RETROVIRUS MEDIATED GENE EXPRESSION

IN

HAEMATOPOIETIC CELLS

by

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Abstract

The expression of retroviral vectors in cell lines and primary cells was investigated, with emphasis on autocrine stimulation of haematopoietic cells by GM-CSF. In this study, two distinct retroviral vectors were used which allowed a dual approach to the investigation of expression of exogenous sequences in haematopoietic cells. Retroviral expression of GM-CSF in the growth factor dependent FDCP1 cell line resulted in factor independent colony growth, the frequency of which was related to the vector used. The characterisation of cell lines derived from viral infection of FDCP1 cells revealed that these cells required the concentration of self produced growth factor to attain a critical level before colony development occured. As a result of virus infection, FDCP1 cells which express the retrovirally encoded GM-CSF have a proliferative advantage over the parental cell line.

Finally, a number of variables which might influence the success of retroviral infection of primary murine bone marrow cells were investigated. Initial results suggest that bone marrow cells can be infected with retroviral constructs containing the neomycin gene, the efficiency of which may be related to the origin of the vector.

Thus, this study demonstrates the feasability of using retrovirus vectors to express exogenous sequences in haematopoietic cells. It also provides a system which can be used to evaluate the efficiency with which new vectors will express genes in primary haematopoietic cells.

Abbreviations

bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CFC	colony forming cell
CFU	colony forming unit
CM	conditioned medium
CSF	colony stimulating factor
EDTA	ethylenediamine tetra-acetic acid
FD	factor dependent
FI	factor independent
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/macrophage colony stimulating factor
IL-3	interleukin 3
kb	kilobases
LTR	long terminal repeat
LTMC	long term marrow culture
M-CSF	macrophage colony stimulating factor
MOPS	sodium morpholinopropane sulphonic acid
MPSV	myeloproliferative sarcoma virus
MLV	murine leukaemia virus
MSV	murine sarcoma virus
SDS	sodium dodecyl sulphate

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Chapter 1.1

MAINTENANCE AND REGULATION OF THE HAEMATOPOIETIC SYSTEM

Introduction

The amenability of the haematopoietic tissues for the development of both culture and assay systems has been a key point in studies addressing the mechanisms of differentiation. Thus it has been established that during the process of blood cell formation, stem cells proliferate and differentiate into large numbers of short lived mature cells (Whetton and Dexter 1986). The relationships between these cell types are depicted in Figure 1.1. The mechanisms by which cells as diverse as erythrocytes, macrophages, granulocytes, platelets, megakaryocytes and lymphocytes are derived from a common stem cell has been investigated by in vitro and in vivo clonal assays (Metcalf 1984). These assays have been used to characterise the group of glycoprotein growth factors known collectively as the colony stimulating factors (CSFs) which have been shown to be essential in vitro for proliferation and differentiation of the haematopoietic cells (Sieff 1987, Metcalf 1984).

The Cellular Composition of the Haematopoietic Compartment

Stem Cells.

The haematopoietic hierarchy can be subdivided into three compartments: the stem cell, progenitor cell and mature cell compartments (Metcalf 1984). Stem cells are defined as a pluripotent population capable of producing the entire range of functional mature haematopoietic cells (Till and McCulloch 1980). The stem cell compartment is maintained by self renewal, a property which discriminates this compartment from others (Metcalf 1984). The murine haematopoietic stem cell has been characterised and studied using an in vivo assay (see below).

Murine in vivo stem cell assay.

In 1961 Till and McCulloch established a method for analysing the radiation sensitivity of normal murine bone marrow. This assay relied on mice (whose haematopoietic system had been destroyed by irradiation), receiving an intravenous injection of a small number of bone marrow cells from a healthy donor mouse. Murine haematopoietic

This figure is reproduced from Metcalf, D. (1984)
The precursor cells in each haemopoietic lineage.
Two subsets of self-generating stem cells exist
(CFU-S-1 and CFU-S-11). These give rise to
committed progenitor (colony forming) cells
restricted to one differentiation lineage, or two
in the case of granulocyte-macrophage progenitor
cells. All stem and progenitor cells can form
colonies in semi-solid cultures. CFU-S-1 form
blast cell colonies, CFU-S-11 (Mix-CFC or
Multi-CFC) form mixed colonies usually containing
erythroid cells. All other colony-forming cells
form colonies of restricted differentiation
lineage. Some macrophages, mast cells and T- and
B- lymphocytes retain substantial proliferative
capacity and also form colonies _in_ _vitro_. The
existence and exact origin of stem cells common to
T- and B-lymphocytes are unresolved questions.
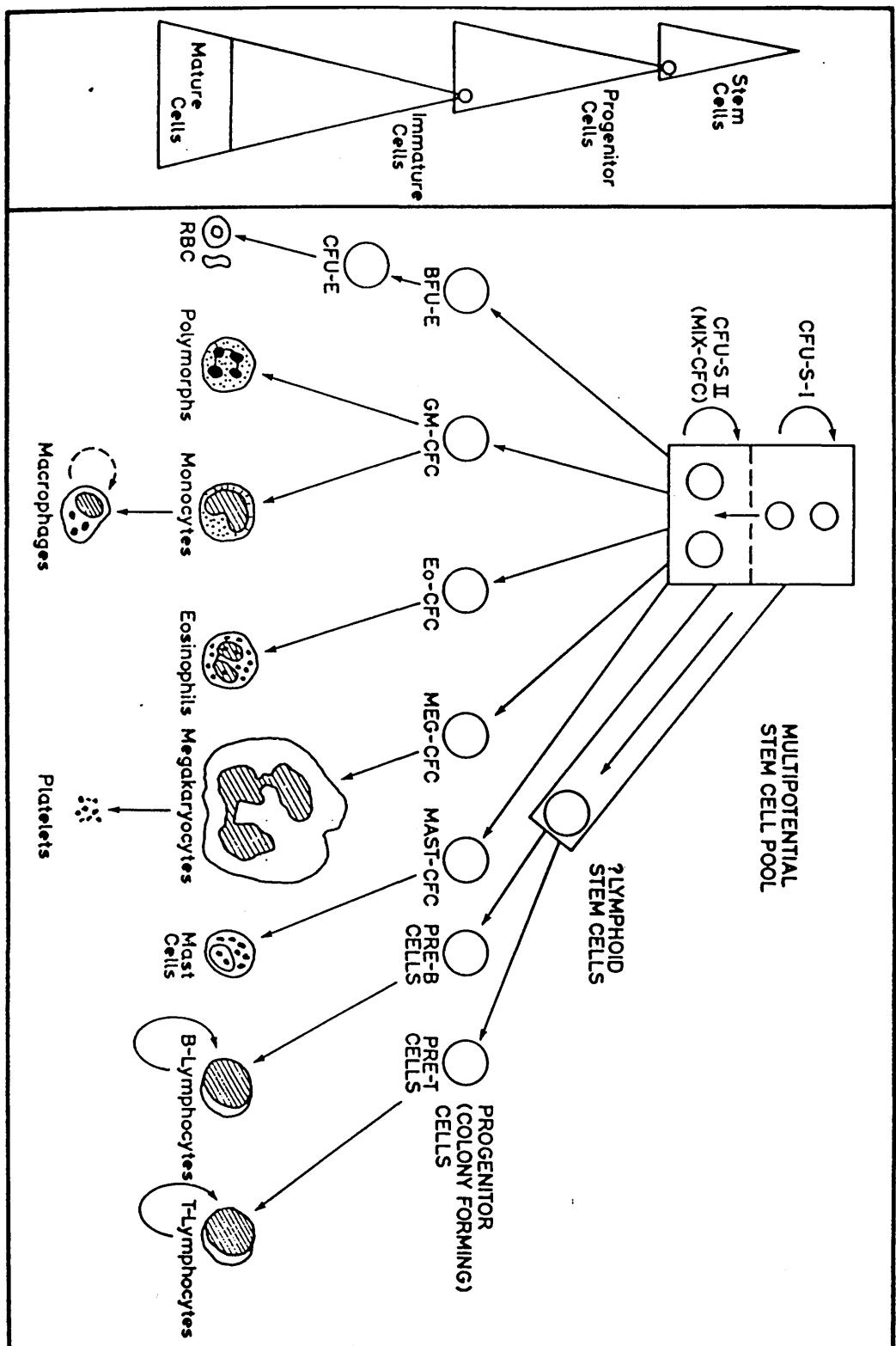
tissue was thus discovered to contain a population of cells that have the capacity to form macroscopic colonies of proliferating haematopoietic cells in the spleens of irradiated recipient mice. However, the bone marrow contains many different cell types, and so the precise cellular origin of the colonies is speculative. Due to this uncertainity these cells are termed colony forming units (CFU) or colony forming units- spleen (CFU-S). Analysis of colony morphology over several days after transplantation showed that on day one the colonies consisted mainly of early and late erythroblasts, undifferentiated cells and small numbers of myelocytes and metamyelocytes. However by day eleven erythropoiesis and granulopoiesis were equally well advanced and megakaryocytes were also observed. This system has allowed the analysis of self renewal and differentiation capacities of the CFU.

Clonal Nature Of The Spleen Colonies

Till and McCulloch (1961) predicted that colonies were clonal by showing a linear relationship between the small number of cells injected (10^4-10^5) and the small numbers of colonies observed (7-12 per 10^5 cells injected). However a minimum transplant of 10^4 cells were required before colonies were observed and this was assumed to be due in part to the presence of a high proportion of differentiated cells in the bone marrow which do not participate in colony formation and in part to lodging and development of CFU in regions other than the spleen. Clonality was formally proven by using donor bone marrow that had been exposed to ionizing radiation (Barnes et al 1968, Becker et al 1963), which causes random chromosome abnormalities in cells, resulting in each individual cell (including CFUs) possessing unique chromosome markers (Barnes et al 1968, Becker et al 1963). The clonal progeny from different CFU s could therefore be distinguished on a cytogenetic basis (Barnes et al 1968, Becker et al 1963).

 Proliferation, Differentiation and Self Renewal of CFU-S

During the first 10 days spleen colonies show extensive proliferation and attain a size of approximately 10^6 cells (Trentin 1971). Up to ten days most colonies exhibit only one differentiated cell type, but from days 11 to 14 an increasing number of colonies consist of two or more different differentiated cell phenotypes

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(Trentin 1971) . Initially it was the high proliferative capacity and the multilineage development that made the CFU a candidate for a stem cell in this system (Trentin 1971) . Siminovitch et al. (1963), studied the self renewal properties of CFU by retransplanting individual primary colonies into secondary hosts. If self renewal occurs within the developing colony then spleen foci should be observed in secondary recipients. In fact colony forming cells are found within individual primary colonies but their distribution is extremely heterogeneous, with 24 out of 36 individual colonies showing no capability to form spleen colonies, while 12 out of 36 show varying degrees of self renewal from 1 to 36 colonies per spleen. This heterogeneity could be a reflection of an intrinsic property of the cell population being assayed. Thus it may be possible that not every CFU capable of forming a primary colony is able to self renew or alternatively variation could be due to sampling errors between spleens. The observation that heterogeneity in numbers of CFU-S per spleen between spleens was not so great using pooled colonies, suggested that the observed differences may be a property of the cells themselves (Siminovitch et al. 1963).

Heterogeneity among spleen colonies with respect to self renewal

Siminovitch et al.(1963) examined a number of parameters in order to explain the heterogeneity described above. It was observed that when self renewal was tested in colonies of different ages ranging from day 10 to 14, the number of CFU s within the colonies increased with age although heterogeneity was present at all time points. Colony size also varied but not enough to explain the overall heterogeneity observed, leaving the question open as to the cause of this variation.

