

Thomson, A (1991) The Cytokine Handbook. (A. Thmoson, ed., Academic Press, London, 1991).

Todaro, G.J., & De Larco, J.E. (1978) Growth factors produced by sarcoma virus-transformed cells. *Cancer Res.* 38, pp. 4147

Tohyama, K., Lee, K.H., Tashiro, K., Kinashi, T., & Honjo, T. (1990) Establishment of an interleukin-5-dependent subclone of an intereukin-3-dependent murine hemopoietic progenitor line, LyD9, and its malignant transformation by autocrine secretion of interleukin-5. *EMBO J.* 9, pp. 1823-1830.

Trojan., J., Blossey, B., Johnson, T.R., Rudin, S.D., Tykocinski, M., Ilan, J., & Ilan, J. (1992) Loss of tumourigenicity of rat glioblastoma directed by episomebased anti-sense cDNA transcription of insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA* 89, pp. 4874-4878.

Trojan., J.,Johnson, T.R., Rudin, S.D., Ilan, J., Tykocinski, M.,& Ilan, J. (1993) Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I mRNA. *Science* 259, pp. 94-97.

Vandenabeele, P., Jayaram, B., Devos, R., Shaw, A., & Fiers, W. (1988) Interleukin 1-alpha acts as an autocrine growth factor for RPMI 1788 and Epstein-Barr virus-transformed human B-cell line. *Eur. J. Immunol.* 18, pp. 1027-1031.

Vink, A., Coulie, P.G., Wauters, P., Nordan, R.P. & Van Snick, J. (1988) B cell growth and differentiation activity of interleukin-HP1 and related murine plasmacytoma growth factors. Synergy with interleukin 1. *Eur. J. Immunol.* 18, pp. 607-612.

Wakasugi, H., Rimsky, L., Mahe, Y., et al. (1987) Epstein-Barr virus-containing B-cell line produces an interleukin-1 that it uses as a growth factor. *Proc. Natl. Acad. Sci. USA* 84, pp. 804-808.

Waldmann, T.A. (1991) The interleukin-2 receptor. *J. Biol. Chem.* 266, pp. 2681-2684.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J.S., & Deuel, T.F. (1983) Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. *Nature* 304, pp. 35-39.

Weissman, D., Parker, D.J., Rothstein, T.L., Marshak-Rothstein, A. (1985). Methods for the production of xenogeneic monoclonal antibodies in murine ascites. *J. Immunol.* 135, pp. 1001-1003.

Werge, T.M., Biocca, S. & Cattaneo, A. (1990) Intracellular immunization Cloning and intracellular expression of a monoclonal antibody to the $p21^{ras}$ protein. *FEBS Lett.* 274, 193-198.

Wodnar-Filipowicz, A., Heusser, C.H., & Moroni, C. (1989) Production of the hemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. *Nature* 317, p. 255.

Wong, P.M.C., Chung, S.-W., & Nienhuis, A.W. (1987) Retroviral transfer and expression of the interleukin-3 gene in hemopoietic cells. *Genes and Dev.* 1, pp.358-365.

Yamada, G., Kitamura, Y., Sonoda, H., Harada, H., Taki, S., Mulligan, R.C., Osawa, H., Diamantstein, T., Yokoyama, S., & Taniguchi, T. (1987) Retroviral expression of the human IL-2 gene in a murine T cell line results in cell growth autonomy and tumourigenicity. *EMBO J.*6, pp. 2705-2709.

Yamamoto, S. Hattori, T., Matsuoka, M., Ishii, T., Asou, N., Tagaya, Y., Yodoi, J.,& Takatsuki, K (1986) Induction of Tac antigen and proliferation of myeloid lekemic cells by ATL-derived factor: comparison with other agents that promote differentiation of human myeloid or monocyte leukemic cells. *Blood* 67, pp. 1714-1720.

Young, D.C., Demetri, G.D., Ernst, T.J., Cannistra, S.A., & Griffin, J.D. (1988) In vitro expression of colony-stimulating factor genes by human acute myeloblastic leukemia cells. *Exp. Hematol.* 16, pp. 378-382.

Young, D.C. & Griffin, J.D. (1986) Autocrine secretion of GM-CSF in acute myeloblastic leukemia. *Blood* 68, pp. 1178-1181.

Young, D.C., Wagner, K., & Griffin, J.D. (1987). Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. *J. Clin. Invest.* 79, pp. 100-106.

Zhang, X.G., Klein, B., & Bataille, R. (1989). Interleukin-6 is a potent myeloma-cell growth factor in patienst with aggressive multiple myeloma. *Blood* 74, pp. 11-13.

APPENDIX 1: PROLIFERATIVE RESPONSES OF FACTOR-DEPENDENT CELL LINES

The factor-dependent cell lines HT-2, R6X, 41E5, and FDC-P1 were used in these studies. Typical factor-response curves are presented for each line, and the factors to which they are known to respond (figures 1.37 - 1.40). Absolute quantitation of growth factors has not been stressed in the body of the thesis, since each assay included an internal control, and the conclusions drawn were based on these internal comparisons. International conventions have established the defintion of a "unit" of bio-activity for a growth factor as the amount of factor present in a solution that gives rise to half the maximal proliferation of which the assay cell is capable in response to that growth factor. The World Health Organisation has organised an international collaborative effort to develop reference standard preparations of hemopoietic growth factors. After at least 5 years of work, the first standard reference preparations have recently become available - for human GM-CSF and G-CSF (Dr H. Ziltener, Biomedical Research Centre, personal communication).

The need for these standards is highlighted by the differences seen in apparent activity of a preparation according merely to the duration of the bio-assay. A decrease in apparent bioactivity is seens with time, in terms of a shift to the right of the proliferation curve (in the form titrations are here presented). Actual results obtained with a source of IL-3 and the R6X cell line are presented in figure 1.41. The bio-assay was plated in three separate Terasaki microtiter plates, and one plate harvested, after identical thymidine pulse duration on each of the next 3 days.The apparent bio-activity of each asaay was approximated in each case, as shown. The bio-activity estimations ranged from between 512 and 1024 units of activity as determined on day 1, to between 64 and 128 units as determined on day 3, although each assay was in fact performed with the identical titration of IL-3. This decrease with apparent bio-activity over time is expected since cell number in each well increases in response to net proliferation-inducing levels of factor, and the factor is also consumed by the cells it stimulates.

Several of the growth factors used in these studies were applied in the form of conditioned media of cell lines transfected with vectors allowing expression of the factors (Karusayama and Melchers, 1988, and see Materials and Methods). These lines were kindly donated by Dr. Fritz Melchers of the Institute of Immunology, Basel, Switzerland.

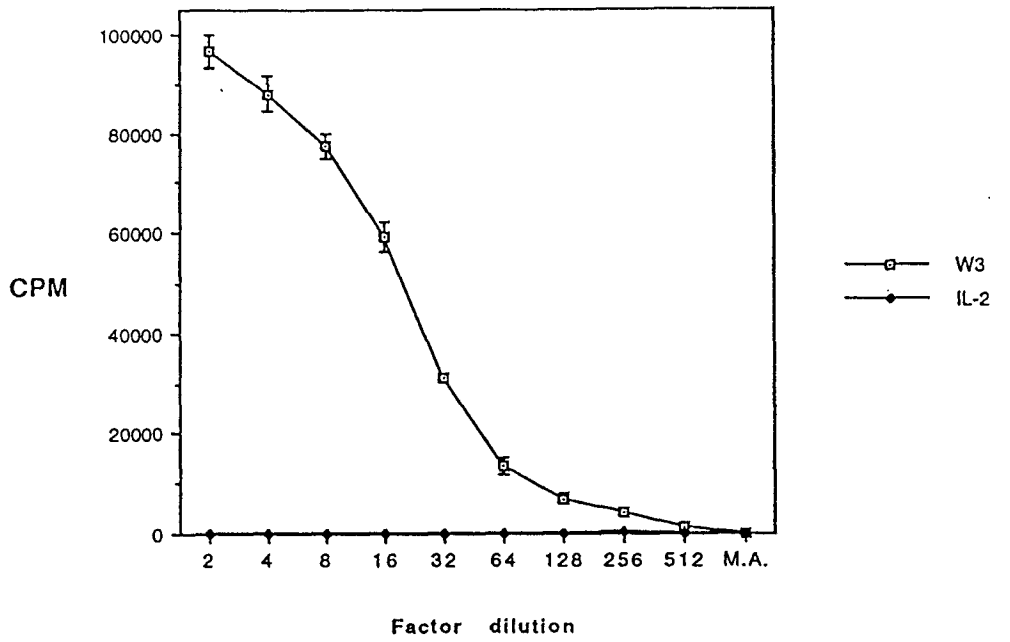


Fig. 1.37 Typical growth-factor responses of R6X cells W3 - WEHI-3B conditioned medium (source of IL-3). The IL-2 titration is the same as that shown in figure 1.38. 500 cells per well. 48 hour incubation plus 10 hour ^{3}H-thymidine pulse.

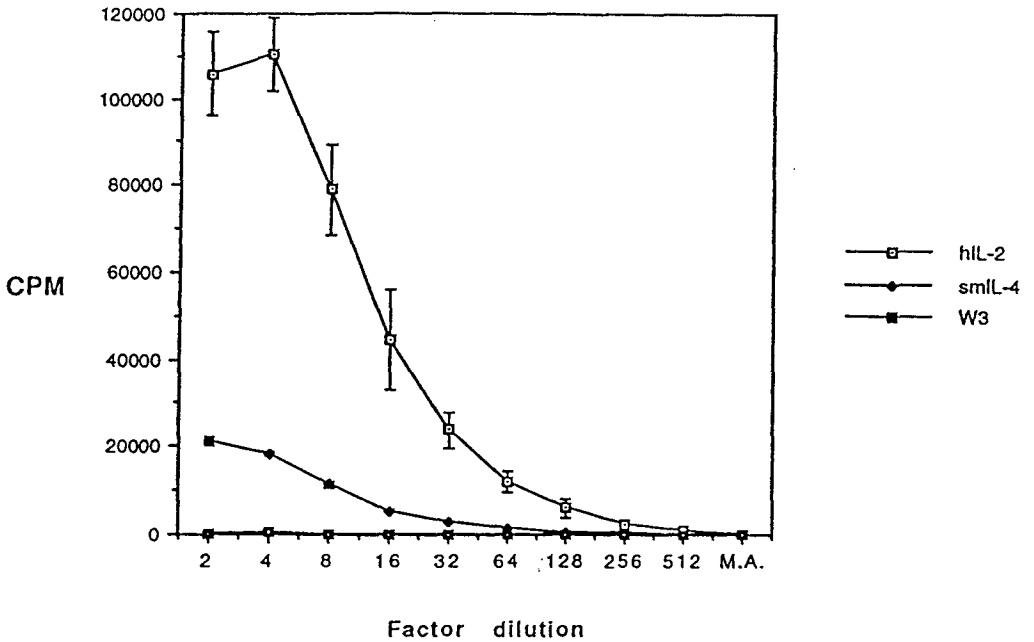


Fig. 1.38. Typical growth-factor response of HT-2 cells hIL-2 - recombinant human IL-2. Starting concentration approximately 40 units per ml, acording to manufacturer. smIL-4 - synthetic mouse IL-4 (kindly donated by Dr. Ian Clark-Lewis, Biomedical Research Centre). W3 - WEHI-3B conditioned medium (source of IL-3). 1000 cells per well. 48 Hour incubation plus 12 hour ^{3}H-thymidine pulse.

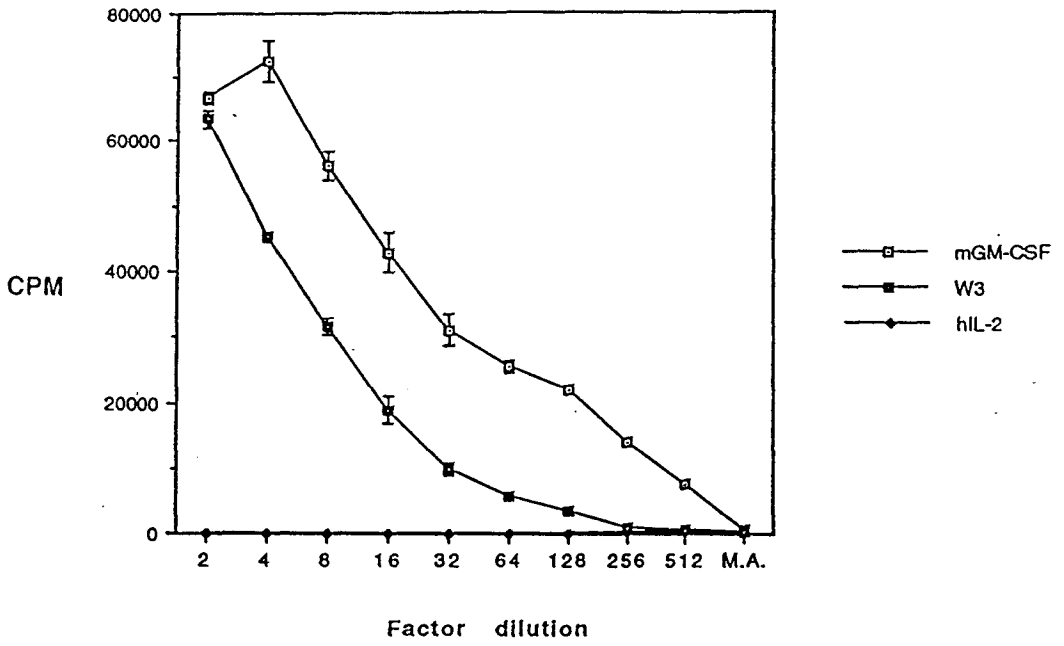


Fig. 1.39 Typical growth-factor responses of FDC-P1 cells mGM-CSF - affinity-purified mouse GM-CSF produced by stimulated T-cells (kindly donated by Dr. Hermann Ziltener, Biomedical Research Centre); W3 - WEHI-3B conditioned medium (source of IL-3) The IL-2 titration is the same as that shown in figure 1.38. 500 cells per well. 60 hour incubation plus 8 hour ^{3}H-thymidine pulse.

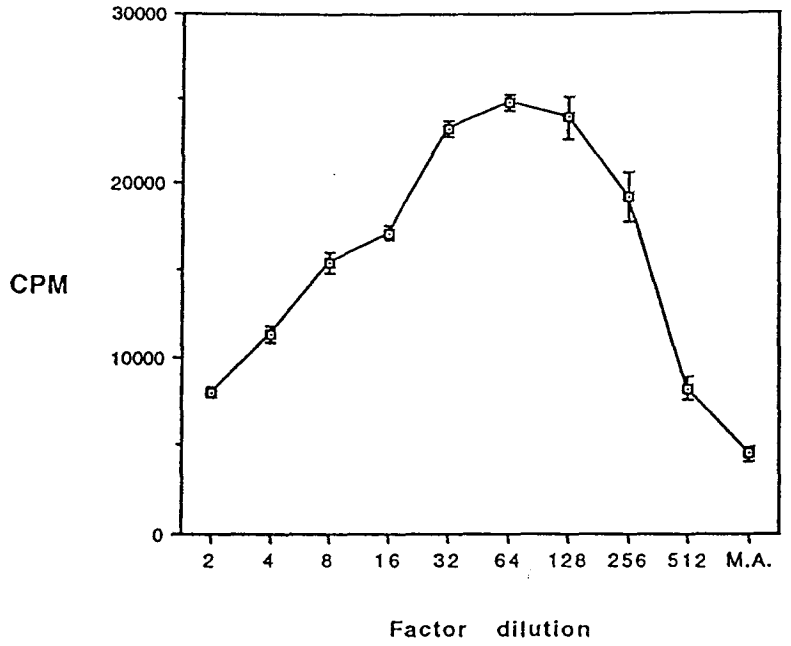


Fig. 1.40. Typical growth-factor response of 41E5 10 -fold concentrated conditioned medium of Psi2 fibroblasts was used as a source of mouse IL-6. Higher concentrations appear to inhibit proliferation. In the presence of 10% FCS (as used here), 41E5 cells at this density show some thymidine incorporation in the absence of a known source of IL-6. 500 cells per well. 60 hour incubation plus 10 hour ^{3}H-thymidine pulse.

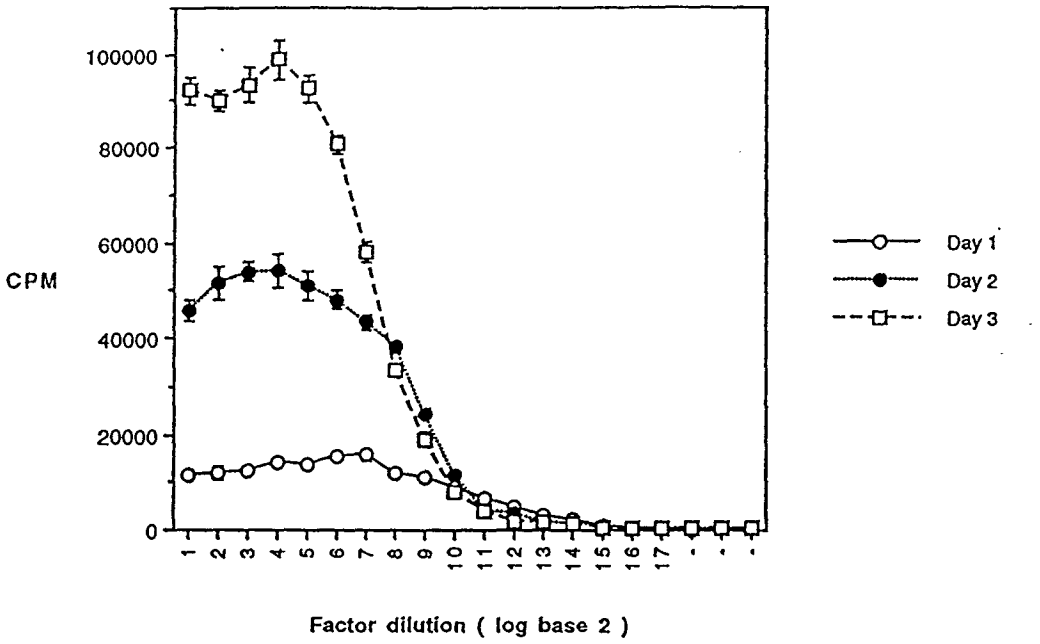


Fig. 1.41. Apparent decrease of bio-activity with duration of assay The curves result from harvest on 3 consecutive days of the same inital titration of IL-3 on R6X cells (500 per well). Pulse duration was 8 hours in each case. Note the shift in the position of the one unit value - i.e. the point on the horizontal axis corresponding to the half-maximal stimulation point on the curve.

APPENDIX 2: PURIFICATION OF ANTIBODIES

The DMS1 and DMS2 antibodies were obtained from secreting hybridoma cells in storage at the Biomedical Research Centre. Antibodies were prepared either from tissue culture supernatants or from mouse ascites. Supernates were first concentrated by Amicon ultrafiltration, using membranes with a molecular weight cut-off of 15000 M_r, and then passed over sheep anti-mouse-IgG affinity columns [prepared by Dr. Hermann Ziltener of the Biomedical Research Centre]. For preparation of mouse ascites, 5 x 10^6 cells were injected into the peritoneal cavities of pristane-primed mice, and the mice sacrificed and ascites collected when abdominal swelling was apparent. The antibody-content of ascites preparations was enriched by initial precipitation with ammonium-sulphate, and dialysed against 3 changes of 200 volumes of phosphate-buffered saline before further purification. The purification steps were monitored by enzyme-linked immuno-sorbent assay (ELISA), in which the antibody in solution was captured on plates that had been coated with sheep anti-mouse IgG, and detected with commercially obtained peroxidase-labelled goat anti-mouse IgG (which showed no background cross-reactivity with the sheep antibodies). Typical results of column-purification are presented in figure 1.42. Resultant preparations were assessed by small-scale gel-electrophoresis (Phast mini-gel system). The only bands visible on silver-staining of these gels were those of the sizes of IgG heavy and light chains, and the intensity of these bands suggested that the preparations were at least 95% pure immunoglobulin. The protein content of these antibody preparations was quantitated by the Bradford method, using commercially obtained bovine serum albumin for construction of the standard curve.

Rat antibodies were obtained from hybridoma cells, either as tissue-culture supernatants or as mouse ascites. For preparation of mouse ascites, the protocol of Weissman et al. (1985 - see Materials and Methods) was followed, in which mice were immuno-suppressed by sub-lethal irradiation and injection of hydrocortisone. The 11B11 (anti-mIL-4) antibody, has a kappa light chain, and was thus affinity-purified on a mouse anti-rat-kappa affinity column (prepared as above, from antibody produced in ascites by 11B11 hybridoma cells, and coupled to cyanogen-bromide activated sepharose beads). 11B11 was produced by Dr. Junichi Ohara (Ohara and Paul, 1985). IL-4-specific neutralising activity of the preparation used is shown in figure 1.43. The neutralising rat anti-mouse IL-6 monoclonal 6B4 (Vink et al., 1988) was prepared similarly. A specificity control for the prearation of 6B4 used here is shown in figure 1.44.

The PC61 rat anti-mouse-IL-2-receptor antibody has a lamda light chain, however, and attempts to purify PC61 ascites preparations by ion-exchange chromatography and protein A chromatography proved unsuccessful (yielding protein "peaks" too broad to provide any enrichment of the antibody).

Therefore a rabbit anti-rat immunoglobulin antibody was prepared at the Biomedical Research Centre (with the assistance of Mr. John Babcook), by injecting two rabbits sub-cutaneously with a combination of 11B11 antibody and dialysed, ammonium-sulphate concentrated PC61 ascites preparations. The anti-rat antibody titre of the rabbit serum was monitored by ELISA with 11B11-coated plates, and when the titre appeared to plateau, the rabbits were sacrificed, and serum collected. The immunoglobulin fraction of the serum was purified by protein A affinity chromatography, and coupled to cyanogen-bromide activated sepharose beads after densitometric quantitation. Affinity columns prepared with these beads were used to purify PC61 antibodies from ascites preparations. Purification was monitored by ELISA with commercial anti-rat antibodies that showed no background cross-reactivity against either rabbit or mouse immunoglobulin.

The rabbit antibodies to mIL-3 (Rab7) and mGM-CSF (Rab39) were produced by Dr. Hermann Ziltener and John Babcook of the Biomedical Research Centre. The antigens used to raise these antibodies were synthetic peptides of mIL-3 and GM-CSF synthesised by Dr. Ian Clark-Lewis of the Biomedical Research Centre. Factor-specific neutralising activities of these antibodies are shown in figures 1.45 and 1.46.

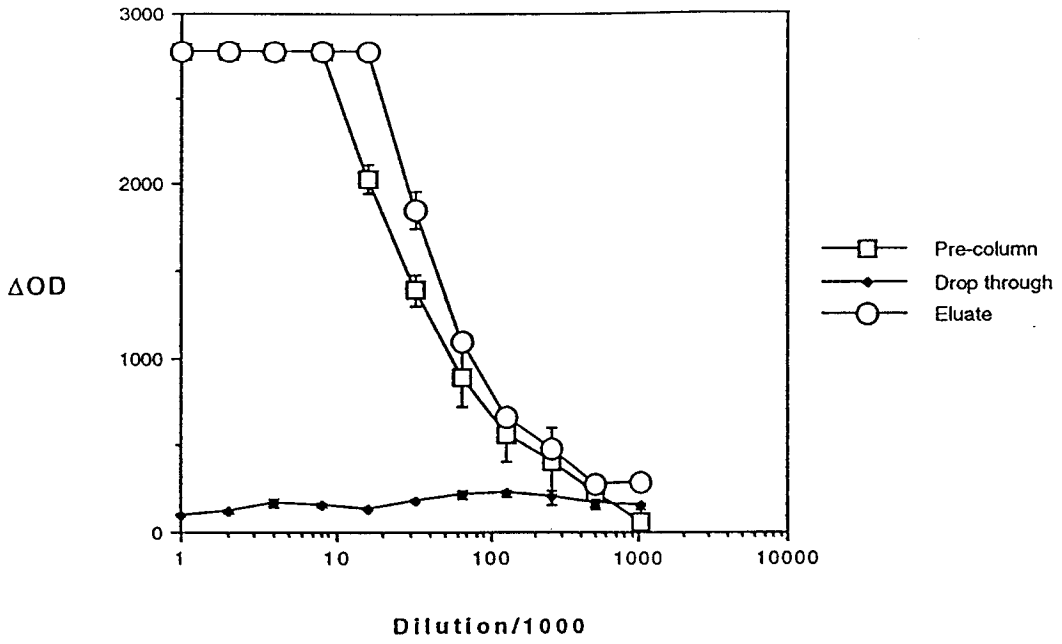


Fig. 1.42. ELISA monitoring of affinity-column purification of DMS antibodies.

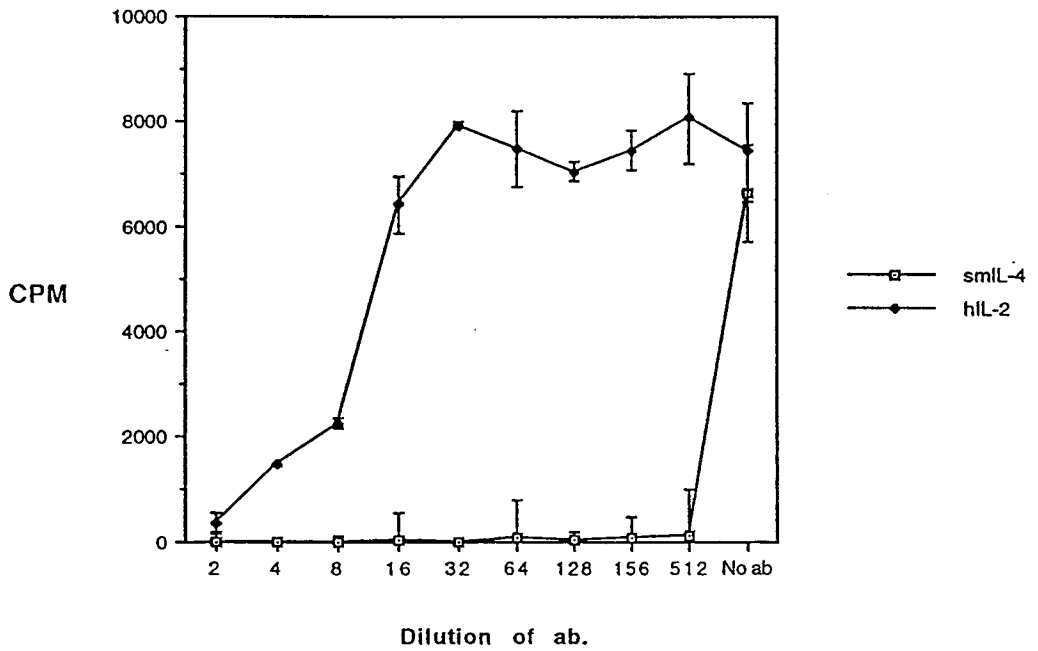


Fig. 1.43. Specific inhibtory activity of the 11B11 antibody preparation used in these studies. Antibody was titrated in the presence of approximately one unit of synthetic mouse IL-4 (kindly donated by Dr. Ian Clark Lewis, Biomedical Research Centre) or an amount of recombinant human IL-2 that produced a similar proliferative resonse. HT-2 cells - 1000 per well. 48 hour incubation plus 8 hour ^{3}H-thymidine pulse.

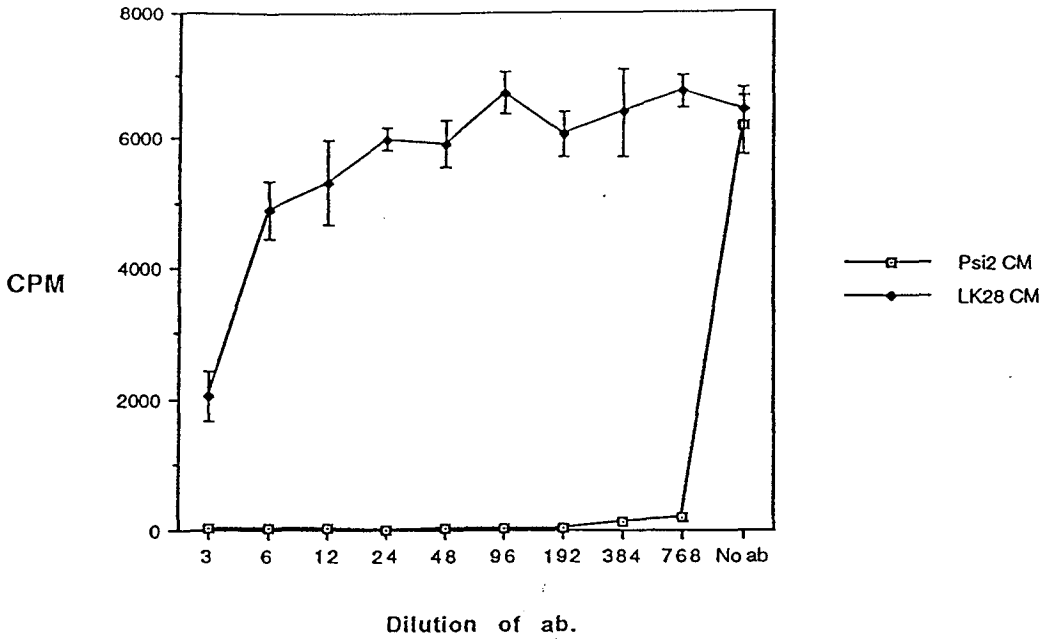


Fig. 1.44. Specific inhibtory activity of the 6B4 antibody preparation used in these studies. Antibody was titrated on cells of the IL-6-responsive cell-line B9 (ref.) in the presence of either human or mouse IL-6. LK28 CM - conditioned medium of a B-lymphoblastoid human cell line; Psi2 CM - 5-fold concentrated conditioned medium of Psi2 mouse fibroblasts. 500 cells per well. 40 hour incubation plus 8 hour ^{3}H-thymidine pulse.

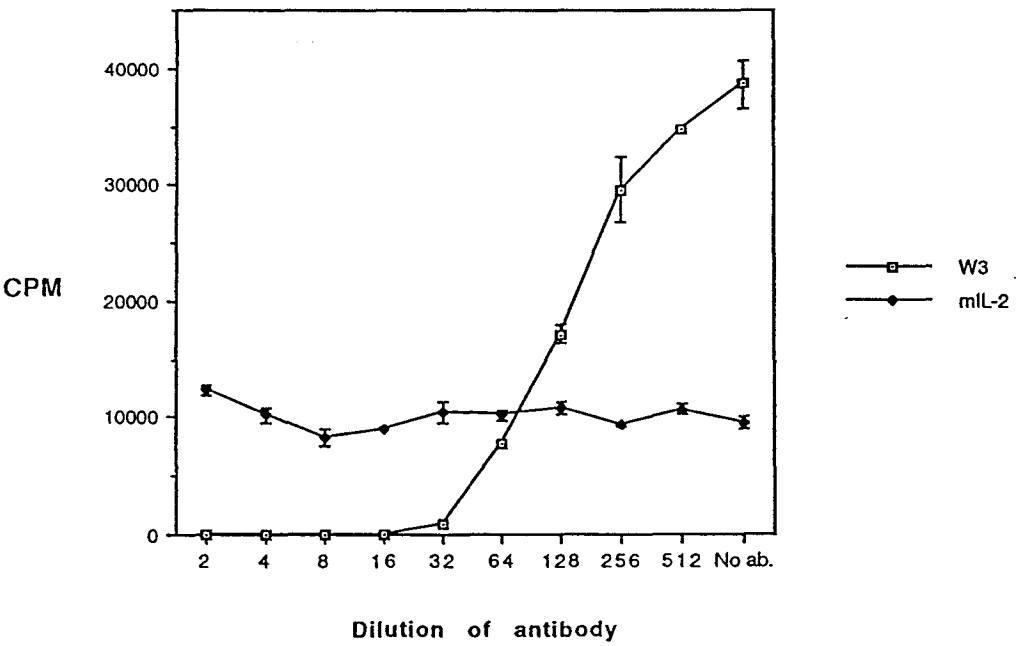


Fig. 1.45. Specific inhibtory activity of the Rab7 antibody preparation used in these studies Antibody (as serum) was titrated on FD.C/2 cells in the presence of approximately one unit of IL-3, or a smaller amount of murine IL-2. Initial concentration of serum was 1/30. 500 cells per well. 60 hour incubation plus 8 hour ^{3}H-thymidine pulse.

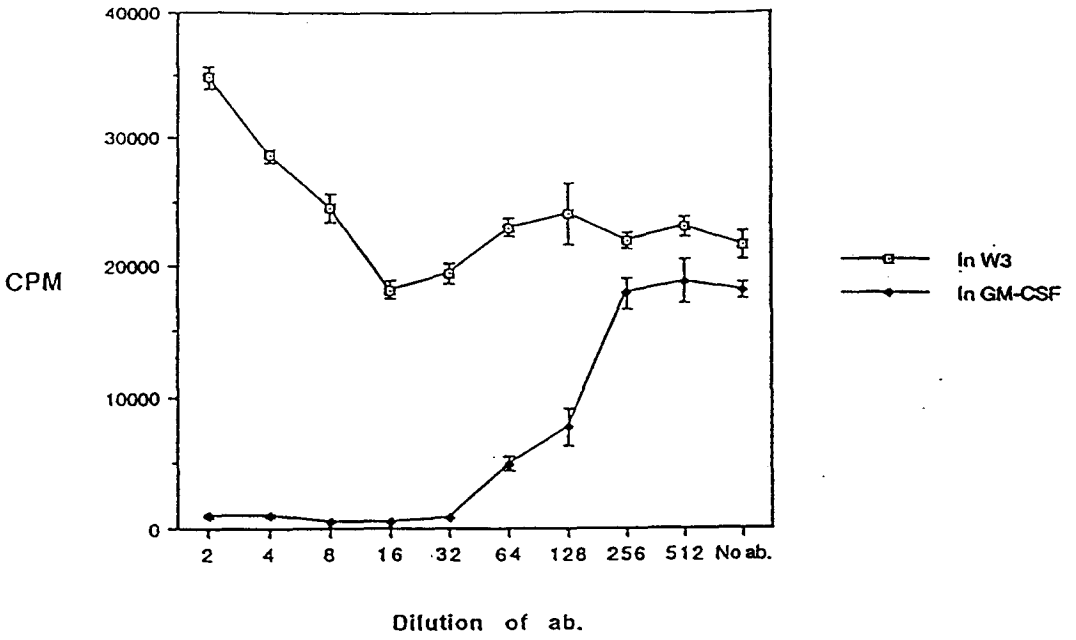


Fig. 1.46. Specific inhibtory activity of the Rab39 antibody preparation used in these studies. Antibody (as serum), was titrated on FDC-P1 cells in the presence of approximately one unit of either IL-3 or synthetic murine GM-CSF (courtesy of Dr. Ian Clark-Lewis, Biomedical Research Centre). W3 - WEHI-3B conditioned medium (source of IL-3). Note the apparent stimulatory activity at higher concentrations of anti-serum. 500 cells per well. 60 hour incubation plus 6 hour ^{3}H-thymidine pulse.