The bone marrow cells which can form primary colonies constitute a mixture of cells with varying degrees of self renewal capacity (Siminovitch et al.1963). If this is a heritable trait of the CFU, then serial transplantation should result in predictable self renewal patterns. This experiment is difficult to carry out as the self renewal capacities of the CFC decrease during repeated passage , perhaps due to dilution out of CFC or accessory cells rather than any alteration in phenotype of the CFC themselves (Schofield and Dexter 1985, Spooncer et al 1985, Ross et al 1982). Alternatively it may be

that not all the spleen colonies arise due to proliferation of a multipotential cell. Single lineage colonies which are observed especially during early time points of colony formation (Trentin 1971) ,may not have reached their full developmental capacity or indeed passed the point where mixed lineages were obvious, so they may appear to be lineage restricted although having arisen from a multipotential cell. It is difficult to assess what is a multilineage colony since it is unlikely that a few macrophage cells would be noticed in a colony that is predominantly erythroid.

The lower self renewal capacity of cells in day 10 colonies compared to that in day 14 colonies suggests the presence of a lineage restricted, low self renewing progenitor cell population capable of forming spleen colonies (Siminovitch et al. 1963). Colony formation may be a dynamic event with subpopulations exhibiting different characteristics each contributing to repopulation of the spleen.

Finally, heterogeneity among CFC with respect to self renewal, might occur during the development of the colony due to alterations in the balance between self renewal and differentiation. Colonies showing no self renewal capacity may have received a strong stimulus to differentiate and the use of irradiated mice may provide localised environments within the spleen which are biased towards differentiation or self renewal (Siminovitch et al. 1963). This point raises the question of the role of the microenviroment in stem cell maintenance, self renewal and differentiation (See Chap.1.3).

Models for haematopoietic development based on the CFU assay.

The haematopoietic inductive microenvironment (HIM) model of Trentin (1971), proposes the multipotential stem cell interacts with the environment to direct the course of development, a theory which is based on the spacial distribution and phenotypic heterogeneity of foci within the spleen. Trentin (1971) observed that spleen foci consisting of predominantly one mature cell phenotype, could on transplantation into secondary recipients, generate spleen foci consisting of many cell morphologies. He concluded that the initial lineage restriction was due to local enviromental influences at the site of the focus, and that the re-seeded CFC became influenced by their individual localities. Till and McCulloch 1980, Till et al. 1964, favour an alternative interpretation of the data available on

heterogeneity within spleen colonies. Their model is called the stochastic model of stem cell proliferation and suggests that each CFC has a random (stochastic) decision either to self renew or differentiate. This infers that the control of stem cell development is very flexible. However if a population of CFC s are examined as a whole then regulation would appear tightly regulated as any individual random decision would be obscured. Under normal conditions the number of mature cells in the blood remains fairly constant (Burgess and Nicola 1983), so if the mature cell compartment was originally derived from very few cells, and a stochastic mechanism was responsible for developmental decisions, then the number of mature cells would fluctuate at random. The only two situations in which a stochastic mechanism could work would be if many stem cells contribute to the maintainance of haematopoiesis, so negating heterogeneity caused by small sample numbers, or, if haematopoiesis was maintained by a few stem cells, the circulating cell numbers would be kept constant by a feedback mechanism acting after their production. This could be achieved by mature cell storage in tissues and organs.

Recent work by Leminschka et al.(1986) has enabled the number of stem cells actively contributing to the mature cell compartment to be assessed. Mice received transplants of bone marrow infected with a retroviral vector. The random integration of retroviruses into host DNA provides a unique marker system in which cells derived from a common precursor can be identified due to the integration site of the virus. These results indicate that very few stem cells contribute to the mature cell compartment at any one time. Although this is preliminary data subject to the sensitivity of Southern analysis, it argues against a stochastic mechanism for the maintainance of haematopoiesis.

Summary of CFU-S experiments.

The observed heterogeneity of CFU s is likely to be due to several causes. The bone marrow used for the initial injection is a mixed population of cells and the cell giving rise to the colonies is undefined (Till and McCulloch 1961). It is likely that the cell population capable of generating spleen colonies is heterogeneous itself and not all are stem cells Siminovitch et al. (1963). If sub-populations could be purified sufficiently or cell lines generated, then this could be examined in more detail. Attempts have

been made to do this, but purification manipulations appear to alter the cells characteristics (Schofield and Dexter 1985, Spooncer et al 1985, Ross et al. 1982).

Different regions of the spleen may provide local environments which regulate differentiation or at least influence the probability of a stochastic decision (Trentin 1971). Microenvironmental control is difficult to examine in vivo and experiments using in vitro culture systems have been designed to address the uncertainty around the control of haematopoiesis. These will be discussed in Chapter 1.3.

All experiments described above were carried out using irradiated mice as an in vivo culture system for studying haematopoietic development, which is clearly not the normal situation. It should be re-emphasised that the cells leading to colony formation are not necessarily the same as those which maintain normal haematopoiesis. These experiments were not specifically designed to examine long term reconstitution of the haematopoietic system and there is a variable degree of animal mortality following irradiation which could lead to experimental bias.

In vitro assays for multipotential cells.

The in vivo stem cell assay has been used to determine factors influencing haematopoietic differentiation (McCulloch et al. 1964, Curry et al. 1967). The assay is however difficult to manipulate and early time points of colony formation are not readily observable (Till and McCulloch 1980). The methodology for achieving clonal growth of bone marrow cells in vitro was originally developed independently by Bradley and Metcalf 1966 and Pluznik and Sachs 1965. In vitro colony assays have permited the analysis of progenitor and stem cell populations which have colony forming capabilities under the appropriate growth conditions. Colony formation takes place within a semi solid matrix such as soft agar or methocellulose when a source of colony stimulating factor (CSF) is added (Metcalf 1984).

Mix-CFU in vitro Assay for mixed erythroid colony growth

Although CFU-S cannot be formally proven as the cell that maintains haematopoiesis, its proliferative, self renewal and differentiation properties are indicative of a stem cell. Multilineage colony growth was first described by Johnson and Metcalf

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in 1977.   Foetal liver cells were used as a source of haematopoietic cells and colony growth was analysed after stimulation by pokeweed mitogen stimulated spleen cell conditioned media (SCM).   Up to 649 erythroid colonies per 10^5 cells were formed depending on the stage of foetal liver development, although only about 50% of these erythroid colonies show mixed lineages.   Erythroid development from lineage restricted progenitors has been shown to be dependent on the presence of erythropoietin (Epo). Any erythropoiesis observed could therefore be due to Epo contamination of the SCM or human plasma which might stimulate a progenitor cell popoulation rather than a stem cell. However, an _in vivo_ assay for Epo failed to detect contamination, which suggested that mixed colony formation was due to direct stimulation of a multipotential cell.

When Metcalf _et al_.(1979) examined the self renewal properties of 7 day mixed colonies from foetal liver by recloning primary colonies in agar,   secondary colonies were observed at a frequency of 1 in 10 with a low or variable replating efficiency.   These colonies were small and were mostly granulocytes and/or macrophages, with only 8 out of 76 daughter colonies being mixed erythroid.   When pools of seven day mixed erythroid colonies were injected into irradiated mice to assay for CFU-S, low numbers were detected (10 CFU-S/ 10^5 cells). These early attempts to characterise _in vitro_ assays for the stem cell population were in part successful, as mixed lineages were detected and self renewal was observed although only at a low level and with some loss of multipotentiality.

Johnson revised this assay in 1980 (Johnson 1980) and made several important changes.   Originally human plasma was used, which resulted in variation between batches in the ability to form and maintain mixed colonies from both foetal and adult tissue.   When foetal calf serum selected for granulocyte/macrophage colony growth was used, and the SCM concentrated 10 fold, erythroid colony growth could be detected in adult bone marrow at a frequency of 22 to 44 colonies per 10^5 cells, in comparison to less than 10 colonies per 10^5 cells with human plasma.   The cell population detected by this assay is termed mix-CFC (Johnson 1980), or alternatively CFU-mix (Harra and Noguchi 1982) or CFU-GEMM (Nakahata and Ogawa 1982).   Its relationship to the CFU-S has been established not only by its colony forming characteristics but also by cell cycle and physical characteristics.

Johnson (1980) showed that 12.5% of adult bone marrow mix-CFU were in S-phase as determined by tritiated thymidine or hydroxyurea suicide experiments, a value similar to that for CFU-S in cycle reported by Vassort et al. 1973. In contrast CFU-GM progenitor cells show a reduction of 31% Johnson (1980). The physical characteristics of the different cells were also analysed. Johnson (1980) and Vassort et al (1973) demonstrated that both CFU-S and mix-CFU have similar bouyant densities of 3.6 to 5.3 mm/h, whereas CFU-GM progenitors which sediment at 4.6 to 6.1 mm/h. The ability of mix-CFU to generatate multilineage colonies and self renew suggests it is a stem cell population, which is heterogeneous and has a very low incidence of true multipotential cells (Johnson 1980). It should be stressed that only 40-50% if the total erythroid colonies are multilineage, and on average only one CFU-S is observed in a single mix-CFU colony (Johnson 1980). It is possible however that the observed heterogeneity and low incidence may not be entirely due to the cell population itself, but may in part be due to culture conditions (Johnson 1980).

Blast Cell Colony Assay

In 1982 Nakahata and Ogawa (Nakahata and Ogawa 1982) identified a new class of colony forming unit which showed stem cell characteristics and formed what are described as blast cell colonies. When bone marrow or spleen cells are incubated for over 10 days in the presence of SCM and Epo most of the developing colonies show differentiation. However, beyond two weeks these colonies begin to disintegrate. Surviving colonies contain 40-1000 homogeneous cells with no apparent terminal differentiation (blast cell colonies). All colonies yielded mixed colonies consisting of granulocytes, erythrocytes, macrophages and megakaryocytes (GEMM colony) after replating (Nakahata and Ogawa 1982). The cells from the primary colony also show high replating efficiencies, and secondary and tertiary blast cell colonies producing further blast cell and GEMM colonies with high efficiency, highlighting the extensive self renewal capacity of these cells. Self renewal was also examined after transfer of the blast cell colonies into irradiated mice. These experiments revealed as many as 47 CFU-S per stem cell colony. The numbers of CFU-S and CFU-GEMM within stem cell colonies closely

correspond to each other and they are therefore perhaps overlapping populations. However when identical analysis was performed on GEMM colonies, no stem cell colonies were observed on replating, and only low numbers of CFU-S were observed within GEMM colonies. The data obtained in GEMM colonies is similar to that obtained by Metcalf et al. (1979) on the clonies from mix-CFC and the populations they describe are probably identical. Nakahata and Ogawa(1982) propose that because of lack of differentiation and the large numbers of secondary GEMM colonies within stem cell colonies, the stem cell colonies arose from a more primitive cell than the GEMM.

The data showing that stem cell colonies occur at a low frequency and are heterogeneous in their self renewal and GEMM forming capacities (Nakahata and Ogawa 1982), has been used to substantiate the stochastic model for stem cell self renewal and differentiation (Nakahata et al. 1982, Ogawa et al. 1983). What would the possible role of the environment in the culture system be? Since colony formation will not occur unless a source of stimulator is added, (in this case SCM), (Nakahata and Ogawa 1982). The growth factor added may be considered to allow cell survival only and not influence the decision to self renew or differentiate. The data could also be explained by the presence of a heterogeneous population which requires SCM to "activate" a pre-existing commitment which has occurred earlier in the cells history either stochastically or induced. Alternatively the SCM and microenvironments within the culture dish could take a more active role in decision making. Soluble factors may act on the heterogeneous population influencing commitment and/or subsequent development. The actions of soluble growth factors will be discussed in more detail in another section.

Colony Forming Unit Type A (CFU-A) Assay

Recently Pragnell and colleagues (1988), have described a new in vitro assay that detects a multipotential cell population, (CFU-A), which has similarities to CFU-S. By incubating a low density of normal bone marrow cells, with a source of GM-CSF and M-CSF, macroscopic mixed lineage colonies develop over 11 days. The multipotential cells (CFU-A) which give rise to such colonies have been shown to have similar cell cycle characteristics to CFU-S, and also respond to specific proliferation regulators of CFU-S. The CFU-A assay is attractive in its simplicity to perform

(normal bone marrow and defined growth factors) and score(macroscopic colonies at a high incidence of 150-200 colonies per 10^5 cells).

The in vitro multipotential cell assays described, have established a methodology by which early cell development can be studied. There are however doubts as to the exact nature of the cell population they detect and both in vivo and in vitro assays show considerable variability in certain characteristics.

Progenitor Cell Compartment and its relationship to Haematopoietic Growth Factors.

The in vitro growth of haematopoietic cells is dependent on a source of colony stimulating factor (Metcalf 1984). Prior to the establishment of in vitro assays for multipotential cells, growth conditions were described that permitted colony growth displaying only one, or at most two, mature differentiated cell types (Pluznick and Sachs 1965, Bradley and Metcalf 1966). These colonies were assumed to be the progeny of early cells which had restricted lineage potential and were termed progenitor cells (Metcalf 1984). This characteristic was used to distinguish them from multipotential stem cells as assayed in vivo (Metcalf 1984). The development of progenitor assays is inextricably linked to the investigation of the interaction of colony stimulating factors with haematopoietic cells (Metcalf 1984). The class of progenitor under examination is defined by the morphology of the resultant colony , which in turn, is dependent on the source and type of colony stimulating factor (CSF) (Metcalf 1984). The actions of CSF (or growth factors) will be described shortly.

In order to resolve the relationship between murine in vivo stem cells and colony forming cells in vitro, cell separation studies must be performed on the heterogeneous haematopoietic population. If a single population of cells with the ability to proliferate in both stem and progenitor assays could be purified , then it would be likely that the assays characterise the the same cells. If, however, cell populations could be separated that showed colony forming ability in only one or other assay, this would provide good evidence for the presence of sub-populations within the haematopoietic compartment and relationships and roles in haematopoietic development could be investigated.

Cell Separation Studies

Worton et al. (1969) used cell density and cell size as parameters to separate murine bone marrow. Fractions from equilibrium density gradient and velocity sedimentation gradients were analysed in the CFU-S and the in vitro progenitor colony forming assay using conditioned media from mouse L cells as a source of stimulator. Two populations of cells were distinguished by cell density and cell size, and CFC progenitors had a higher sedimentation velocity (4.9mm/h) than the CFU-S (3.9mm/h). Although these populations overlapped, the results led Worton et al.(1969) to conclude that at least some of the cells that can form spleen colonies are not detected in the in vitro culture assay and that some of the cells that form colonies in vitro are not detected in the spleen assay. The overlapping distributions of these suggest that perhaps there is a sub-population of cells in the region of overlap which is detected by both assays. This overlap may reflect true variation with each population or it may be the result of variation in the detection efficiency of the two assays used.

Improvements in techniques such as fluorescent activated cell sorting has enabled cell populations to be separated much more efficiently. Fluorescent activated cell sorting (FACS) separates cells according to light scattering properties (Visser and Bol 1981). This property, coupled to either lectin or antibody affinity has clearly defined the existance of subsets of early haematopoietic clonogenic cells (Visser and Bol 1981).

Nicola and Johnson (1982) separated two fractions of clonogenic cells from foetal liver which differed in their lectin binding properties and thus were amenable to fluorescent activated cell sorting. The two fractions were termed the pre-colony forming cell (preCFC) fraction and the CFC fraction. The pre CFC fraction contained many CFU-S but very few cells with the ability to form colonies in vitro (CFCs). In liquid culture this preCFC fraction generated many CFC after stimulation for 5-7 days with either pokeweed mitogen stimulated spleen cell conditioned media (SCM) or postendotoxin serum. In contrast, the CFC fraction contained very few CFU-S but a high frequency of CFC cells. Stimulation of this fraction in liquid culture for 2 days by colony stimulating factors generated large numbers of differentiated progeny and by 4 days few CFC could be

detected.   Thus multipotential cells could be separated from progenitor (CFC) cells and under the appropriate conditions multipotential cells may generate lineage restricted colony forming cells.

It is clear that sub-populations of haematopoietic cells exist. The observation by Nicola and Johnson (1982) that pre CFC (multipotential cells) can generate lineage restricted CFC, offers an experimental system within which factors influencing transitions from multipotential to lineage restricted cells can be examined. The fact that cells are physically separate from those forming spleen colonies in irradiated mice, confirms the theory that the haematopoietic compartment is made up of sub-populations.

Response of Haematopoietic Cells to Colony Stimulating Factors.

In vitro colony growth is totally dependent on a source of colony stimulating factor (Metcalf 1984). Colony growth has been used to define and purify a family of glycoproteins collectively known as the colony stimulating factors (Sieff 1987). Stimulatory activity has been identified in many sources including murine organs and many established cell lines, (Burgess et al.1986, Metcalf and Johnson 1978, Nicola et al.1979, Shrader and Clark-Lewis 1982, Pragnell et al.1988, Stanley and Heard 1977, Schrader and Crapper 1983, Koury and Pragnell 1982), with the most predominant activity resulting in granulocyte and/or macrophage colony growth and   termed granulocyte-macrophage colony stimulating factor (Metcalf 1985b. Purification and molecular cloning has so far identified four distinct proteins capable of sustaining granulocyte/macrophage development, namely G-CSF, M-CSF, GM-CSF and IL-3 (Metcalf 1986, Fung et al.1984, Yang et al. 1986, Nicola et al.1985, Nagata et al.1986, Wong et al.1987, Wong et al. 1985, Stanley et al.1985, Miyatake et al 1985). The full range of these growth factor activities will now be discussed in relation both to one another and other relevant regulation factors, such as erythropoietin (Metcalf and Johnson 1979) and H-1 (Mochizuki et al 1987).

Colonies which differ in their composition of single lineage mature cells, may arise from a single growth factor interacting with different pre-committed progenitor cells, or alternatively, a group of regulatory factors, which themselves differ, could operate on a rather

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more homogenous target cell population, actively influencing the developmental decisions of the cell. These two scenarios in which haematopoietic growth factors might interact with responsive cells to regulate colony formation need not be mutually exclusive and the use of pure growth factors and fractionated cell populations has permitted experiments designed to address such questions to be attempted.

Chapter 1.2

Biological Activities of Granulocyte-Macrophage Colony Stimulating Factor

Introduction

Early observations by Metcalf (1970) indicated the importance of colony stimulating factors (CSFs) in haematopoietic colony development. For example Metcalf (1970) demonstrated that single cell suspensions of bone marrow cells immobilised in soft agar were capable of limited proliferation (up to 16 cells) but died unless CSF from human urine was added. Supplementation by growth factor tends to sustain cellular proliferation with approximately 10% of the initiated clusters of cells progressing to colonies, (clusters are defined as groups of less than 50 cells, whereas colonies comprise of more than 50 cells). The colony morphology follows a predictable pattern and during the first two days colonies are granulocytic but by day seven, macrophage colonies predominate. The number and rate at which new colonies appear increases with the concentration of CSF suggesting that CSF is at least a trigger for colony formation. The delayed addition of CSF to the cultures revealed a 50% loss of viability after only one day and so CSF is also necessary for survival. Colony growth decreases and ceases by day seven but this is not due to growth factor depletion or instability. Experiments in which prior incubation of CSF and re-use of agar previously used to support colony growth were performed which led the author (Metcalf 1970) to rule out these possibilities. Although new colony growth will occur after the addition of fresh cells to agar that has already sustained colony growth, the CSF does show some depletion, which suggested to Metcalf(1970) that perhaps the CSF was actively metabolised.

Analysis of CSF-progenitor cell interactions

The ability to recognise mature granulocytes and macrophages within colonies allowed for the purification of the proteins responsible for this activity (Burgess et al 1986). The lung cells from mice previously treated with endotoxin (MLCM) are a good source of granulocyte, macrophage stimulating activity (Burgess et al 1986, Burgess et al.1977). One of the proteins isolated from MLCM is called granulocyte-macrophage colony stimulating factor (GM-CSF) (Burgess et

al 1986). The protein was initially purified 3500 fold from MLCM and used to stimulate colony growth from murine marrow (Burgess et al 1977). At high concentrations (greater than 20 μg/ml), Burgess et al (1977) observed 3 colony types consisting of granulocytes, macrophages and mixed granulocyte/macrophages. The proportions of each colony type were slightly different from that after stimulation of bone marrow cells by unfractionated MLCM. MLCM stimulates almost equal proportions of all three cell types whereas the pure GM-CSF showed a slight bias towards macrophage colonies. The colony proportions altered more obviously after the concentration of GM-CSF was decreased. This led to the frequency of granulocyte colonies decreasing until at low concentrations (less than 70pg/ml) only macrophage colonies developed (Burgess et al 1977).

It can be concluded from this series of experiments (Burgess et al 1977) that GM-CSF is able to interact with cells in the progenitor compartment that are either bipotential granulocyte-macrophage colony forming cells (GM-CFC s) or unipotential granulocyte CFC s (G-CFC s). The observation that a single pure growth factor stimulated three different colony types (Burgess et al 1977) was held to substantiate previous data on the heterogeneity of the progenitor compartment (Metcalf and MacDonald 1975).

Heterogeneity of the progenitor compartment

Two years previously Metcalf and MacDonald (1975) had used velocity sedimentation to analyse the heterogeneity among bone marrow colony forming cells. They found that the larger, more rapidly sedimenting cells required less stimulation by MLCM in order to produce colonies than the more slowly sedimenting smaller cells. In fact when colony morphology was examined, macrophage colonies formed only from the rapidly sedimenting fractions whereas the proportion of granulocyte colonies rose in cultures of more slowly sedimenting cells. Initially two distinct sub-populations appear to have been separated and these respond to different levels of MLCM. Most colonies which were granulocytic in composition change with the appearance of a secondary population of macrophage cells until the whole colony consists of macrophages. Therefore although the sedimentation technique separates cells which differ in their response to CSF concentration , this may represent a compartment of cells which

differ in their rate of transformation during colony growth from granulocyte to macrophage, rather than qualitative difference in their capacity for differentiation. Macrophage colonies are formed at low CSF concentration and it is the large colony forming cells (CFC s) which respond to low levels of CSF, and so this may explain why such colonies develop. There may then be a prexisting heterogeneity in the bone marrow within which various CSF s might preferentially stimulate selected subpopulations. This data sugests that two such populations which respond to MLCM may exist: a progenitor restricted to the macrophage pathway is activated at low CSF concentration and a bipotential GM-CSF progenitor which, at high concentrations of CSF, will develop into granulocytes and macrophages.

The Influence of GM-CSF on Colony Composition

Metcalf (1980) studied the ability of GM-CSF to influence colony composition by analysis of paired daughter cells derived from a single cell, initiated in a high concentration of GM-CSF. Semi-pure GM-CSF was titred so that 2,500 units stimulated colonies that were 58% granulocyte and 50 units stimulated colonies that were 76% macrophage. Bone marrow cells were incubated in the presence if 2,500 units of GM-CSF for 16 to 24 hours, after which the daughter pairs from dividing cells were removed, separated and transferred to new culture dishes. Subsequent development proceeded in either 50 units or 2,500 units of GM-CSF. Eight out of forty one pairs of daughter cells treated this way generated granulocyte colonies when transferred into high GM-CSF concentration and macrophage containing colonies when moved into low concentrations of GM-CSF. A further six out of forty one pairs gave granulocyte-macrophage mixed colonies on transfer into high GM-CSF concentrations. In comparison 39 out of 48 pairs produced colonies that were identical in composition when both daughter cells were transferred into high levels of GM-CSF (Metcalf 1980). As previously discussed, low levels of GM-CSF predominantly stimulate macrophage colonies and high concentrations of GM-CSF results in granulocyte or mixed granulocytes/macrophage colonies (Metcalf and MacDonald 1975). The data on transfer of paired daughter cells from high GM-CSF concentration suggests that at the time of transfer these cells are still able to respond to the new environment

since in some cases, daughter cells transferred into low GM-CSF conditions develop into macrophage colonies (Metcalf 1980). These results imply that for GM-CSF to influence the morphology of colony formation it may have to remain constant for a period of longer than the initial 16 to 24 hours (Metcalf 1980). This data (Metcalf 1980) does seem to indicate that the progeny of progenitor cells are responsive to environmental changes and so represent a point at which developmental regulation can occur.