A NEW TECHNIQUE IN GENE DELETION

FOREWORD

Much of the work reported in this part of the present thesis was presented in a paper entitled "Tissue- and site-specific DNA recombination in transgenic mice", published in Proceedings of the National Academy of Sciences of the U.S.A Vol. 89, pp 6861-6865, August 15, 1992. The present author was first author on this publication. Daniel Chui was a co-author, and Jamey D. Marth the senior author.

Since a proportion of the work reported here was performed by colleagues of the present author, strict attention has been paid to accurate attribution of the work involved. These attributions are indicated by inclusion of the name of the researcher who carried out the work in square brackets, when practicable, and in some cases the impersonal third person construction usual in theses has been abandoned in favour of simple third person construction naming the contributor of the relevant pieces of work. Where no such attribution is made, it is to be inferred that the present author performed the work described.

INTRODUCTION

1) FORMATION OF THE CONCEPT (TWO TECHNICAL PROBLEMS WITH A COMMON SOLUTION)

A) A problem in the study of pathogenesis.

In their 1990 review "Autocrine growth factors and tumourigenic transformation" (Lang and Burgess, 1990), Lang and Burgess give examples of spontaneously arising and experimentally induced tumours in which autocrine growth factor production is found. Subsequently they state:

> 'Regardless of all the above examples, it is still not proven that growth factor production is essential for the maintenance of neoplastic transformation in animals.'

Clearly, the only way to ultimately prove this would be to remove from a pre-existing tumour the ability to make or use autocrine factor, and then assay it's ability to behave like a tumour. It might be possible to suppress autocrine factor production by expressing anti-sense message to the autocrine factor within the tumour cells, or inhibit the activity of autocrine factor by exogenously applying antagonist, or

even by arranging it that the tumour cells themselves expressed the antagonist. These strategies, however, suffer from what one might call the "problem of incompleteness"; one might envisage that one or both of these strategies would fail to inhibit the tumour-behaviour, and one would be left asking the question: was all the potentially autocrine activity inhibited? Were there a few molecules of autocrine factor still active within the cells, molecules that were for technical reasons simply beyond the ability of anti-sense or anagonist to inhibit? Was this a complete experiment?

This problem of incompleteness can be given a much deeper context: It is widely believed that oncogenesis is, in general, a multi-step process - that it is the culmination of a series of genetic changes in the normal function of the cells from which tumours arise. In some instances, it is even possible to mimic this series of changes in primary cells in vitro, by the stepwise, or simultaneous, addition of several activated oncogenes (reviewed, for instance, in Hunter, 1991). Here again, one might ask of the tumour that finally arises - which genetic changes must still be in effect for the tumour to be continue to be a tumour? Are all of them required, or were some merely there to allow the developing tumour to overcome some patho-physiological hurdle? Indeed this sort of question can be given even broader scope - in any pathological process involving more than one genetic change, which changes are required for maintenance or exacerbation of the pathological state? How can this question be addressed experimentally? Again, by reversing the effects of a genetic change, and here again, the experiment would have to involve a complete reversal of the effect of the given genetic change(s) for the results to be interpretable, in the event that the pathology persisted after the "reversal". The answers to such questions would be essential to evaluating the feasibilty of particular gene therapies for tumours and other pathological conditions.

The only way to carry out such experiments "cleanly" would be to "sabotage" the gene in question. Currently, such a technique of "gene-sabotage" is used in creating mice that lack the function of a chosen gene, in order to study the effects of the mutation in the development and function of the animal. These "gene-targetting" experiments (for a review see Capecchi, 1989) rely on homologous recombination between the endogenous gene and a piece of transfected DNA.The recombination is designed such that a crucial portion of the endogenous gene is replaced or displaced by a marker gene which can be used to select for success of the gene transfer. Typically, a polymerase chain reaction (PCR) analysis is then performed to determine in which cells the incoming DNA has actually undergone homologous recombination rather than simply integrated into the genome at an irrelevant site. This manipulation is carried out on embryo stem (ES) cells which can then be introduced into blastocysts, and will then contribute to all the tissues of the mouse, including those of the germ-line. Such experiments are highly labour-intensive, since homologous recombination ocurrs in the minority of instances, and there is no guarantee that a given embryo stem cell so selected will indeed

contribute to the germ-line of the resulting mice. It is only because the technique allows conclusions about the function of genes to be drawn in such a precise way ("this is what happens if the gene is absent - definitely absent, no function of the gene there at all"), that it has become one of most respected techniques in analysis of gene function in mammals. Conceivably, this technique might be applied to the questions being considered above, but this would involve a considerable amount of manipulation of tumour cells in vitro, and the deliberate induction of homologous recombination may simply not be possible in some cells, or occur at a frequency that makes such experiments impractical.

B) Problems in the study of development

The technique of inducing mutations in mice by gene-targetting is so powerful as to be rapidly replacing that of creating transgenic mice in which a gene function is overexpressed, or expressed ectopically, in terms of the "cutting edge" of the study of gene function in mammals. It is prone to one limitation, in particular, that may, in some cases, leave the investigator almost empty handed. This may arise in those instances where total absence of function of the gene in question leads to embryonic lethality, even when animals hemizygous for the (null) mutation display no phenotype. Several genes already targetted have resulted in embryonic-lethality for homozygous-null progeny.(Lee et al, 1992; Stanton et al, 1992; Li et al., 1992) In such cases, the investigator may be able to examine early, even pre-implantation embryonic tissue, and thereby determine that the gene is essential for a given process to take place at a given embryonic stage, in a given tissue, and that failure of this process to take place is the cause of the embryonic death.

Moreover, the investigator might be able to go on to make and implant blastocyst chimeras in which embryonic cells from the still-viable homozygous null early embryos (or from ES cells in which both copies of the gene have been "targetted") compete with wild-type cells for subsequent contribution to the various tissues of the resulting animal. In such an animal, one might expect only wild-type cells to be present in those tissues whose development requires the function of the gene, whereas tissues in which the gene's function is immaterial to development would consist of a mixture of wild-type and homozygous-null cells. Although at first glance, this might seem an elegant solution to the problem of embryonic-lethality, it would only allow one to ask in which tissues is the presence of the gene essential for normal development, in the case of genes whose function was cell-autonomous, or at least tissue-autonomous, or whose non-tissue-autonomous function was critically dose-dependent. One might imagine a scenario in which the function of gene *alpha* in tissue A is required for the normal development of tissue B. In the absence of expression of *alpha*, tissue B is present but not fully developed, and this is what one would see in the non-chimeric homozygous-null animal, were

it not for the fact that the total absence of gene *alpha* resulted in embryonic lethality at day 3 post-conception. In the chimeric animal, however, the wild-type cells that make up a portion of tissue A do express ALPHA, and sufficient *alpha* product is available that no abnormality of tissue B is noted. An additional shortcoming of the chimeric strategy is that it would not allow one to address the question of what effect the absence of a gene would have in a mature tissue whose presence was dependent on the function of that gene during development.

C) The solution

Both the problems discussed above would be overcome if one had the ability to remove a defined portion of genetic material from a cell at will. While it would be overstating the case to claim that this is indeed possible for any gene in any cell, there is a method described in the literature whose further development may lead to the realisation of this possibility.

There exists a family of site-specific DNA recombinases members of which are involved in the life cycle of bacteriophage, and the maintenance of copy number of the yeast 2μ plasmid. The activity of these recombinases is such that they will remove from a target sequence DNA that lies between the two directly repeated copies of a specific sequence (which varies from one recombinase to another). Such a recombinase might be used to address the problem of multi-step pathogenesis. The genetic mutation one wished to study (i.e. one of the multiple "steps") might be introduced with a construct in which the mutation sequence is flanked by the specific sequences that would allow later removal of the mutation sequence when exposed to the recombinase activity. Similarly, in the context of homozygous embryonic-lethality of gene-targetting events, one might breed a mouse in which such a potentially embryonic-lethal genotype has been "rescued" by the presence of a copy of the targetted gene flanked by the specific sequence which would allow eventual removal of the gene in tissue(s) and at a time determined by the expression of the recombinase. The subject of this portion of the thesis is a project designed to determine whether such a recombinase could function in a site-specific and tissue-specific fashion in transgenic mice

2) LITERATURE REVIEW

Since the work reported here is by way of a demonstration of the feasibility of a novel technique, a detailed review of DNA recombination has not been undertaken. It has been the author's intention to discuss briefly the literature relevant to understanding the determinants and mechanisms, to the

extent they are known, of site-specific recombination, and such information in the literature as might allow one to predict the scope of the potential applications of the technique described here.

A) Background.

Site-specific recombination may be distinguished from homologous ("general") recombination, in terms of features of the substrate DNA's, the recombination machinery itself, and the structural mechanism of the recombination (Craig, 1988). Homologous recombination (the mechanism that gene-targetting relies upon) involves exchange between regions of DNA sharing extensive homology, and the crossing over or exchange point may lie anywhere within the regions of homology. In contrast, site-specific recombination results in rearrangements of DNA sequences that lack such regions of homology but the recombination points are determined with some precision, within small regions of the DNA. Two distinct forms of site-specific recombination can be recognised: a) transposition, in which there is no homology between the recombination sites, DNA synthesis occurs, and the recombination is non-reciprocal; and b) conservative site-specific recombination, in which the recombination point lies within a short sequence of identity shared by the DNAs involved, breakage and joining of strands occur in the absence of net change in the quantity of DNA present, and recombination is reciprocal (Craig and Kleckner, 1987; Campbell, 1962). It is this latter form of site-specific recombination that forms the subject of this review.

B) Basic structural features.

Although the structural mechanisms of the CRE site-specific recombinase system will be discussed in some detail later, (section D), some of the fundamental topology of such systems will be described at this point, so as to facilitate comprehension of subsequent sections.

Site-specific recombinase activity was first described in the context of the closed-circular double-stranded DNA chromosomes of bacteria and bacteriophage so the topological illustrations of this activity are usually of the form found in figure 2.1 The action of a conservative site-specific recombinase on two separate DNA circles each containing a single target site will result in a single circle containing the DNA from both the former circles, and bearing two identical or nearly identical target sites in direct repeat orientation (fig. 2.1, part a). The reverse reaction also occurs, forming two circles from one larger one in which the target sites occur as direct repeats (fig. 2.1, part b). In the

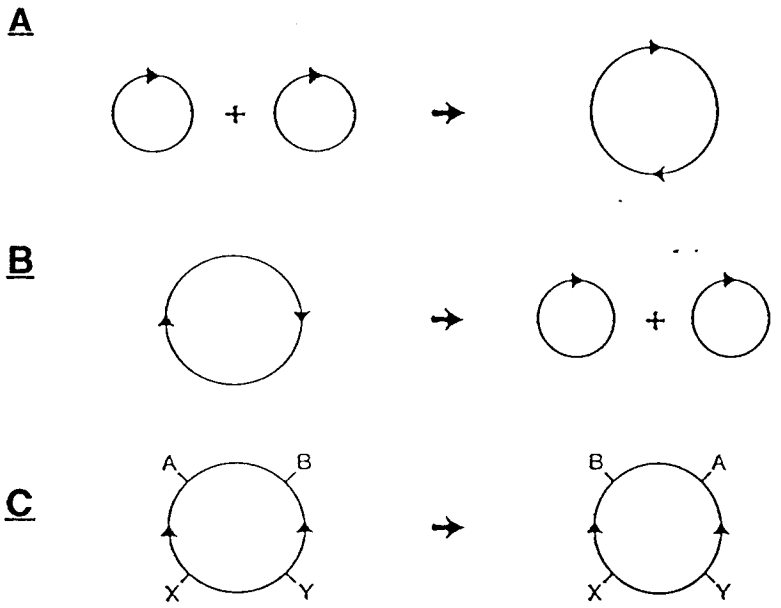


Fig. 2.1. Topology of recombinations mediated by the conservative site-specific recombinases (after Craig, 1988). A) Single target sites on two separate circular DNA molecules. B) Two target sites in the same orientation on a single circular DNA molecule. C) Two target sites in the opposite orientation on a single circular DNA molecule.

case of a circular DNA molecule containing two target sites in opposite orientation, the DNA between the target sites is inverted with respect to the remainder of the circle (fig. 2.1, part c). The implications of these mechansims with respect to DNA in topologically linear forms will become clear in the course of the subsequent discussion.

C) Conservative site-specific recombination in nature.

This form of recombination was first observed during the study of lambdoid bacteriophages. Campbell (Campbell, 1962) first suggested that lambda uses such a mechanism to integrate into the host *E. coli* chromosome during lysogeny. In this case, recombination between the phage sequence *attP* and the host sequence *attB*. is mediated by the phage protein INT, and requires the host protein Integration Host Factor (Thompson and Landy, 1989; Weisberg and Landy, 1983). For this recombination to take place, the *attP* site must be present as supercoiled DNA. A further host protein, FIS, modulates excision of the phage (Thompson et al., 1987). Several other bacteriophage, e.g. P2 and 186 (Kalionis et al., 1986) and P4 (Pierson and Kahn, 1987) use a similar mechanism.

The replicative cycle of bacterial Tn3-like transposons involves two separate steps of site-specific recombination, a non-conservative step and a conservative resolution step mediated by the transposon-encoded resolvase (reviewed in Grindley and Reed, 1985; Hatful and Grindley, 1988). The cyanobacterium *Anabena* uses a site-specific recombination mechanism, in the absence of a supply of reduced nitrogen, to excise DNA segments that interrupt the coding sequences of nitrogen-fixing genes (Haselkorn, 1989; Haselkorn et al., 1986). There is a family of recombinases whose members function to invert the DNA between their recombination sites in *S. typhimurium* and several bacteriophage: the HIN recombinase of *S. typhimurium* changes the orientation of a promoter which results in an alteration of flagellar antigens; the GIN and CIN proteins of bacteriophage Mu and P1, respectively, bring about DNA inversions that allow modulation of the host-range of the bacteriophage (reviewed in Glasgow et al., 1989, Hatful and Grindley, 1988, and Johnson and Simon, 1989). The transposon resolvases and these latter "invertases" require their target sites to be present as supercoiled DNA. Another system of site-specific inversion is found in *E. coli*, which can modulate the presence of fimbriae that allow adhesion to eukaryotic cells, by way of the *fimA* promoter and the *fimB* and *fimE* genes (Glasgow et al., 1989; Klemm, 1984 & 1986; Abraham et al., 1985).

The bacteriophage P1 displays a site-specific recombinase function, in addition to that of CIN mentioned above. Rather than integrate into the host chromsome during lysogeny, as do the lambdoid prophage, P1 is mostly (but see below) maintained as a low-copy-number plasmid (often at

only one copy per cell). Copies of the plasmid tend to form dimers after replication, by homologous recombination, and the resulting failure of copies of the plasmid to segregate into daughter cells would rapidly lead to loss of the prophage from the population. P1 produces an enzyme called CRE that promotes recombination between target sites called *lox* to promote separation of such dimers, and maintain segregation of prophage copies (Austin et al., 1981).

Whereas all the naturally occurring conservative site-specific recombination systems so far mentioned are of prokaryotic origin, such systems have also been described in eukaryotes. The best characterised of these is the FLP system of the 2μm plasmid of yeast. This plasmid, often found in *Saccharomyces cerevisiae* carries the gene encoding the recombinase, and two copies of the target *frt* (*F*LP *r*ecombination *t*arget) in opposing orientation. Expression of the FLP allows the plasmid to increase its copy number per cell, despite obeying the eukaryotic rule of one replication initiation per cell cycle (reviewed in Cox, 1989). Structurally and functionally similar mechanisms have been found in 2μm-like plasmids of other yeasts (Toh-e et al., 1982; Toh-e et al., 1984).

Three of the recombination systems so far mentioned, namely the lambda INT, the P1 CRE, and the 2μm plasmid FLP, share a common mechanism of action at the biochemical level: they attach to broken DNA through 3' phosphodiester linkage to a tyrosine residue, and generate staggered breaks with 5' protruding ends of 6-8 base pairs (Andrews et al., 1985; Craig and Nash, 1983; Gronostajski and Sadowski, 1985; Hoess and Abremski, 1985; Pargellis et al., 1988; Senecoff et al., 1985). Additionally, the INT, CRE, and FLP proteins share a marked sequence homology in a carboxy-terminal domain of about 40 amino acids which has been shown to be responsible for catalytic activity, as deletion removes recombination activity without affecting DNA-binding. Although the target sites of INT, CRE, and FLP share some sequence similarity, the target specificity of the enzymes is thought to be determined by sequences outside the 40 amino acid homologous domain (Pargellis et al., 1988; Parsons et al., 1988; Prasad et al., 1987; Wierzbicki et al., 1987). This latter domain includes a perfectly conserved tyrosine, which has been shown to be the site of DNA linkage (in the case of INT, at least, Pargellis et al., 1988). Many other recombinase proteins share this 40 amino acid domain, and these are collectively termed the "integrase" family of site-specific recombinases (Argos et al., 1986; Pargellis et al., 1988).

Another well-known eukaryotic site-specific recombination system is that of V(D)J recombination in vertebrate lymphocytes, generating the diversity of immunoglobulin and T-cell receptors (reviewed in Gellert, 1992). This system shares with previously mentioned systems some features of the fundamental topological rearrangements illustrated in fig. 2.1, although the mechanism here is somewhat more complex than that of the integrases. In the presence of a pair of chromosomal signal

sites in opposing orientation between protein coding regions, intervening DNA is excised. (Circular DNAs resulting from such excisions have been isolated - Roth et al., 1992). When two signal sites in the same orientation are each followed by coding regions, intervening DNA is inverted leaving the "coding joint" and the "signal joint" present in the chromosome (Weichhold et al.; 1990, Korman et al., 1989; Malissen et al., 1986). There are, however, major differences between V(D)J recombination and conservative site-specific recombination as exemplified above, viz: recombination is not strictly conservative, in that there is often loss or gain of several base pairs of DNA at the point of recombination (contributing to the diversity of antigen-binding sites generated - Tonegawa, 1983; Lieber et al., 1988); and the end-products of V(D)J recombinations that are involved in coding for antigen receptors (as opposed to the excised prodicts) are not themselves substrates for further recombination (Lewis et al., 1985). Many of the factors involved in V(D)J recombination are as yet unknown, although the products of several genes are known to be required. Two of these, *rag1* and *rag2* (for "recombinase activation gene", Oettinger et al., 1990) are known to be necessary but not sufficient for recombination to occur. Of those V(D)J recombination factors so far characterised, only RBP-Jκ, has been shown to share some amino acid sequence simlarity with prokaryotic recombination enzymes - similarity with the 40-amino acid carboxy-terminal catalytic domain characteristic of the integrase family (Matsunami et al., 1989). The expression of RBP-Jκ and of the *rag* products, however, is not restricted to cells in which specific recombination activity has been shown to occur. Interestingly, highly conserved homologs of RBP-Jκ have been found in *Xenopus laevis*, and *Drosphila melanogaster* (Furukawa et al., 1991). Although the amino acid sequence identity between the mouse and fly products is 75%, and, like mouse RBP-Jκ, the fly product specifically binds mouse Igκ recombination recognition sequences *in vitro* (Furukawa et al., 1992, Schweisguth and Posakony, 1992), *D. melanogaster* does not have an immune system, nor, as a far as is known, does it display any V(D)J-like DNA rearrangement.

D) The CRE/*LOX* system

Early studies of the coliphage P1 had suggested that its genetic map was linear. The physical form of its genome was known, however, to be circular. Moreover, when the phage integrated into the host chromosome (albeit at low frequency), the ends of the integration were found to be the same as the ends of the genetic map, and the site of integration was always the same (Scott, 1968; Walker and Walker, 1975; Chesney and Scott, 1978; Chesney et al., 1979). The simplest explanation for these phenomena is that there is a hot-spot for recombination in the phage genome, and some homologous target in the *E.coli* genome. Sternberg et al. (Sternberg et al., 1981; Sternberg and Hamilton, 1981) looked for the hot-spot using an EcoRI fragment of the phage DNA spanning the

ends of the genetic map. They made hybrid phage by inserting this into a lambda phage with mutations in the known recombinases of lambda, and grew the products in mutant *E. coli.* lacking recombinase activity. These workers were able to demonstrate both the presence of the recombination hot-spot and a recombinase-activity encoding sequence within the EcoRI fragment of P1. They named the recombination spot "*loxP*" for *lo*cus of crossing over in *P*1, and the recombination-producing sequence "*CRE*" for *c*auses *re*combination. Using molecules in which the EcoRI fragment was oriented differently with respect to flanking markers, they showed that recombination only occured between molecules when the RI fragments of the two were in the same orientation. By deletion analysis, they were further able to localise the *loxP* site. Sequence of the site was obtained (Sternberg and Hamilton, 1981; Hoess et al., 1982), as was sequence of the site of integration in the host chromosome, and of the resulting phage-chromosome junctions (fig. 2.2) The significant features of these *lox* sites are the 13 bp inverted repeat and the asymmetrical spacer (8 bp long in the *loxP* site) between these repeats. Although surrounding sequences differed, it was not possible to determine whether any features of the DNA outside the 34 bp core were required for recombination.

Deletional mutation of the *loxP* site revealed its likely role in maintaining copy number of phage in daughter cells as the host divides (Austin et al., 1981, and see above, section C). Further studies allowed the minimal *loxP* site that allowed efficient recombination to be defined as lying within a 60 bp region encompassing the 34 bp core (Abremski et al., 1983). With partially purified CRE-containing bacterial extracts, it was shown that *in vitro* recombination between *loxP* sites could be achieved in the presence of either Mg ions or spermidine, and that recombination took place whether the substrate was present as supercoiled, relaxed circular or linear DNA. In addition, intramolecular as well as intermolecular recombination between *loxP* sites was demonstrated, with DNA between the sites excised if the sites were in the same orientation, and inverted if the sites were in opposite orientation (ibid.).

The CRE coding sequence had been shown to lie within a 1.5 kb fragment of the P1 DNA, and cloning of this fragment into a high-level bacterial expression vector allowed purification of CRE to apparent homogeneity - a single band on SDS-PAGE (Abremski and Hoess, 1984). Column elution, gel electrophoresis and density-gradient centrifugation suggested that the CRE product was an asymmetric monomeric protein of 35 kD. In the presence of magnesium ions (at the same concentration as that required to demonstrate *in vitro* bioactivity, i.e. 10mM), the sedimentation coefficient shifted from 3.0 to 4.0 S, suggesting the possibility that CRE was dimerising under these conditions. Although purified CRE bound to any DNA sequence, its efficiency of binding (% bound versus amount of CRE present) was 20-fold higher when the target DNA contained a *loxP* site.

***Lox*P** cctctcagacctaATAACTTCGTATAGCATACATTATACGAAGTTATattaagggttatt

***Lox*R** cctctcagacctaATAACTTCGTATAGCAGGAAGTTATCCGAAGcgatgagagttatccc

***Lox*L** ccaaagtgagtgatatgtTTCGGATAACATACATTATACGAAgttatattaagggttatt

***Lox*B** tatgtTTCGGATAACAGGAAGTTATCCGAAgcgat

Fig. 2.2. Sequences of various *Lox* sites found in the bacteriophage P1 life cycle (After Sternberg 1981) Regions involved in inverted repeats are shown in upper-case. Regions of bacterial origin are underlined. *Lox*P represents the sequence present in the P1 phage, *Lox*R and *Lox*L the two sides of integration recombinants between phage and bacterial DNA, and *Lox*B the reconstructed original bacterial site. The bold areas in the *Lox*P sequence represent the inverted repeats.

Moreover, heparin interfered with binding to all DNA except that which contained *loxP* sequence. Pretreatment of CRE with SDS prevented binding to *loxP*-containing DNA. If the sample was treated with SDS AFTER the CRE and DNA were mixed, the binding to DNA containing a single *loxP* site was reversed, whereas binding to DNA containing two *loxP* sites was not, even when the amount of SDS was more than doubled, suggesting that the complex of CRE with two *loxP* sites was of a different nature from that of CRE with a single *loxP* site. It appeared to the authors that, as evidenced by the molar ratios of the amount of purified CRE and targets sites used and the amount of recombinant product obtained, the recombinase activity was stoichiometrically related to the amount of CRE present, and that more than one molecule of CRE was required per recombination event. It was admitted by the authors, however, that they were unable to determine what proportion of the purified CRE consisted of active molecules. Calculation of physical parameters showed that two CRE molecules would together be more than large enough to bind to the whole 34 bp core of the *loxP* region.

Hoess and Abremski subsequently undertook a study of the CRE-mediated protection of *loxP* sequence from nuclease digestion (Hoess and Abremski, 1984). CRE protected the core 34bp sequence from digestion by both neocarzinostatin and DNAse1. In addition one bp outside this region was protected from neocarzinostatin, whereas 3-5 bp outside the region were protected from DNAse1 activity. The authors attributed this difference to steric hinderance which would tend to affect the larger DNAse1 more than the smaller neocarzinostatin. 1/2 *loxP* sites (consisting of one of the 13bp repeats plus 4bp of the 8bp spacers, although unable to undergo recombination with a full *loxP* site, were still protected from nuclease digestion by CRE. Since CRE was unable to bind to sequence significantly beyond the 34 bp core, it seemed very likely that the "orientation" of the *loxP* site was determined by the asymmetrical 8bp spacer sequence.

The point of strand cleavage and exchange mediated by CRE was determined *in vitro* by the same workers (Hoess and Abremski, 1985). It lies one base internal to start of the 8 bp spacer at either end and the cleavege results in a 6 bp 5' overhang (prior to the ligation event). The breakage occurs on the 3' side of a phosphate moiety and the CRE protein becomes covalently attached to this phosphate, preserving bond energy and thereby rendering unnecessary the presence of high-energy cofactors. This bond is transient since the products of recombination are not attached to CRE molecules. Several topoisomerases, lambda INT and FLP have been shown to utilise the same mechanism (Liu and Wang, 1979 ; Gellert, 1981 ; Craig and Nash, 1983; Gronostajski and Sadowski, 1985, Andrews et al., 1985). The authors had previously been unable to detect topoisomerase activity in CRE preparations (Abremski and Hoess, 1984). Using a mutant *loxP* site in which one base of the 8bp spacer had been deleted and which allowed a much slower recombination reaction than

that of the wild-type site, they were now able to demonstrate topoisomerase activity of the enzyme (Abremski et al., 1986).

Sternberg et al. (Sternberg et al., 1986) sequenced the CRE gene, showing that the coding sequenced predicted a protein of 343 amino acids. Comparison of the predicted sequence to the sequence of 5 other phage recombinases revealed regions of homology shared by all these recombinases toward the carboxy-terminal end of the proteins, with three residues completely conserved across all six sequences (Wierzbicki et al., 1987, Parsons et al., 1988). One of these is a tyrosine at postion 324, the others being histidine (289) and arginine (292). The FLP recombinase had been shown to share with CRE the mechanism of transient covalent linkage to a 3'phosphate of the DNA during the recombination process, and it had been shown that the linkage to FLP was to a tyrosine (Gronostajski and Sadowski, 1985). Although it was not known which tyrosine of FLP was involved, the conservation of tyrosine 324 (also found in FLP) suggested that this might be the residue immediately involved in the covalent attachment of CRE to *loxP* (Wierzbicki et al., 1987).

The INT system of phage lambda had been shown to work through an intermediate Holliday junction (Hsu and Landy, 1984). Using a set of mutants of CRE, Hoess and colleagues (Hoess et al., 1987) were able to isolate a structure with electron-microscopic appearances suggestive of a Holliday junction from interaction of some of these mutants with *loxP* .Additionally, some of the CRE mutants that were themselves unable to recombine linear substrates could nevertheless complete the recombination of a pre-formed *loxP* -*loxP* Holliday junction.

Brian Sauer (Sauer, 1987) first showed that CRE could function in eukaryotic systems. There was some precedent for this, in as much as two other prokaryotic DNA-binding proteins had been shown to function in eukaryotic cells - bacterial lexA protein (Brent and Ptashne, 1984), and the restriction enzyme EcoRI (Barnes and Rine, 1985). Sauer engineered an expression vector in which the expression of CRE was placed under the control of the tightly-regulatable yeast GAL1 promoter. As target sequence he chose a yeast selectable marker (LEU2) which he flanked by *loxP* sites in direct orientation, so that in the presence of CRE activity the marker would be lost. Taking advantage of homologous recombination in yeast, he placed the target sequence at two different chromosomal locations in the yeast genome. Having created stable transfectants of these yeast, under non-inducing conditions, that carried the CRE expression vector, he showed that after one hour of induction, more than 90% of cells had lost the selectable marker. He was able to show, by Southern analysis, that the loss had been due to recombination at or near the *loxP* sites, and in three examples analysed by sequencing, that the recombination had produced a result identical to that produced by

the action of CRE on the same target sequence in *E. coli*. The length of the LEU2 sequence flanked by the *loxP* sites in these experiments was approximately 2.6 kb.

Sauer and colleagues also demonstrated (Sauer et al., 1987) that the CRE/*lox* system could be used to insert DNA into a virus at a pre-integrated *loxP* site.Viral DNA bearing the site was incubated with circular plasmid DNA also bearing a single *loxP* site in the presence of CRE protein. The plasmid sequence could be recovered from the viral DNA by further treatment with CRE. Although this experiment was *in vitro* as opposed to being in the context of a cell nucleus, the length of plasmid sequence here shown to be susceptible to CRE-mediated excision, was 3.4 kb.

In the context of the nucleus of a mammalian cell, Sauer and Henderson (Sauer and Henderson, 1988) showed that CRE was able to mediate recombination of targets present on episomally replicating plasmids. Mouse C127 fibroblasts were infected with a herpesvirus (pseudo-rabies virus) into which had been inserted a 3.1 kbp *loxP*-flanked plasmid fragment. Expression of the CRE protein in the transfected cells resulted in excision of the 3.1 kb fragment as determined by Southern analysis. Retention of the expected single *loxP* site in the resultant episomes was demonstrated by showing that such episomes recovered from the cells were able to undergo CRE-mediated recombination with another *loxP* -containing plasmid *in vitro*. Although no estimate was given of the copy number of the target viral episomes per cell, the authors state that up to 25% of the virus recovered from plate stocks of infected cells expressing CRE protein had undergone CRE-mediated recombination. Importantly, the authors observed NO recombinant products in virus grown in cell that did not express CRE, and had previously shown the frequency of homologous recombination between *loxP* sites in the virus to be less than 1×10^{-5} (Sauer et al., 1987).

The same workers then demonstrated that CRE could function on chromosomally integrated DNA in mammalian cells (Sauer and Henderson, 1989). The target DNA had the structure shown in figure 2.3. In this construct, expression of the G418-resistance selectable marker encoded by the *neo* gene would be inhibited by the 2.6 kb yeast *leu2* gene sequence flanked by *loxP* sites and situated between the SV40 promoter and the *neo* coding sequence. This inhibition of *neo* expression would result from the presence of the several ATG sequences in the yeast gene, which would act as false translational starts with respect to the *neo* sequence.The construct was made by inserting the *lox*P-flanked sequence into the vector pSV2neo. After transfecting mouse fibroblasts with this construct, a cell line was obtained that was less sensitive to G418 than the parental untransfected cells, but still markedly more sensitive to G418 than transfectants made with unmodified pSV2neo. Southern analysis of this line showed that it contained a single integrated copy of the construct shown. The construct had been subjected to CRE-mediated recombination *in vitro*, and fibroblasts transfected

Fig. 2.3. Transfection construct of Sauer and Henderson (after Sauer and Henderson, 1989)

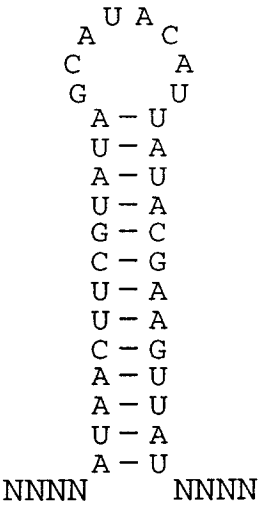


Fig. 2.4. Potential stem-loop structure in the RNA resulting from transcription through a *LoxP* site

with the resultant structure were shown to be resistant to high levels of G418 at about 1/2 the frequency of transfectants made with the original pSV2neo vector. The authors suggest that this may have been due to the presence of the single *loxP* site left by the recombination between the SV40 promoter and the *neo* sequence, as the message resulting from transcription would be able to form a stem-loop structure at this point by virtue of the complementarity of the two 13 bp sequences within the *loxP* sequence (fig. 2.4), and such a stem-loop might interfere with translation (Kozak, 1986). After introducing a CRE expression vector into the target cell line, these investigators were able to show that up to 50% of transfectants became resistant to the same level of G418 as would result from transfection with pSV2neo.