Analysis of Commitment to Single Lineages by Haematopoietic Growth Factors

Interaction of GM-CSF and Macrophage CSF (M-CSF)

M-CSF is a 70,000 molecular weight glycoprotein which preferentially stimulates macrophage colony formation (Stanley et al 1983). This growth factor has been examined together with GM-CSF in order to compare the populations of cells stimulated and the manner in which they exert their differentiation properties. Using pure GM-CSF and partially purified M-CSF, Metcalf and Burgess (1982) used a similar approach to that described for the analysis of the influence of GM-CSF on colony composition. Cultures were initiated using either M-CSF or GM-CSF and daughter and grand-daughter cells were either separated after 24 hours or colonies removed after 2 - 3 days and re-cloned in either M-CSF or GM-CSF. Colony morphology was observed and noted over the subsequent incubation period. Colonies maintained in GM-CSF were composed of approximately equal frequencies of granulocyte, macrophage and mixed granulocyte/macrophage colonies, whereas control colonies maintained in M-CSF were 90% macrophage. If M-CSF was used as an initial stimulus for 2 - 3 days, transfer to either GM-CSF or M-CSF resulted in the colonies being 90% macrophage. GM-CSF-initiated colonies gave approximately 50% granulocyte and granulocyte/macrophage colonies when transferred to either stimulus. These experiments indicate that some commitment is occurring during the 2 - 3 day initial incubation (Metcalf and Burgess 1982).

When these types of experiments were carried out after an initial incubation of only 24 hours (Metcalf and Burgess 1982), cells which had undergone only 1 or 2 cell divisions did not show such clear induction of commitment.During the re-cloning of paired daughter cells

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granddaughter cells and colonies, not all survived transfer into different growth conditions (Metcalf and Burgess 1982). Twelve percent of the cells initiated in M-CSF failed to respond to GM-CSF and so were considered to be monoresponsive to M-CSF. The freqency of monoresponsive cells to GM-CSF is approximately 32% by a similar calculation (Metcalf and Burgess 1982). This indicates that at least two-thirds of the granulocyte-macrophage proge nitor cells in the adult marrow are bi-responsive to GM-CSF or M-CSF (Metcalf and Burgess 1982). These data is difficult to interpret as failure to transfer may be due to conditioning of a previously biresponsive cell by the initial stimulus to become monoresponsive and so this frequency of biresponse is probably an underestimate (Metcalf and Burgess 1982).

Lazar et al (1985) have examined the regulation by growth factors of granulocyte/macrophage production using a genetic approach. They observed that two inbred mouse strains C57BL/6J and LP/J differed in their response to CSF. When SCM was used as a source of GM-CSF (Lazar et al 1985) the dose response curves for bone marrow colony forming cells (CFC) for both strains showed a similar plateau value. However the LP/J marrow had a higher response at low levels of SCM. This response was not seen when mouse L-cell conditioned medium was used as a source of M-CSF (Lazar et al 1985) both strains of mice had identical dose response curves. Lazar and his colleagues (1985) interpreted these results to mean that the two CSF's act independently on bone marrow progenitors.

Subsequently, however, Lazar et al (1985) discounted the possibility that two factors used (GM-CSF and M-CSF) were acting on independent populations of progenitors. Concentrations of both factors that gave maximal colony formation were mixed. If both factors were acting on separate populations, the numbers of colonies should be additive (or higher) than the numbers generated by the individual factors. However mixing L-cell conditioned media with SCM showed no significant increase in colony numbers over those observed for the single factors, and so it seems likely that both CSF s act on a single population of progenitors which differs in its response to M-CSF and GM-CSF in LP/J mice. The conclusion that a single biresponsive population of progenitors is the main target for both GM-CSF and M-CSF agrees with the previously discussed work (Metcalf and Burgess 1982).

The increased sensitivity of progenitor cells from the LP/J strain of mice to lower levels of GM-CSF in comparison to C57BL/6J mice, without any obvious differences in total numbers of colony forming cells present in the marrow, could be explained on the basis that the C57BL/6J mice have progenitor cells with receptors which display an altered affinity for GM-CSF, resulting in fewer colonies being produced at low CSF concentrations (Lazar et al 1985). At high concentrations, differences between strains would perhaps be minimal since all receptors would be saturated regardless of affinity (Lazar et al 1985). It is of interest that the preliminary comparisons of circulating blood cells levels did not reveal any significant strain deficiency, which could mean that the CFC response to growth factors is not significant in determining the final numbers of circulating cells. It is possible that the levels of CSFs may be regulated by strain differences , for example LP/J mice may have decreased levels of GM-CSF in comparison to C57BL/6J.

Proliferative Efects of GM-CSF and Granulocyte-CSF (G-CSF)

The discussion has so far been limited to the activities of GM-CSF and M-CSF. Two factors which appear to act on an overlapping population of progenitor cells in vitro. A third growth factor, G-CSF, is associated with the ability to stimulate granulocyte colony growth from bone marrow cells (Metcalf and Nicola 1983) and can induce differentiation of both murine (Metcalf and Nicola 1982) and human leukaemic cells (Vellenga et al. 1987).

Metcalf and Nicola (1983) used G-CSF purified from MLCM to examine its proliferative effects on normal mouse haematopoietic cells. This preparation stimulated small numbers of granulocyte colonies. If the plates were examined after two or three days many proliferating cells which, by day seven had died, were found to be present. It was suggested that G-CSF may sustain the early proliferative divisions of cells destined to differentiate down lineages other than the granulocytic, and without the appropriate lineage specific stimulus these cells die. In order to study this, replating experiments were carried out in which cells initiated for two days in either G-CSF or GM-CSF were transfered to new growth conditions. Thus when proliferating cells, initiated in G-CSF for two days, were transfered to similar G-CSF growth conditions, only 9% of

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the cells survived to produce granulocytic colonies. However, on transfer to dishes containing GM-CSF, 40% of the cells survived. Reciprocal transfer experiments, using GM-CSF initiated cells, resulted in approximately 90% of the proliferating cells surviving transfer . These observations suggest that G-CSF can initiate, but not sustain, the proliferation of many granulocyte-macrophage colony forming cells (GM-CSF's) and is capable of stimulating only a subpopulation of progenitors to form colonies. The effect of G'-CSF on multipotential mix-CFC's and BFU-E erythroid progenitors was also examined by overlaying the G-CSF initiated foetal liver cells with either SCM or erythropoietin. SCM will stimulate erythroid or mixed erythroid colony formation, whereas erythropoietin will not (Metcalf and Nicola 1983). If G-CSF is used as an initial stimulus and erythropoietin is added two days later, no erythroid development is observed. In contrast the delayed addition of SCM resulted in reasonable numbers of erythroid containing colonies as well as eosinophil colonies. It was therefore suggested that G-CSF either stimulates the initial proliferation of multipotential cells, or it permits their survival but not proliferation (Metcalf and Nicola 1983). To discriminate between these two possibilities individual cells were mapped on culture plates and their fate followed during the course of the experiment. The results obtained indicated that most of the mixed and erythroid colonies that developed following addition of SCM arose from single cells that had survived but not proliferated under the influence of G-CSF (Metcalf and Nicola 1983).

In order to define the population of cells that are stimulated to form granulocyte colonies in response to G-CSF, and to relate this to the progenitor populations already known to respond to GM-CSF and M-CSF, colony numbers were counted, either after stimulation of bone marrow by G-CSF alone, or in combination with M-CSF and GM-CSF (Metcalf and Nicola 1983). The combination of G-CSF with either of the other two factors resulted in the formation of more colonies than would have been predicted by the addition of colony numbers resulting from stimulation by either factor alone, suggesting that G-CSF may interact with a sub-population of bone marrow progenitors distinct from either GM-CSF or M-CSF (Metcalf and Nicola 1983).

GM-CSF was previously shown to be capable of stimulating limited proliferation of multipotential precursor cells but unable to

generate mature erythroid of mixed erythroid colonies (Metcalf and Johnson 1979, Metcalf et al 1980). The above data adds G-CSF to a list of factors that have the capacity to allow survival and proliferation of a wider range of cells that was initially suspected from the predominant colony type they generated in vitro.

Proliferative and Differentiation Effects of GM-CSF

The GM-CSF cDNA has recently been cloned (Gough et al., 1984). The cDNA has been expressed in both eukaryotic (Metcalf et al., 1986b) and prokaryotic systems (Metcalf et al. 1986a, DeLamarter et al. 1985, Robinson et al. 1987) thus allowing large quantities of recombinant protein to be purified and its activity compared to native GM-CSF (Metcalf et al. 1986a). The use of prokaryotic expression systems for growth factor production has an advantage over eukaryotic tissue culture systems since there is less likely to be contaminating cellular protein or serum proteins which may act on haematopoietic cells. Proteins produced by bacteria do not however undergo post-translational modification and so cannot be assumed to have the same activity as native GM-CSF (Metcalf et al 1986a). Metcalf et al (1986a) have directly compared pure native GM-CSF, with E.coli produced recombinant GM-CSF (rGM-CSF). Both forms show almost identical activities and stimulate granulocyte and/or macrophage colony formation from both adult and foetal haematopoietic tissue. At high concentrations (greater than 80 pg/ml) both forms stimulate eosinophil colonies, and, at very high concentration (greater than 640 pg/ml) megakaryocyte colonies. The observation that GM-CSF stimulates megakaryocytes could be due to contaminating factors in the high concentrations of GM-CSF used, however, Robinson et al (1987) have also noted megakaryocyte colony formation after simulation by rGM-CSF produced by E.coli at a concentration of around 9 ng/ml. This is a 10-fold lower concentration than that used by Metcalf and his colleagues (1986a). These two independent reports suggest that megakaryocyte colony stimulating activity may also be a function of GM-CSF.

In addition, at high concentration both native and rGM-CSF showed a weak erythroid response on adult bone marrow (Metcalf et al 1986a). This was not observed for foetal liver progenitors. However, as expected, both forms could support the initial proliferation of

erythroid and multipotential cells.

Summary

      The availability of _in vitro_ colony assays have enabled the CSF responsive cells to be identified and analysed. CFC's can be identified by the fact that they divide a short period of time after growth factor stimulation and are recognisable as doublets (Metcalf 1980). Single cell transfer of paired daughter cells has established that a single cell can give rise to a colony consisting of both granulocytes and macrophages (Metcalf 1980, Metcalf and Burgess 1982). This cell is termed the granulocyte-macropage colony forming cell (Metcalf and Burgess 1982). This system has been utilised to determine the effect of sequential exposure to different growth factors (Metcalf and Burgess 1982), and has contributed greatly to our present understanding of the various cell populations in the bone marrow and the range of factors they interact with.

      Both GM-CSF and M-CSF appear to act on a largely overlapping population of progenitors but they differ in effect on the final colony composition (Metcalf and Burgess 1982 , Lazar _et al_ 1985). The use of rGM-CSF has highlighted its ability to stimulate eosinophil and megakaryocyte colony formation (Metcalf _et al_ 1986a, Robinson _et al_ 1987). Thus the interaction between GM-CSF and its target cell population is complex. GM-CSF acts in a concentration dependent manner on GM-CFC's (Metcalf 1980) and can stimulate proliferation of what is likely to be an even earlier cell than the GM-CFC which has the potential to generate erythroid or mixed erythroid colonies (Metcalf _et al_ 1980, Metcalf _et al_ 1986a). Stimulation by a particular growth factor such as GM-CSF or M-CSF not only permits survival and proliferation but can also in some way commit the developing cells within a clony to become lineage restricted (Metcalf and Burgess 1982). Subsequent stimulation of committed cells by another growth factor cannot alter the type of differentiation pathway the cells have entered (Metcalf and Burgess 1982).

      A third factor, G-CSF, shares with GM-CSF the capacity to stimulate a wider range of progenitor cells than its name implies (Metcalf and Nicola 1983). On its own it is unable to sustain fully differentiated colony growth other than granulocytic colonies (Metcalf and Nicola 1983). Granulocytic growth appears to result from

stimulation by G-CSF of a sub-population of GM-CFC's distinct from
that stimulated by GM-CSF or M-CSF alone (Metcalf and Nicola 1983).

Colony stimulating factors have a wide variety of activities.
Not only do they provide a permissive milieu for progenitor cell
survival and proliferation in a partially lineage indifferent manner,
but they can direct cells along particular differentiation pathways.
In fact, once cells are committed to a particular differentiation
pathway, CSF s may again act in a rather lineage indifferent manner to
promote colony formation. The above discussion is an obvious
simplification of the ongoing processes due to differences in specific
activities exhibited by different factors and the inherent
heterogeneity of the haematopoietic compartment.

Interleukin 3 (IL-3)
Introduction

GM-CSF is capable of stimulating the early cell divisions of a
multipotential cell that on subsequent stimulation by an appropriate
factor can form a mixed erythroid colony _in vitro_ (Metcalf _et al_
1980). By itself GM-CSF shows only a weak and perhaps artifactual
ability to generate erythroid or mixed erythroid colonies (Metcalf _et_
_al_ 1986a). The _in vitro_ multipotential stem cell assay requires
stimulation of the mix-CFC by lectin stimulated ← spleen cell
conditioned media to generate erythroid colonies (Johnson 1980). This
activity is also detected in the conditioned media from the murine
WEHI 3B myelomonocytic cell line (Bazill _et al_ 1983). The injection
of cells derived from mix-CFC colonies can lead to the formation of
spleen colonies suggesting that at least some of the mix-CFC
correspond to multipotential stem cells (Johnson 1980), and it is
these cells that may be targets for the CSF in SCM or WEHI 3B
conditioned media, (Iscove _et al_., 1982). The conditioned media from
SCM exhibits four biological activities as assessed by colony growth.
These are granulocyte/macrophage, eosinophil, megakaryocyte and
erythroid colony stimulating activities (Clark-Lewis _et al_ 1984).
These activities may be a result of a variety of different factors
acting on haematopoietic precursor cells or alternatively, one
molecule may be active on an early multipotential cell and its
progeny. This activity has been variously referred to as burst
promoting activity (BPA), multi-CSF, interleukin 3 (IL-3), mast cell

growth factor (MCGF), P-cell stimulating factor and haematopoietic growth factor (Ihle et al. 1983).

Purification of The Molecule Responsible For BPA

Protein purification of SCM indicated that some of the eosinophil and granulocyte/macrophage activity could be attributed to separate factors but most of the eosinophil and granulocyte/macrophage activity was associated with erythroid and megakaryocyte colony stimulation (Cutler et al. 1985). These activities have subsequently been purified to homogeneity from SCM (Cutler et al.,1985) and WEHI conditioned media (Ihle et al., 1982), with retention at all stages of the multilineage activity. The cDNA coding for IL-3 has been cloned from both the WEHI 3B (Fung et al. 1984) and normal T cells (Yokota et al. 1984) and it seems that IL-3 production is closely associated with T cells (Schrader and Clark-Lewis 1982, Young et al. 1987). These cells may therefore be important in haematopoietic regulation. Proliferation and molecular cloning has enabled the range of actions ascribed to IL-3 to be examined in detail and these will be discussed below.

Biological Activities of IL-3
Stem Cell Interactions

Interest in IL-3 was stimulated when it was observed that IL-3 was the only CSF that supported the proliferation and differentiation of such a diverse number of mature cell types which sugested it might interact with a multipotential stem cell (Hapel et al 1985). Current experimental evidence to substantiate a direct interaction with a stem cell is poor. However, the availability of pure recombinant IL-3 (rIL-3) (Sieff et al. 1987) should enable further experiments to be carried out. Schrader and Clark-Lewis (1982) described an in vitro liquid culture system in which factors that modulate stem cell survival can be monitored by incubation with bone marrow for seven days. Stem cell numbers were subsequently measured by standard in vivo spleen cell focus forming assay (Till and McCulloch 1961). The conditioned media from SCM and a T-cell hybrid produced an activity distinct from GM-CSF or IL-2, which was found to promote survival of CFU-S in liquid culture. A source of GM-CSF used as a control did not stimulate CFU-S survival. It is likely

considering the source, molecular weight and biological activities of this factor that it is identical to IL-3 (Schrader and Clark-Lewis 1982).

Nicola and Johnson (1982) used FACS to separate haematopoietic cells into preCFC and CFC fractions, (as described previously). The pre CFC fraction contained the majority of CFU-S and very few CFCs, and the initial CFC fraction contained very few CFU-S but had many progenitor CFCs. Stimulation of this fraction by SCM resulted in mature differentiated cells being produced with the loss of CFCs. The production of lineage restricted progenitors from the CFU-S containing fraction was found to be dependent on SCM and therefore the SCM was considered to elicit a differentiation effect on the stem cells present in the preCFC fraction allowing them to produce progenitor cells (Nicola and Johnson 1982). Using a source of GM-CSF (MLCM), no such effect was observed. Therefore a factor or factors present in SCM but not MLCM could interact with members of the stem cell compartment. Pure IL-3 from SCM added to this assay system resulted in a similar pattern of differentiation as impure SCM (Cutler et al. 1985).

The in vitro blast cell colony assay (Nakahata and Ogawa 1982) has been used to assess the role of IL-3 in multipotential cell proliferation and differentiation. As described in a previous section the blast cell colony assay detects multipotential cells present at low frequency in haematopoietic tissues by their response to SCM (Nakahata and Ogawa 1982). It is possible to substitute IL-3 for SCM in the assay (Suda et al. 1985). Koike et al. (1986b) demonstrated that blast cell colonies were sensitive to low levels of IL-3, whereas the multilineage and progenitor colonies of differentiated cells were dependent on high levels of IL-3. If the addition of low levels of IL-3 was delayed until seven days after the commencement of incubation, blast cell colonies proliferate as if stimulated from day one. Although a reduction of colonies derived from more mature progenitors was observed, the number of blast cell colonies was the same as in control cultures. Koike and colleagues (1986b) concluded from these results that IL-3 provides a permissive environment for the proliferation of an early population of stem cells , but is not required for their survival if they are non-cycling. This conclusion assumes that stem cells enter the cell cycle at random and then

require IL-3. The blast cell colonies observed after delayed addition of IL-3 are apparently derived from multipotential cells that have remained out of cycle until IL-3 was supplied. The lack of any detectable drop in blast cell colony numbers between control and delayed addition plates implies that very few stem cells are cycling and any that are cycling prior to IL-3 addition would be lost but not observed due to experimental variance. The observation that mature colonies require higher concentrations of IL-3 to survive than blast cell colonies implies a declining sensitivity to IL-3 as stem cells develop into mature cells. How growth factors interact physically with cells and how specific differentiation messages are transmitted will be discussed in Chapter 1.4. However declining sensitivity may be due to changes in receptor number or affinity for IL-3 as the cell differentiates or , an alternative possibility could be that early progenitor cells transduce the signal elicited by IL-3 more efficiently (Koike et al 1986b).

Differentiation Patterns of Cells Responding to IL-3
IL-3 and Erythropoiesis

Most in vitro systems used to study colony growth require various concentrations of sera which may contain erythropoietin or other growth factors which could act synergistically with, or mask, the effect of any added growth factor. Suda et al. (1986) used a serum free system to study the combination of pure IL-3 and erythropoietin (Epo) on an enriched source of haematopoietic progenitors. In the absence of Epo, IL-3 alone could produce multilineage colonies of which 3 out of 29 contained a low percentage of erythroid cells. No colonies were formed by Epo alone, but the combination of IL-3 plus Epo produced colonies of which 31 out of 33 contained erythroid cells. Single cell transfer experiments showed that IL-3 was able to maintain the growth of multipotential cells and support the terminal differentiation of neutrophils, macrophages, eosinophils and megakaryocytes without additional growth factors. Terminal differentiation of erythroid cells were not observed as added Epo is necessary for maximal erythroid differentiation.

The detection of erythroid or mixed erythroid colonies is in part dependent on the source of haematopoietic tissue. The authors cited above use the spleens from adult mice treated with the

cytotoxic drug 5-fluorouracil (5FU), which has been demonstrated to cause a transient enrichment for early precursor cells (Bradley and Hodgson 1979). Foetal liver is another source of enriched early precursor cells (Metcalf and Johnson 1979) and it has been reported that erythroid colonies could be detected in the absence of detectable Epo (Suda et al.,1985;1986,Johnson and Metcalf 1977, Hapel et al 1985, Metcalf et al 1987a). It cannot be ruled out that in these cases IL-3 stimulates the release of Epo from cells present within the agar matrix resulting in erythropoiesis (Zucali et al 1987, Mangan et al 1982, Sieber and Sharkis 1982). Alternatively even though some experiments were carried out " serum free", the growth factor preparation may be contaminated by other CSFs or bacterial endotoxin.

With respect to the ability of IL-3 to stimulate erythroid development, the data is confusing (Suda et al. 1985;1986 ,Johnson and Metcalf 1977, Hapel et al 1985, Metcalf et al 1987a, Sieff et al., 1980). IL-3 could conceivably be capable of supporting terminal differentiation of erythroid cells, but this may be only a sub-population of the cells which can generate erythroid colonies under other stimulatory influences. The proportion of pre-erythroid cells that are responsive to IL-3 on its own may be very small and vary between tissues of the adult and foetal mouse. The observed erythropoiesis could also be a result of an indirect action of IL-3 on a varying populations of cells that respond to IL-3 by producing Epo, or purely artifactual due to contaminating material. At present it seems reasonably clear that Epo is necessary for optimal erythroid or mixed erythroid colony growth in conjuction with IL-3 or GM-CSF (Sieff et al., 1985, 1986).

IL-3 TARGET CELL POPULATION

Although IL-3 has been demonstrated to generate colonies of diverse mature cell types which suggests it can act on a multipotential cell (Clarke-Lewis et al 1984), M-CSF was considered to act predominantly on a unipotential or bipotential progenitor cell that has been derived form a multipotential cell (Metcalf and Burgess 1982). Koike et al. (1986a) have tried to determine a link between cell populations on which various factors act. M-CSF generated more macrophage containing colonies from normal bone marrow than IL-3. When a population of bone marrow cells enriched for primitive cells

was subjected to stimulation, IL-3 produced more macrophage colonies.
Thus it was interpreted by Koike et al. (1986a) that both IL-3 and
M-CSF could support macrophage colony growth, but IL-3 acted on a more
primitive cells than M-CSF. In order to define whether the two
populations were separate or overlapping, IL-3 and M-CSF were mixed
and macrophage containing colonies counted. The number of colonies
after stimulation by both factors together was close to or in excess
of the sum of the number of colonies supported by M-CSF or IL-3 alone,
which suggests either that the target cell populations are different,
or alternatively, there is a population of cells that require both
factors for their proliferation. In addition to the increase in
colony numbers, it was noted that the combined growth factors resulted
in larger colonies than generated by the separate factors, and so even
if the two factors act on distinct populations their activities may
overlap. The delayed addition of M-CSF to IL-3 initiated cultures
indicated that factors can act sequentially on developing colonies to
increase the final colony size.

Bartelmez et al (1985) analysed liquid cultures enriched for
primitive or mature cells for their responses to IL-3 and M-CSF .
Addition of IL-3 to the culture of primitive cells resulted in an
increase in the total number of M-CSF binding cells , whereas addition
of M-CSF did not. In cultures of more mature cells, M-CSF generated
approximately 17 times more M-CSF binding cells than IL-3. Taken
together this data indicates that IL-3 can act on a more primitive
cell than M-CSF to generate macrophage colonies. There are probably
two distinct populations that respond to either IL-3 or M-CSF, but
IL-3 may generate the more mature progenitors that M-CSF can
stimulate. This would explain why IL-3 initiated colonies respond to
M-CSF to generate larger colonies than either factor alone (Koike et
al 1986a). In contrast,as previously discussed, the populations of
cells that GM-CSF and M-CSF stimulate show almost a complete overlap
(Metcalf and Burgess 1982) and so comparison of target cell
populations may represent a distinguishing point between IL-3 and
GM-CSF.