The information relating to the CRE/*lox* system to this point was sufficient to suggest that it might be a suitable candidate for creating a site-specific recombination system in transgenic animals. Similar information was available in relation to FLP, another well-characterised conservative site-specific recombinase (reviewed in Cox, 1988, and Futcher, 1988). Like CRE, FLP would function on DNA in any topology, in the absence of high-energy cofactors, and the absence of any host proteins. The target of FLP, (*frt*) is also a 34 bp sequence with an asymmetrical 8 bp spacer between inverted repeats of a 13 bp sequence. The only feature of CRE activity which had not been duplicated experimentally at the time of initiating the work here reported was its ability to act on DNA placed into the context of the mammalian genome. This criterion was decisive, in the absence of any other, as to which system to choose for engineering site-specific, tissue-specific recombination in transgenic mice.

3) EXPERIMENTAL STRATEGY AND DESIGN OF CONSTRUCTS

Dr. Jamey Marth of the Biomedical Research Centre undertook to support the current research, the aim of which was to test the function of the CRE-LOX system in transgenic mice. An experiment was designed in which it would be possible to assess the deletion of DNA from the genome of transgenics as a function of expression of the CRE enzyme in a given tissue at a given stage of development. This was to be achieved by first creating two transgenic mouse lines, one expressing the CRE enzyme, and the other carrying a target sequence flanked by LOX sites, and then inter-breeding mice of these two lines to make doubly transgenic mice in which the CRE might function to delete the LOX-flanked target.

As this was to be the first instance of the expression of CRE (or any site-specific recombinase) in a mammal, it was decided that the target sequence should be one the presence or absence of which

could be easily determined, but which would not itself affect the development or viability of cells or tissues of the animal. This latter feature was incorporated into the experimental desgn so that a) whatever the outcome of the experiment, one could at least clearly determine whether the expression of the CRE enzyme in transgenic mice would itself result in any phenotype, and b) so that one might be able to assess the efficiency of CRE-mediated deletion. Moreover, it was deemed desirable that the target sequence should be one which could theoretically be used as a "reporter" in subsequent experiments - i.e. by including it between the LOX sites along with the gene of interest whose presence or absence might be less easy to determine. This latter criterion was met by the *E. coli lacZ* (ß-galactosidase) gene, hereinafter referred to as *ßGAL* This gene had the advantage, as a reporter gene, that its expression could be assayed in cells that would remain viable, and that cells expressing the *ßGAL* product could be sorted by FACS for subsequent manipulation. Additionally, a construct including this gene, and made by G. MacGregor, a recognised authority in the use of this gene as a reporter (MacGregor et al., 1992), was available at the Biomedical Research Centre.

One caveat involving the *ßGAL* sequence, however, was that it was almost 1kb larger than any sequence previously published as having been susceptible to CRE-mediated deletion. Additionally, since it is common for the ends of transgene fragments to be truncated in the integration process (Covarrubias et al., 1986), a construct design in which one of the two LOX sites was 5' of the promoter would involve the risk of losing this LOX site during the integration event, resulting in a loss of recombination ability at this end of the transgene. In view of this, it was decided that the *ßGAL* sequence should itself be immediately surrounded by LOX sites in the transgene construct. This would entail the presence of a LOX site between the promoter and the *ßGAL* coding sequence Since, in one orientation, the LOX sequence contained an ATG, which would create a false translation start site if interposed between promoter and coding sequence, it was clear that the LOX sites would have to be oriented so as to avoid this potential cause of poor expression of *ßGAL*. There remained the possibility that the mere presence of a LOX site between promoter and coding sequence would inhibit expression (see above, Section 2 D). There was good evidence, however, that a LOX site in this position WOULD permit expression from the coding sequence, in eukaryotes (Sauer, 1987), and in mammalian cells, in particular (Sauer and Henderson, 1989).

Of known tissue-specifc transgene expression constructs, p1017 (Chaffin et al., 1991) was one with which Dr. Marth had already worked, having himself been responsible for the cloning of the gene from which the promoter in p1017 was derived (Marth et al., 1985). Transgenic mice made with derivatives of p1017 were known to display thymocyte-specific expression of transgenes.(Chaffin et al., 1991; Garvin et al., 1990; Cooke et al., 1991). p1017 was therefore selected as the vector for the CRE

transgene, with the hope that transgenics expressing CRE in a thymocyte-specific fashion might be of subsequent value in addressing immunological questions.

There was debate, however, as to the vector that would be used to obtain expression of ßGAL. The present author proposed the use of a derivative of p1017 in which the *LCK* promoter was replaced by a cytomegalovirus Immediate Early (CMV-IE) gene promoter. This promoter had been shown to be capable of driving expression of transgenes in a wide variety of tissues of transgenic animals, including lymphoid tissue. It was envisaged that the various tissues in which *ßGAL* was expressed, in the resulting transgenics, would act as "internal" positive controls in animals in which the *ßGAL* expression in thymocytes had been abrogated in virtue of the CRE-mediated deletion of *ßGAL* sequence from the thymocyte genome. The presence of such "internal" controls would provide visually dramatic evidence of tissue-specificity (X-Gal or fluorescent staining of thymocytes as compared to cells of other tissues). This scheme would also counter suggestions that a LOX site between promoter and coding sequence may indeed be inhibitory, should *ßGAL* expression NOT be detectable in thymocytes of transgenics. Dr Marth felt that there would be time-constraints impinging on the publishability of results of these experiments, and that there would be too great a risk involved in using a vector not previously shown in his own laboratory to function adequately as a transgenic vector. Consequently, it was decided to use the same p1017 vector for the LOX-flanked *ßGAL* target, in the expectation that *ßGAL* would be expressed in thymocytes only, and that tissue-specificity of CRE-mediated recombination could be determined by Southern analysis of DNA from various tissues.

Debate also arose as to the question of which technique should be used to make the *ßGAL* transgenics: blastocyst implantation of electroporated ES cells, or the more traditional technique of pro-nuclear injection of fertilised oocytes. The former is much more time-consuming than the latter, and has a much higher failure-rate, in terms of achieving germ-line transmission of the transgene. An advantage of the ES-cell method would be that it almost always involves single-copy integration of the transgene construct, as against the multi-copy integration which is the almost inevitable result of the pro-nuclear injection method (C.Ong and J. Marth, unpublished observations, and Covarrubias et al., 1986). A single-copy integration would, one might expect, make for simpler assessment of the function of CRE in deleting that single copy of the *ßGAL* sequence - one would simply be assessing in what proportion of thymocytes the *ßGAL* was deleted. As Southern analysis might prove essential to the assessment of CRE function, this analysis would be greatly simplified if there were a single *ßGAL* sequence in each cell, as compared to multiple such sequences. Possible anomalies arising at the termini of integration sites, and affecting restriction sites used in Southern analyses, would be more readily comprehensible, and might even be identified in ES cells prior to creation of transgenic mice. Despite these advantages, it was again felt that time constraints due to likely competition

warranted the utilisation of the traditional pro-nuclear injection technique. Less DNA would be used for each micro-injection than customary, in the hope of selecting transgenic founder mice bearing relatively few copies of the LOX-flanked*βGAL* transgene target.

RESULTS

1. CREATION OF TRANSGENE CONSTRUCTS

The plasmids pBS31, and pBS64 were kindly donated by Dr. Brian Sauer of E.I DuPont de Nemours and Co., Inc., Molecular Biology, Central Research and Development Department, Wilmington, DE, U.S.A. The plasmid pCMVß was kindly donated by Dr.F. Jirik of the Biomedical Research Centre

A) p1017CRE

p1017 was cut with BamHI, and the ends made blunt. The CRE coding sequence was obtained from pBS31 as a 1.5 kb fragment, by digestion with XhoI and XbaI. After making these ends blunt with Klenow, and ligation into p1017, orientation in recombinants was determined by digestion of mini-prep DNA with BamHI and XbaI , as there is a unique, assymetrically placed BamHI site within the CRE sequence and there is a unique XbaI site in p1017 at a convenient position (fig.2.5). The original BamHI cloning site in p1017 lies within the first exon (in 5' untranslated sequence) of the hGH sequence. A eukaryotic ribosome-binding site sequence is therefore present 5' of this point.

B) p1017LOX^{2}ßGAL

i) pLOX^2

Since the vector pBS64 contains a single LOX site, it was first necessary to create a derivative that contained two LOX sites in the same orientation separated by a unique cloning site. In the desired derivative, the two LOX sites would be surrounded by sites that, after insertion of DNA between the LOX sites, would allow the removal of a fragment containing both LOX sites as well as the intervening DNA. To this end pBS64 was cut with BamHI, the ends made blunt with Klenow, and then self-ligated to create pBS64Δ (fig.2.6). pBS64Δ was then cut with HindIII, the ends made blunt with Klenow, and then cut again with XmnI (which cuts within the coding sequence of the ampicillin resistance gene - Amp). The larger resulting fragment of pBS64Δ, containing one LOX site and a portion of the Amp coding sequence, was purified by gel electrophoresis. The XmnI-AvaI fragment of the original pBS64, containing the complementary part of the Amp coding sequence and one LOX site, was purified by gel electrophoresis. The plasmid pLOX^2 was constructed by ligating the two purified fragments,

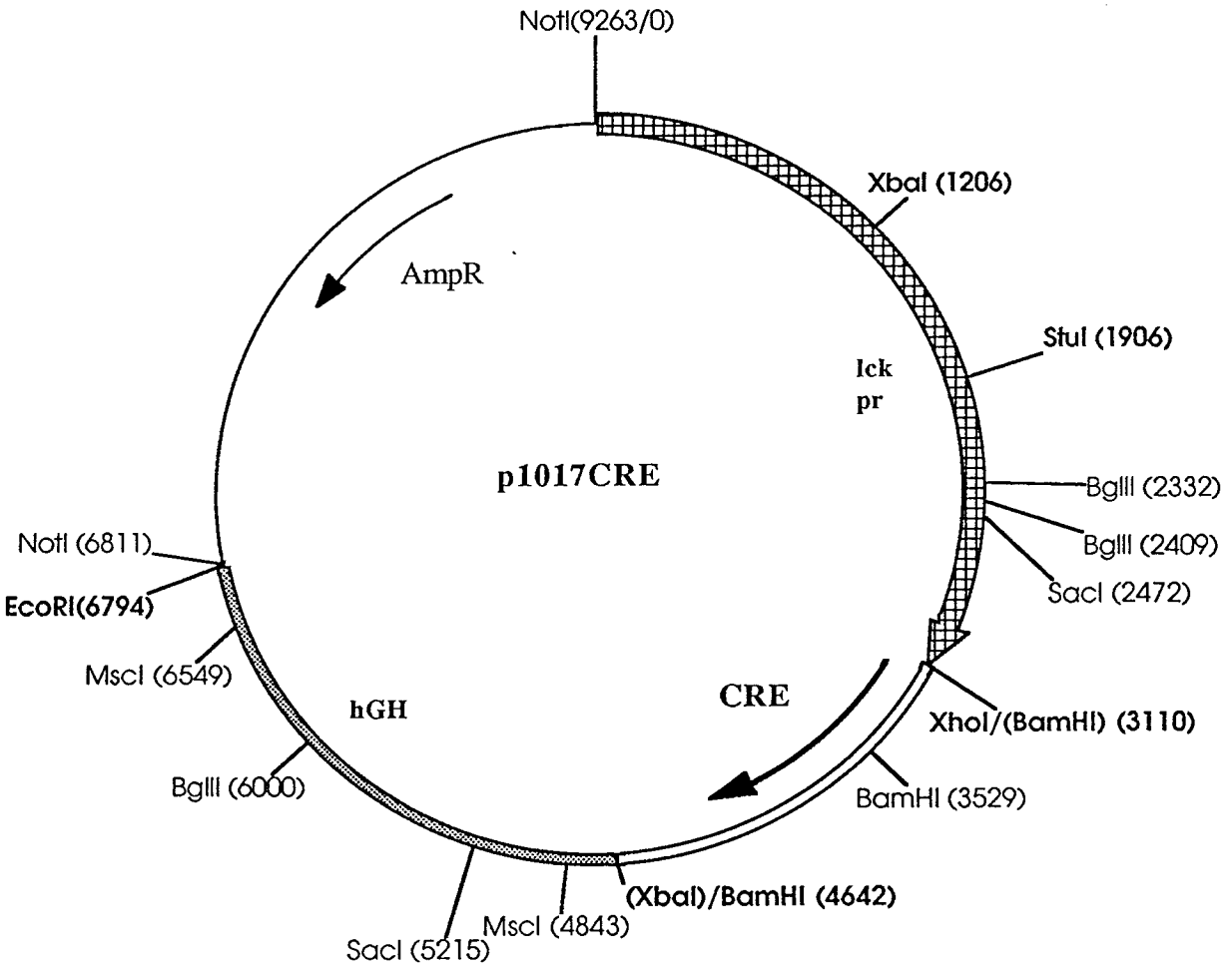


Fig. 2.5. p1017CRE The 1.5 kb XhoI-XbaI fragment of pBS31 was blunt-ligated into the BamHI site of p1017. XhoI and BamHI sites were recreated as shown. Sites used for subsequent Southern analyses are also shown, as well as the NotI sites used to separate the transgene from the plasmid backbone.

which, in the appropriate orientation, would recreate the Amp gene, allowing efficient selection of the desired transformants on ampicillin plates (fig. 2.6). Since having the correct sequence of LOX sites in the correct orientation with respect to each other would be essential to the interpretation of experiments performed with derviatives of pLOX^2, it was deemed appropriate to determine the sequence of the relevant portion of the vector. Figure 2.7 shows the sequence obtained using a commercially available primer to the SP6 promoter. Although the sequence was obtained from only a single strand, it was felt that since the sequence corresponded exactly with that expected, it was unnecessary to sequence the same region of the opposite strand.

ii) pLOX^{2}ßGAL

The E.coli *ßGAL* coding sequence was obtained as a 3.5kb fragment from pCMVß (MacGregor and Caskey, 1989) after digestion with NotI and gel purification. After blunting the NotI ends of the.ßGAL fragment, this was ligated into the blunted BamHI site of pLOX^2, to obtain the plasmid pLOX^{2}ßGAL (fig. 2.8). Orientation of the *ßGAL* sequence was determined by PvuII digestion and SacI digestion of recombinant plasmid DNAs.

iii) p1017LOX^{2}ßGAL

The LOX-ßGAL-LOX fragment of pLOX^{2}ßGAL was obtained by gel purification after digestion with EcoRI and HindIII, and after blunting the ends, this fragment was ligated into the blunted BamHI site of p1017 to form the plasmid p1017LOX^{2}ßGAL (fig. 2.9). Correct orientation of the LOX-ßGAL-LOX sequence with respect to the LCK promoter of p1017 was determined by digestion of recombinant DNAs with NdeI. At the time of construction of this vector, a survey of the literature suggested that the longest sequence removed from between LOX sites by the CRE enzyme (or, indeed, by any site-specific recombinase from between its target sites) had been less than 3 kb in length (Sauer and Henderson, 1989)

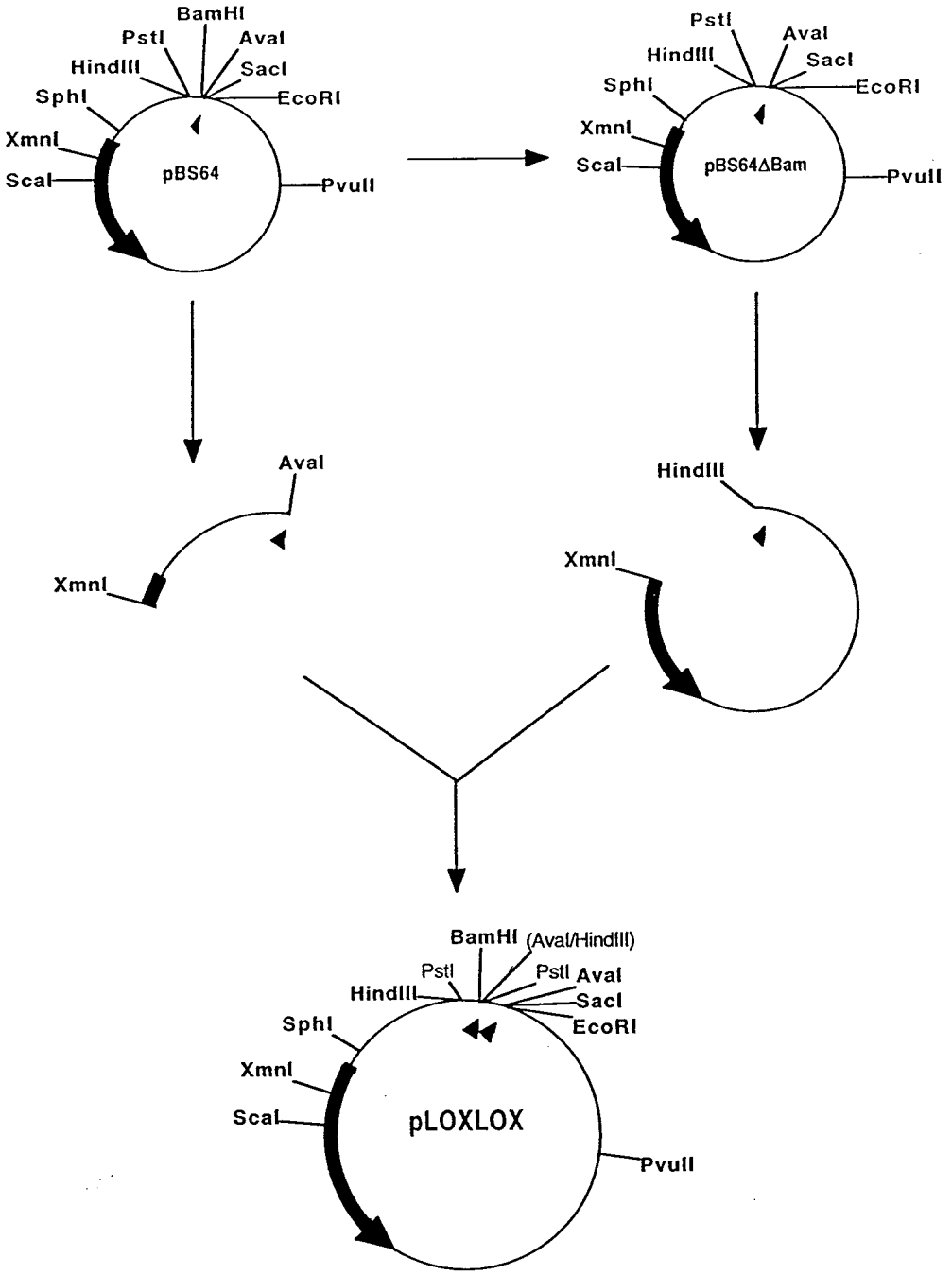


Fig. 2.6. Creation of pLOX2 A portion of pBS64 was ligated to the complementary portionof a derivative of pBS64 in which the BamHI site had been destroyed by filling in with Klenow. Ligation products in which the fragments were joined in the desired orientation recreated the ampicillin-resistance sequence, allowing efficient selection on Amp plates. The LOXP sequences are indicated as arrowheads.

CTGCAGGTCGAGGGACCTA**ATAACTTCGTATAGCATACATTATACGA**
PstI

AGTTATATTAAGGGTTCCGGATCCGGAGCTTGGGCTGCAGGTCGAGGGA
BamHI ΔHindIII/ΔAvaI PstI

CCTA**ATAACTTCGTATAGGCATACATTATACGAAGTTAT**ATTAAGGG

TTCCGGATCGATCCCCGGGCGAGCTC GAATTCGTAATCATGTC
ΔBamHI SmaI SacI EcoRI

Fig. 2.7. Determined sequence of a portion of the vector pLOX^2. The two LOXP sequences are shown in bold, and the sites destroyed by blunting are indicated with a 'Δ' before the site name. The remaining unique BamHI cloning site is titled in bold.

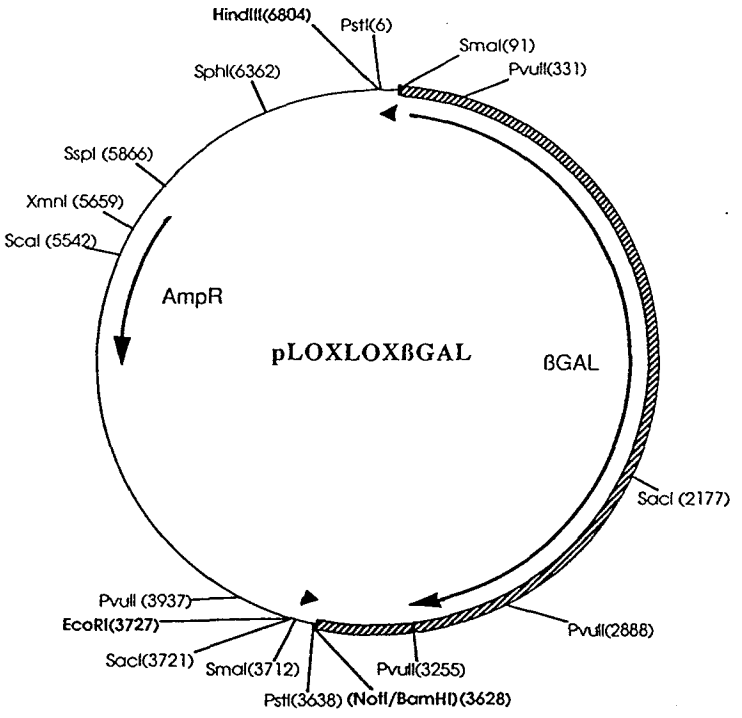


Fig. 2.8. pLOX^{2}ßGAL. The 3.5 kb NotI fragment of pCMVß containing the ßGAL coding sequence was blunt-ligated into the unique BamHI site of pLOX^2. PvuII and SacI sites used to determine orientation are shown. The LOXP sequences are indicated as arrowheads. The "negative" direction of these arrowheads with respect to the orientation of the ßGAL coding sequence is used to highlight the importance of this orientation of the sequences to avoid an ATG between the destined position of the promoter and the ßGAL coding start site.

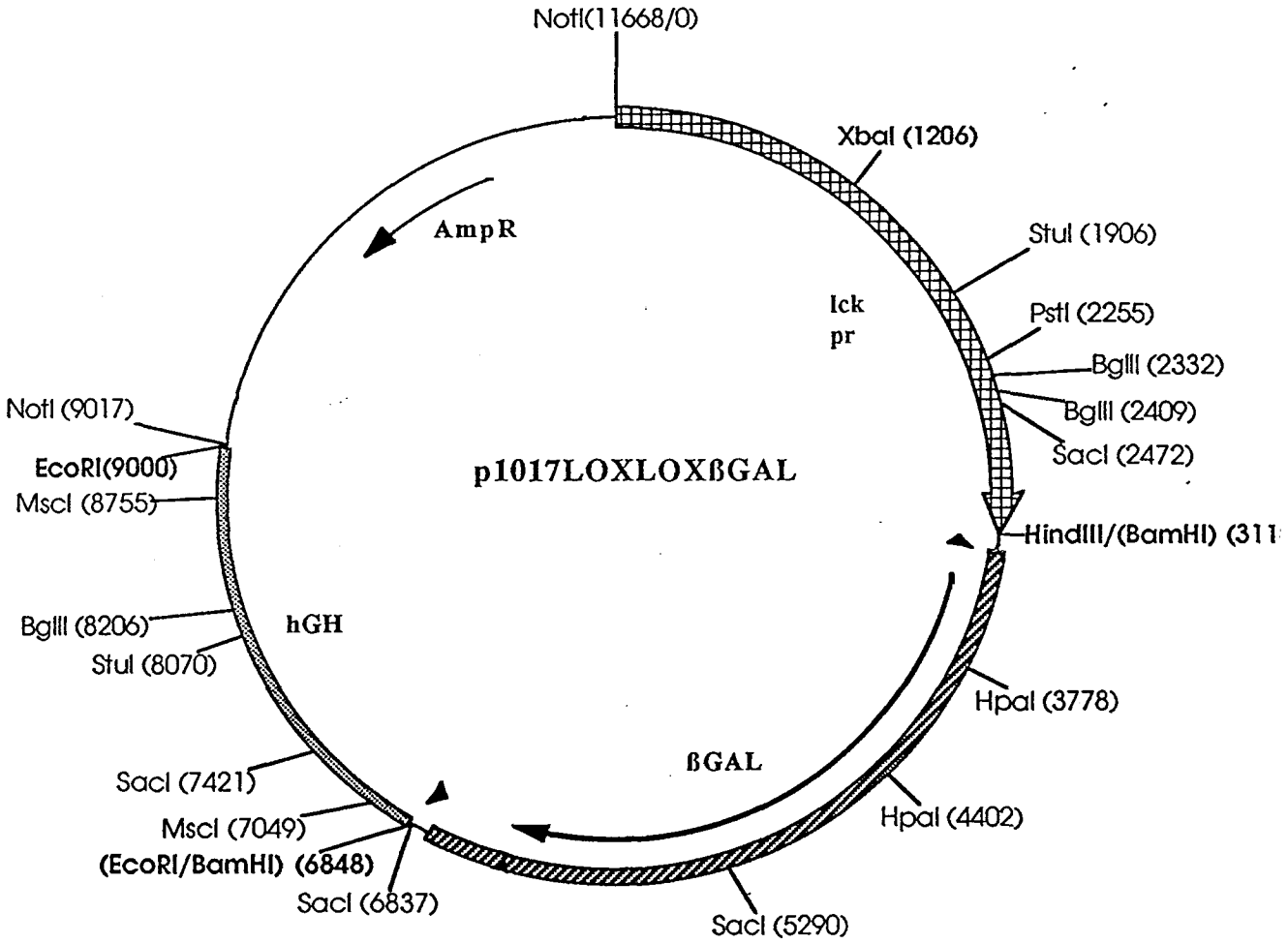


Fig. 2.9. p1017LOX^{2}ßGAL The 3.7 kb HindIII-EcoRI fragment from pLOX^{2}ßGAL was blunt ligated into the unique BamHI site of p1017. The NdeI sites used to determine orientation are shown. Sites used for subsequent Southern analyses are also shown. The HindIII site at the 5' end of the ligation was recreated, probably due to a two base "chew-back" of the BamHI cut end, attributable to contaminating activity in the phosphatase or Klenow preparations. LOX sites are indicated by the unattached arrowheads. The ~600 base pair fragment between the HpaI sites indicated was used as the probe for ßGAL.

2. CREATION OF OTHER PLASMIDS

A) pUC19CRE

In order to facilitate subsequent isolation and manipulation of the CRE coding sequence, a XhoI -MluI fragment containing the CRE sequence was purified from pBS31, the ends made blunt, and ligated into the blunted BamHI site of pUC19, forming pUC19CRE (fig 2.10, A). The orientation of the CRE sequence within pUC19 was determined by SspI digestion. Since the MluI/BamHI blunt junction recreated the BamHI site, and there is a BamHI site within the CRE sequence, BamHI releases a fragment of CRE sequence of approximately 690 bp in length from pUC19CRE, and this fragment was gel-purified and used as a CRE probe in dot-blot, Southern and Northern analyses.

B) pUC19LCKX

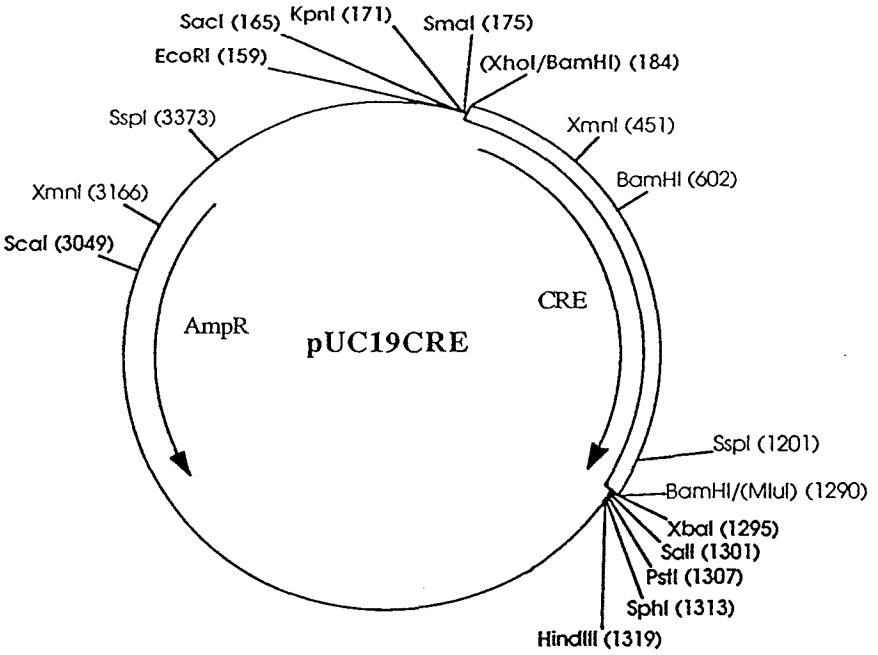
In order to facilitate subsequent isolation of the LCK promoter fragment for use as probe in Southern analyses, a 640 bp SacI-XhoI fragment of p1017CRE, containing the most 3' portion of the LCK promoter sequence was gel-purified and ligated into pUC19 that had been cut with SacI and SalI. The resultant plasmid, pUC19LCKX, is depicted in figure 2.10, part B. The approximately 640 bpSacI-HindIII fragment of pUC19LCKX was gel-purified and used as probe in subsequent analyses.

3. CREATION OF TRANSGENIC MICE AND DETERMINATION OF THE PRESENCE OF TRANSGENES

A) Purification and injection of transgene fragments.

All transgene fragments were separated from vector DNA by NotI digestion and gel purification prior to pronuclear injection, since it had been reported that presence of such DNA could inhibit expression of transgene sequences (Jaenisch, 1988). These NotI fragments of the plasmids are hereinafter referred to as the "transgene constructs". [Pronuclear injection of these fragments into fertilised embryos of ICR outbred mice was performed by Daniel Chui. 2 to 4 pg was injected in a volume of approximately 1 to 2 pl].

A



B

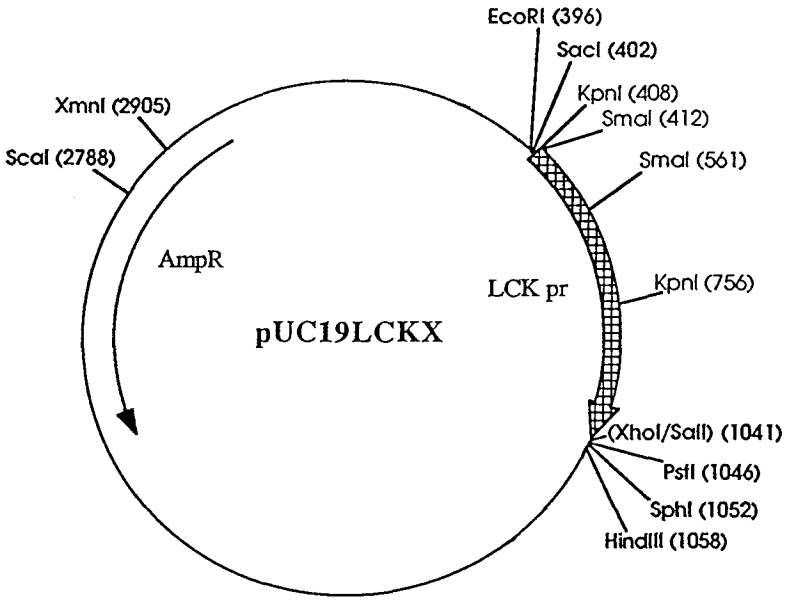


Fig. 2.10. Sub-cloned fragments used for probes A) pUC19CRE. A 1.1 kb XhoI-MluI fragment of pBS31, containing the CRE coding sequence was blunt-ligated into the BamHI site of pUC19. The SspI sites used to determine orientation are shown. The BamHI site was recreated at the BamHI/MluI junction as expected, but the XhoI site was not, possibly due to some "chew-back" by contaminating activity in the Klenow preparation. B) pUC19LCKX. A 640 bp SacI-XhoI fragment of p1017CRE, containing the most 3' portion of the LCK promoter sequence, was ligated into pUC19 that had been cut with SacI and SalI. The XhoI-SalI junction destroys both sites.

B) Determination of the presence of transgenes

The DNA fragment used as a probe for the presence of the CRE transgene was described above. A sequence comparison between the E. coli and Mus musculus Beta-galactosidase coding sequences was performed in order to determine which portion of the ßGAL sequence would serve as a suitable probe . In the absence of any obvious region of high sequence similarity, a HpaI fragment of approximately 630 base pairs (see fig. 2.9) was chosen for use as a probe. In addition, the EcoRI-HindIII fragment of pLOX[2] was gel-purified for use as a probe for the presence of LOX sequences. A preliminary assay of the suitability of these fragments as probes was performed by hybridisation to various plasmid DNAs in dot-blots (fig 2.11). In each case the probe hybridised to all and only those target DNAs that contained the relevant DNA sequences.

In some early analyses, a portion of the hGH sequence (an approximately 620 bp SmaI-EcoRI fragment purified from p1017) was used as a probe. [Dot-blots of tail-tissue DNA were routinely performed with an hGH probe by Daniel Chui, to distinguish transgenic mice created with derivatives of p1017 from their wild-type littermates]. The appearance of unexpected bands in several Southern analyses probed with this fragment led to the eventual abandonment of this fragment as a probe. These additional bands may well have been due to cross-hybridisation of the fragment with mouse growth hormone sequences, since the human and mouse growth hormone sequences display significant homology (fig. 2.12).