Sieff and his colleagues (1987) carried out a direct
comparison between the ability of IL-3 and GM-CSF to sustain the
proliferation of BFU-E erythroid progenitor cells in the presence of
Epo. Using recombinant gibbon IL-3, human GM-CSF and human marrow or

foetal liver cells, the authors showed that IL-3 generated 25% more BFU-E erythroid colonies than GM-CSF. The addition of both IL-3 and GM-CSF to bone marrow cells together did not result in stimulation of more BFU-E than IL-3 alone. It was therefore suggested that there are two populations of BFU-E one of which is responsive to IL-3 alone and another population which responds to both IL-3 and GM-CSF. The relationship between IL-3 and G-CSF was examined in the same study (Sieff et al 1987). G-CSF showed no capacity to produce erythroid colonies and the addition of both IL-3 and G-CSF together did not result in any increase in BFU-E colonies above those stimulated by IL-3 alone (Sieff et al 1987). In comparison to this both factors together generated more granulocyte colonies than either IL-3 or G-CSF alone (Sieff et al 1987), suggesting there may either be distinct sub-populations of cells that respond to G-CSF or IL-3 or a population that requires both. The granulocyte colonies which develop after dual stimulation are larger than those which are stimulated by single factors which implies that at least some colonies respond to both IL-3 and G-CSF during their development. The comparison between GM-CSF and G-CSF previously discussed (Metcalf and Nicola 1983) noted that GM-CSF probably acts on a distinct population from G-CSF, or both act together on a single population. In this respect GM-CSF and IL-3 have a similar relationship to the progenitor cell population specific for granulocyte colonies.

SUMMARY

IL-3 is a growth factor that has a broad range of proliferative effects on primitive haematopoeitic cells (Metcalf et al 1987). It can directly stimulate granulocyte, macrophage, eosinophil and megakaryocyte colony formation (Metcalf et al 1987). In addition it can stimulate multipotential precursors of mixed erythroid colonies (Suda et al 1986) . Both G-CSF (Metcalf and Nicola 1983) and GM-CSF (Metcalf et al 1980) promote the survival and proliferation of a number of immature cells, a function not initially apparent from the colonies they produce. GM-CSF is most closely related to IL-3 when the wide range of colonies it stimulates is considered (Metcalf et al 1986a). However, they can be distinguished quite clearly after comparison between the target cell populations these factors interact with on their own or in conjuction with other factors . Although both IL-3 and GM-CSF stimulate early multipotential cells, IL-3 probably

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influences a slightly more primitive cell (Nicola and Jonson, 1982). It is difficult to make direct comparisons between IL-3 and GM-CSF as the information for the most part must be completed from different sources. With the improvement of techniques and the availability of pure growth factors, systematic studies should now be possible to elaborate on the relationships between different CSFs.

Chapter 1.3

Introduction To Long Term Marrow Culture (LMTC)

LTMC (Dexter et al 1977a,b) are established by forming an adherent layer of stromal cells in vitro. This layer has been shown to comprise of a mixture of different bone marrow derived endothelial cells, adipocytes and macrophage cells (Dexter et al 1977a,b, Allen and Dexter 1984). The adherent layer is established when bone marrow cells are inoculated into tisssue culture flasks and incubated in the presence of carefully selected serum (Dexter et al 1977a,b). During the first three week establishment period the numbers of non-adherent cells, CFU-S and progenitor cells decline and after three weeks the culture is re-plenished with fresh bone marrow (Dexter et al 1977a,b). These cultures will now support the long term proliferation and differentiation of stem cells, including CFU-S, for several months (Dexter et al 1977a,b). During this period the ratio of stem cells:CFU-GM:mature cells is approximately the same as found in vivo (Boettiger et al 1984).

Multipotential CFU-S are found both in the adherent stromal layer and the non-adherent cells (Dexter et al 1977a). On feeding, half the culture medium is removed thus depleting the system of non-adherent stem cells (Dexter et al 1977a). Subsequently the numbers of non-adherent CFU-S rise again, an increase which is mirrored by an increase in the proportion of cycling CFU-S stem cells (Dexter et al 1977a,b, Dexter et al 1980b). One day after feeding, 40% of the CFU-S are in cycle but by day seven this has dropped to 10% (Dexter etal 1977b). The self renewal of adherent stem cells thus replaces stem cells lost by release into the culture medium (Dexter et al 1980b). The increasing number of non-adherent cells could participate in a feedback mechanism switching off proliferation of stem cells (Dexter et al 1977b).

Role of the environment in haematopoesis

An absolute requirement for an adherent stromal in LTMC (Dexter et al1977a,b), suggests that specific cellular interactions are necessary for culture development (Allen and Dexter 1984). Some of the most compelling evidence supporting the role of the environment in haematopoeisis comes from the study of genetically determined haematopoietic defects (Dexter and Moore 1977, Tavassoli et al 1973,

Bernstein 1970). Macrocytic anaemia is observed in two strains of mice carrying different mutations, (w/w^V and sl/sl^d mice) (Bernstein 1970). The anaemia observed in w/w^V mice is caused by an intrinsic defect in the stem cell population and bone marrow from these mice is therefore unable to rescue irradiated recipient mice (Dexter et al 1980b, Bernstein 1970). In contrast, the abnormal haematopoeisis observed in sl/sl^d mice is due to a defective microenvironment (Dexter et al 1980b, Bernstein 1970). Bone marrow cells from sl/sl^d mice will form spleen colonies in irradiated mice but the anaemia in these recipients cannot be cured by injection of normal bone marrow stem cells (Dexter et al 1980b, Bernstein 1970, Trentin 1971). The anaemia observed in w/w^V mice (normal environment, defective CFU-S) can be corrected by injection of stem cells from sl/sl^d mice (defective environment, normal CFU-S) (Bernstein et al 1968). However the anaemia in sl/sl^d mice can only be alleviated by transplanting w/w^V haematopoietic environment tissue i.e. spleen tissue (Bernstein 1970, Tavassoli et al 1973. These in vivo effects can be reproduced in vitro using LTMC (Dexter and Moore 1977). These observations therefore underline the importance of the stromal microenviroment for the maintenance of normal haematopoiesis.

In an attempt to define more rigorously the complex interactions between stem cells and the microenvironment, Dexter and his colleagues have used FACS purified stem cells (Spooncer et al 1985) and multipotential cell lines (Wyke et al 1986) to repopulate feeder layers consisting of mouse fibroblast 3T3 cells. Addition of FACS purified stem cells to monolayers of 3T3 cells results in their proliferation and differentiation as if the cells had been seeded on to normal stromal cells (Roberts et al 1987). Although the FACS purified cells used in these experiments contained between 50% and 100% CFU-S, the survival and differentiation on 3T3 cells could be a result of "contaminating" bone marrow cells (Roberts et al 1987). The availability of cell lines which retain the characteristics of multipotential stem cells has made the investigation of cell to cell interactions simpler (Wyke et al 1986). These lines, termed FDCP-mix, have been established from LTMC after infection with v-src retrovirus preparations, and can differentiate into mixed lineage colonies (Wyke et al 1986). FDCP-mix cells are dependent on the presence of IL-3 for their survival and proliferation in vitro (Wyke et al 1986). However,

they can be maintained on established bone marrow stromal layers without the addition of IL-3 (Roberts et al 1987). The 3T3 culture system described above also supported the normal development of FDCP-mix cells in the absence of IL-3 but could not support another IL-3 dependent cell line, FDCP-2 (Dexter et al. 1980a, Roberts et al. 1987). Since both of these cell lines require IL-3 for their growth in vitro, it seems likely that IL-3 is not the active factor maintaining FDCP-mix cells in the 3T3 system (Roberts et al. 1987). It is possible however that the FDCP-2 cell line cannot utilise any IL-3 in the form in which it is presented by the 3T3 cells (Roberts et al. 1987). In fact, the conditioned media from 3T3 cells was found to be unable to support FDCP-mix or FACS CFU-S cells (Roberts et al. 1987). These results suggest that if any critical growth factor is released, then it is present in very small amounts. It is most likely therefore, that cell to cell interaction is an essential aspect of stem cell maintenance (Roberts et al 1987). This simplified system again highlights the absolute requirement for cell to cell contact and membrane intergrity for stem cell survival and differentiation. It is even possible that metabolic activity may not be an absolute requirement in the short term. If growth factors are involved in this system they may be membrane bound, and so be available to stem cells even after gluteraldehyde fixing of the feeder layer (Roberts et al 1987).

Stromal Cell Production of CSF Activity

There is some evidence that cells which reside in the marrow , but which are not colony forming cells (Hultner et al. 1982, Chan and Metcalf 1972 , and bone marrow stromal cell lines (Naparstek et al 1986, Tsai et al 1986) can release haematopoietic growth factors, which would seem to contradict the results obtained for LTMC outlined above. Although it can be argued that cell lines have undergone many changes during their establishment and that any growth factor release is an abnormal. However, it is also possible that in normal bone marrow or in LTMC the growth factors are either membrane bound, or released in very low levels that cannot be detected using the current assays. The cell to cell contact in LTMC may therefore optimise the transfer of growth factors and allow specific homeostatic activities to occur that maintain the regulated balanced development. Recently

Gordon and her colleagues (1987) demonstrated that although CSF activity could not be detected from the conditioned media of LTMC stromal cells layers, dialysed salt extract from these cells contained detectable CSF activity. These salt treated stromal layers were then tested for their ability to bind exogenous GM-CSF. These experements showed that that not only did stromal material bind exogenous GM-CSF, but that bone marrow stromal extracellular matrix also had this capacity (Gordon et al 1987). Thus the compartmentalisation of growth factors within the stromal layer and subsequent interaction with target cells via cell to cell contact can be envisaged. This data goes some way to reconciling the apparent discrepancies in the roles attributed by haematopoietic growth factors in different in vitro culture systems.

The possibility that membrane bound forms of haematopoietic growth factors exist, and can interact with target cell populations over short distances can now be tested. The cDNA for human macrophage-CSF (M-CSF) has been introduced into 3T3 fibroblasts resulting in the release of detectable levels of CSF activity in the growth medium (Rettenmier et al 1987). Using an antibody to M-CSF , the kinetics of protein synthesis were analysed and M-CSF was found in the culture medium after a lag period during which the protein was only detected in an intracellular form (Rettenmier et al 1987). Indeed, the secreted protein was found to be smaller in size than the intracellular protein , thus these results sugest that the release of mature protein into the culture medium may require proteolysis (Rettenmier et al 1987). It is of notable interest that trypsin treatment of M-CSF expressing 3T3 cells increased the detectable amount of M-CSF in the culture medium (Rettenmier et al 1987). The membrane bound form of M-CSF could thus be envisaged as exerting its effect only at points of cell to cell contact (Rettenmier et al 1987). The demonstration that such a membrane bound form of M-CSF exists in this case supports this hypothesis.

The Role of Diffusable Regulators in LTMC

Clonal growth of haematopoietic cells in vitro (Metcalf 1984) and the short term survival of CFU-S (Schrader and Clark-Lewis 1982) has been shown to be dependent on a source of colony stimulating factor (CSF). It therefore follows that LTMC may produce their own CSF in order to maintain the culture. However both conditioned media from

feeder layers or "populated" cultures fail to support colony growth (Dexter et al. 1977d). It is possible that the levels of CSF are too low to be detected, or that any CSF released is metabolised very quickly. Therefore in an attempt to optimise conditions Dexter and his colleagues (1977d) added CSF in the form of mouse embryo or heart conditioned media to the LTMC. Feeding the cultures with exogenous conditioned media caused a dramatic decrease in CFU-S and progenitors after only one week, followed by their virtual disappearance after three weeks in culture (Dexter et al. 1977d). These results suggest that perhaps the balance between self renewal and differentiation observed in the culture is affected by the levels of CSF added resulting in a shift in the balance towards differentiation . More recently, the addition of pure GM-CSF or L-cell conditioned media to cultures did not result in any such decline in stem cell self renewal, nor did the addition of antisera raised against L-cell growth factor disturb the cultures in any detectable way (Dexter et al 1980b). These results imply that cultures are sensitive to some sources of CSF. Other sources, such as GM-CSF, have no detectable effect and as a result the GM-CFC progenitor cells may be under very tight control in this system (Dexter et al. 1980b). It is possible that any effect that occurs may be masked by dynamic changes in cell numbers due to homeostatic regulation in response to GM-CSF activity. However these experiments do not give any positive evidence for the role of CSF in the maintainance of LTMC.

Early observations by Lord et al. (1976) demonstrated the presence of inhibitor of stem cell proliferation in normal bone marrow, which was also detected in LTMC (Dexter et al 1980b). Dexter et al. (1980b) observed that after feeding LTMC, the proportion of cycling CFU-S increased from 10% to 35% is associated with two activities detected after Amicon filtration of LTMC conditioned media. Amicon filtration separated two activities, a stimulator of stem cell proliferation in fraction III, and an inhibitor of stem cell proliferation in fraction IV (Dexter et al 1980b). The stimulatory activity in fraction III observed from the first day after feeding conditioned media was found to be active at 50μg/ml and could increase the steady state (10%) of CFU-S in cycle to greater than 35% (Dexter et al. 1980b). In comparison seven day conditioned media also stimulated stem cell proliferation but only at a level of 100 μg/ml.

Similarly fraction IV from day one post-feeding showed an inhibitory effect at 200 μg/ml, whereas the corresponding factor from day seven media was more active in its inhibition at a lower concentration (Dexter et al 1980b). The relative activities of these factors, with stimulator being more effective in day one media and inhibitor of stem cell proloferation more active in day seven media, corresponds to the percentage of CFU-S in cell cycle during this period (Dexter et al 1980b). These experements showed that the proportion of stem cells in S-phase is high immediately after feeding (day one) and then drops to 10% by day seven, suggesting that modulation of the stem cell inhibitor and stimulator levels is required in order to maintain haematopoeisis in LTMC (Dexter et al 1980b). The removal of stem cells during feeding leads to the production of stimulator, an effect which is still observed when fresh media containing cells previously removed, were returned to the cultures (Dexter et al 1980b). It is therefore unlikely that the cells themselves provide the stimulus. The authors suggest that perhaps a disruption in cell to cell interactions caused either by simple mechanical agitation, differentiation, migration,or death might be the signal for inhibitor/stimulator production (Dexter et al 1980b).

It is possible to extend this hypothesis to the regulation of normal haematopoiesis in the bone marrow where the proliferation of stem cells also can be influenced by inhibitor and stimulator levels . Inhibitor/stimulator levels may thus fluctuate in a regulated manner in order to achieve the correct number of stem cells required to maintain haematopoiesis. The same effect could also be achieved if the level of say inhibitor remained constant and a corresponding alteration in the level of stimulator occured in response to stem cell demands. An alternative hypothesis sugests that different regions of the bone marrow produce these proteins and cells move from niche to niche during development they become exposed to different microenvironments, and different developmental stimuli.

Summary of the role of growth factors and microenvironment in haematopoeitic development.

Conclusions as to how haematopoietic development may be controlled differ depending on the experimental system used. LTMC experiments appeared to suggest that CSF s are not required in

haematopoietic development (Dexter et al 1977a,b), whereas in vitro assays with semi-solid media are CSF dependent (Metcalf 1984). The characterisation of stimulators and inhibitors of stem cell proliferation in LTMC (Dexter et al 1980) and bone marrow cells (Lord et al 1976, Lord and Wright 1980), as well as CSF activity from stromal cells (Tsai et al 1986) has begun to resolve many inconsistencies between the two systems. For example the bone marrow stroma was previously considered to be a physical niche within which stem cells develop (Till et al 1964) but is now considered as an environment in which biochemical interactions can take place (Allen and Dexter 1984). Molecules which modulate stem cells or stem cell progeny may be distributed in compartments or concentrations throughout the stroma, as may the clonogenic population itself. It is quite conceivable that the levels of these regulatory molecules are not static but are modulated in response to the demands on the system (Lord and Wright 1980, Dexter et al 1980). During normal steady state haematopoiesis it is arguable whether much control is necessary if the rate of production of new mature cells is equal to that of cell death.

Whether the identified glycoprotein CSFs are responsible for the maintainance of normal haematopoiesis in vivo is also debatable. Certainly GM-CSF activity has been detected in the serum and tissues of mice (Burgess et al 1986, Metcalf and Johnson 1978, Nicola et al 1979) but IL-3 has only been detected after stimulation of T-cell populations in vitro (Schrader and Clark-Lewis 1982 Young et al 1987). The presence of these activities could be connected with the role of some CSF's in mature granulocyte and macrophage activation (Lopez et al 1983, Grabstein et al 1986).

Continuous infusion or repeated injection of GM-CSF (Metcalf et al 1987b) and IL-3 (Metcalf et al 1987a, Kindler et al 1986) into mice shows that exogenous CSF can elicit a response in vivo but these experiments are difficult to interpret. It is debatable whether endogenous CSF would ever be maintained in vivo at a continuous level similar to that achieved by exogenous administration. Whether intraperitoneal or intravenous injection of CSF ever reaches the "normal" sites for haematopoiesis is not clear. It is important to remember that the cells which can respond to CSF experimentally are not necessarily the same as the ones which do respond in vivo during normal haematopoiesis, a caveat which applies to many of the

experiments described so far.

Although growth factors can be functionally distinguished by criteria such as target populations and the predominant colony morphology, they do appear to overlap in their functions (Chap.1.2). It is possible that _in vivo_ their full developmental activities are never exploited except under very rare circumstances. Therefore each factor may in effect be considerably more restricted in activity. IL-3, for example, may be an "emergency" factor which can stimulate primitive cells to differentiate along several lineages. Thus, if there was a sudden loss of mature cells, IL-3 could initiate pan-lineage stimulation which could then become directed by interactions with lineage restricted factors such as M-CSF. It could however have a restricted role in maintaining a particular class of stem cell survival or proliferation, without commiting the cells to any differentiation pathway.

The possibility exists that a cell at any given point in the differentiation pathway may have a different growth factor requirement either for survival, cell cycle status or differentiation commitment, and this may vary from stage to stage. The declining sensitivity of IL-3 to stem cells during differentiation may be an example of this (Koike _et al_ 1986a. The differing requirements of distinct progenitor subtypes to achieve maximal colony stimulation (Sieff _et al_., 1987) may therefore relect the "stemness" of the progenitor cell being assayed. As cells progress down the differentiation pathway they may require growth factors which previously had little effect, but which can now either instruct the cell to develop down a particular pathway or act as a trigger to release a previously determined programme of differentiation events. At any given progenitor stage, a cell could show differing sensitivities to alternative growth factors and so not only would the presence or absence of a factor be important but the balance of various factors factors may be vital to the cell during its developmental course.

If all primitive cells were responsive to growth factors, very strict compartmentalisation would have to be enforced in the marrow, which permit restricted and orderly cell/growth factor interaction. It is apparent that not all cells respond to any given factor, and that several factors act on separate populations with various degrees of overlap (Chap. 1.2). This means that even though

CSF may be present, a cell may not be able to respond to it. The specific interaction necessary could be envisaged to occur by cells displaying the appropriate surface receptors for the growth factors, these may be maturity or lineage restricted. These are discussed below.

Chapter 1.4.

Growth Factor/Receptor Interactions

The production of mature granulocytes and macrophages in vitro has so far been shown to be dependent on four molecularly distinct CSFs (Chap.1.2). These have overlapping biological activities, and clonal analysis experements have shown that the same progentor cell is stimulated by more than one CSF (Chap.1.2). This implies either that a progenitor cell may have receptors for more than one CSF, or that several growth factors act through the same receptor. However, as discussed in chapter1.2, this latter possibility is unlikely. The relationships between the different CSF receptors in terms of their cell lineage and stage specific expression has been examined using radioiodinated derivatives of G-CSF, M-CSF, GM-CSF and IL-3. The binding of the CSFs to their receptors has been analysed, and the results have led to a possible explanation as to how the overlapping biological actions of the CSFs are achieved.

Cellular Distributions of Receptors

Cross linking experiments using iodinated M-CSF has identified the M-CSF receptor as a protein of molecular weight 165kD homologous to the viral oncogene v-fms (Sherr et al 1985). Bone marrow macrophages have been shown to express around 5×10^4 M-CSF receptors at $2^{\circ}C$ (Guilbert and Stanley 1986). This temperature causes irreversible binding without permitting any metabolic processess such as membrane turnover to influence the number of receptors and allows the total number of M-CSF receptors to be established (Guilbert and Stanley 1986). On shifting the cells from $2^{\circ}C$ to $37^{\circ}C$ there is a rapid disappearance of surface ^{125}I-M-CSF (with a half life of a few minutes) which is due, in the main, to internalisation (Guilbert and Stanley 1986). A minor component is dissociated from the cell surface (Guilbert and Stanley 1986). Receptor replacement takes 1-3 hours but the relative contribution by receptor recycling and de novo synthesis is uncertain (Guilbert and Stanley 1986). Byrne et al (1981), Bartelmez et al (1985) have shown that binding of M-CSF is restricted to mononuclear phagocytic cells and their precursors. Of particular interest is the observation that with bone marrow preparation, enriched for primitive cells, there is very little binding of ^{125}I-M-CSF when compared to a population which contained many

progenitor type cells which bound M-CSF (Bartelmez et al 1985). The increased binding capacity of the more mature cells has been attributed to the fact they express high numbers of M-CSF receptors per cell (Bartelmez et al 1985) and they therefore concluded that the numbers of M-CSF receptors per cell increased with cell maturation (Bartelmez et al 1985). Using iodinated G-CSF, Nicola and Metcalf (1985) have demonstrated by autoradiography and morphological examination, that there was an increase in receptor numbers per cell, on more mature cells in experiments when all cells of the neutrophil granulocyte lineage were labelled. Crosslinking of G-CSF to its receptor has revealed a protein of molecular weight 150kD which is present at an average of 80-160 molecules per bone marrow cell (Nicola and Metcalf 1985).

The binding distribution of ^{125}I-IL3 reflects its multilineage activity, since essentially all neutrophils, granulocytes, eosinophil granulocytes and macrophages are labelled (Nicola and Metcalf 1986). The labeled population express approximately 117-130 receptors per cell (Nicola and Metcalf 1986). In contrast to both M-CSF and G-CSF binding there was an observed decrease in labelling with the increasing maturity of the cells (Nicola and Metcalf 1986), There was however a small heterogeneous population of cells which exhibited high labelling (Nicola and Metcalf 1986). This pattern could explain the observed functional heterogeneity displayed by IL-3 (Chap.1.2) and also the declining sensitivity of cells to IL-3 as they mature (Koike et al.1986). Park et al(1986a) noted that crosslinking of IL-3 to the IL-3 dependent myeloid cell line FDC-P2 detected two putative receptor molecules of molecular weight 72.5kD and 113kD respectively, both of which show the same affinities for IL-3. These molecules could either represent two distinct receptors or the higher molecular weight protein could be a complex of the 72.5kD receptor bound to a 40.5kD protein which may be part of the IL-3 receptor complex (Park et al1986a). Although both bands are observed on crosslinking to intact cells, only the 72.5kD receptor protein is crosslinked when purified cell membranes are used (Park et al 1986a). If the cytoplasmic protein (40.5kD) does interact with an integral membrane protein (72.5kD) then this could be dislodged during preparation of the plasma membranes (Park et al 1986a). It could of course be crosslinked fortuitously and may be unrelated to the receptor for IL-3 (Park et al

1986a).