The presence of transgene DNA was determined by probing dot-blots of tail-tissue DNA with the CRE and ßGAL probes. [The initial analysis of tail-tissue DNA from potentially transgenic mice was performed by Daniel Chui. Once mice bearing each transgene were isolated and subsequent breeding commenced, collection and analysis of tail DNA was shared by Daniel Chui and myself]. Two founder CRE-transgenic mouse were isolated from 4 litters totalling 19 mice screened. All experiments here reported were performed with progeny of one of these Cre founders. Two LOX-ßGAL-LOX founder mice were isolated from 3 litters totalling 21 mice screened. These founder mice were derived from implants numbered "86" and "87" in the Marth laboratory, and the LOX-ßGAL-LOX progeny of breeding these mice are referred to as being of the "86" or "87" lines respectively.

C). Absence of abnormal phenotypes in transgenic mice

Neither mice bearing the CRE nor those bearing the LOX-ßGAL-LOX transgenes displayed any grossly discernible phenotype.Since the p1017LOX-ßGAL-LOX transgene was expected to cause

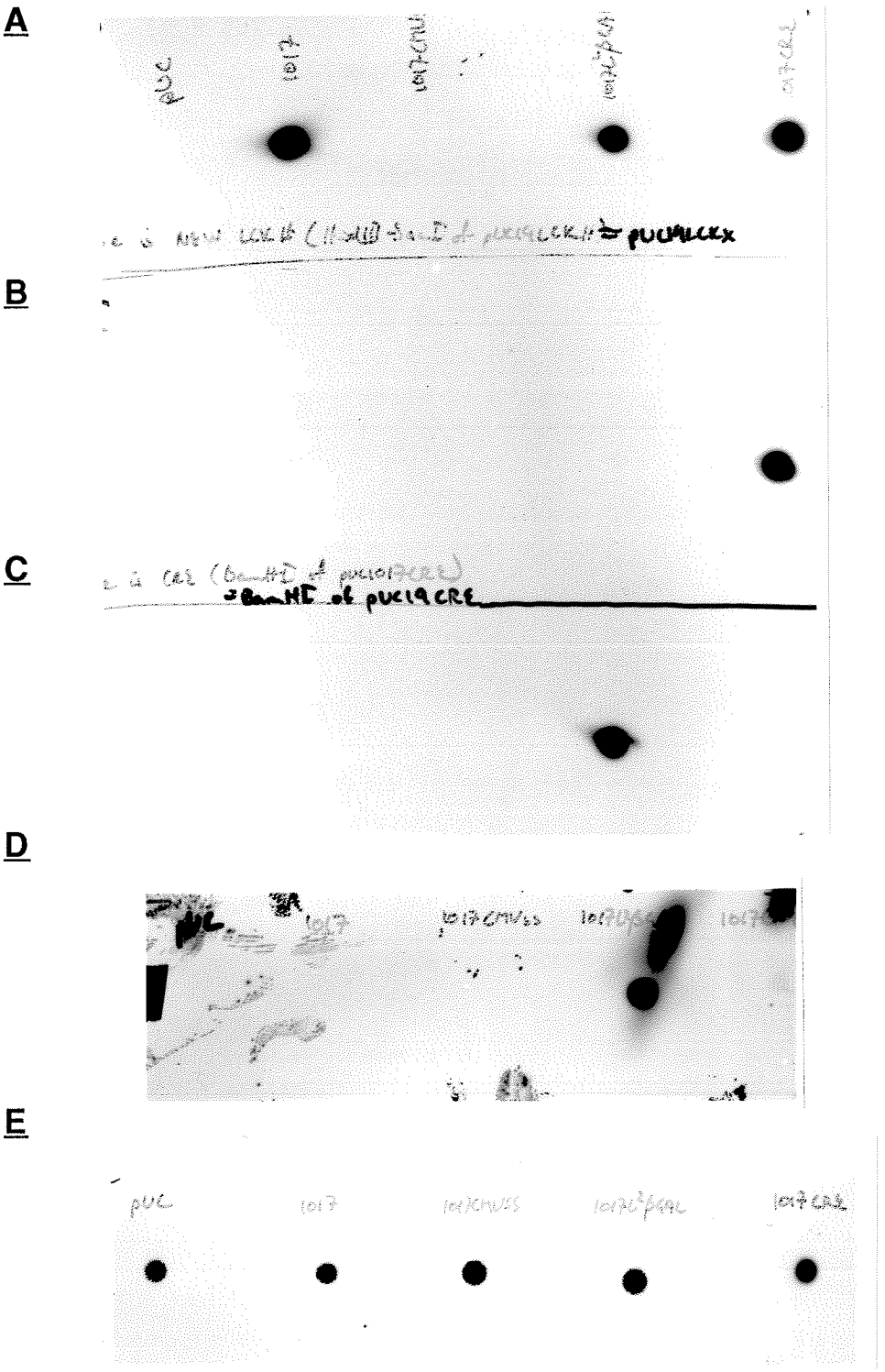


Fig. 2.11. Controls for dot-blots Each panel shows an autoradiograph of hybridisation of the various probes used to dot blots of approximately 1 ng of the following plasmids: pUC19, p1017, p1017CMVSS (A p1017 derivative in which the LCK promoter had been replaced by the hCMV promoter), p1017LOX^{2}βGAL, and p1017CRE. A) Probe is LCK fragment. B) Probe is CRE fragment. C) Probe is βGAL fragment. D) Probe is LOX fragment. E) Probe is pUC19. In every case the probes detected all and only the expected plasmids.

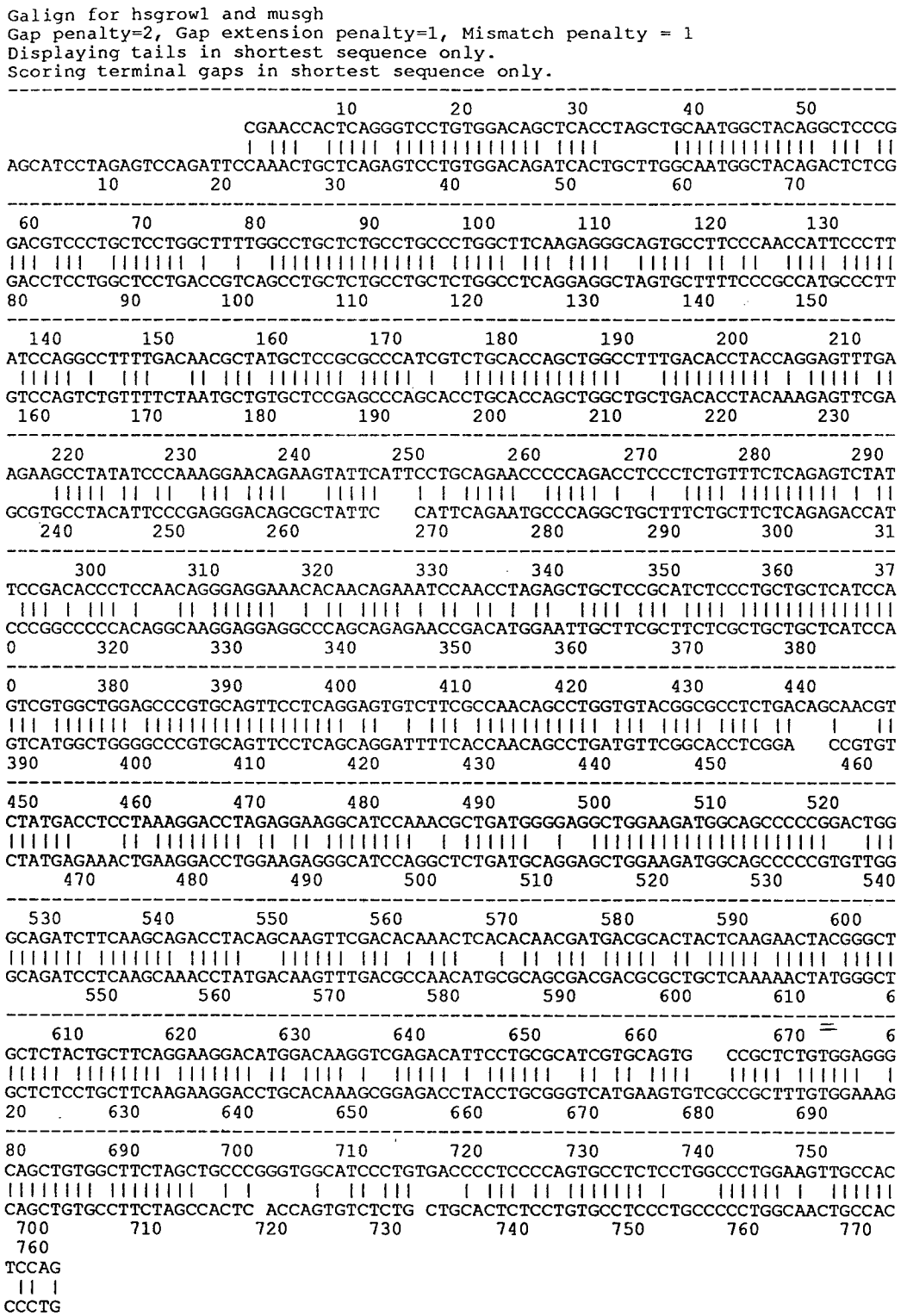


Fig. 2.12. An alignment of the mouse and human growth hormone cDNA sequences

expression of βGAL in thymocytes of mice bearing this transgene, thymocytes from several mice of both the 86 and 87 LOX-βGAL-LOX-transgenic lines were collected and assayed for βGAL activity. Several assays of increasing sensitivity were carried out on these thymocytes, viz. staining using X-Gal as substrate, and direct micoroscopic and FACS-assisted fluorescence assays using fluorescein-di-ß-D galactoside as substrate. In no instance, however, was a difference observed between wild-type thymocytes and those from LOX-βGAL-LOX-transgenic mice. This apparent failure to express βGAL may have been attributable to the relatively low copy number of the transgene structure (see section 4B), since lack of expression is often observed with p1017-derived transgenes present in low copy number (Jamey Marth, Biomedical Research Centre, personal communication; see also Appendix 1). Subsequent assays of the function of CRE in the CRE-trangene-bearing animals were therefore based upon Southern analysis.

4. CREATION AND ANALYSIS OF CRE/LOX-βGAL-LOX DOUBLY TRANSGENIC MICE

A) Creation of doubly transgenic mice

Mice bearing the CRE transgene were mated with those bearing the LOX-βGAL-LOX transgene. The progeny of such breedings were initially analysed by probing dot blots of tail DNA. As no expression of the CRE gene was expected in tail tissue, and no consequent removal of the βGAL portion of the locus was likely, it was felt safe to assay for the presence of the LOX-βGAL-LOX transgene by probing dot-blots with the βGAL fragment.There was no indication from the dot-blots that the βGAL portion of the transgene construct was present in the tail-tissue DNA of significantly less than the expected number of progeny of LOX-βGAL-LOX-transgenic parents (i.e. 50% of the progeny of a mating in which one parent was heterozygous for the LOX-βGAL-LOX "locus"). An example of these dot-blot analyses is given in fig.2.13, and a portion of the genealogy of the CRE/LOX-βGAL-LOX families is given in fig 2.14. Both the CRE and LOX-βGAL-LOX transgene integrations were transmitted in simple Mendelian fashion, as autosomal "loci". Most of the mice analysed in this work were heterozygous with respect to both the CRE and the LOX-βGAL-LOX transgene integrations.

B) Initial analysis of integration structures

A Southern analysis of DNA digested with either EcoRI or XbaI was performed to determine, at a rudimentary level, the structure of each transgene integration (fig. 2.15). EcoRI and XbaI each cut

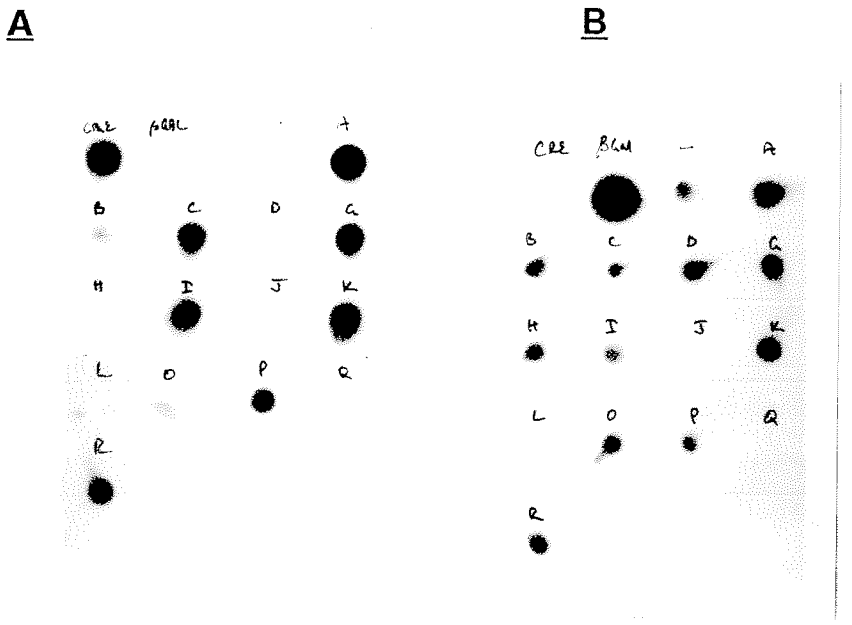


Fig. 2.13. Examples of dot-blots used to genotype transgenic progeny Approximately 5µg of tail-tissue DNA is dotted in each spot, except in spots labelled "CRE" or "ßGAL" which consist of approximately 1ng of the plasmids p1017CRE and p1017LOX^{2}ßGAL, respectively. The blot is of tail DNAs of a litter that resulted from the mating of a CRE/LOX-ßGAL-LOX transgenic mouse to a LOX-ßGAL-LOX transgenic mouse. Some progeny will therefore be expected to bear two "alleles" of the LOX-ßGAL-LOX integration. A) Probe is CRE fragment. B) Probe is ßGAL fragment.

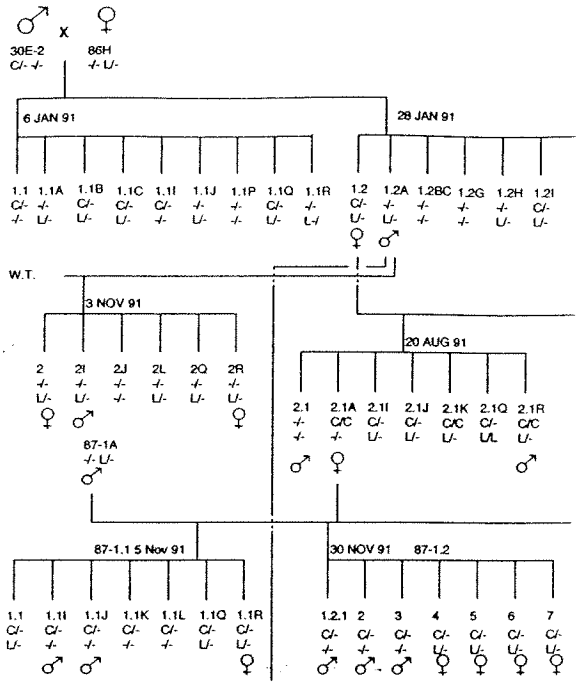


Fig. 2.14. A portion of the genealogical tree of the mice used in these experiments The symbols "C" and "L" refer respectively to integration array "alleles" of the CRE and LOX-ßGAL-LOX transgenes. Birth dates of litters are indicated.

only once within each copy of the transgenic constructs (see figs. 2.5 and 2.9). The appearance of prominent bands of the size of the transgene constructs in the Southern analysis of tail DNA from a CRE-transgenic animal (about 7 kb) and a LOX-ßGAL-LOX-transgenic animal (of the 86 line, about 9 kb) strongly suggests that both the CRE and LOX-ßGAL-LOX loci consist of a series of (at least two) copies of the respective transgene construct and that the great majority of these copies are oriented in a tandem head-to-tail array with respect to each other, as depicted in figure 2.16. This sort of array is commonly observed in transgene integration structures (reviewed in Brinster and Palmiter, 1986, Jaenisch, 1988, and Hanahan, 1989).

In order to provide some assessment of the number of copies of each transgene construct within each of the integration arrays, Southern analysis was performed with a combination of restriction enzyme and probe that would allow densitometric comparison of intensity of transgene bands with bands resulting from hybridisation to the endogenous mouse *lck* gene (e.g. fig. 2.17). From such analyses it was estimated that the 1017LOX-ßGAL-LOX construct was present in about 8 copies in each of the 86 and 87 lines, and that about 110 copies of the 1017CRE construct were present in the CRE transgenic line. (This latter figure is a much rougher approximation than the former , and is likely to be an underestimate, due to the loss of linearity of the increase in intensity of band with increasing copy-number at such high levels). For reasons unkown, both Cre founders obtained were of very high copy number.

C) Demonstration of hybridisation of the CRE and ßGAL probes to fragments of appropriate size in DNA of transgenic animals.

The approximate structure of the majority of the integrated DNA in the transgenic mouse genomes having been established, the ability of probes other than the LCK probe to hybridise to bands of the appropriate size was confirmed. In this instance, both tail and thymocyte DNAs were analysed, and these Southerns provided preliminary evidence suggesting that the LOX-ßGAL-LOX sequence was altered in the thymocytes of doubly transgenic animals in a manner consistent with the predicted action of the CRE enzyme (figs 2.18 and 2.19). It appeared that the amount of ßGAL-hybridising DNA was significantly diminished in thymocytes of doubly transgenic mice as compared to tail tissue of the same animals. The hybridisation of these probes to DNA fragments of the expected size suggested that the performance of the restriction enzymes in particular, and of the Southern analyses in general was adequate for analysis of the function of CRE in these transgenic mice.

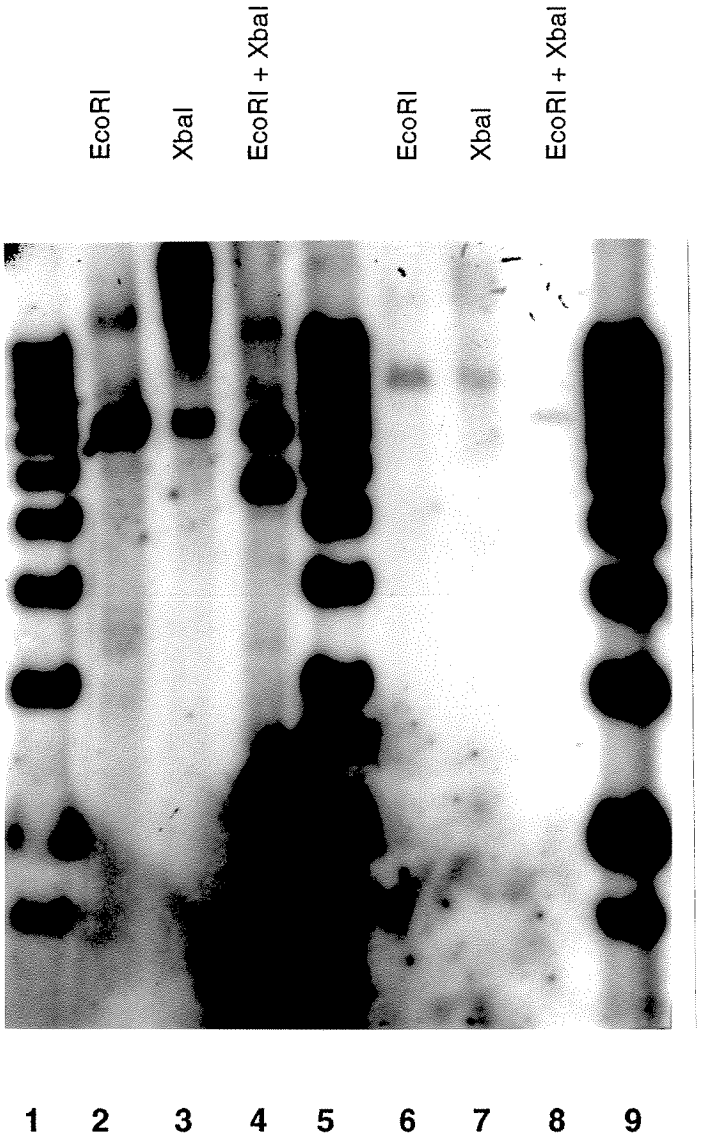
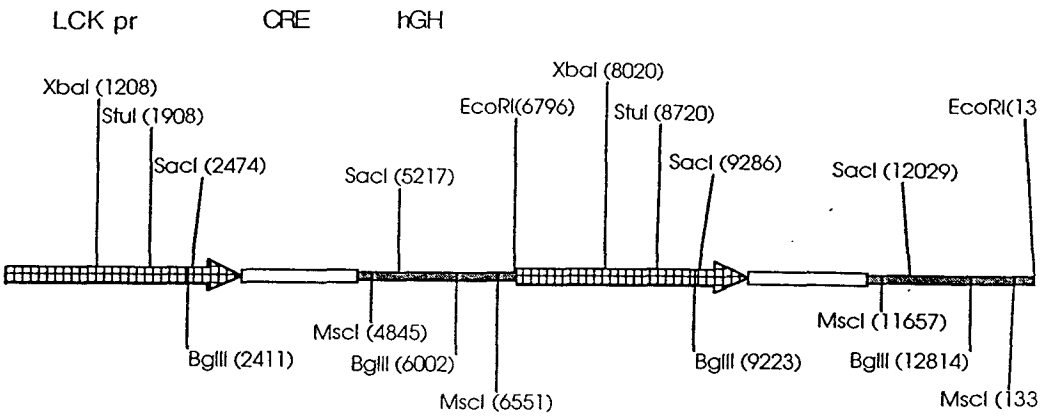


Fig. 2.15. Southern analysis of tail DNA from a CRE and a LOX-βGAL-LOX transgenic mouse Digests are as shown above each lane. Lanes 1,5, and 9 - 1kb ladder. CRE mouse - lanes 2,3,4; LOX-βGAL-LOX mouse - lanes 6,7,8. Probe is a 600 bp SmaI-EcoRI fragment of the polyA signal region of the hGH gene from p1017. Despite the extensive artifact, informative bands are present. The very faint bands visible in lanes 2 and 4 between 3 and 4 kb are likely to be due to cross-hybridisation of the probe to mouse growth hormone sequences (see fig. 2.12). EcoRI and XbaI single digests show prominent bands of about 7kb and 9kb in the CRE transgenic and LOX-βGAL-LOX transgenic DNAs respectively. The persistence of bands of these sizes in the double digests (lanes 4 and 8) despite more intense bands at lower molecular weights, suggests partial digestion. The appearance of the high molecular weight smear in lanes 3 and 7, as well as the signal present at the lane origin in lane 3 is consistent with the partial digestion being due to XbaI. The molecular size markers used in all Southern analyses (the "1kb ladder") consist , from top to bottom, of bands of these approximate sizes: 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.6, 1.0, 0.5 kilobases (kb), and several bands below 0.5 kb, not shown in most of these analyses. The lowest moelcular size marker here visible is the1.6 kb band.

A



B

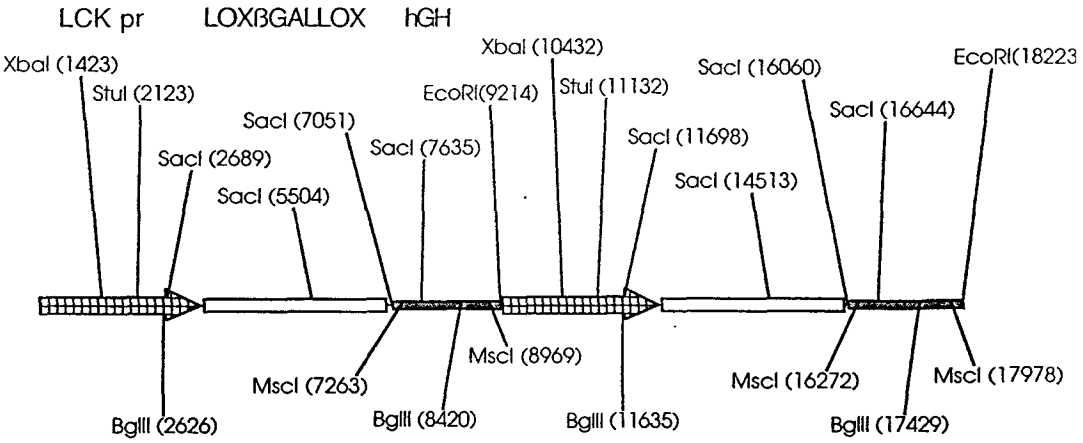


Fig. 2.16. Illustration of head-to-tail integration arrays of the two transgene constructs Sites used for Southern analyses are shown, as well as their distances in base-pairs from the 5' end of the array as illutrated. A) p1017CRE array. B) p1017LOXßGALLOX array.

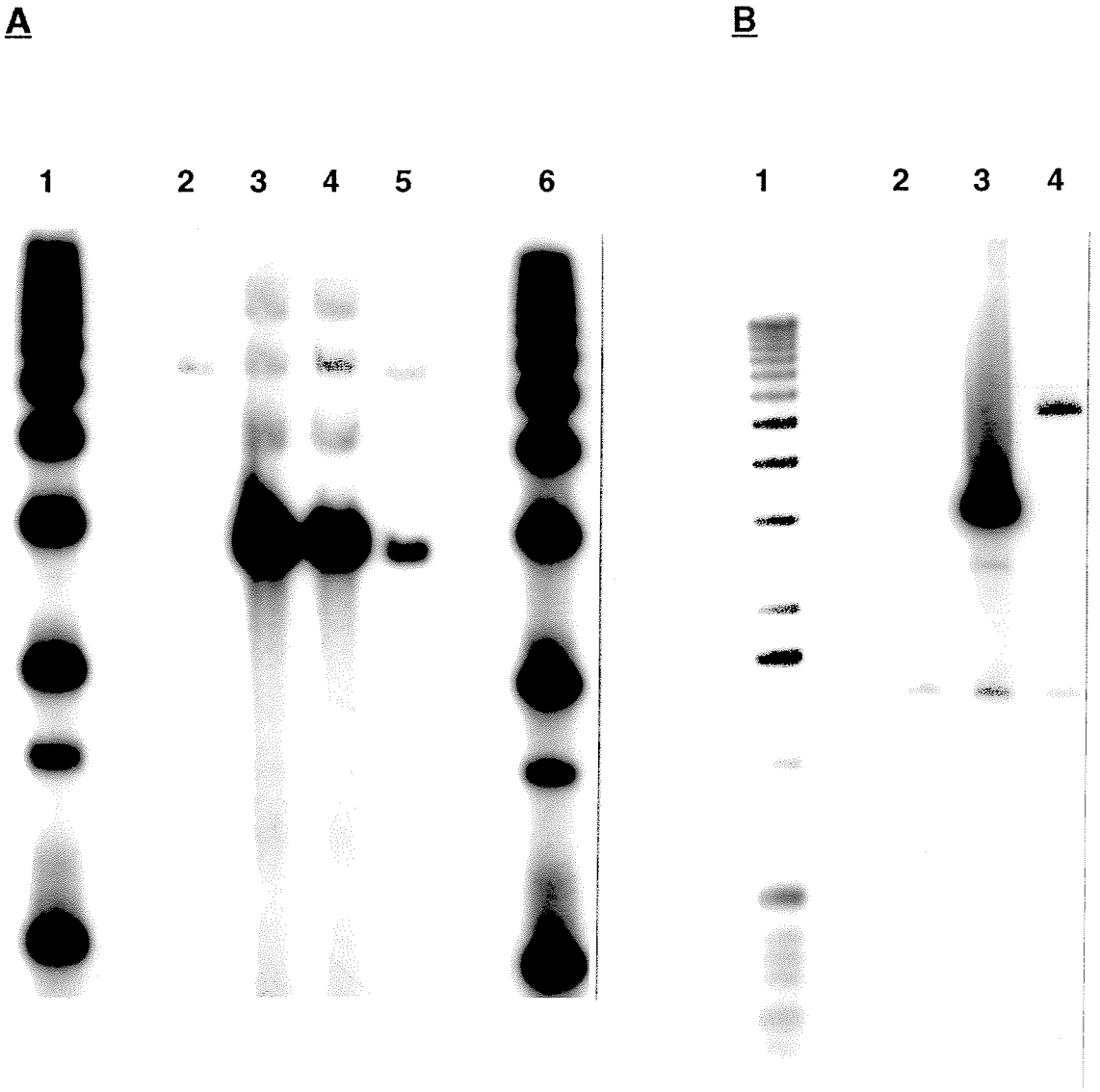


Fig. 2.17. Two of the Southern analyses used for approximate densitometric quantitation of transgene copy number Probe is LCK fragment. A) SacI digest of mouse tail DNAs. Lanes 1 and 6 - 1kb ladder, lane 2 - wild-type, lane 3 - doubly transgenic, lane 4 - CRE transgenic, lane 5 - LOX-ßGAL-LOX transgenic. The band resulting from hybridisation to the endogenous *lck* gene is at approximately 5kb. B) StuI/MscI double digests. Lane 1 - 1kb ladder, lane 2 - wild-type thymocyte DNA, lane 3 - CRE transgenic thymocyte DNA, lane 4 - LOX-ßGAL-LOX transgenic thymocyte DNA. The band resulting from hybridisation to the endogenous *lck* gene is at approximately 1.4 kb. LOX-ßGAL-LOX mice analysed here were from the 86 line. [Daniel Chui performed similar Southerns to determine copy number in the 87 LOX-ßGAL-LOX line.] Additional bands not identified here are discussed in Appendix 2.

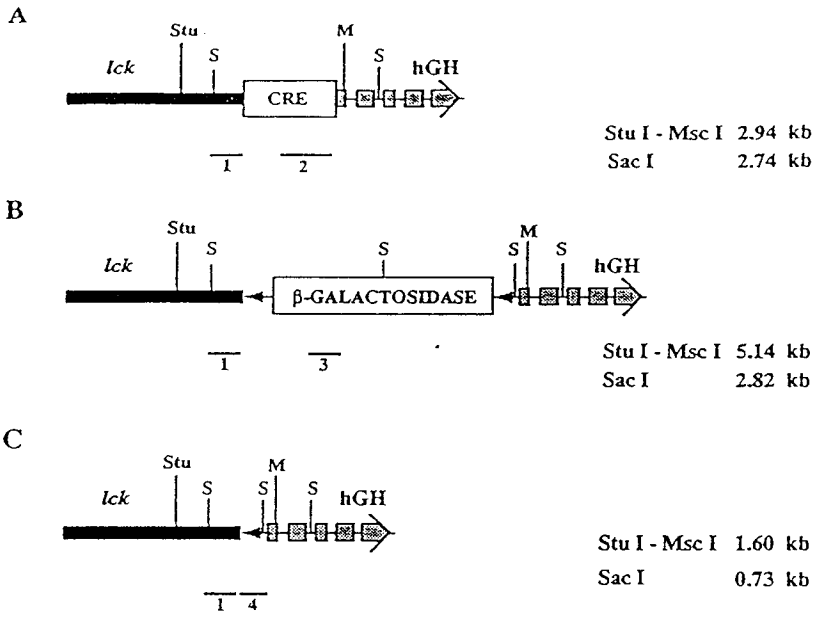


Fig. 2.18. Illustration showing expected band sizes from Southern analyses of transgene constructs and the expected recombination result [Figure prepared in collaboration with Dr. Jamey Marth.] Probes used in Southern analyses are represent by numbered lines. 1 - LCK fragment, 2 - CRE fragment, 3 - βGAL fragment, 4 - the LOX sequence. A) p1017CRE construct. B) p1017LOX-βGAL-LOX construct. C) Expected recombination result. S - SacI, M - MscI.

D) Demonstration of the function of CRE in the thymocytes of doubly transgenic mice

A Southern blot [performed by Daniel Chui] probed with the LOX fragment, showed that, in thymocytes of a doubly transgenic mouse, the band representing the intact 1017LOX-ßGAL-LOX array was greatly diminished in intensity when compared with the band from tail tissue of the same animal. Moreover, in these doubly transgenic mouse thymocytes a recombinant band of a size consistent with the action of CRE on the LOX-ßGAL-LOX target sequences was present (fig. 2.20, part A). Such bands, the result of CRE-mediated recombination, will hereafter be referred to as "recombinant" bands. A faint hybridisation signal was present, however, in Southern blots probed with the LOX fragment in all lanes containing DNA derived from mice bearing the CRE transgene. As the LOX fragment was obtained in such a manner as to include a small region of the polylinker of the pLOX^2 plasmid, and the copy number of the CRE transgene was so high, it was postulated that this faint band may have been the result of some copies of the CRE transgene integration array containing residual pUC backbone. This may have arisen due to incomplete digestion of the p1017CRE plasmid with the enzyme, NotI, used to separate transgene construct from pUC vector sequence (p1017 is a pUC derivative), and some remaining pUC sequences may have been sufficiently similar to the pLOX^2 polylinker to result in a hybridisation signal.

Use of the LCK fragment as probe would, depending on the restriction digest, allow one to see all transgene fragments involved in the experiment (i.e. both transgene arrays and results of recombination) and the endogenous *lck* gene. The ability to see the latter would allow comparison of evenness of loading and transfer between samples in preparation of Southern blots. This fragment was therefore used for most subsequent anlyses. A Southern analysis of the DNAs shown in figure 2.20, part A, digested with SacI and probed with the LCK fragment, again showed a recombinant band of a size (approximately 0.7 kb) consistent with the expected action of CRE in thymocyte DNA of doubly transgenic mice (fig 2.20, part B). Similarly, recombinant bands of approximately the expected size were found on Southern analysis of thymocyte, but not tail, DNA of another doubly transgenic mouse (fig 2.21). In this case, SacI and BglII digests were performed , the BglII digest providing the possibility of assessing the loss of hybridisation to the ßGAL-transgene fragment (at approximately 6 kb), in addition to the appearance of a recombinant band (at between 2 and 3 kb). To remove any remaining doubts that this recombinant band might be due to an artefact, an analysis was performed on DNAs digested with StuI and MscI together, and probed with the same LCK fragment, a combination which would allow visualisation of the CRE and LOX-ßGAL-LOX transgene fragments, a recombination result, and an endogenous *lck* gene fragment in the same lane (fig 2.22). This analysis again showed diminution in intensity of the signal due to the LOX-ßGAL-LOX transgene (at , and the

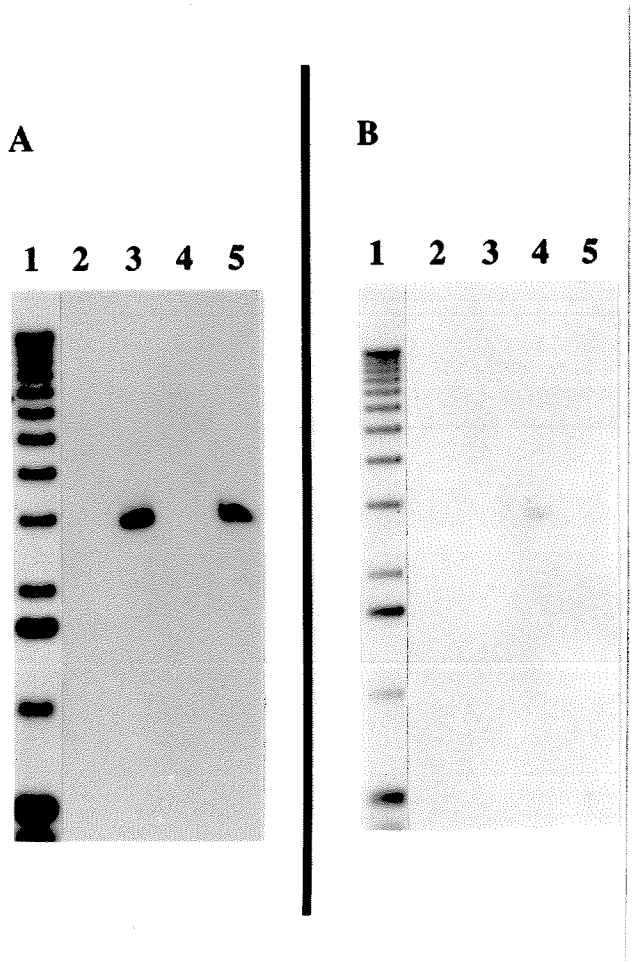


Fig. 2.19. Southern analysis of thymocyte DNA samples A) Digest is StuI/MscI, probe is CRE fragment. Lane 1 - 1 kb ladder, lane 2- wild-type, lane 3 - CRE transgenic, lane 4 - LOX-βGAL-LOX transgenic, lane 5 doubly transgenic. B) Digest is SacI, probe is βGAL fragment. Samples identical to those in A. No hybridisation is visible in the sample from the doubly transgenic animal.The smallest size markers visible here are the 0.5 kb bands.

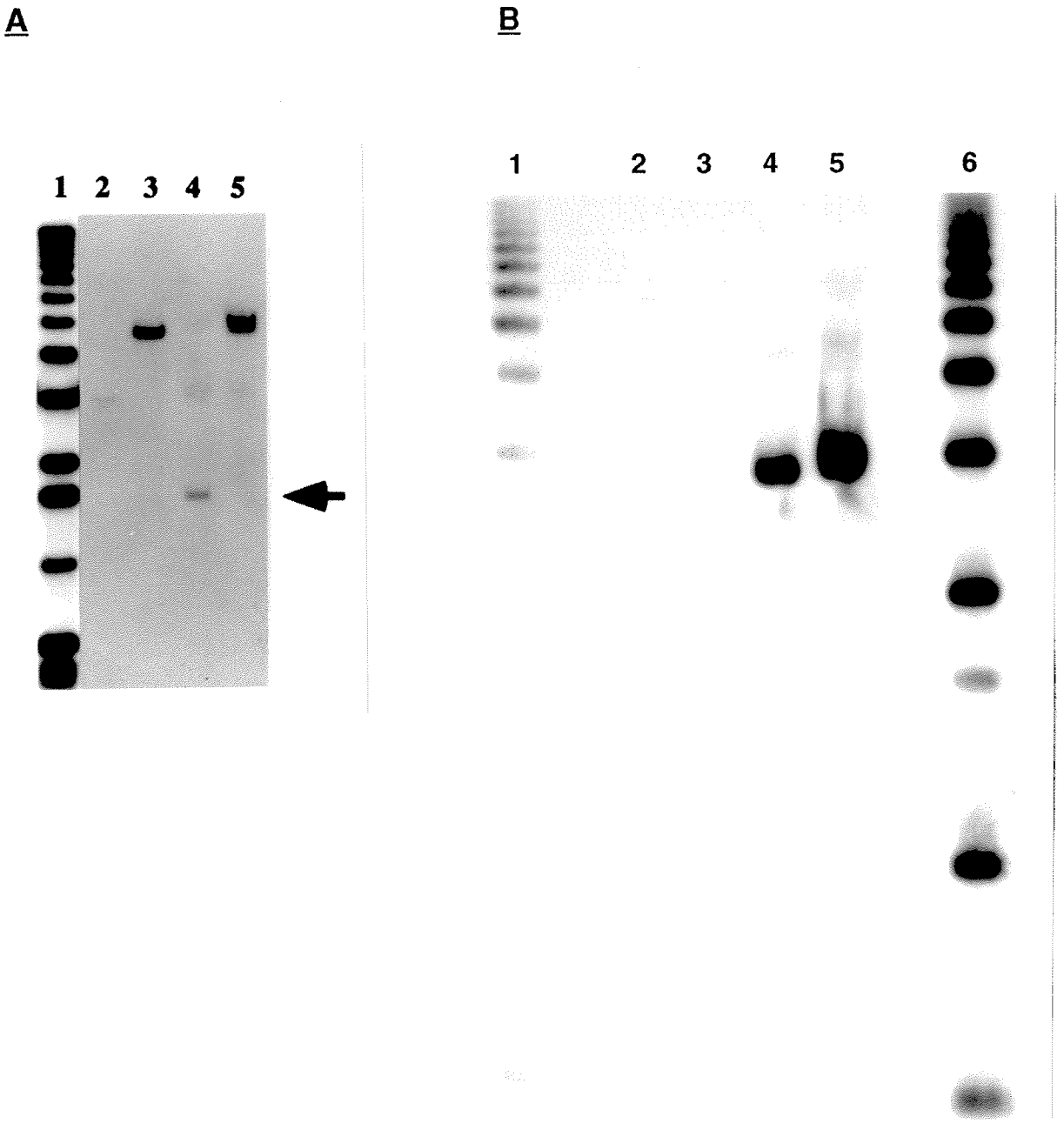


Fig. 2.20. Southern analyses showing the results of recombination in doubly transgenic CRE/LOX-βGAL-LOX thymocyte DNA A) Digest is StuI/MscI, probe is LOX fragment. Lane 1 - 1kb ladder, lane 2 - CRE transgenic, lane 3 - LOX-βGAL-LOX transgenic, lane 4 - double transgenic, lane 5 - tail DNA from the same mouse as lane 4. The band indicated by the arrow in the thymocyte DNA of the doubly transgenic mouse (but absent from the tail DNA of the same animal) is consistent with CRE-mediated recombination between LOX sites in the LOX-βGAL-LOX transgene array. B) Digest is SacI, probe is LCK fragment. Lanes 1 and 6 - 1kb ladder, lane 2 - wild-type mouse, lane 3 LOXβGALLOX transgenic, lane 4 - CRE transgenic, lane 5 - double transgenic. A band consistent with CRE-mediated recombination is again (faintly) visible in the doubly transgenic thymocyte lane at. approximately 0.7 kb. The endogenous *lck* band is apparent at about 5 kb. The smallest size markers visible are the 0.5 kb bands.

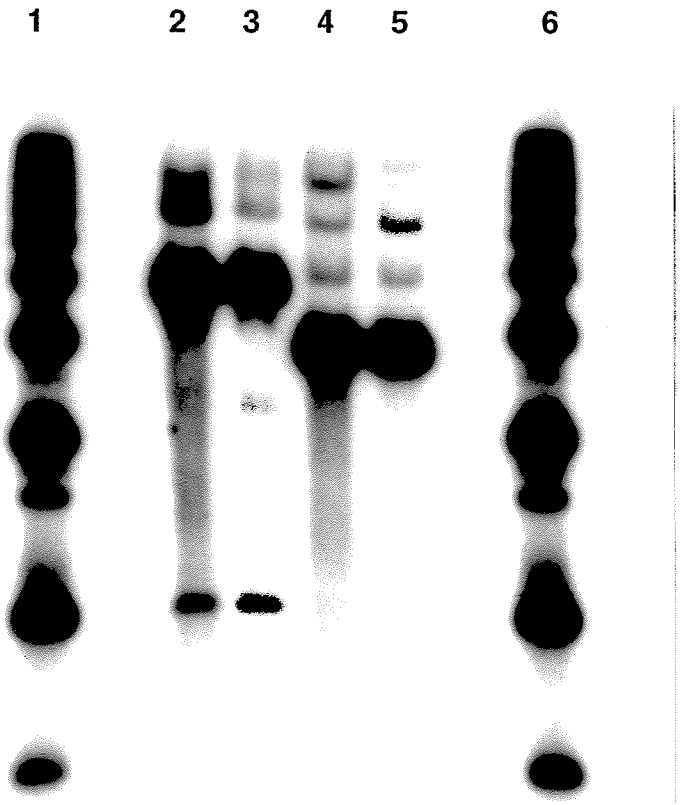


Fig. 2.21. Southern analysis of tail and thymocyte DNAs from another doubly transgenic mouse (86-1.2R). Lanes 2 (tail) and 3 (thymus) show BglII digests. Lanes 4 and 5 show SacI digests of the same DNA samples. Probe is LCK fragment. The band resulting from hybridisation to the endogenous *lck* gene is at approximately 1 kb in BglII digests. Both digests show recombinant bands of a size consistent with CRE-mediated recombination in thymocyte DNA, at between 2 and 3 kb in the BglII digest and at about 0.7 kb in the SacI digest. The smallest size marker visible here is the 0.5 kb band.

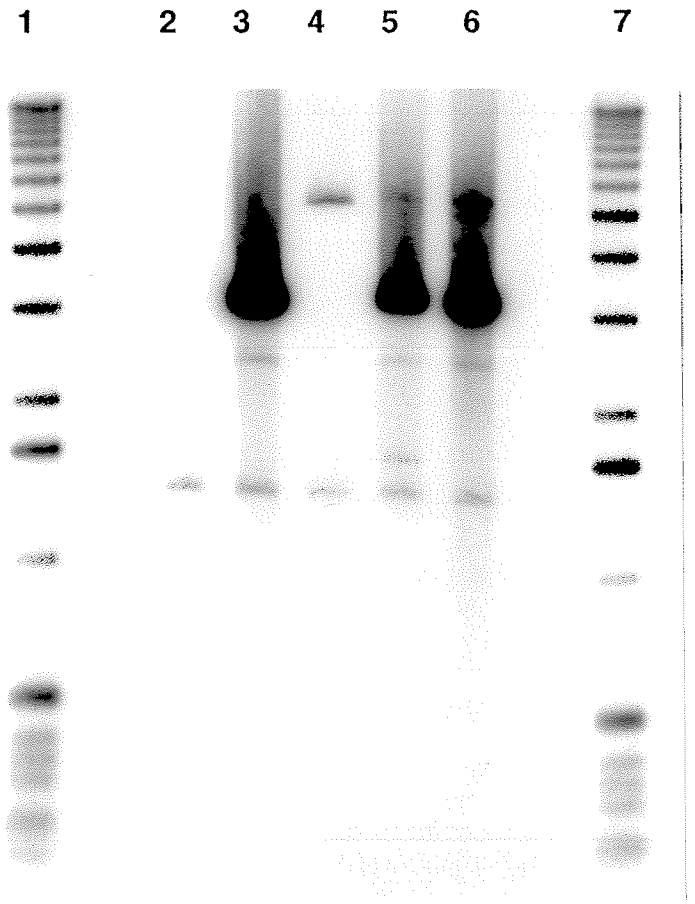


Fig. 2.22. Southern analysis of DNA showing the recombination result with the StuI/MscI digest Probe is LCK fragment. Lanes 1 and 7 - 1kb ladder, lane 2 - wild-type thymocyte, lane 3 - CRE transgenic thymocyte, lane 4 - LOX-βGAL-LOX transgenic thymocyte, lane 5 doubly transgenic thymocyte. Lane 6 shows the tail DNA of the same mouse as lane 5. The recombinant band at approximately 1.6 kb in lane 5 is consistent with CRE-mediated recombination in this thymocyte DNA. The entire 1kb ladder is visible here, the most intense band towards the bottom of the figure being the 0.5 kb band.

concomitant appearance of a recombinant band of the the size predicted to follow from excision of the βGAL sequences from between the LOX sites in the transgene array (at approximately 1.6 kb).

E) Investigation of the recombination by PCR

Oligonucleotide primers were designed [by Dr. Jamey Marth] to allow amplification of sequence between the most 3' end of the LCK promoter, and the second exon of the hGH sequence present in the p1017-derived transgene constructs. Using these primers, Daniel Chui was able to amplify a fragment of between 500 and 600 bp in length from thymocyte, but not tail, DNA of doubly transgenic mice. This sequence was not present in thymocyte DNA from wild-type or singly transgenic mice. The amplified sequence was shown to hybridise to the to the LOX fragment probe (fig. 2.23). It would be expected that such amplified sequence would be larger than sequence amplified from the empty p1017 plasmid using the same primers. This was shown to be the case, and it was estimated that the difference in size was consistent with the presence of a single LOX site (fig. 2.24, part A). Additionally, it was shown that digestion of this fragment with two restriction enzymes resulted in fragments of the approximate size expected following CRE-mediated recombination. (fig 2.24, parts B and C)

F) Demonstration of tissue-specificity.

To this point, evidence correlating CRE expression with the appearance of the recombination pattern in Southern analyses, had been provided largely on an animal-to-animal basis (i.e. recombination was only seen in animals bearing the CRE transgenes), although it will be noted that in no case was a pattern suggestive of recombination observed in tail-tissue DNA. To support the assertion that this recombination was a result of the action of the CRE enzyme, further correlation between CRE expression and the appearance of recombination of genomic DNA was sought. The question now put was whether the correlation held from tissue to tissue within individual doubly transgenic animals.

In order to determine in which tissues the CRE gene was expressed, Daniel Chui performed both Northern analysis and Western blotting analysis on tissues of several CRE-transgenic and doubly transgenic mice (fig. 2.25). This survey included extracts prepared from tail, kidney, liver, spleen and brain, as well as thymocytes. Whether assayed at the RNA or protein levels, CRE product could be detected in thymocyte extracts, but not in extracts of any of the other tissues surveyed. This was in keeping with the properties previously attributed to the LCK promoter used in the p1017 construct,

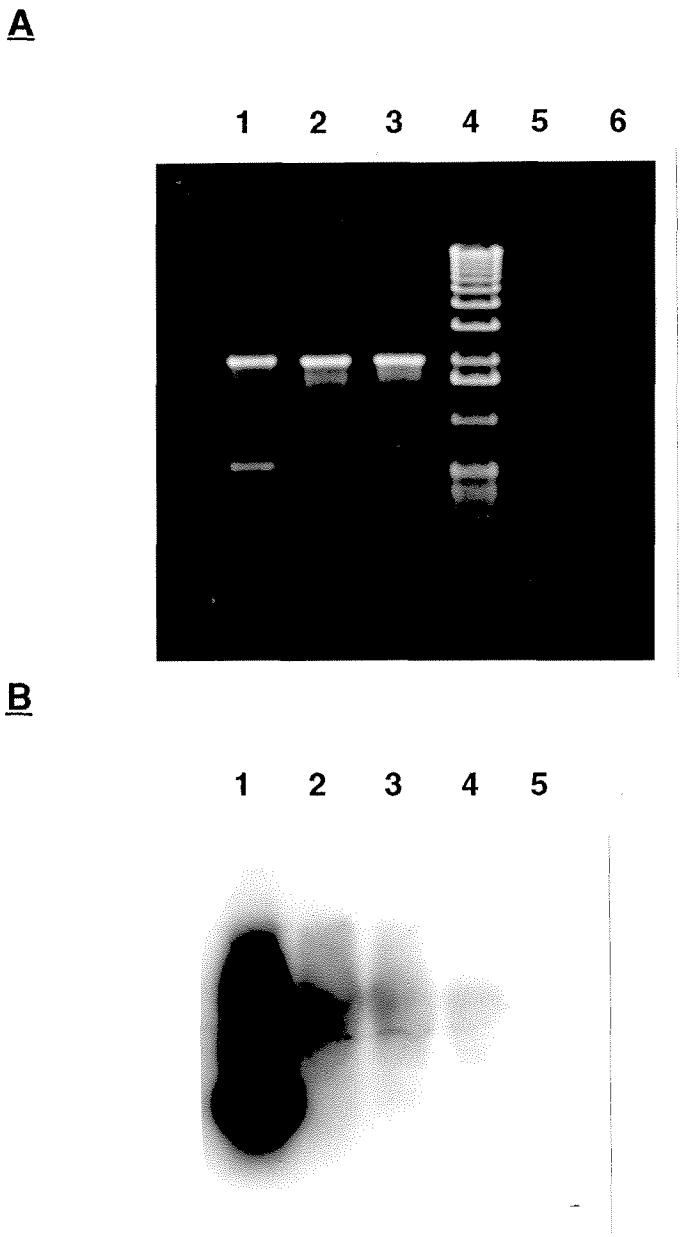


Fig. 2.23. Southern analysis of PCR amplified fragments from CRE transgenic and doubly transgenic mice [Courtesy of Daniel Chui] A) Ethidium-stained gel of PCR from genomic DNAs. Lane 1 - doubly transgenic thymocyte, lane 2 - doubly transgenic tail, lane 3 - CRE transgenic thymocyte, lane 4 - 1kb ladder, lane 5 - LOX-ßGAL-LOX transgenic thymocyte, lane 6 wild-type thymocyte. The entire 1kb ladder is visible. The result of the recombination event is evident as the band running at slightly above 0.5 kb in lane 1. The strong band at approximately 2 kb represents the sequence amplified from the many copies of the CRE transgene. The reason for the failure to amplify the expected 3.7 kb band, containing the LOX-ßGAL-LOX sequence, from tail of doubly transgenic and thymocyte of LOX-ßGAL-LOX transgenic mouse is unknown. B) LOX probe of the gel shown in part A. Some cross-hybridisation to the amplified CRE product is apparent in the singal in lanes 2,3, and 4, but the most prominent hybridisation is to the 0.5 kb fragment in lane 1.

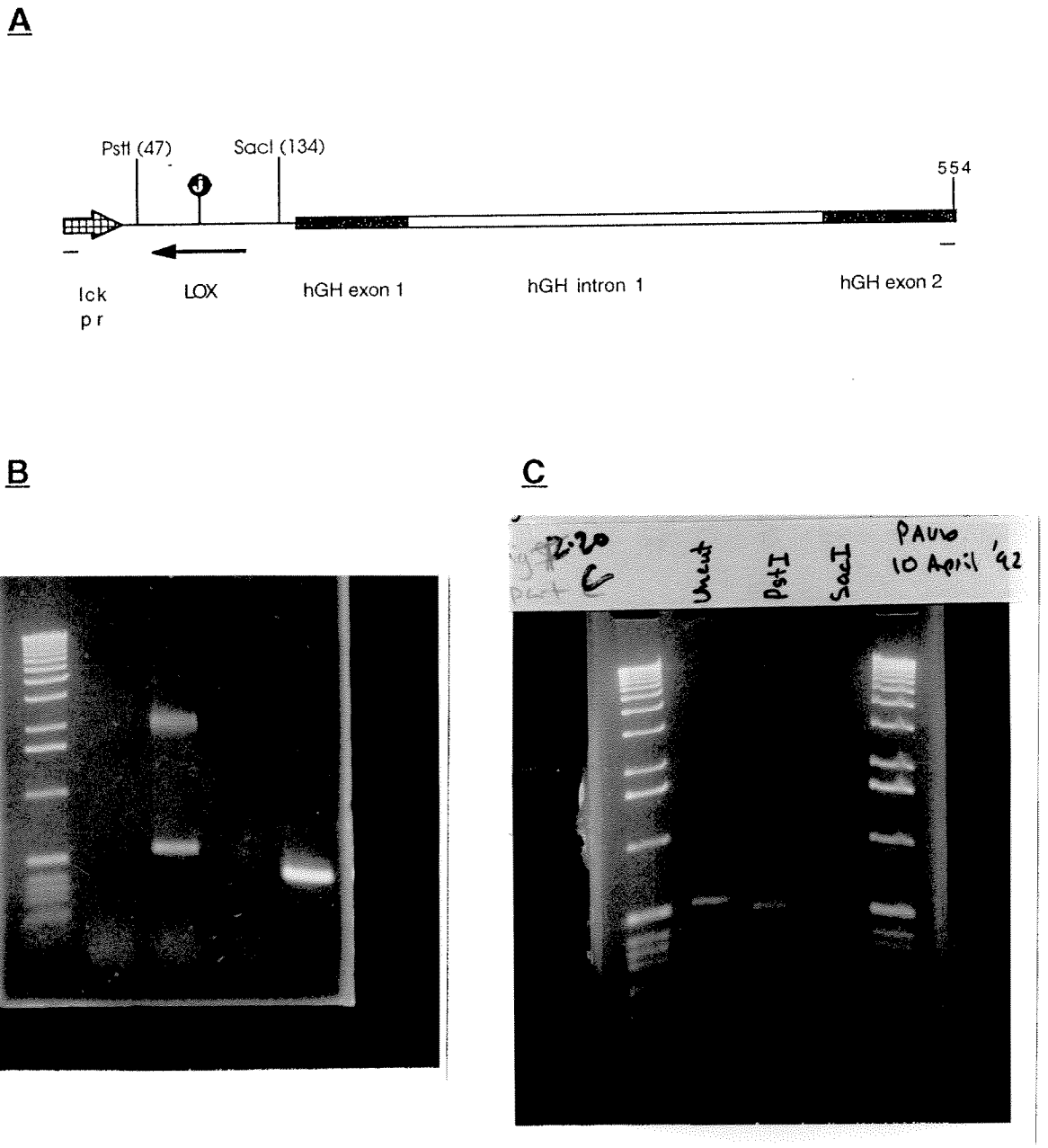
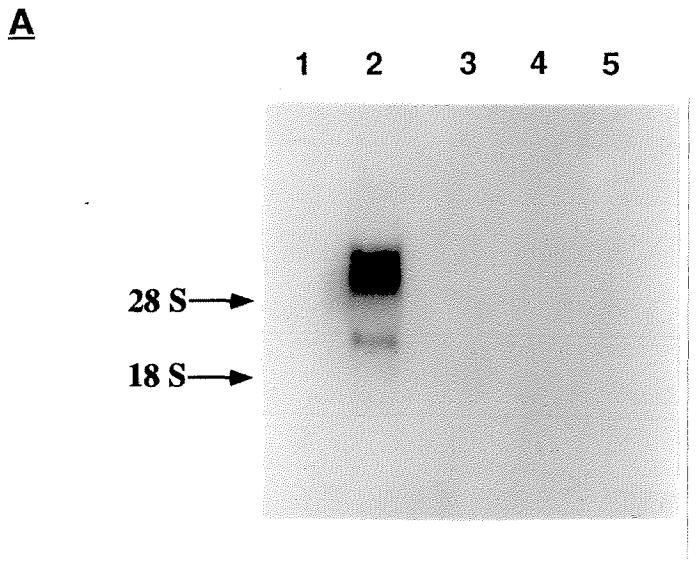


Fig. 2.24. Restriction enzyme analysis of PCR-amplified fragment from CRE/LOXßGALLOX doubly transgenic mouse thymocyte DNA A) Diagram of the fragment expected to be amplified from doubly transgenic thymocyte DNA after CRE-mediated recombination. The symbol "J" marks the expected point of recombination, and the recreated LOX site is shown, as are the sites used for restriction digests. The exon/intron structure of this region of the hGH sequence is also indicated. B) Ethidium stained gel showing results of PCR amplification from doubly transgenic thymocyte DNA (lane 2) and a plasmid control (lane 3). Lane 1 - 1kb ladder. The band at approximately 2 kb respresents amplification from the CRE transgene (see fig. 2.19, part A). The difference in size between the smaller band found in the thymocyte sample and that amplified from plasmid is consistent with the inclusion of the recombination remnant between the priming sites in the thymocyte sample, see A. C) Ethidium stained gel showing results of digestion of the PCR amplifed fragment from doubly transgenic thymocyte DNA. The sizes of the digest fragments are consistent with the results of CRE-mediated recombination.



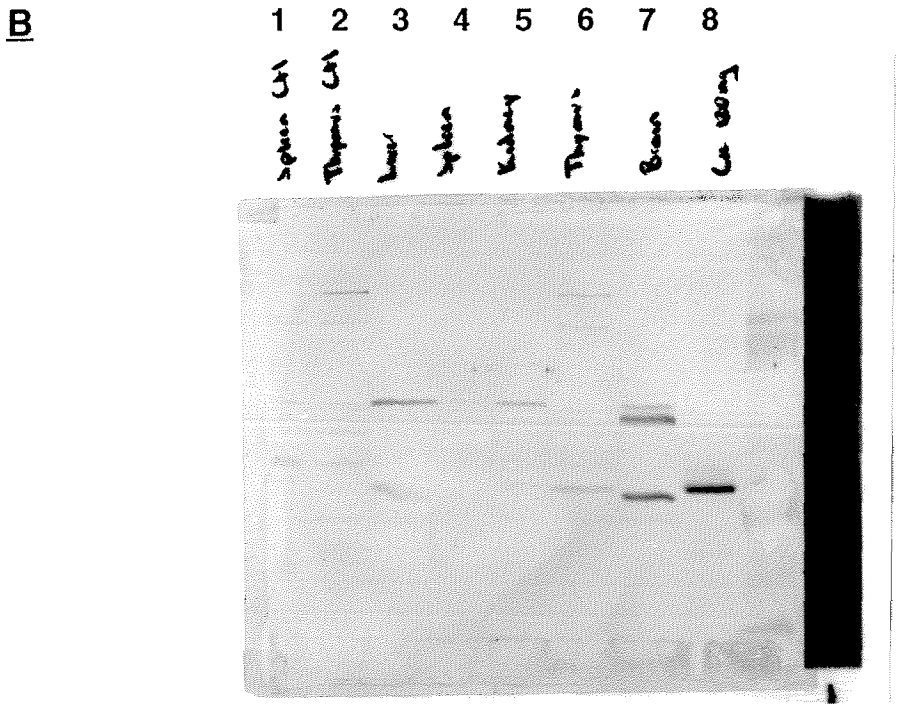


Fig. 2.25. Tissue distribution of CRE expression A) Northern analysis [courtesy of Daniel Chui] showing CRE-hybridising message in various tissues of a doubly transgenic mouse. Lane 1 - brain, lane 2 - thymocyte, lane 3 - kidney, lane 4 - spleen, lane 5 - liver. The positions of the 28S and 18S ribosomal RNA bands as determined from prior ethidium staining are shown. B) Western [courtesy of Daniel Chui] of extracts of the same tissues probed with rabbit polyclonal antibody to CRE. Lanes 1 and 2 contain samples from a wild-type mouse, lanes 3-7 from a CRE transgenic, lane 8 contains 120 ng of purified CRE protein. Although several apparently cross-reactive bands appear in various tissues, a band of the same size as the purified protein is ONLY apparent in thymocyte extract. Approximate molecular masses are indicated at the side of the figure.

and to p1017-derived transgenic constructs, in particular (Chaffin et al., 1991; Garvin et al., 1990; Abraham et al., 1991; Cooke et al., 1991). Both Daniel Chui and I performed surveys of genomic DNAs from these same tissue samples (fig.2.26). In no case was any suggestion of recombination found in DNA from tail, kidney, liver or brain. In longer exposures, however, a trace of the recombinant band suggestive of CRE-mediated recombination was apparent in analyses of spleen-derived DNA from these animals. Whereas this finding discounted the proposal that there was perfect correlation between sites of CRE expression and sites of the appearance of the results of recombination, it nevertheless supported the claim that the apparent recombination seen in Southern analyses was the result of the activity of the CRE enzyme - see below.

G) The recombination result is heritable through mitosis.

Although disappearance of the "intact" LOX-ßGAL-LOX transgene fragments was not noted in Southern analyses of DNA from splenic tissue of doubly transgenic animals, some of these analyses revealed faint "new bands" suggestive of CRE-mediated recombination. Since no CRE expression was detected in spleen, it was reasoned that the recombination may have taken place in those splenic cells that were derived from thymocytes i.e. the majority of splenic T cells. To address the question of whether the recombinant band component of splenic DNA was due to the presence of T-cells, the splenic T-cell population was enriched by FACS sorting of CD4+ and CD8+ cells.Southern analysis of DNA from the resulting population revealed an enrichment for the pattern characteristic of CRE-mediated recombination (loss of LOX-ßGAL-LOX and gain of the recombinant band) in comparison with DNA from the initial splenic population (fig. 2.27). Subsequently, cells from the CD4+/CD8+ splenic population were cultivated in the presence of ConA and IL-2 for two weeks, and extracts of the resultant population were subjected to both Northen and Southern analysis [performed by Daniel Chui]. These analyses revealed the persistence of the recombination pattern and the absence of detectable CRE expression. Whereas it was formally possible that CRE expression had nevertheless occurred both in the splenic T-cell population and in the cells during in vitro cultivation, it was considered more likely, given the previously determined pattern of expression from p1017-derived transgenes, that these findings represented evidence that the results of Cre-mediated recombination were passed to daughter cells through mitoses.

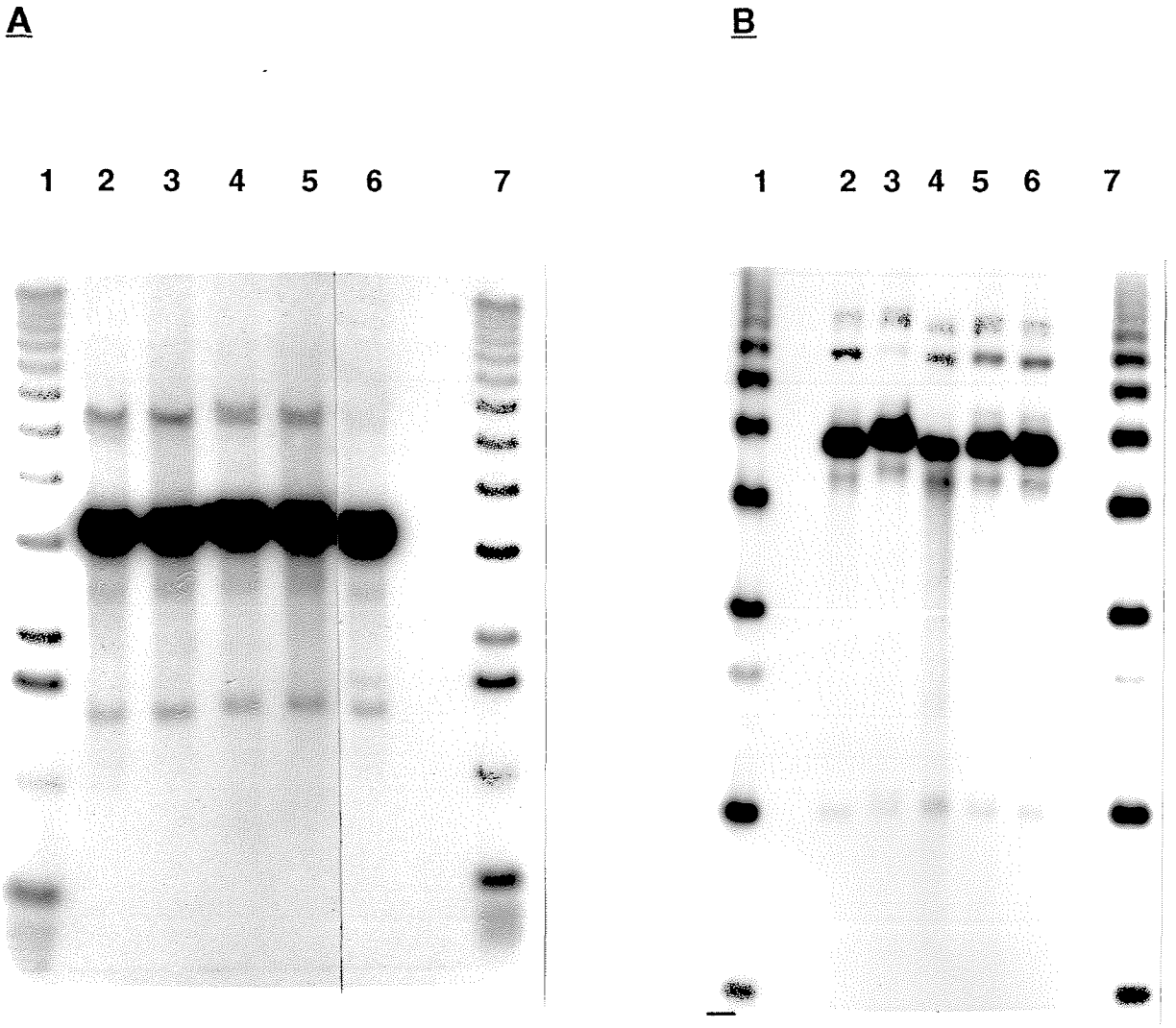


Fig. 2.26. Tissue distribution of recombination A) Southern analysis [courtesy of Daniel Chui] of DNAs from the same tissues sampled in fig. 2.25. Digest is StuI/MscI, probe is LCK fragment. Lanes 1 and 7 - 1kb ladder, lane 2 - tail, lane 3 - liver, lane 4 - brain, lane 5 - kidney, lane 6 - thymocyte. Hybridisation to endogenous *lck* results in bands at approximately 1.4 kb. The smallest marker size visible is the 0.5 kb band. The thymocyte DNA shows loss of the intact ßGAL fragment (of approximately 5.2 kb) and appearance of a recombinant band at approximately 1.6 kb. B) Southern analysis of tissues of another animal (86-2.1l). Digest is BglII, probe is LCK fragment. Lanes 1 and 7 - 1kb ladder; lane 2 - brain; lane 3 - thymus; lane 4 - tail; lane 5 - spleen; lane 6 - kidney. The smallest size marker visible is the 0.5 kb band. Hybridisation to endogenous *lck* results in bands at approximately 1 kb. (Although disappearance of the intact ßGAL band at approximately 6 kb is evident in thymocyte DNA from this animal, the expected recombinant band at between 2 and 3 kb is not seen. For a possible explanation of this, see Appendix 2.) From figures 2.25 and 2.26, evidence of recombination is seen to parallel evidence of CRE expression.

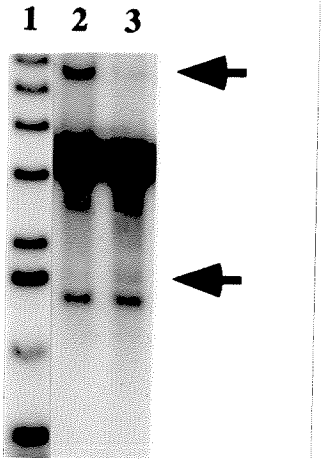


Fig. 2.27. Southern analysis of DNA from spleen and spleen-derived T-cells of a doubly transgenic mouse [Courtesy of Daniel Chui] DNA is from the same mouse as that analysed in Fig. 2.26 part A). Digest is StuI/MscI, probe is LCK fragment. Lane 1 - 1kb ladder, lane 2 - splenocyte DNA, lane 3 - spleen-derived T-cell DNA. T-cell DNA shows loss of the intact βGAL band (upper arrow) and presence of the expected recombinant band at approximately 1.6 kb consistent with the results of CRE-mediated recombination (lower arrow). Hybridisation to endogenous *lck* sequence is apparent in the bands below 1.6 kb. The lowest size marker visible is the 0.5 kb band.

H) Assessment of the efficiency of recombination.

As determined by Southern analyses in which any remnant LOX-ßGAL-LOX transgene fragment and the recombinant band could be distinguished, it would appear that the amount of recombination that had taken place as a percentage of the total "available" target sequence, varied from animal to animal (as shown, for example, in figure 2.28). Evaluation of the many possible explanations of this variability was deemed to be beyond the resources of the laboratory in which these experiments were performed. It was, however, possible to obtain an estimate of the efficiency of the recombination in terms of the number of copies of the ßGAL fragment removed from the target array in several animals. Southern analyses were selected in which both tail and thymocyte DNAs from the same LOX-ßGAL-LOX transgenic line were present, and in which both the LOX-ßGAL-LOX and endogenous *lck* fragments could be distinguished to allow "normalisation" of loading and of transfer efficiency. Densitometric comparisons of the intensity of the LOX-ßGAL-LOX fragment signals from tail and thymocyte preparations were performed. The calculations derived from the Southern blot shown in figure 2.28, for instance, revealed the loss of LOX-ßGAL-LOX fragment hybridsation signals of 87%, 99%, and 97% in the thymocyte preparations of three mice from the 86 line, in comparison to the tail DNA of one of these mice. If the estimate of 8 copies per cell of the LOX-ßGAL-LOX transgene in mice of this line were accepted, one would be led to conclude that in order to remove more than 87.5% of the hybridisable ßGAL DNA from a tissue, all 8 copies must be removed from at least some of the cells in that tissue. Accepting this estimate, then, it was proposed that in two of these three mice, at least some thymocytes had lost all copies of the ßGAL target sequence. Indeed, the efficiency of the recombination may have been greater than the estimates obtained from these Southern analyses, since the thymocyte DNA used could not be prepared from all and only those thymocytes in which CRE was expressed, but was "contaminated" with DNA from cells, such as stromal cells, and other non-T-cells which are members of the thymic population, and which do not express transgene sequences driven by the LCK promoter. Of 22 animals screened from 7 litters, in various analyses, all showed some evidence of Cre-mediated recombination.

I) Length of sequence involved in recombination

In those Southern analyses in which the loss of the LOX-ßGAL-LOX transgene signal was nearest to 100% (e.g. figure 2.28), the intensity of the recombination product signal approached roughly half that of the endogenous *lck* gene signal. (To some extent, the corollary also appeared to be true - in instances in which removal of the LOX-ßGAL-LOX fragment was less complete, the intensity of the recombinant band signal was greater than half that of the endogenous *lck* gene signal. See, for

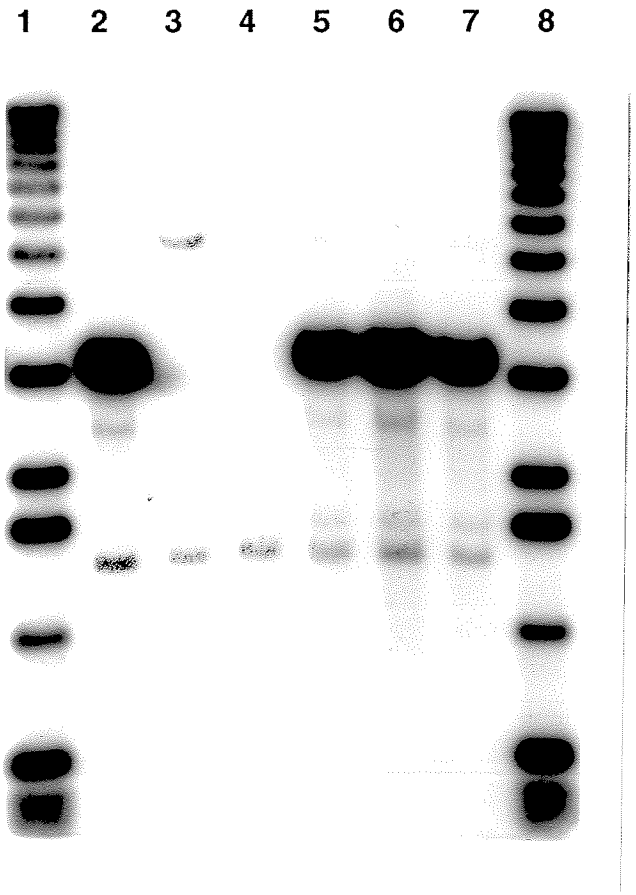


Fig. 2.28. Southern analysis of thymocyte DNAs showing recombination results in three more doubly transgenic mice [courtesy of Daniel Chui]. Digest is StuI/MscI, probe is LCK fragment. Lanes 1 and 8 - 1kb ladder, lane 2 - CRE transgenic, lane 3 - LOX-βGAL-LOX transgenic, lane 4 - wild-type, lanes 5,6,7 - three doubly transgenic mouse thymocyte DNAs. The intense bands twoards the bottom of the figure in the marker lanes are the 0.5 kb markers. Loss of the intact LOX-βGAL-LOX fargments (at approximately 5.2 kb) and appearance of the recombinant bands at approximately 1.6 kb is apparent in the thymocyte samples. Densitometric analyses "normalised" for loading and transfer on the basis of the intensity of the hybridisation to the endogenous *lck* gene were performed, and the percentage of available target DNA removed was calculated to assess the efficiency of recombination, see text.

example, figure 2.29 part B) This would be the result expected had a single copy of the LCK-promoter-containing recombination product remained in each cell after the action of the recombinase (whereas each cell would contain two copies of the endogenous *lck* gene). This, in turn, could only occur if all copies of the LCK promoter lying between LOX sites, within the 1017LOX-βGAL-LOX transgene array, had been removed from the CRE-expressing cells (see figure 2.16, part B). From no Southern analysis was it possible to infer that any fragment removed from chromosmal DNA had subsequently integrated elsewhere in the genome, and it was postulated that such excised DNA was degraded.

The length of the βGAL sequence between the LOX sites in the 1017LOX-βGAL-LOX construct was approximately 3.5 kb. These experiments had demonstrated that sequences of this length (almost 1000 bp longer than had been documented in prior reports) were susceptible to CRE-mediated recombination. In addition, the present data demonstrated that the approximately 5.1 kb of adjoining hGH and LCKpromotor sequences lying between LOX sites in the head-to-tail array were also susceptible to CRE-mediated recombination. Indeed, it is possible that the recombination event reusulting in the apparent deletion of the entire array of some 8 copies of the transgene construct may have taken place between the two outermost LOX sites in the array - over a distance of approximately 70 kb - or in steps involving any pair of LOX sites within the array.

J) Recombination between target sites in another chromosomal context

Following the experiments conducted to this point, the possibility remained that the function of CRE was limited to some chromosmal target sites. It would clearly not have been possible to demonstrate that CRE could act on LOX sites at any chromosomal location. The hypothesis that the apparent recombination was due merely to an accident of the particular chromosomal context of the LOX-βGAL-LOX transgene integration in the 86 transgenic line, was, however, clearly refutable. To this end, mice of the CRE transgenic line were mated to mice of the 87 LOX-βGAL-LOX transgenic line. In this line, the transgene array was present in a different chromosomal context from that of the 86 line, as determined by a) the size of "flanking" fragments seen on Southern analyses (figs. 2.29 and 2.30, see also Appendix 2), and b) the independent segregation of the 86 and 87 "alleles" amongst progeny of an 86/87 doubly transgenic parent. The results of the CRE-87 line mating were doubly transgenic progeny in which thymocyte-specific recombination in the LOX-βGAL-LOX array was apparent (figs. 2.29 and 2.30), showing that CRE could act on targets in at least one other chromosomal context.

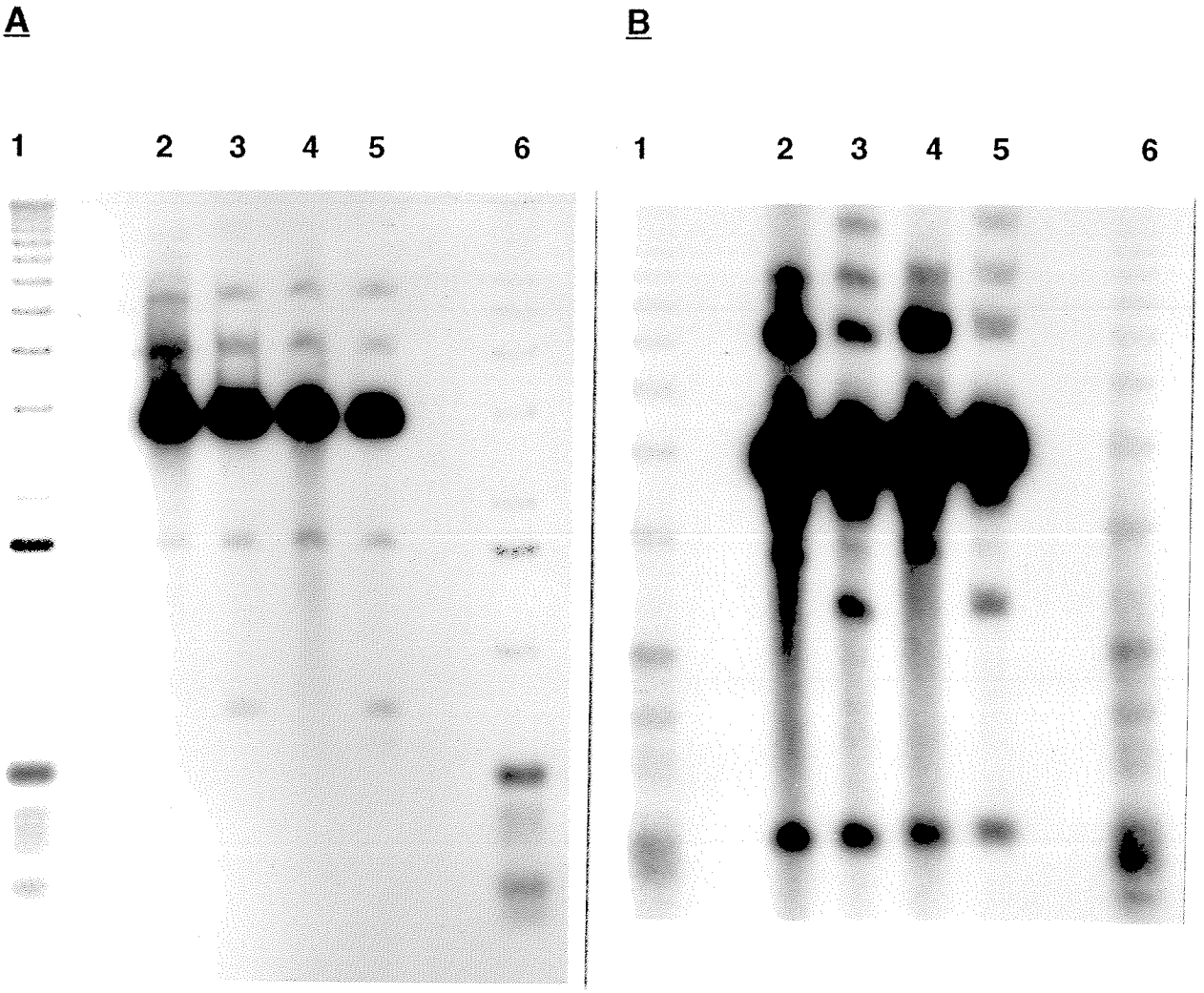


Fig. 2.29. Southern analyses showing CRE-mediated recombination of target sequence in another chromosomal context A) Tail (lanes 2 and 4) and thymocyte DNAs (lanes 3 and 5) from two doubly transgenic mice of the 87 line (lanes 2 and 3 - 87-1.1, lanes 4 and 5 - 87-1.1Q). Lanes 1 and 6 - 1kb ladder. Digest is SacI, probe is LCK fragment. The entire 1kb ladder is visible, with the 0.5 kb band being the most intense marker apparent. In addition to the CRE band at approximately 3 kb, and the endogenous *lck* band at approximately 5 kb, both animals show the expected recombinant band in thymocyte samples at approximately 0.7 kb. The band in all samples at approximately 1.6 kb is due to an anomaly of the LOX-βGAL-LOX integration in the 87 line (see Appendix 2). B) The same DNA samples as shown in A, digested with BglII and probed with LCK. The endogenous *lck* hybridisation is seen in all samples at approximately 1 kb, towards the bottom of the figure. The main CRE bands are seen at approximately 4 kb, and "flanking bands" due to CRE at slightly below the main band, between 3 and 4 kb, and also at approximately 8kb. The expected intact LOX-βGAL-LOX band is seen at approximately 6 kb, and an additional band due to the anomalous LOX-βGAL-LOX integration in the 87 line is present in the tail samples. A recombinant band of the expected size is present in both thymocyte samples at approximately 1.7 kb. An additional recombinant band is seen in thymocyte samples at approximately 10-11 kb, again due to the anomalous LOX-βGAL-LOX integration in the 87 line.

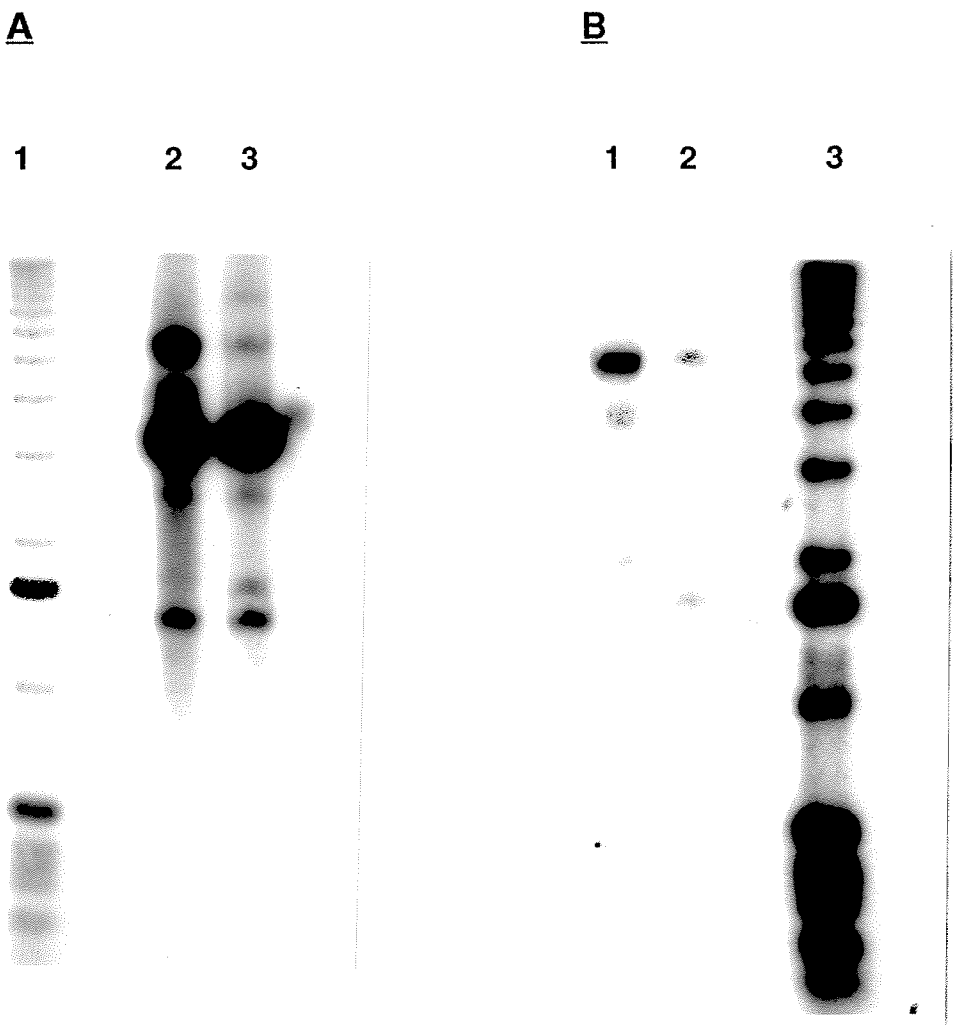


Fig 2.30. Southern analyses of the function of CRE and nature of the LOX-βGAL-LOX integration in the 87 line Tail and thymocyte DNAs of one of the mice shown in figure 2.29 (mouse 87-1.1Q). A) Digest isStul/MscI, probe is LCK fragment. Lane 1 - 1kb ladder, lane 2 - tail, lane 3 - thymocyte. The entire 1kb ladder is visible, with the 0.5 kb band being the more intense band toward the bottom of the figure. B) Digest is Stul/MscI, probe is LOX fragment. Lane 1 - tail, lane 2 - thymocyte, lane 3 - 1kb ladder.

DISCUSSION

1) CONCLUSIONS DRAWN FROM THE DATA

The results of these experiments permit the conclusion that the CRE-LOX recombination system can be used to generate tissue-specific and developmentally stage-specific deletion of DNA from the genome of transgenic animals. The ability to direct this activity in time and space depends upon elements of the transgene construct (primarily the promoter) used to drive the expression of the CRE enzyme. As more and more genes are analysed, more and more tissue-specific and developmentally stage-specific transgene constructs will become available, thereby increasing the range of questions that can be addressed with the CRE-LOX system.

In spite of the fact that CRE is an enzyme that evolved in a prokaryotic context (derived, as it is, from bacteriophage), it functions efficiently in the eukaryotic context of transgenic mice. Notably, CRE lacks an obvious nuclear localisation signal, and the mechanism by which it enters the nucleus to gain access to its target sites is unknown. The experiments described here suggest that CRE can reduce an array of some 8 LOX-flanked sequences to a single LOX-site-containing sequence in the transgenic mouse genome, although it is not possible to determine from these experiments whether this takes place through direct recombination between the outermost pair of LOX sites in the array, or through a sequence of step-wise recombination events involving LOX sites within the array. DNA excised by this system does not appear to reintegrate into the genome. The results of the recombination event are passed through mitoses to daughters of the cells in which the recombination event occurred.

Expression of CRE *per se* (in thymocytes, at least) does not appear to have any affect on the phenotype of transgenic mice. Furthermore, LOX sites do not undergo recombination in the absence of CRE activity, as evidenced by a) the absence of the recombination pattern in Southern analyses of tissues other than thymocytes in doubly transgenic mice, b) the absence of the recombination pattern in any Southern analysis of any tissue examined from singly transgenic mice, and c) the failure to find bands indicative of recombination in DNA from such control tissues following PCR amplification. This lack of spontaneous recombination is consistent with the previous demonstration that the presence of such short directly repeated sequences does not lead to spontaneous recombination in cells (Bollag et al., 1989).

It is possible to conclude that fragments of DNA of at least 5.1 kb in length are susceptible to CRE-mediated deletion, whereas previous published reports were limited to fragments of less than 3 kb.

This finding considerably increases the range of CRE-LOX experiments that can be sensibly designed. Although one cannot conclude from the data here presented that CRE can function at LOX sites located ANYWHERE in the genome of transgenic mice, the data at least suggest that this may be the case, since two different apparently random chromosomal locations of LOX-flanked targets were susceptible to the function of CRE.

2) QUESTIONS RAISED BY THE DATA, AND EXPERIMENTS ADDRESSING THESE QUESTIONS

Several important questions have been raised in the foregoing discussion of the data. Additional questions arise from consideration of the data in the context of the literature relating to site-specific recombinases.

1) How does CRE function in terms of entering the nucleus in the absence of a known nuclear-localisation signal? The size of the nuclear pore is sufficient to allow the passage of a spherical protein of 50 to 60 kd (Paine et al., 1975; Peters, 1983), so the CRE protein of 38 kd may simply diffuse into the nucleus. Alternatively, the protein may access chromosomal targets during the nuclear membrane breakdown that occurs during mitosis. At least this latter possibility could be assessed by performing an experiment in which, say, cells of a mouse fibroblast line would be transfected with a construct containing the CRE sequence under the control of an inducible promoter, and a selectable marker such as a neomycin resistance cassette. The same fibroblasts would be transfected with a second construct in which a reporter sequence e.g. ßGAL , would be flanked by LOX sites and driven by a "constitutive" promoter, such as the CMV-IE promoter. After selection of stable transfectants, the cells would be selected for continued expression of the reporter gene , and such cells would be analysed by Southern blotting to establish the integrity of the LOX-flanked construct. Such cells would then be brought to growth-arrest (by growth to confluence or serum-starvation in the case of mouse fibroblasts) and the expression of CRE induced. A survey of the cells with respect to subsequent expression of the reporter, supported by Southern analysis or PCR to demonstrate the predicted CRE-mediated recombination result would then indicate whether CRE could function in cells not undergoing mitosis. The efficiency of such function could be compared to the efficiency in the same transfectants that were not growth-arrested. In this latter case clonal analysis would have to be undertaken to prevent distortion of the results due to cell multiplication, and the possibility of some accidental growth advantage or disadvantage to cells in which recombination had occured. An even more powerful design would involve the "target" construct carrying its LOX sites not around the reporter gene itself, but around an inhibtor of reporter expression placed between the promoter and

the reporter sequence (see 6, below). Removal of this inhibitor would allow expression of the reporter in cells in which recombination had taken place, providing a positive marker of the experimental outcome, rather than a negative one. Such experiments would be subject to the considerations raised in question 2, below.

2) Can CRE indeed function on LOX sites located anywhere in the genome? This question could not be directly addressed experimentally. The hypothesis that CRE can indeed function at sites located anywhere in the genome could be refuted by the finding of a failure to function of CRE on LOX sites that were located in the genome at an interval the size of which had been previously shown to be susceptible to CRE function.

3) What is the largest space between LOX sites across which CRE can function to delete the intervening DNA in the genomic context? This question would be difficult to address experimentally. One might design a series of constructs of progressively increasing inter-LOX size, and assess the ability of CRE to delete the intevening DNA *in vitro*. The assessment of function in the context of the genome, however, would depend on the location of all the potential target constructs in the identical chromosomal context so as to avoid confusion of this question with question 1, above. Ironically, the literature has suggested that one way in which to insert a sequence at a pre-defined chromosomal location is to first of all insert (by transfection of cells) a single LOX site, and then make use of the action of CRE to integrate a LOX-flanked sequence at the previously integrated single LOX site (Sauer and Henderson, 1990).

4) Does the presence of a LOX site between promoter and coding sequence reduce expression of that sequence, albeit permitting SOME level of expression? This question might be addressed by simply designing an expression construct for some reporter sequence whose product is quantifiable e.g. chloramphenicol acetyl transferase (Gorman et al., 1982), or ßGAL (Hall et al., 1983), with or without a LOX sequence between promoter and coding sequence. The amount of product produced by cells transfected with these constructs could then be assessed. Once again, chromosmal location might influence the amount of product independently of the presence of the LOX site, and in this instance it would clearly be impossible to use the strategy of CRE-mediated insertion into a pre-existing LOX site to create a uniform context for transfected construct. Two approaches might be used to avoid this potential hazard a) the use of another recombinase system with properties similar to those of CRE (e.g. the FLP-FRT system, see O'Gorman et al., 1991) to create the predetermined site of integration in the genome, or b) a less elegant "brute force" approach, creating many different transfected lines with each construct and using a statistical argument to support a conclusion from the results so obtained.

5) Could the CRE-LOX system be used to induce inter-chromsomal recombination in transgenic animals, as has been demonstrated in the FLP-FRT system between homologous chromsomes of Drosophila (Gorman et al., 1982)? Could this then be used to create models of pathology associated with chromosomal translocation, in which the events immediately following the translocation could be studied since the developmental timing of the translocation would be predetermined by virtue of the properties of the promoter driving CRE expression? The feasibility of inducing recombination between LOX sites on different chromosomes can be assessed with the transgenics already available at the Biomedical Research Centre, and experiments to address this question are ongoing at the time of writing.

6) Could the CRE-LOX system be used as a technique to advance study of cell-lineage in vertebrates? The possibility of using the FLP-*frt* system for this purpose is discussed in some detail in O'Gorman et al., 1991. The experimental outline might be briefly described as follows: since the results of recombination are passed through mitosis to daughter cells, the recombination could be used as a means of removing a block to the expression of a reporter gene, and the reporter gene would then be expressed in all daughter cells of the cell in which the recombinase had originally acted. More concretely,with respect to the CRE-LOX system, a reporter construct would be designed in which a DNA fragment, say a small cDNA with polyA signal, or even a small gene (Sauer and Henderson, 1989), would be interposed between promoter and the reporter coding sequence, the interposed fragment being flanked by LOX sites. The promoter in this construct would be such as to drive expression in any cell type (candidate promotors currently avaliable include the CMV-IE gene promoter, and the Elongation Factor II gene promoter). A transgenic mouse would be made with this construct. A second transgenic would be made in which CRE expression was driven by a promoter that would only allow expression in a defined cell type at a defined stage of development. Breeding the two transgenics would result in doubly transgenic progeny in which the block to transcription of the reporter sequence would be removed in only those cells in which CRE was expressed, so the reporter would be expressed in these cells AND in all the daughter cells of these cells. One would then be able to derive a cell-lineage map for these cells.

Although the problem of a LOX site between promoter and coding sequence remains in this design, O'Gorman and colleagues (O'Gorman et al., 1991) have shown that an FRT recombination site can be placed slightly 3' of the initiating ATG of the ßGAL coding sequence, and providing this does not interrupt the reading frame of translation, ßGAL activity will still be expressed from single-copy integrations of such constructs in mouse cell lines. (driven by the CMV-IE promoter). (Although these authors quantitated the ßGAL activity in the transfectants, they did not undertake a comparison of ßGAL activity in transfectants made with constructs containing or lacking the FRT site.) The FRT

recombination site has features very similar to those of the LOX sequence, and is of the same size, so it seems likely that such a solution might be adapted to the CRE-LOX system. A useful variation would be to chose a cDNA as the "blocking" fragment which was itself a "reporter" sequence, so that the transgenic line derived with the reporter construct could be checked for the ablity of its promoter to drive expression in ALL tissues, prior to breeding with the CRE line. Expression of CRE in the doubly transgenic mice would then, in effect, substitute one reporter for another (differently assayed) reporter.

The primary conceptual inspirations for development of a CRE-LOX system in transgenic animals were discussed in the introduction (section 1). Briefly stated, the system would, it was hoped, allow one to address the following questions: a) is a particular gene's expression required for maintenance of a pathological state, and b) can an embryonic-lethal gene-targetting experiment be salvaged, allowing interesting results to be drawn from a tissue-specific gene ablation? Experiments aimed at addressing both questions are currently in progress within the Biomedical Research Centre.

BIBLIOGRAPHY

Abraham, J.M., Freitag, C.S., Clements, J.R., & Eisenstein, B.I. (1985) An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli. Proc. Natl. Acad. Sci. USA* 82, pp.5724-5727.

Abraham, K.M., Levin, S.D., Marth, J.D., Forbush, K.A., & Perlmutter, R.M. (1991) Thymic tumourigenesis induced by overexpression of $p56^{lck}$. *Proc. Natl. Acad. Sci. USA* 88, pp. 3977-3981.

Abremski, K., Hoess, R., & Sternberg, N. (1983) Studies on properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* 32, pp. 1301-1311.

Abremski, K.,& Hoess, R.(1984) Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J. Biol. Chem.* 259 pp. 1509-1514.

Andrews, B.J., Proteau, G.A., Beatty, L.G., & Sadowski, P.D. (1985) The FLP recombinase of the 2 μ circle DNA of yeast: interaction with its target sequences. *Cell* 40, pp. 795-803.

Argos, P., Landy, A., Abremski, K., Egan, J.B., & Haggard-Ljungquist, E. (1986) The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO J.* 5, pp. 433-440.

Austin, S. Ziese, M. & Sternberg, N. (1981) A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell* 25, pp. 729-736.

Barnes, G., & Rine, J. (1985) Regulated expression fo endonuclease *EcoRI* in *Saccharomyces cerevisiae*: nuclear entry and biological consequences. *Proc. Natl. Acad. Sci. USA* 82, pp. 1354-1358.

Bollag, R.J., Waldmann, A.S., & Liskay, R.M. (1989) Homologous recombination in mammalian cells.*Annu. Rev. Genet.* 23, pp. 199-225.

Braun, R.E., Peschon, J.J., Behringer, R.R., Brinster, R.L., & Palmiter, R.D. (1989) Protamine 3'-untranslated sequences regulate translational control and subcellular localisation of growth hormone in spermatids of transgenic mice. *Genes Dev.* 3, pp. 793-802.

Brent, R., & Ptashne, M. (1984) A bacterial repressor protein on a yeast transcriptional terminator can block upstream activation of a yeast gene. *Nature* 312, pp. 612-615.

Brinster, R.L. & Palmiter, R.D. (1986) Introduction of genes into the germ line of animals. In *The Harvey Lectures* 80 (Liss, New York), pp. 1-38.

Campbell, A.M. (1962) Episomes. *Adv. Genet.* 11, pp.101-45.

Capecchi, M.R. (1989) Altering the genome by homologous recombination. *Science* 244, pp. 1288-1292.

Chaffin, K.E., Beals, C.R., Wilkie, T.M., Forbush, K.A., Farr, A.G., Davison, B.L., & Perlmutter, R.M. (1991) Dissection of thymocyte signalling pathways by *in vivo* expression of pertussis toxin ADP-ribosyltransferase. *EMBO J.* 9, pp. 3821-3829.

Chesney, R.H., & Scott, J.R. (1978) Suppression of a thermosensitive *dnaA* mutation of *Escerichia coli* bybacteriophage P1 and P7. *Plasmid* 1, pp. 145-163.

Chesney, R.H., Scott, J.R., & Vapnek, D. (1979) Integration of the plasmid prophages P1 and P7 into the chromosome of *Escerichia coli* . *J. Mol. Biol.* 130, pp.161-173.

Cooke, M.P., Abraham, K.M., Forbush, K.A., & Perlmutter, R.M. (1991) Regulation of T cell receptor signalling by a *src* family protein tyrosine kinase ($p59^{fyn}$). *Cell* 65, pp. 281-291.

Covarrubias, L., Nishida, Y., & Mintz, B. (1986) Early postimplantation embryo lethality due to DNA rearrangements in a transgenic mouse strain. *Proc. Natl. Acad. Sci. USA* 83, pp. 6020-6024.

Cox, M.M. (1988) FLP site-specific recombination system of *Saccharomyces cerevisiae*. In *Genetic Recombination* Kucherlapati, R. & Smith,G.R. eds. Am. Soc. Microbiol., Washington D.C. pp. 429-443.

Cox, M.M. (1989) DNA inversion of the 2 μm plasmid of *Saccharomyces cerevisiae*. In *Mobile DNA* , Berg, D.E. & Howe, M.M. eds., Am. Soc. Microbiol., Washington D.C. pp. 661-670.

Craig, N.L. (1988) The mechanism of conservative site-specific recombination. *Annu. Rev. Genet.* 22, pp.77-105.

Craig, N.L. & Kleckner, N. (1987) Transposition and site-specific recombination. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, eds. F.C. Neidhardt et al., Am. Soc. Microbiol., Washington D.C. Vol. 2 pp.1054-1070.

Craig, N.L., & Nash, H.A. (1983) The mechanism of phage I site-specific recombination: site-specific breakage of DNA by *int* topoisomerase. *Cell* 35, pp. 795-803.

Dale, E. & Ow, D.W. (1991) Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* 88, pp. 10558-1052.

Furukawa, T., Kawaichi, M., Matsunami, N., Ryo, H., Hishida, Y., & Honjo, T. (1991) The Drosphila RBP-J_K gene encodes the binding protein for the immunoglobulin J_K recombination signal sequence. *J. Biol. Chem.* 366, pp. 23334-23340.

Furukawa, T., Maruyama, S., Kawaichi, M., & Honjo, T. (1992) The Drosphila homolog of the immunoglobulin recombination signal-binding protein regulates peripherlal nervous system development. *Cell* 69, pp. 1191-1197.

Futcher, A.B. (1988) The 2 μm circle plasmid of *Saccharomyces cerevisiae. Yeast* 4, pp. 27-40.

Garvin, A.M., Abraham, K.M., Forbush, K.A., Farr, A.G., Davison, B.L., & Perlmutter, R.M. (1990) Disruption of thymocyte development and lymphomagenesis induced by SV40 T-antigen. *Int. Immunol.* 2, pp. 173-180.

Gellert, M. (1981) DNA topoisomerases. *Annu. Rev. Biochem.* 50, pp. 879-910.

Gellert, M. (1992) Molecular analysis of V(D)J recombination. *Annu. Rev. Genet.* 22, pp.425-446.

Glasgow, A.C., Hughes, K.T., & Simon, M.I. (1989) Bacterial DNA inversion systems. In *Mobile DNA* , Berg, D.E. & Howe, M.M. eds., Am. Soc. Microbiol., Washington D.C. pp. 637-660.

Golic, K.G. (1991) Site-specific Recombination Between Homologous Chromosomes in *Drosophila Science* 252, pp. 958-961.

Gorman, C.M., Moffat, L.F., & Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2, pp.1044-1051.

Grindley, N.D.F., & Reed, R.R. (1985) Transpositional recombination in prokaryotes. *Ann. Rev. Biochem.* 54, pp.863-896.

Gronostajski, R.M., & Sadowski, P.D. (1985) The FLP recombinase of the *Saccharomyces cerevisiae* 2 μ plasmid attaches covalently to DNA via a phosphotyrosyl linkage. *Mol. Cell. Biol.* 5, pp. 3274-3279.
Hall, C.V., Jacob, P.E., & Ringold, F.Lee (1983) Expression and regulation of Escherichia coli lacZ gene fusions in mammalian cells. *J. Mol. Appl. Genet.* 2, pp. 101-9.

Hanahan, D. (1989) Transgenic mice as probes into complex systems.*Science* 246, pp. 265-1274.

Haselkorn, R. (1989) Excision of elements interrupting nitrogen fixation operons in cyanobacteria. In *Mobile DNA* , Berg, D.E. & Howe, M.M. eds., Am. Soc. Microbiol., Washington D.C. pp. 735-742.

Haselkorn, R., Golden, J.W., Lammers, P.J., & Mulligan, M.E. (1986) Developmental rearrangement of cyanobacterial nitrogen-fixation genes. *Trends Genet.* 2, pp.255-259.

Hatfull, G.F , & Grindley, N.D.F. (1988) The resolvases and DNA-invertases: a family of enzymes active in site-specific recombination. In *Genetic Recombination* Smith, G.R., & Kucherlapati, R., eds., Am. Soc. Microbiol., Washington D.C. pp. 357-396.

Hoess, R.H., Ziese, M., & Sternberg, N. (1982) P1 site-specific recombination: Nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. USA* 79, pp. 3398-3402.

Hoess, R., & Abremski, K. (1984) Interaction of the bacteriophage P1 recombinase Cre with the recombining site *loxP*. *Proc. Natl. Acad. Sci. USA* 81, pp. 1026-1029.

Hoess, R.H., & Abremski, K. (1985) Mechanism of strand cleavage and exchange in the Cre-*lox* site-specific recombination system. *J. Mol. Biol.* 181, pp.351-362.

Hoess, R., Wierzbicki, A, & Abremski, K.(1987) Isolation and characterization of intermediates in site-specific recombination. *Proc. Natl. Acad. Sci. USA* 84, pp. 6840-6844.

Hsu, P.L., & Landy, A. (1984) Resolution of synthetic *att*-site Holliday structures by the integrase protein of bacteriophage lambda. *Nature* 311, pp. 721-726.

Hunter, T.(1991) Cooperation between oncogenes. *Cell* 64, pp. 249-270.

Jaenisch, R. (1988) Transgenic animals. *Science* 240, pp. 1468-1474.

Johnson, R.C., & Simon, M.I. (1987) Enhancers of site-specific recombination in bacteria. *Trends Genet.* 3, pp.262-267.

Kalionis, B., Dodd, I.F., Egan, J.B. (1986) Control of gene expression in the P2-related temperat*E. coli*phages. III DNA sequence of the major control region of phage 186. *J. Mol. Biol.* 191, pp.199-209.

Klemm, P. (1984) The *fimA* gene encoding the type-1 fimbrial subunit of *Escherichia coli* : nucleotide sequence and primary structure of the protein. *Eur. J. Biochem.* 143, pp.395-399.

Klemm, P. (1986) Two regulatory *fim* genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli*. *EMBO J.* 5, pp. 1389-1393.

Korman, A.J., Maruyama, J., & Raulet, D.H. (1989) Rearrangement by inversion of a T-cell receptor d variable region gene located 3' of the d constant region gene. *Proc. Natl. Acad. Sci. USA* 86, pp. 267-271.

Kozak, M. (1986) Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA* 83, pp. 2850-2854.

Lang, R.A., & Burgess, A.W. (1990) Autocrine growth factors and tumourigenic transformation. *Immunol. Today* 11, pp. 244-249.

Lee, E. Y.-H. P., Chang, C.-Y., Hu, N., Wang, Y.-C., Lai, C.-C., Herrup, K., Lee, W.-H., & Bradley, A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* 359, pp288-294.

Lewis, S. Gifford, A., & Baltimore, D. (1985) DNA elements are asymmetrically joined during the site-specific recombination of kappa immunoglobulin genes. *Science* 228, pp. 677-685.

Li, E. , Bestor, T.H., & Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69, pp. 916-926.

Lieber, M.R., Hesse, J.E., Mizuchi, K., & Gellert, M. (1988) Lymphoid V(D)J recombination: nucleotide insertion at signal joints as well as coding joints. *Proc. Natl. Acad. Sci. USA* 85, pp. 8588-8592.

Liu, L.F., & Wang, J.C. (1979) Interaction between DNA and *Escherichia coli* DNA topoisomerases. I. Formation of complexes between the protein and superhelical and nonsuperhelical duplex DNAs. *J. Biol. Chem.* 254 pp. 11082-11088.

MacGregor, G.R. & Caskey, C.T. (1989) Construction of plasmids that express *E. Coli* ß-galactosidase in mammalian cells. *Nucleic Acids Res.* 17, p. 2365.

MacGregor G.R., Nolan, G.P., Fiering, S., Roederer, M., & Herzenberg, L. (1989) Use of *E.coli lacZ* (ß-Galactosidase) as a reporter gene. In *Methods In Molecular Biology* 7 (Humana, Clifton, New Jersey).

Malissen, M., McCoy, C., Blanc, D., Trucy, J., Devaux, C. et al. (1986) Direct evidence for chromosome inversion during T-cell receptor ß gene rearrangements. *Nature* 319, pp. 28-33.

Marth, J.D., Peet, R., Krebs, E.G., & Perlmutter, R.M. (1985) A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. *Cell* 43, pp. 393-404.

Matsunami, N., Hamaguchi, Y., Yamamoto, Y, Kuze, K., Kanagawa, K., et al. (1989) A protein binding to the J kappa recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif. *Nature* 342 pp. 934-937.

Oettinger, M.A., Schatz, D.G., Gorka, C., & Baltimore, D. (1990) *Rag*-1 and *Rag*-2, adjacent genes that synergistically activate V(D)J recombination. *Science* 248, pp. 1517-1523.

O'Gorman, S., Fox, D.T., & Wahl, G.M. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251, pp. 1351-1355.
Paine, P.L., Moore, L.C., & Horowitz, S.B. (1975) Nuclear envelope permeability. *Nature* 254, pp. 109-114.

Pargellis, C.A., Nunes-Duby, S.E., Moitoso de Vargas, L. & Landy, A. (1988) Suicide recombination substrates yield covalent I Int-DNA complexes and lead to identification of the active site tyrosine. *J. Biol. Chem.* 263, pp. 7678-7685.

Parsons, R.L., Prasad, P.V., Harshey, R.M., & Jayaram, M. (1988) Step-arrest mutants of FLP recombinase: implications for the catalytic mechanism of DNA recombination. *Mol. Cell. Biol.* 8, pp.3303-3310.

Peters, R. (1983) Nuclear envelope permeability measured by fluorescence microphotolysis of single liver-cell nuclei. *J.Biol. Chem.* 258, pp. 11427-11429.

Pierson, L.S. III, & Kahn, M.L. (1987) Integration of satellite bacteriophage P4 in *Escherichia coli*: DNA sequences of the phage and host regions involved in site-specific recombination. *J. Mol. Biol.* 196, pp.487-496.

Prasad, P.V., Young, L.-J., & Jayaram, M.(1987) Mutations in the 2-micrometer circle site specific recombinase that abolish recombination without affecting substrate recognition. *Proc. Natl. Acad. Sci. USA* 84, pp. 2189-2193.

Roth, D.B., Nakajima, P.B., Menetski, J.P., Bosma, M.J., & Gellert, M. (1992) V(D)J Recombination in mouse thymocytes: double-strand breaks near T cell receptor d rearrangement singales. *Cell* 69, pp. 41-53.

Sauer, B. (1987) Functional expression of the *cre-lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae. Mol. Cell. Biol.* 7, pp. 2087-2096.

Sauer, B., Whealy, M., Robbins, A., & Enquist, L. (1987) Site-specific insertion of DNA into a pseudorabies virus vector. *Proc. Natl. Acad. Sci. USA* 84, pp. 9108-9112.

Sauer, B., & Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* 85, pp. 5166-5170.

Sauer, B. & Henderson, N.(1989) Cre-stimulated recombination at *loxP*-containing DNA sequences placed into the mammalian genome. *Nucleic Acids Res.* 17, pp. 47-161.

Sauer, B. & Henderson, N. (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol.* 2, pp. 441-449.

Schweisguth, F., & Posakony, J.W. (1992) *Suppressor of hairless*, the Drosphila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates.*Cell* 69, pp. 1199-1212.

Scott, J.R. (1968) Genetic studies on bacteriphage P1. *Virology* 36, pp. 564-574.

Senecoff, J.F., Bruckner, R.C., & Cox, M.M. (1985) The FLP recombinase of the yeast 2-μm plasmid: characterisation of its recombination site.*Proc. Natl. Acad. Sci. USA* 82, pp. 7270-7274.

Stanton, B.R., Perkins, A.S., Tessarollo, L. , Sassoon, D.A., & Parada, L.F. (1992) Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev.* 6, pp. 2235-2247.

Sternberg, N., & Hamilton, D. (1981) Bacteriphage P1 site-specific recombination. I. Recombination between *loxP* sites. *J. Mol. Biol.* 150, pp. 467-486.

Sternberg, N., Hamilton, D., Austin, S., Yarmolinsky, M., & Hoess, R. (1981) Site-specific recombination and its role in the life cycle of bacteriophage P1. *Cold Spring Harbour Symp. Quant. Biol.* 45, pp. 297-309.

Sternberg, N., Sauer,B., Hoess, R., & Abremski, K. (1986) Bacteriophage P1 *cre* gene and its regulatory region. Evidence for multiple promoters and for regulation by DNA methylation. *J. Mol. Biol.* 187, pp. 197-212.

Thomas, K.R., & Capecchi, M.R. (1987) Site-directed mutagenesis by gene targetting in mouse embryo-derived stem cells. *Cell* 51, pp. 503-512.

Thompson, J.F., Moito de Vargas, L., Koch, C., Kahmann, R., & Landy Q. (1987) Cellular factors couple recombination with growth phase: Characterisation of a new component in the I site-specific recombination pathway. *Cell* 50, pp.901-908.

Thompson, J.F. & Landy, A (1989) Regulation of bacteriophage lambda site-specific recombination. In *Mobile DNA* , Berg, D.E. & Howe, M.M. eds., Am. Soc. Microbiol., Washington D.C. pp.1-22.

Toh-e, A. , Tada, S., & Oshima, Y. (1982) 2μm DNA like plasmids in the osmophilic haploid yeast *Zygosaccharomyces rouxii. J. Bacteriol.* 151, pp.1380-1390.

Toh-e, A. , Araki, H., Utatsu, I., & Oshima, Y.(1984) Plasmids resembling 2μm DNA in the osmotolerant yeasts *Saccharomyces bailii* and *Saccharomyces bisporus. J. Gen. Microbiol.* 130, pp.2527-2534.

Tonegawa, S. (1983) Somatic generation of antibody diversity. *Nature* 302 pp. 575-581.

Walker, D., & Walker, J.T. (1975) Genetic studies of coliphage P1. I. Mapping by use of prophage deletions. *J.Virol.*16, pp. 525-534.

Weichhold, G.M., Klobeck, H-G., Ohnheiser, R., Combriato, G., & Zachau, H.G. (1990) Megabase inversion in the human genome as physiological events. *Nature* 347, pp. 90-92.

Weisberg, R.A. & Landy, A. (1983) Site-specific recombination in phage lambda. In *Lambda II,* eds. R.W.Hendrix et al., Cold Spring Harbor Lab., New York, pp.211-250

Wierzbicki, A., Kendall, M., Abremski, & Hoess,R. (1987) A mutational analysis of the bacteriophage P1 recombinase Cre. *J. Mol. Biol.* 195, pp.785-794.

APPENDIX 1: EXPRESSION OF SEQUENCES 3' OF THE LCK PROMOTOR IN THE LOX-ßGAL-LOX TRANSGENIC ANIMALS

As no ßGAL enzymatic activity was detectable in mice bearing the 1017LOX-ßGAL-LOX transgene, the functional integrity of the ßGAL coding sequence used in this construct was assessed as follows: The plasmid pCMVß (MacGregor and Caskey, 1989) was modified so as to include a neomycin-resistance cassette from pMC1Neo-PolyA (Thomas and Capecchi, 1987). This derivative was electroporated into cells of mouse hemopoietic lines and transfected cells were shown to generate histochemically detectable ßGAL activity by X-Gal staining.

Daniel Chui carried out Northern analysis of 5µg samples of total cellular RNA from thymocytes of the LOX-ßGAL-LOX transgenic mice, and was unable to detect a signal. Consequently, an oligonucleotide primer was designed by Dr. Jamey Marth to allow amplification of *ßGAL* sequence in conjunction with the hGH exon 2 primer used for PCR amplification of the recombination product (see section above). Daniel Chui was able to show that after reverse transcription from LOX-ßGAL-LOX transgenic thymocyte RNA template, a sequence could be amplified which was of the length of the expected portion of the *ßGAL* message, plus the LOX sequence and the spliced hGH sequence, and which hybridised specifically to the LOX probe . Amplification from the RNA was not possible without the initial reverse-transcriptase step. Even if the amplification obtained after reverse-transcriptase had been due to contaminating transgene DNA, the resulting band would have been of a size distinguishably larger than that in fact obtained since the transgene DNA included intronic sequence of approximately 250 bp in length which was absent from the corresponding RNA (see fig. 2.24, part A). A quantitative PCR analysis (fig. 2.31) of the amplified sequence suggested that the message was present in as few as 3 to 4 copies per cell, a figure consistent with the lack of detectable ßGAL activity.

In the product of the CRE-mediated recombination in doubly transgenic mouse thymocytes, the LCK promoter might theoretically drive expression of the hGH product. The structure of this product would consist of (5' to 3') the LCK promoter, a single LOX site formed by recombination of LOX sites, and the entire coding sequence of human growth hormone, as well as its poly-adenylation signal. Since very sensitive assays are available for the presence of hGH, and such assays have been used for the detection of hGH in transgenic mice (Braun et al., 1989), extracts of thymocytes of doubly transgenic mice were subjected to such assays. The doubly transgenic mouse thymocytes used in these assays had been shown by Southern analysis to harbour the CRE-mediated recombination product. Using a polyclonal antibody kindly provided by Dr Richard Palmiter (University of Washington, Seattle), thymocyte extracts were subjected to Western blot analysis (fig. 2.32).

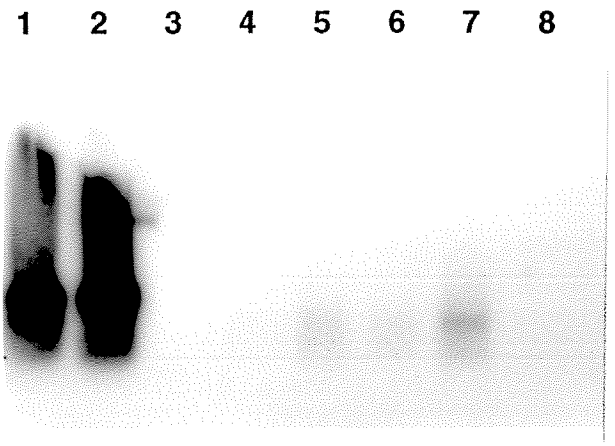


Fig. 2.31. PCR amplification of RNA from LOX-ßGAL-LOX thymocytes [courtesy of Daniel Chui]. Lanes 1 - 3: LOX-ßGAL-LOX transgenic thymocyte DNA (without reverse transcription). Lane 1 - 1 ng, lane 2 - 500 pg, lane 3 - 100 pg, lane 4 - LOX-ßGAL-LOX transgenic thymocyte RNA without reverese transcription. Lanes 5 to 8 with reverse transcription. Lane 5 - 1 ng LOX-ßGAL-LOX transgenic thymocyte total RNA, lane 6 - 1 ng LOX-ßGAL-LOX transgenic thymocyte total RNA from another animal, lane 7 - 2 ng LOX-ßGAL-LOX transgenic thymocyte total RNA, lane 8 - wild-type mouse thymocyte RNA. Probe is LOX fragment. The signal from the RNA runs at a smaller size than that from the DNA, as expected.

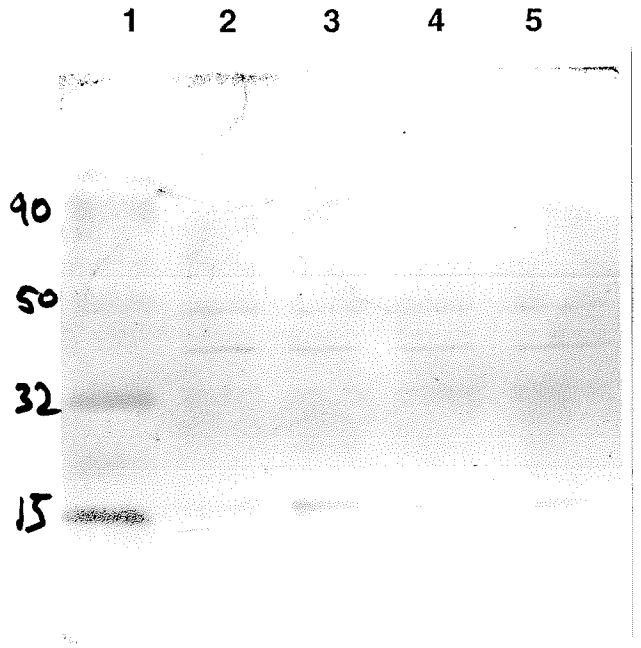


Fig. 2.32. Western analysis of thymocyte samples for detection of hGH Approximate molecular masses in kD are marked on the left of the blot. Lane 1 - molecular mass markers, lane 2 - wild-type, lane 3 - CRE transgenic, lane 4 - LOX-ßGAL-LOX transgenic, lane 5 - doubly transgenic. No band of the expected size of human growth hormone (20 - 22 kD) is seen in any lane.

This failed to reveal an hGH-specific signal in any thymocyte extract. As a positive control for hGH protein was not available at the Biomedical Research Centre, thymocyte extracts were sent to the Division of Clinical Chemistry at Vancouver General Hospital, where radio-immuno-assay was routinely performed to assess hGH levels in patients. Such assays, although capable of detecting as little as 1.5 ng/ml of assay sample (Joan Treppanier, V.G.H., personal communication), failed to show the presence of hGH in extracts of doubly transgenic thymocytes prepared at a concentration of total protein from 3×10^7 thymocytes per milliliter.

While it is possible that the presence of a LOX site 3' of the LCK promoter inhibited expression of downstream sequence, as previously discussed, an alternative explanation of this finding can be proposed. The structure of the 1017LOX-ßGAL-LOX transgene integration was previously investigated (at least in the 86 line, see fig 2.16) by Southern analysis. This analysis went as far 5' as the XbaI site in the LCK promoter, as no further-5'-cutting enzyme was used in digests. The size of the predominant hybridising band in this analysis would suggest that the majority of the LCK promoter was intact in the majority of copies of the integrated construct. There is, however, no means of determining from this analysis whether the LCK promoter present in the most 5' copy of the construct within the integration array was intact even as far as the XbaI site. It is possible, for instance that much of the promoter was truncated in this copy, and the resulting EcoRI and XbaI fragments are too large to be clearly distinguishable as separate "flanking" bands, especially since it seems likely that the XbaI digestion in this analysis was incomplete. This most 5' copy of the LCK promoter is the one expected to remain in the recombination product after complete collapse of the LOX-ßGAL -LOX array. It seems possible, accordingly, that the portion of the LCK promoter remaining in the recombination product might be insufficient ot allow expression of 3' sequence. This question would be amenable to further investigation by PCR using oligonucleotide primers directed to various portions of the LCK promoter, and by Southern analysis with probes made from the most 5' portion of the LCK promoter present in p1017.

APPENDIX 2: MINOR BANDS AND "FLANKING" SEQUENCES

Transgenic mice are usually made in order to achieve the expression of a gene product in a tissue in which it is not normally expressed, at a time in development at which it is not normally epxressed, and/or at significantly higher levels than normal. Often Southern analysis of transgenic tissue is not reported, or merely referred to with respect to the estimated copy number of the integrated transgene. It has not usually been necessary to examine the precise structure of transgene arrays. The present project, however, made extensive use of Southern analysis of transgene arrays, in

determining experimental results. In the course of these analyses, numerous anomalies of transgene integration - which are likely to be present in more "usual" transgenic experiments, unbeknownst to the investigators - became apparent. In some instances these rendered more difficult the explanation of bands appearing on Southern analyses, and in some analyses, a precise description of integration structures that would account for all bands was lacking. Nevertheless, it was felt appropriate that where explanation of the "minor" bands was possible, this should be attempted, without interrupting the presentation of data and conclusions based largely on the "major" bands visible in Southern analyses. The explanations offered here are, in most cases, the simplest of several available, and are presented to show that reasonable explanations that do not detract from the validity of the conclusions drawn from the data, are possible.

A) The 1017CRE integration structure.

i) Figure. 2.16, lanes 2-4 The hGH probe shows predominant bands at between 7 and 8 kb in size as expected (fig. 2.16, part A) in both the single EcoRI digest and the XbaI digest. The XbaI digest lane shows a) some hybridisation present at the point of "origin" of the lane, extensive smearing above the predominant band, and a diminshed intensity of the band with respect to that in the EcoRI lane. These XbaI appearances are all suggestive of partial digestion, as is the appearance of the 7-8kb band in the double digest (lane 4). The EcoRI digest shows a minor band at a point slightly above the.largest marker (i.e > 12 kb). In a simple head-to tail array integration,.a single such "flanking" band would be expected at a position determined by the nearest chromosomal EcoRI site on the 5' side of the integration (with respect to the orientation of the transgene). Similarly, an XbaI flanking band would be expected at the 3' side of the integration, and such a band may be visible (depending on the quality of the reproduction) in lane 3. That this XbaI "flanking" band appears of similar size to the EcoRI "flanking" band, is unimportant, since size discrimination is very poor at this "upper" end of gels, and an actual similarity of size would be coincidental. Since this hGH probe might be expected to hybridise weakly to mouse growth hormone, sequnces, no firm concluisons can be drawn from any additional bands that may be apparent on this Southern analysis.

ii) Figure 2.17, part A, lanes 3 and 4.

This analysis is of tail DNA, so no recombination products are present. The LCK probe has picked up a single band in wild-type mouse DNA, due to the presence of the 2 copies of the enodogenous LCK gene. Digestion appears complete. The band resulting from the presence of the 1017βGAL

transgene (lane 5) is, as expected, of approximately the same size as that from the 1017CRE construct (fig. 2.16). Therefore, no difference is seen between the bands from the doubly transgenic animal (lane 3) and the 1017CRE transgenic (lane 4). In both lanes 3 and 4 two "minor" bands appear at sizes of approximately 4 kb and 8-9 kb. The intensity of these bands suggests a copy number of the corresponding DNA fragment of the order of that of the endogenous *lck* gene. The simplest explanation for these bands is that they represent true flanking sequences at EITHER end of the integration array, involving a truncation of the terminal copies of the transgene construct at points central to the outer SacI sites at each end (i.e 3' to the most 5' expected SacI site, and 5' to the most 3' expected SacI site) Such true flanking sequences would, however, be expected to produce bands of approximately HALF the intensity of the endogenous *lck* band. Although more elaborate explanations for these additional bands might be entertained, it is not clear that the appearances of this Southern warrant further interpretive effort.

iii) Figure. 2.17 part B, lane 3 (and figure 2.18, lanes 3,4, and 5; figure 2.26, part A; figure 2.27, lanes 2 and 3; figure 2.28, lanes 2,5, 6, 7.).

These Southerns show thymocyte DNA from wild-type and singly transgenic mice, probed with the LCK probe, and doubly digested with StuI and MscI. The appearances suggest close to complete digestion. The endogenous *lck* gene fragment runs at between 1kb and 1.6 kb. In this instance, in addition to the endogenous *lck* band, and a predominant band near 3kb that would be expected from the 1017CRE array (fig. 2.16), a band of somewhat lesser intensity than that of the endogenous *lck* band is present at between 2 and 3 kb. Such a fragment is consistent with the hypothesis of both 3'and 5' true flanking sequences, suggested above, and, in the simplest interpretation, would be due to the truncation at the 5' end, since a truncation here at a point 3' to the SacI site would also be 3' to the unique StuI site in the construct. At the 3' end SacI site, it is possible that a truncation exists between the MscI site and the SacI site, so that no flanking sequence from this end would be apparent with the StuI/MscI double digest.

iv) Figure.2.21, lanes 2 and 3.

These lanes contain DNA from a doubly transgenic animal, digested with BglII. Digestion appears near to complete. The explanation of minor bands offered above leads one to expect two "flanking" bands of the 1017CRE array to be present. The additional LCK-hybridising band immediately below the

expected 1017CRE band at between 3 and 4 kb (see fig. 2.16) may represent one such band, and the blurry signal at about 7-8 kb the other.

B) The 86 line 1017LOX-ßGAL-LOX array.

i) Figure 2.15, lanes 6,7,8.

The hGH probe of singly transgenic 86 line DNA shows the majority of the integration structure to be of the form illustrated in fig. 2.16 part B. The appearance of the Southern suggests that the XbaI digest was incomplete. There is a faint suggestion of a flanking band in the EcoRI lane. Such a band would be expected at the 5' end of the integration array.

ii) Figure 2.17, part A, lane 5; figure 2.17, part B, lane 4; figure 2.28, lanes 3, and 5-7.

Both SacI digest and StuI/MscI double digests probed with the LCK probe show bands of several times the intensity of the endogenous *lck* band at the sizes expected. No other bands are visible with these digests

iii) Figure 2.20, part C, lanes 3 and 4.

This Southern, although a SacI digest probed with the LCK fragment, shows a band of very low intensity in comparision to that of the endogenous *lck* fragment, at about 4-5 kb in lane 3. This appearance suggests it may be due to incomplete digestion. The same applies to the DNA from doubly transgenic tail in lane 4. In this case the additional bands at between 4 and 5 kb, and above 7 kb, may be due to incomplete digestion of the CRE array.

iv) Figure 2.26, part B, lanes 2-6.

This tissue survey from a doubly-transgenic 86 line animal shows almost complete disappearance of the ßGAL array fragment from thymocytes compared with the band of expected size at between 5 and 6 kb in DNA derived from other tissues. This BglII digest, probed with the LCK fragment, also shows the endogenous *lck* band at about1 kb. However, the expected recombination product at between 2

and 3 kb is NOT seen in this autoradiograph (nor in a longer exposure of the same blot). This contrasts with the Southern of figure 2.21, another BglII digest probed with the LCK fragment,.which does show a band of this size in thymocyte DNA from another doubly transgenic 86 line animal. BglII digests of thymocyte DNA from a third doubly transgenic 86 line animal (not shown) failed to reveal the band of the expected size of the recombination product. A possible explanation for this anomaly is as follows: a truncation of the 1017LOX-βGAL-LOX construct has occured between the SacI and BglII sites at either the 5' or 3' ends of the array, such that the recombination product of BglII digestion IN animals in which the whole array has been collapsed by recombination is either too large to transfer from the gel to nitrocellulose at sufficient levels to be visible, or is hidden by another band. The reason that a recombination product of the expected size is nevertheless visible in the Southern of figure 2.21, is that in this animal the array was not completely collapsed, and the product seen is the result of recombination in copies of the transgene that are internal to the terminal copy which suffered the truncation. Thus, the recombination product of expected size is still present, whereas in mice in which the array recombination has gone to completion (in the majority of thymocytes), the copies responsible for the visibility of this band have been "recombined out".

C) The 87 line 1017LOX-βGAL-LOX array.

i) Figure 2.29, part A, lanes2-5.

Ths SacI digest of DNA from two doubly transgenic animals of the 87 line, probed with the LCK fragment, shows a band of near 1.6 kb present at equal intensity in both tail and thymocyte samples. This band has never been seen in digests of DNA from singly transgenic 1017CRE animals, and appears despite the presence of the expected recombination product in thymocytes of both animals. It mayhave been that this anomaly was due to a truncation or other integration "pathology" that removed a LOX site, since these appearances suggest this SacI fragment was not amenable to recombination.

ii) Figure 2.29, part B, lanes 2-5.

The BglII digest of the same DNA samples as discussed in section i, above, probed with the LCK fragment, also suggests an integration anomaly. Some degradation is evident, especially in tail DNA, but digestion appears near to complete. Although the expected recombination product is present, at between 2 and 3 kb, there is a hint of a band in tail DNA at nearer to 3 kb, which seems fainter in

thymocyte DNA, and the additional appearance in thymocyte DNA of a band at about 10kb The intensities of the new bands in relation to that of the endogenous *lck* band, and of the original and remnant ßGAL bands, suggest that the array collapse may not have gone to completion in these animals.

iii) Figure 2.30, part A, lanes 2 and 3; part B, lanes 1 and 2.

A StuI/MscI digest was performed on the tail and thymocyte DNAs derived from one of the two animals of sections i and ii, above. Aliquots of these digests were analysed by probing either with the LCK fragment (part A) , or the LOX probe (part B). The LCK probe shows the expected recombination product at near 1.6 kb, and an additional product in thymocyte DNA of about 7-9 kb. The LOX-probed blot of the same samples shows at least three LOX-hybridising fragments in tail, which seem to diappear or diminish in intensity,in thymocyte DNA: that of the expected size (at 5-6 kb), and much more faint bands at near 4kb and near 2 kb. Correspondingly, as the recombination product of expected size (near 1.6 kb) appears in thymocyte DNA, so also does a faint signal at the 7-9 kb area. An additional ver faint signal is apprent in both tail and thymocyte DNAs, unchanged, at about 3-4 kb. This latter is unlikely to correspond to the band of about 1.6 kb seen in the 87 line SacI digest (section i, above), since the intensity relative to that of the LOX-ßGAL-LOX array band is much less than that seen in the SacI digest. (On the other hand, use of the LOX probe would be expected to give a much lower relative intensity due to relative exaggeration of the intensity of the LOX-ßGAL-LOX array band, as every copy of the transgene construct in this array harbours TWO LOX sites.) This very faint signal may reflect weak hybridisation of the LOX probe to some pUC sequence present in a few copies of the CRE transgene construct, due to the presence of some polylinker sequence in the LOX fragment used as probe. The complexity of the 1017LOX-ßGAL-LOX integration structure in the 87 line seems to defy a simple explanation ialong the lines of the common integration structure anomalies (Brinster and Palmiter, 1986).

MATERIALS AND METHODS

1) TISSUE CULTURE

A) Passaging and proliferation assays

All cell culture was performed in RPMI 1640 medium (Terry Fox Laboratories, Vancouver B.C.) supplemented with 10% v/v fetal or newborn bovine serum (Hyclone, Logan, Utah) and 50 μM 2-mercaptoethanol (Aldrich, St. Louis, MO). Cells were incubated in humidified 37^{o} C incubators in an atmosphere of 5% CO_2 in air. Factor dependent cell lines were passaged by making 5 serial 1 volume to 5 volumes dilutions in fresh medium on the day of passage (every 5 - 7 days). Passaging was usually performed in 60 x 15 mm culture dishes (Corning, Corning, NY,), and occasionally in the 1 ml wells of 24-well tissue culture plates (Falcon, BD Labware, Lincoln Park NJ). Pipetting of cells was done with calibrated serological pipettes from Baxter (Burnaby, B.C.) and a Pipetaid pipettor (Drummond Scientific Co., Brodwall, PA). Adherent cells were passaged and assayed in tissue-culture treated dishes and trypsinised as necessary after washng the cells free of serum in two changes of 10 mls of PBS. All washing of mammalian cells (other than prior to electroporations) was done at room temperature in a Canlab (Edmonton, Alberta) Labofuge B centrifuge, at 1200 rpm approximate average relative centrifugal force of 400) for 4 to 5 minutes. Centrifugation was performed in 10 ml polypropylene or polystyrene tubes (Disposable Products, Adelaide, Australia) or 15 ml polystyrene or 50 ml polypropylene tubes (Falcon). Passaging medium for factor-dependent cells contained super-saturating amounts of the appropriate growth-factor, generally between 4 and 10 units as determined on the cell line being passaged (Part 1, Appendix 1 and section B, below). Cells were thus maintained in log-phase growth, generally at below 2 x 10^5 cells per ml. AUTOSTIMULATORY cells were passaged as described in the text.

Cells were counted in a 1:1 mixture of suspended cells in PBS containing eosin (0.2% w/v - BDH, Vancouver, B.C.) or trypan-blue (0.4% BDH) by examination in a hemacytometer with an Olympus microscope. At least 200 cells were counted for each enumeration. Cells were washed prior to all assays by repeated centrifugation with 3 changes of 8 mls of medium or Hanks' buffered salt solution containing 2% v/v newborn bovine serum, and one centrifugation in assay medium, prior to enumeration. Medium for proliferation assays was always supplemented with fetal rather than newborn bovine serum. Titrations were performed by making serial 2-fold dilutions of growth-factor, supernate, antibody etc. in the wells of round-bottomed 96-well plates, or in the wells of 24-well tissue-culture

plates (Falcon), and applying 5µl of each dilution to the wells of 60-well Terasaki microtiter plates (Disposable Products,), in triplicate. Resuspended cells were then applied in 5µl of medium. Plating in Terasaki wells was performed with an EDP automated pipettor (Woburn, MA). When the assay had been plated out, approximately 200 -300 µl of medium was applied to the inner circumference of the Terasaki plates to maintain adequate hydration of the culture wells. After the initial period of incubation, 250 nCi (approximately 9.25 kBq) of ^{3}H-thymidine (ICN, St. Laurent, QUE) was added in 5 µl of medium to each well, and incubation allowed to proceed further. In the last 30 - 60 minutes before harvesting the assay, Terasakis were inverted to encourage cells to gather in the culture liquid drop, facilitating their harvesting onto pre-punched glass-fibre sheets (Titertek, Flow Laboratories Inc, Mississauga, ONT). Harvesting was performed using tap water, a Venturi device and a specialised harvesting platform allowing culture liquid to be washed through the sheets with double-distilled water, and subsequent fixing of DNA to the sheets with methanol. Scintillation cocktail (BetaMax, ICN) was applied, and counting was performed using a protocl of 2 minutes count per sample in a humidified room, which minimised interference due to accumulation of static electrical currents on the glass-fibre or the beta-counter (Taurus Autopmatic Scintillation Counter, Micromedics - ICN).

Pipetting of volumes of less than one milliliter, for cell-biology, biochemistry, and molecular biology applications, except for the plating of Terasaki assays (above), was performed with Socorex (Renes, Switzerland) or Gilson (Villiers-Le-Bel, France) pipettors. Pipette tips were from Island Scientific (Seattle, WA)or Costar (Cambridge, MA).

Soft agar (Difco, Detroit, MI) cloning was performed by making a 3% w/v slolution of agar in sterile double-distilled water, heating the agar by flame or in a micro-wave oven until it had dissolved and then allowing it to cool to 37^{o} C prior to adding one volume of agar to nine volumes of medium containing cells and appropriate growth factor. The mixture was then mixed by pipetting and plated into 60 x 15 mm tissue culture dishes which were rapidly transferred to a metal surface in a 4^{o} C environment, to discourage cells from sinking to the bottom of the dishes before the agar had gelled. Plucking of colonies from agar was performed on the stage of an Olympus dissecting microscope with a pulled Pasteur pipette, in a sterile laminar outflow cabinet. Ficoll density-gradient centrifugation was performed with Ficoll-Hypaque (Pharmacia, Baie d'Urfe, QUE), according to the manufacturers instructions.

B) Sources of growth-factors

Several of the growth-factors used were produced as symthetic poly-peptides by Dr. Ian Clark Lewis of the Biomedical Research Centre, as indicated in the text and figures. Recombinant human IL-2 was

the kind gift of Cetus Corporation. Murine IL-2 was obtained as the supernatant of a hybridoma cell line (X63-Ag8/653) that had been engineered to secrete IL-2 by transfection with an expression vector carrying the murine IL-2 cDNA., the kind gift of Dr. Fritz Melchers of the Basel Institue of Immunology. Similar supernatant was also used as a source of IL-3. These supernates were obtained from 24 - 48 hour growth at high densities (approximately 10^6 per ml), and were filtered through 0.22 µm Millex GV syringe filters (Millipore, Bedford, MA) prior to use. Supernatant of the myeloid leukemia cell WEHI-3B was also used as a source of mIL-3, in this instance concentrated 10-fold prior to use as a 2% v/v solution in passaging medium. Recombinant mIL-4 was from Cetus corporation. Mouse IL-6 was obtained as the supernatant of the fibroblast cell line Psi2, and human IL-6 as supernate of the lymphoblastoid cell line LK28, derived by Dr. John Schrader at the Walter and Eliza Hall institute of Medical Research, Melbourne, Australia. All concentrations were performed with Amicon concentrators (Amicon Canada, Oakville, ONT).

G418 was used as Geneticin powder (Gibco BRL, Burlington, ONT). The manufacturer's specification of active weight was used to calculate the concentrations used. Hygromycin B was in powder form from Boehringer Mannheim (Boehringer Mannheim Canada, Laval, QUE), and concentrations were calculated on a simple weight per volume basis. The fluoresecent dye PKH2 was from Molecular Probes Inc. (Eugene, OR). Polybrene was obtained from Sigma. (St. Louis, MO).

C) Antibodies

Monoclonal antibodies were derived from tissue-culture supernatants of hybridoma cells or ascites. For production of ascites of mouse monoclonal antibodies (DMS1, DMS2 and Mouse Anti-Rat Kappa - MAR 18.5), Balb/C mice were primed by intraperitoneal injection of pristane (Aldrich) 5 days to 5 weeks before intraperitoneal injection of 1 - 5 x 10^6 hybridoma cells. For production of ascites of rat antibodies (PC61, 11B11, and 6B4), Balb/C mice were pristane primed and treated with sub-lethal gamma irradiation and hydrocortisone injection, prior to introduction of hybridoma cells, according to the protocol of Weissman et al., 1985. Mice were sacrificed in carbon dioxide when abdominal swelling due mto ascites was apparent, but before the animals displayed other signs of the tumour burden. Rabbit antibodies Rab7 and Rab39, were used as serum and were the kind gift of Dr. Hermann Ziltener. The rabbit anti-rat immunoglobulin anti-serum was raised as indicated in Part 1, Appendix 2 by initial subcutaneous injection of approximately 50 µg of partially purified rat immunoglobulins in Freund's complete adjuvant (Difco) , followed by booster injections containing approximately the same amount of protein in incomplete adjuvant after 2 weeks, and every 4 weeks thereafter, for a total of 6 injections.

Fluorescence activated cell sorting and analysis was done by incubating approximately 10^6 target cells in 1 ml of a solution of 2% newborn bovine serum in PBS, containing primary antibody for 30 minutes to 1 hour at 4° C with periodic agitation to prevent settling of cells. Primary antibody was used at 1 µg per ml (in the case of PC61) or 1:2000 v/v dilutions of commercial CD4 and CD8 antibodies (Becton-Dickinson, San Jose, CA) rehydrated according to the manufacturers specifications. After incubation, cells were washed twice in 10 mls of the incubation solution without antibody, and then resuspended in a similar 1:2000 v/v solution of appropriate fluorescein-coupled secondary antibodies (Kierkegaard Perry Laboratories, Gaithersburg, MD) rehydrated according to the manufacturers instructions. After further incubation at 4° C for an hour, cells were again washed three times, and resuspended in 2% serum in PBS prior to sorting or analysis in the University Hospital Acute Care Unit facility with a FACStar sortet (Becton-Dickinson) by Mr. Dan Zecchini.

D) Gene transfer into cells.

Retroviral gene transfer was performed as described in the text. Cells were prepared for electroporation as follows: 1 to 2 x 10^7 cells were washed by centrifugation in PBS twice, to remove serum, and then resuspended in 0.8 mls of PBS. If plasmid was linearised prior to electroporation, restriction digest was followed by standard phenol/chloroform extraction, precipitation with ethanol, and washing by centrifugation in 70% ethanol (as per Maniatis et al., 1982). All plasmids were resuspended at a concentration of 15 to 20 µg in 20 µl of sterile double-distilled water. The cell suspension was pipetted into the electroporation cuvette (Bio-rad, Hercules, CA), and the 20 µl plasmid suspension added to it. The mixture was allowed to sit in ice for 5 to 10 minutes and then gently agitated to resuspend cells prior to electropration. Electroporations were performed as specified in the text, using a Bio-rad Gene Pulser. After electroporation the cuvette was returned to ice for 5 to 10 minutes prior to transfer with a disposable soft pipette into 10 mls of pre-warmed medium in a 10 ml tissue culture flask. After 48 hours of incubation, cells were harvested and resuspended at a concentration of 5 x 10^4 to 1 x 10^5 viable cells per ml in medium containing G418 or hygromycin, and the suspension distributed into the wells of 96-well tissue culture plates. Cuvettes were reused after soaking overnight in double-distilled water\, rinsing with 70 % ethanol, and subjecting to 2.5 x 10^6 rads of gamma irradiation in a Gammacell unit (Nordion).

2) BIOCHEMISTRY

A) Antibodies

Rabbit antibodies were used as serum, or purified by protein-A sepharose (Pharmacia) affinity chromatography (as per Goding, 1986). Ascites and supernates were concentrated by ammonium sulphate precipitation prior to further purification by antibody affinity chromatography (ibid.). Affinity columns were prepared with cyanogen bromide activated sepharose beads (Pharmacia) according to the manufacturers instructions. Columns were run in PBS and eluted in 0.1M glycine pH 2.5. Protein was quantitated for coupling to beads, by reading absorption at 260 nm wavelength using a Hewlett-Packard (Edmonton, Alberta) spectrophotometer. Other quantifications were performed using the Bradford modification of the Lowry method (Bradford, 1976) with reagent from Bio-rad, and a standard curve made with bovine serum albumin (Sigma). Readings were performed in a Phillips Pye Unicam spectrophotometer (Cambridge, England).

Enzyme linked immuno-sorbent assay (ELISA) was performed at room temperature in round-bottom96-well polyvinlychloride trays (Falcon) coated overnight with the appropriate capture reagent diluted in PBS supplemented with 0.5% sodium azide. Incubation of sample and developing antibodies were for 1 to 2 hours each at room temperature. Sample titrations (serial two-fold dilutions in triplicate) and subsequent antibodies were applied in 5% skim-milk powder in PBS with azide except the distilled water wash prior to addition of peroxidase substrate. Commercial entibodies were used at concentration recommended by the manufacturers (Kierkegard Perry Laboratories, or Calbiochem, La Jolla, CA). The reactions were developed with 0.5 mg/ml of the chromogenic substrate 2,2"-azinobis(3-ethylbenzthiazoline sulfonic acid) (Sigma,) in citrate buffer (pH 6.5) containing 0.006% v/v hydrogen peroxide (1/500 dilution of 3% stock kept in the dark at 4^{o} C). Colour was allowed to develop at 37^{o} C for 20 minutes and results were read as comparisons of absorption at 405 vs 490 nm for each well, in an EL309 Microplate Reader (Biotek Instruments Inc., Burlington, VT).

B) Protein analyses

Analytical protein gels were run according to the manufacturers instructions using the Pharmacia Phast mini-gel system with reagents from BDH.

Protein extraction from thymocytes was performed by resuspending PBS-washed cells at 4° Cin an Eppendorf tube (Brinkmann Instruments Inc., Rexfdale, ONT) at a concentration of 2 x 10^6 cells per 80 µl in a solution of 20 mM tris(hydroxymethyl)aminomethane (Tris, pH 8.0), 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% V/V Triton X-100, 137 mM sodium chloride, 1 mM phenylmethylsulphonylfluoride, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 1 µM pepstatin. Buffer reagents for this procedure were from Sigma and BDH, and protease inhibitors from Sigma and Boehringer Mannheim. The cell solution was then centrifuged for 1 minute at 4° C in a desktop refrigerated Eppendorf centrifuge at 14,000 rpm (approximate average relative centrifugal force of 14,000). Supernatant was transferred to another Eppendorf tube, and 20 µl of solubilisation buffer added. Solubilisation buffer was made as follows: to 31 mls of 50 mM Tris (pH 6.8) was added 5 g of sodium dodecyl sulphate (SDS), 25 ml glycerol, 0.5 ml of a saturated solution of bromophenol blue (all from BDH). This solution was stored frozen and prior to use 15 mg/ml of dithiothreitol (Boehringer Mannheim) was added.

3) MOLECULAR BIOLOGY

A) Plasmid manipulations

All plasmids were grown in E. coli strain DH5α. For both liquid and agar cultures, bacteria were grown in either Luria Broth (Gibco-BRL) or 2 x YT (ingredients from Difco), made from powder (as described in Sambrook et al., 1989). Competent cells were prepared using a variant of the method of Maniatis et al. (1982). After the initial incubation in 100 mM calcium chloride, cells were centrifuged, resuspended in 50 mM calcium chloride, and incubated at 4° C overnight. Glycerol (BDH) was then added (from autoclaved 75% w/v stock) to a final concentration of 15%, and 210 µl volumes of the cell suspension were aliquoted into 1.5ml Eppendorf tubes for storage at -70° C. For transformations, 100 or 200 µl of thawed competent cells were added to 10 or 20 µl of ligation reaction respectively, and the mixture was left in ice for 30 to 45 minutes. Then the mixture was transferred for 1.5 minutes to a 42° C waterbath, and returned to ice for 10 minutes prior to incubation with 1.3 ml of broth in a shaking incubator at 37° C for one hour. Cells were recoverd by centrifugation and plated onto agar (1.2 % w/v in 2 x YT) in 100 x 15 mm plastic Petri dishes (Fischer Scientific, Ottawa, ONT.). Agar contained 100 µg/ml ampicillin (Sigma) when selecting for ampicillin-resistant transformants, and 12.5 µg /ml kanamycin (Boehringer Mannheim) when selecting derivatives of the pBMGNeo plasmid.

All small scale manipulations of bacteria, DNA and plasmids were performed in Eppendorf tubes. Centrifugations were performed in desktop Eppendorf high-speed centrifuges at an average relative

centrifugal force of approximately 14,000. Small scale plasmid preparations were performed by alkaline lysis and standard phenol/chlorofom extraction (Maniatis et al., 1982) or the TELT method as described in He et al. (1989), involving lysis of bacteria in a 1:1 mixture of a) standard phenol/chlorofom and b) a solution of 2.5M lithium chloride, 50 mM Tris (pH 8.0), 4% v/v Triton X-100, 62.5 mM EDTA , prior to preciptation of the aqueous phase with ethanol. Large scale preparations were performed by alkaline lysis followed by a variation of the cesium chloride method as described in Maniatis et al. (1982), in which centrifugation was performed in 6 ml Sorvall (Du Pont, Wilmington, DE) polyallomer tubes at 15 ^{o} C at 60,000 rpm in a Sorvall TV865 rotor in a Sorvall RC50 ultra-centrifuge (with an average relative centrifugal force of approximately 315,000). The iso-propanol precipitate from a 1 litre culture was divided between two 6 ml tubes, centrifuged for 4 hours, the ethidium-stained bands pooled, and the result centrifuged for 4 to 16 hours. Bands were extracted in a final volume of less than 4 ml, and ethidium was extracted 3 to 4 times with TE-saturated 1-butanol. The volume of the DNA solution was then brought up to 10 mls with the addition of TE, 200 µl of a 5M solution of sodium chloride in double-distilled water was added, and 20 mls of ethanol added. This solution was then incubated for 3 to 16 hours in a -20^{o} C freezer, prior to precipitation of the plasmid by centrifugation at 19,000 rpm (average relative centrifugal force of 33,000) at 4^{o} C in an SS34 fixed-angle rotor in an RC5 centrifuge (Sorvall). Plasmid pellets were resuspended in TE, and further precipitated with ethanol, and washed by centrifugation in 70% ethanol to reduce volume and partially sterilise the plasmid suspension prior to electropration. Plasmids were stored at 4^{o} C in TE.

Agarose (Gibco BRL) gels for plasmid analysis were run in TBE for ethidium bromide visualisation, or TAE for Southern analysis, as described in Maniatis et al. (1982). These were made as 10 x stock (TAE) or 5 x stock (TBE) and diluted prior to use. Gels were run in International Biotechnologies Inc. gel boxes, using Pharmacia power supplies. Ethidium bromide staining was performed by soaking the gel for 5 to 10 minutes in a solution of approximately 5 µg/ml ethidium bromide in running buffer (made by adding 20 µl of a 10 mg/ml solution in water to approximately 40 ml of running buffer. This staining solution was reused as often as practicable for analytical gels other than those subsequently transferred for Southern analysis, and ethidium was added to the solution periodically, as required. Bands were visualised using a short-wavelength UV transilluminator (UVP Inc., San Gabriel CA), and gels photographed using an MP4 Land Camera (Polaroid, Cambridege, MA), and Polaroid 667 film cartridges. Reagents for these manipulations were from BDH (generally AnalaR grade) except for agarose and cesium chloride (Gibco BRL), and ethidium bromide (Boehringer Mannheim). DNA was quantitated by fluorometry with a Hoefer DNA Mini-Fluorometer (San Francisco, CA), by comparing fluorescence at 460 nm with that of a supplied standard DNA preparation (Hoefer) in a solution of 0.1 µg/ml Hoechst dye 33258 (Aldrich) in water.

Ligations were performed at 4° C over 12 to 16 hours in buffers supplied by the manufacturers of the ligase (New England Biolabs, Mississauga, ONT, or BRL). Fragment ratios for ligations were determined by ethidium bromide visualisation of fragments electrophoresed in agarose, and molar ligation ratios were generally of the order of 3:1 to 5:1 (insert:vector) for cohesive-end fragment ligations, and 5:1 to 10:1 for blunt-end ligations. Restriction digests were performed with enzymes from Pharmacia, BRL, or New England Biolabs, with supplied buffers, according to the manufacturers instructions. Incubation for analytical digests were for 30 minutes to 1 hour in a 37° C waterbath. All digestions were stopped by the addition of sucrose-based (BRL) or Ficoll-based (Pharmacia) loading buffer prepared as described in Maniatis et al. (1982).

Phosphatase treatment of vector ends was performed with alkaline phophatase (Boehringer Mannheim) using between 18 and 24 units per treatment (1 µl of the supplied preparation). Restriction digests were first heat inactivated in a 70° C waterbath for 10 minutes, cooled to room temperature by brief incubation in ice, and centrifuged for a few seconds to precipitate condensation. To every 20 µl of digest was then added 1 µl of 10% w/v SDS, 3.5 µl of 1M Tris (pH 9.0) and the phophatase. The mixuture was incubated in a 37° C waterbath for 30 minutes, and 1 µl of 0.5M EDTA was added, and the mixture then subjected to phenol/choloroform extraction. After ethanol precipitation and 70% ethanol washing, the DNA was resuspended in an appropriate volume of double-distilled water for gel quantitation or fill-in of overhangs with Klenow prior to ligation.

Filling of restriction enzyme ends was accomplished by resuspending plasmid DNA in 15 µl of double-distilled water, adding 2 µl of New England Biolabs restriction buffer 2 (50 mM sodium chloride, 10 mM magnesium chloride, 1 mM dithiothreitol, 10 mM Tris pH7.9), 2 µl of mixed deoxynucleotide triphosphates (10 x stock prepared according to Sambrook et al., 1989) and 1 µl (5 units) of Klenow fragment of DNA polymerase I (New England Biolabs). Incubation was allowed to proceed for 30 minutes in a waterbath at 37° C, and the mixture was then incubated for 15 minutes in a 70° C waterbath to inactivate the Klenow, and an aliquot then taken for gel quantitation prior to ligation.

B) Genomic DNA manipulations and Southern analyses

Fragments for labelling as probes were prepared by gel separation, visualised after ethidium staining in fresh solution, using a 365 nm wavelength UV transilluminator (Spectroline, Westbury, NY)and purified using the Geneclean (Bio 101 Inc., La Jolla, CA) or Sephaglas (Pharmacia) kits, according to the manufactures instructions. 16 µl aliquots containing 30 to 50 ng of fragment each were made in double-distilled water in Eppendorf tubes, and stored at -20° C. Labelling was carried out by the

random priming method. An aliquot was thawed, the tube immersed in boiling water for 5 to 10 minutes, and cooled to room temperature. To the aliquot was added 15 µl of "oligo-mix" (see below), 2 µl of Klenow fragment of DNA polymerase I, and 3 to 4 µl of α-^{32}P dCTP solution (NEN Du Pont, Mississauga, ONT - 111 TBq/mmol, 370 MBq/ml, used before or no more than 14 days after the supplied activity date). After incubation at room temperature for 2 hours, 20 µl of a 1% w/v solution of bromophenol blue (BDH) and 170 µl of TE was added to the reaction. The mixture was then passed by centrifugation through a "spin column" prepared by compacting by centrifugation a slurry of Sephadex G50 (Pharmacia) in TE into a 1 ml syringe over a small wad of glass-wool, to separate probe from unincorporated nucleotides. The labelled probe was collected in a screw-top 1.5 ml tube (Fischer Scientific) and to the contents were added 20 µl of 2M sodium hydroxide (BDH) and 760 µl of SET (1% w/v SDS, 10 mM Tris, 5 mM EDTA, pH 7.5). The labelled probe was heated for 10 minutes in a boiling water bath prior to addition to hybridisation reactions. All handling of radioactive materials was done with a sheet of plexiglass between the experimenter and the material.

"Oligo-mix" was prepared as follows: solution A consisted of 1 ml of oligo buffer (1.25M Tris pH 8.0, 0.125M magnesium chloride - stored at 4° C) 18 µl of 2-mercaptoethanol (Sigma) and 10 µl of each of dATP, dGTP, and dTTP (100mM stocks, Pharmacia). Solution B was 2 M Hepes pH 6.6 (- Terry Fox Laboratories), and solution C consisted of random hexanucleotides (Pharmacia, supplied as "50 A_{260} units") reconstituted in 500 µl of sterile double-distilled water (and stored at -20° C). The mix was prepared by combining A,B, and C in ratios of 1:2.5:1.5 (generally 100 µl : 250 µl : 150 µl). "Oligo-mix" was aliquoted in 50 µl lots in Eppendorf tubes and stored at -20° C.

Mice were obtained from the UBC Animal Facility (DBA/2) or from Harlan-Sprague-Dawley, Indianapolis, Indiana (ICR for transgenic experiments). Mice were anaesthetised in a mixture of fluorothane in air, or Avertin and sacrificed in carbon dioxide. Avertin was made by mixing 10g of tribromoethyl alcohol (Sigma) with 10 ml of tertiary amyl alcohol (Sigma), and stored at 4° C wrapped in foil. Transgenic animals were produced by pronuclear injection of zygotes that were subsequently re-implanted into pseudo-pregnant females (obtained by mating with vasectomised males).

Genomic DNA was extracted from various tissues other than thymus (1 to 3 cm of tail, and an equivalent amount of other tissues) by first cutting the tissue to pieces of less than3 mm in any dimension, and then suspending tissue in 400 µl of extraction buffer (100mM Tris pH 8.0, 50 mM EDTA) in an Eppendorf tube. To this was added 40 µl of a 1 mg/ml stock of Proteinase K (Boehringer Mannheim) and the mixture then incubated overnight in a 55° C waterbath. The mixture was then subjected to phenol/chloroform extraction and the DNA precipitated with ethanol by centrifugation at room temperature for 10 minutes. After a further ethanol wash, DNA was resupended in standard TE,

allowed to go into solution and vigorously pipetted prior to quantitation by fluorometry. Thymus cells were obtained by cutting whole thymus with scissors, and using a syringe plunger to gently force it through a wire mesh into 10 to 20 mls of RPMI or PBS, and centrifuging the cells into a smaller volume. Genomic DNA was then extracted in a similar manner to that described for other tissues. Genomic DNA restriction digests were peformed overnight in a 37° C bacterial incubator in up to 50 µl final volume.

For dot-blots, 4 - 5 µg of undigested tail DNA was spotted in as small a volume as allowed adequate dissolution of DNA for quantitation (generally 4 to 8 µl) onto dry nitrocellulose (Schleicher and Schuell BA 85, Keene, NH) and the spots allowed to air-dry. The nitrocellulose was then soaked twice for 1 minute each time, in denaturing solution, and then twice for 1 minute in neutralising solution (solutions as described in Maniatis et al., 1982). After allowing to air-dry, the DNA was cross-linked to the nitrocellulose with a UV Stratalinker (Stratagene, Cambridge, MA). The nitrocellulose was then placed between sheets of clear plastic (BelArt Products, Pequannock, NJ) which was sealed around it with an Audion Elektro heat-sealer (Packing Aids Corp. San Francisco, CA), so as to form a plastic bag, and sufficient hybridisation solution (pre-warmed in a 55° C water bath, see below) was added to wet the entire surface of the nitrocellulose. The probe was then added and the bag sealed. The bag was massaged to distribute the probe evenly through the liquid, and to force bubbles to accumulate in a corner so they could be heat-sealed away from the nitrocellulose. The bag was then incubated overnight at 42° C between two plates of plexiglass.

For Southern analyses, DNA was quantitated after digestion by fluorometry, and 5 µg was loaded per lane in loading buffer as previously described. Gels were run as for analyses, but in TAE, and comb-sizes used for pouring gels were such as to allow loading of 40 µl per lane. After running gels they were stained with fresh solutions of ethidium bromide, and photographed on a low-wavelength UV box, and allowed to sit over the UV source for 1 to 2 minutes to nick the DNA and improve the efficiency of transfer. The gel was twice immersed for 20 minutes each time in alkaline denaturation solution and then twice for 20 minutes in neutralisation solution, in a plastic lunch box on a rocking platform (Bellco Biotechnology, Vineland. NJ). Capillary transfer to nitrocellulose was then performed as described in Sambrook et al. (1989). The nitrocellulose was allowed to air-dry and transferred to a dry oven at 80° C for 3 hours. The nitrocellulose was then bagged as described above for dot-blots, but pre-hybridisation solution (see below) added without probe. The bag was placed between plexiglass sheets and incubated at 42° C overnight. The following day, the prehybridisation solution was drained from the bag, and replaced with hybridisation solution, probe added, and the bag sealed as before for a further overnight incubation.

After probe hybridisations, nitrocellulose was removed form the bags and rinsed briefly in approximately 50 mls of "blot wash" (see below) in a flat-bottomed Pyrex vessel. The nitrocellulose was then washed twice, adding approximately 100mls of fresh "blot-wash" each time to the vessel and allowing the contents to rise to 60° C by partially immersing the bowl in a 70° C water bath. The nitrocellulose was removed from the vessel, allowed to air-dry on a piece of Whatman (3MM paper, and then wrapped in Saran Wrap (Dowbrands Canada, Paris, ONT). Adhesive paper was placed on the Wrap, and this was then marked with a ^{35}S-containing ink to assure correct orientation of the auto-radiograph. The ink was made by injecting approximately 10 µCi (370 kBq) of ^{35}S-methionine from ICN into the refill cartridge of a Schaefer fountain-pen. The blot was then exposed to XAR5 film in a an X-omatic cassette (Eastmann-Kodak, Rochester, NY) for several hours to several days at -70° C. The auto-radiograph was developed in a Kodak X-omat processor. Densitometry was performed with a Computer Densitometer from Molecular Dynamics Inc., in the Biotechnology Laboratories of the University of British Columbia.

Prehybridisation solution was made by mixing: 80 mls of SET (see above), 160 mls of deionised formamide (nucleic acid grade, Gibco BRL), 100 mls of 20 x SSC, 40 mls of 50 x Denhardt's solution (both as described in Sambrook et al., 1989), 20 mls of 1M sodium phosphate buffer (pH 6.0), 40 mls of 10% w/v glycine, and 40 mls of a 1.5 mg/ml solution of herring sperm DNA. The mixture was stored at 4° C. Hybridisation solution was made by mixing: 167 mls of deionised formamide, 100 mls of 20 x SSC, 12.5 mls of 1M sodium phosphate buffer (pH 6.0), 100 mls of 50% dextran sulphate, 34 mls of 1.5 mg/ml herring sperm DNA, 40 mls of 50 x Denhardt's solution, and 6.25 mls of 10 x SET. This solution was also stored at 4° C. Blot wash, was stored at room temperature, and consisted of 0.1 x SSC and 0.1% w/v SDS in double-distilled water. Ingredients for these reagents were obtained as follows: Ficoll 400 - Pharmacia, polyvinylpyrrolidone - BDH, bovine serum albumin - ICN, deionised formamide - Gibco BRL, sodium citrate - BDH, glycine - Gibco BRL, monobasic and dibasic sodium phophate - BDH, dextran sulphate - Pharmacia, herring sperm DNA - Sigma.

Molecular weight markers for Southerns were radio-labelled as follows. A 10 x stock of buffer was made by mixing 500 µl of 1M tris (pH 7.5), 150 µl of 1M magnesium chloride, 20 µl of 0.5M dithiothreitol, and 330 µl of double-distilled water. The buffer was stored at -20° C. For the labelling reaction, 2 µl of this buffer was added to 1 µl of the 1kb ladder (BRL), 3 µl of a solution of dATP, dGTP and dTTP (3mM with respect to each), 7 µl double-distilled water, 5 µl of α-^{32}P dCTP, and 2 µl of E coli DNA Polymerase I (New England Biolabs). This mixture was incubated for between 1 and 3 hours at room temperature, and free label separated from labelled fragments by spin column, as above.

C) Sequencing, PCR, and Northern blots

Sequencing of the pLOX^2 plasmid was performed with a commercial primer (Pharmacia) recognising the bacterial SP6 promoter. Sequencing was performed by the dideoxy-nucleotide chain termination method (Sanger et al., 1977) using a Sequenase kit (United States Biochemical, Cleveland, Ohio), according to the manufacturers instructions.

For polymerase chain reactions (PCRs), a 10 x buffer was prepared, consisting of: 200 mM Tris (pH 8.0), 15 mM magnesium chloride, 500 mM potassium chloride (BDH), and 0.1% w/v gelatin (Sigma). The solution was stored at 4° C. A 20 x stock of deoxynucleotide triphosphates (Pharmacia) was prepared consisting of a solution that was 5 mM with respect to each dNTP.

Oligonucleotide primers were synthesised by Mr John Babcook at the Biomedical Resaearch Centre with an Applied Biosystems PCRMate synthesiser (Mississauga, ONT), using reagents from Pharmacia and Applied Biosystems. Oligonucelotides were cleaved and purified as follows: ammonium hydoxide solution (BDH, stored at 4° C) was drawn into the synthesis cartridge with a 1 ml syringe, and allowed to soak the matrix for 20 minutes at room temperature. Then the solution was expelled into a screw-top 1.5 ml tube, and this procedure was repeated 2 more times. The tube was then capped, sealed with Parafilm (American National Can, Greenwich, CT), and incubated in a 55° C water bath for 8 to 15 hours. The cap was removed and replaced with Parafilm, into which a few holes were punched with an 18 gauge needle. The contents of the tube were then dried in a Savant Speedvac concentrator (Savant Instrument Inc., Farmingdale, NY). The contents were then resuspended in 200 µl of TE, and passed through a spin-column (as above without dye). Oligos were then quantitated by measuring their absorbance at 260 nm, and a fomula allowing the calculation of the expected absorbance of a 1M solution of the oligo according to base composition (16,000A + 12,000G + 7,000C + 9,600T = absorbance of a 1M solution, where A, G,C, and T are the numbers of each of these nucleotides residues in the oligo).

PCR was then performed by first mixing 5 µl of 10 x buffer, with 5 µl of 20 x dNTP stock, 4 µl of each primer solution (containing approximately 1 µmol of primer), 52 µl of double-distilled water, and 20 µl of solution containing the template DNA in a PCR tube (Perkin Elmer Canada, Etobicoke, ONT) and adding 50 µl of paraffin oil (BDH). The tube was then incubated at 94° C for 5 to 10 minutes in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). While the contents were warming, a solution was made containing 5 µl of 10 x PCR buffer, 3 or 4 µl of double-distilled water, and 1 µl of Taq Polymerase (5 units, Promega, Madison, WI). 30 cycles of PCR were then performed as follows 94° C for 1

minute, 55° C for 1 minute and 30 seconds, 72° C for 2 minutes. Reaction products were then kept at 4° C until 10 - 20 µl aliquots were analysed by gel electrophoresis.

Reverse-transcription PCR was performed using Moloney murine leukemia virus reverse transcriptase from New England Biolabs, with supplied buffer, according to the manufacturers instructions, in a final volume of 20µl, in the thermal cycler. The entire reaction was then used as template for the subsequent PCR step.

Total cellular RNA was prepared from feshly homogenised tissues or thymus cell suspensions in 6 M guanidium thiocyanate (Pharmacia) and fractionated through a cesium chloride (Gibco BRL) cushion, as described in Chirgwin et al. (1979). RNA was denatured with formamide/formaledehyde, for agarose gel electrophoresis, blotting and hybridisation, as described in Marth et al. (1985).

BIBLIOGRAPHY

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quamtities of protein utilizing the principle of dye binding. *Anal. Biochem.* 72, pp. 420-.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979) *Biochemistry* 18, pp.5294-5299.

Goding, J.W. (1986) Monoclonal antibodies: principles and practice. (Second edition, Academic Press, London).

He, M., Wilde, A. & Kaderbhai, M.A. (1989) A simple single-step procedure for small-scale preparation of *Eschericia coli* plasmids. *NAR* 18, p1660.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* 227, pp 680 -

Maniatis. T., Fritsch., E.F. & Sambrook, J. (1982) Molecular Cloning A Laboratory Manual (Cold Spring Harbour Laboroatory, New York).

Marth, J.D., Peet, R., Krebs, E.G. & Perlmutter, R.M. (1985) *Cell* 43, pp. 393-404

Sambrook, J., Fritsch., E.F. & Maniatis. T. (1989) Molecular Cloning A Laboratory Manual (Second edition, Cold Spring Harbour Laboroatory, New York).

Sanger, F., Nicklen, S. & Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467

Weissman, D., Parker, D.J., Rothstein, T.L. & Marshak-Rothstein, A. (1985) Methods for the production of xenogeneic monoclonal antibodies in murine ascites. *J. Immunol.* 135, pp. 1001-1003.