The major action of GM-CSF is stimulation of the production of mature granulocytes and macrophages (Metcalf 1984), and this biological pattern is mirrored by by the physical labelling of cells within the myelomonocytic lineage by ^{125}I-GM-CSF (Walker and Burgess 1985). Radiolabelled GM-CSF not only binds to primary bone marrow cells (Walker and Burgess 1985) but to many myeloid cell lines as well as two T-cell lines (Park et al 1986b). Walker and Burgess (1985) have observed that there appear to be two classes of GM-CSF receptor, a low and a high affinity receptor, present on both bone marrow cells and mature peritoneal exudate neutrophils. Although the low affinity receptor is present in equal numbers per cell in both bone marrow and peritoneal neutrophils, the bone marrow has a greater number of high affinity receptors than the peritoneal cells (Walker and Burgess 1985). Walker and Burgess(1985) propose that the different classes of receptor may reflect different actions GM-CSF may exert on mature and imature cells. The presence of only low affinity binding sites on mature peritoneal neutrophils led to the proposal that the high affinity receptors may be present on the GM-CFC progenitor cells and early myeloid cells which would be replaced by the low affinity receptors as the cells differentiate (Walker and Burgess 1985). The numbers of low affinity receptors per cell is in the range of 30-400 whereas the high affinity receptor could be present up to 1000 molecules per cell (Walker and Burgess 1985). Walker and Burgess (1987) also followed ^{125}I-GM-CSFs interaction with its receptor on WEHI 3B cells. They demonstrated that bound GM-CSF is rapidly internalised (half life 7min) and processed within the lysozomal compartment. The reappearance of GM-CSF receptors on the membrane can in part be attributed to receptor recycling since blocking de novo protein synthesis with cycloheximide does not prevent receptor reappearance, but the majority results from synthesis of new receptors.

Park and her colleagues (1986b) have also studied GM-CSF binding to murine bone marrow cells, as well as a number of myeloid and T-cell lines. They detected a crosslinked receptor protein of molecular weight 130kD in contrast to the bone marrow receptor of molecular weight 51kD reported by Walker and Burgess (1985). Park et al (1986b) suggest that the 51kD protein described by Walker and

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Burgess 1986) is a proteolytic product which corresponds in size to the smallest crosslinked fragments. Park et al (1986b) also crosslinked GM-CSF to bone marrow cell receptors, and this resulted in two proteins corresponding to receptor sizes of 179kD and 69kD. One other myeloid cell line tested also had the smaller peptide and a larger 188kD protein. If the 179kD protein product observed in bone-marrow is the true GM-CSF receptor and the 51kD peptide is the final product of proteolysis (this is disputed by Walker and Burgess 1985) then bone marrow may have a specific protease that modifies the 179kD receptor to a smaller 69kD biproduct. These results (Park et al 1986b) suggest that the 130kD receptor observed in 5 continuous cell lines, including WEHI 3B, could perhaps be an altered gene product, similar to the observed differences between the v-fms (Coussens et al 1986) and v-erb B (Schlessinger 1986) oncogenes and their cellular counterparts which act as receptors for M-CSF and EGF respectively. The variety of different molecular sizes that are associated with ^{125}I-GM-CSF crosslinking may be related to the multiple biological actions of GM-CSF (Metcalf 1984, Chap. 1.2.) The concentration dependent commitment by GM-CFCs in response to GM-CSF (Metcalf 1980) and its activity on fully differentiated cells (Lopez et al 1983, Grabstein et al 1986) may be mediated through independent receptors (Walker and Burgess 1985), although at present this is pure speculation.

The data on identification and cellular distribution of CSF receptors is complex. Binding of the iodinated growth factor reveals the distribution of protein able to interact with CSF. When using myeloid cell lines it must be remembered that establishment of the clone in culture may have resulted in an alteration in the product of interest. It is however difficult to resolve a complex issue involving many receptors with such a heterogenous population as the bone marrow cells, without the aid of cell lines. It is worth noting that the levels of iodinated CSF necessary to observe maximal receptor occupancy have been as high as three orders of magnitude greater than that necessary to produce biological effects (Park et al 1986b). This suggests that only a few of the maximum available receptors are required to stimulate colony formation and it is therefore the interactions occurring at these sites that would be most interesting. The availability of more receptors than required to produce maximal

stimulation may be necessary if growth factor is produced in very small amounts within a 3-dimensional environment. The random distribution of receptors around the cell surface could increase the probability of interaction under limiting growth factor release conditions. Alternatively the excess receptors may provide a cascade system in which growth factors interact with a cell surface receptor stimulating a down regulation of receptors from the cell surface so mimicking receptor occupancy and so amplifying the initial stimulus. The dynamic interactions between CSF receptors will now be discussed.

Modulation of CSF Receptors

Walker and colleagues (1985) observed that the four growth factors M-CSF, G-CSF, GM-CSF and IL-3 did not compete for the same receptor when assays on murine bone marrow were carried out at $0^{\circ}C$. Therefore each individual CSF interacts with a unique receptor. However, preincubation with of one CSF at either $21^{\circ}C$ or $37^{\circ}C$, could consequently modify the binding properties of other CSFs to their isologous receptor in a predictable manner (Walker et al.1985). The down regulation of an unoccupied receptor by the binding of a distinct CSF to its isologous receptor is termed CSF receptor trans-modulation (Walker et al. 1985).

Walker et al. (1985) observed that at high concentrations, IL-3 down regulate its own receptors as well as the receptors for GM-CSF and M-CSF very efficiently, but will only reduce receptor binding to G-CSF by about 50%. GM-CSF binding down modulates its own receptor and the unoccupied receptors for M-CSF and G-CSF although in a similar manner to IL-3 there are still approximately 25% of the G-CSF receptors available for isologous CSF binding (Walker et al 1985). Pre-incubation of bone marrow with G-CSF down modulates the G-CSF receptor and approximately 50% of the M-CSF receptors and M-CSF down modulates its own receptors and approximately 60% of the GM-CSF receptors (Walker et al 1985). The degree to which down modulation occurs is dependent on the concentration of the CSF used during the pre-incubation with bone marrow before iodinated factor is added (Walker et al 1985). The pattern observed at high concentration of GM-CSF or IL-3 reveals a population of G-CSF receptors that are refractory to down modulation (Walker et al 1985). These may either be distributed among all the receptor bearing cells or perhaps

represent a sub-population of cells (Walker _et_ _al_ 1985). It is possible that cells may express the receptor for G-CSF but not IL-3 or GM-CSF and so would not interact with IL-3 or GM-CSF. Clonal analysis of cells that respond to these three factors has already been discussed in chapter1.2, and it was concluded that G-CSF could act on separate populations of cells to that of IL-3 (Sieff _et_ _al_ 1987) or GM-CSF (Metcalf and Nicola 1983). The presence of G-CSF binding sites that do not down modulate is perhaps due to the existence of different subsets of G-CSF receptors or a population of cells that have IL-3 and GM-CSF receptors but these are not linked to the G-CSF receptors (Walker _et_ _al_.1985)

Receptor transmodulation could occur by either internalisation of the receptor or by causing a receptor to alter its affinity to its ligand. There are examples of both types of event in the literature. Transmodulation of the EGF receptor has been observed in 3T3 fibroblast cells treated with PDGF (Bowen-Pope _et_ _al_ 1983) . The observed response of 3T3 cells treated with PDGF is a result of an altered affinity for EGF at the EGF receptor without a change in receptor numbers (Bowen-Pope _et_ _al_., 1983). The M-CSF receptor on murine peritoneal macrophages can be transmodulated by the phorbol ester TPA due to receptor internalisation (Chen _et_ _al_ 1983). TPA can also elicit a down regulation of EGF receptors in HeLa or A431 epithelial cells by causing an alteration in receptor affinity (Bowen-Pope _et_ _al_., 1983, Shoyub _et_ _al_ 1986). Thus the same effector, TPA, can generate a similar response (receptor down regulation) in different ways depending on the receptor. Walker _et_ _al_ (1985) deduce from the binding curves of the various iodinated haematopoietic growth factors to bone marrow cells that transmodulation is occurring via receptor internalisation rather that an altered affinity.

Biological Significance of Receptor Transmodulation

The observed pattern of receptor down regulation is similar to the growth promoting activities of the individual factors (Walker _et_ _al_ 1985). For example IL-3 can stimulate cells to produce granulocyte/macrophage colonies as well as stimulate the early proliferative division of primitive cells and erythroid precursors (Metcalf _et_ _al_ 1987a, Sieff _et_ _al_ 1986). IL-3 elicits the most wide ranging down regulation effects by transmodulating the G-CSF, GM-CSF and M-CSF receptors (Walker _et_ _al_ 1985), although this type of

correlation is most obvious for GM-CSF. In vitro, GM-CSF shows a concentration dependent effect on colony development from GM-CFC progenitors (Metcalf 1980). At low concentrations, mainly macrophage colonies develop, but with increasing concentration of GM-CSF, granulocyte colonies appear (Metcalf 1980). GM-CSF also shows a concentration effect on the down regulation of M-CSF and G-CSF receptors (Walker et al. 1985). At low concentrations of GM-CSF, M-CSF receptors are down regulated, whereas, at increased concentrations G-CSF receptors begin to be down regulated (Walker et al. 1985). If the down regulation of an unoccupied receptor mimics the response of an occupied receptor, then macrophage colony growth at low levels of GM-CSF could be explained by the binding of GM-CSF to its own receptor causing transmodulation of the unoccupied M-CSF receptor resulting in macrophage colony growth (Walker et al 1985 , Nicola 1987). This argument could be applied to IL-3 which causes the down regulation of M-CSF, G-CSF and GM-CSF receptors (Walker et al 1985). In vitro, in the absence of other factors colony growth stimulated by Il-3 occurs when the unoccupied receptors are down regulated (Walker et al 1985, Nicola 1987) . The data described above would explain why G-CSF and M-CSF are lineage restricted in their effects. According to this theory, in the absence of lineage specific factors such as G-CSF or M-CSF, IL-3 and GM-CSF would primarily be mitogenic signals stimulating cell proliferation, with differentiation directed by the down regulation of the more lineage resrtricted receptors (Nicola 1987) .

The above model implies that at some point during the proliferation of primitive cells in response to IL-3 or GM-CSF they must express lineage restricted receptors . Bartelmez et al (1985) demonstrated that bone marrow enriched for early progenitors did not respond to stimulation by M-CSF even though a small number bound ^{125}I-M-CSF. This enriched population of primitive cells could be stimulated by IL-3 to produce cells which showed an increase in the percentage of ^{125}I-M-CSF binding cells. This was shown by autoradiography to be due to an increase in the number of M-CSF binding cells and in receptor density per cell, prior to these cells becoming fully differentiated (Bartelmez et al 1985). The receptor for M-CSF appears to be present on primitive cells before it is capable of responding to M-CSF. The appearance of M-CSF receptors on

the surface of the proliferating cell population would then be available for either transmodulation by IL-3, or isologous receptor down regulation by M-CSF, to produce mature cells.

Despite the obvious difficulties in interpreting the short term effects on a heterogeneous population, the model for transmodulation does explain the observed colony development after CSF stimulation although it may simplify a complex set of interaction occurring in any developing colony. Analysis of this sort on purified cell populations and autoradiographic studies should ease the problems of interpretation as should the use of cell lines.

The Theory of Receptor Transmodulation and its Implication in the Disturbance of Normal Haematopoiesis.

If GM-CSF and IL-3 are primarily mitogenic signals that act as differentiating agents only in the absence of lineage specific factors (Nicola 1987), then it should be possible to disrupt the transmodulation pathway such that the lineage specific receptors are not down regulated, and cells will proliferate in response to IL-3 or GM-CSF but not differentiate. This is exactly what is observed with the factor dependent haematopoietic cell lines (Dexter et al 1980a). Most factor dependent myeloid cell lines require IL-3 for survival (Dexter et al 1980a). If these cells have a disruption in their receptor transmodulation pathway, then it is not surprising that they continue to survive and proliferate in response to the mitogenic stimulus of IL-3, although are unable to mediate transmodulation of lineage specific receptors for differentiation (Nicola 1987). The 32D myeloid cell line is dependent on IL-3 for its survival in vitro (Metcalf 1985a), but will however differentiate if G-CSF is added to the culture (Rovera et al 1987). This observation implies that some of the factor dependent cell lines, although blocked at a certain point in differentiation and perhaps defective in transmodulation, could differentiate if exposed to the appropriate growth factor. Receptor transmodulation has only been examined in one cell line (Walker and Burgess 1985). The WEHI-3BD^{+} myeloid cell line is not dependent on exogenous growth factor for its survival (Metcalf and Nicola 1982) but it does retain receptors for GM-CSF (Walker and Burgess 1985). It does not, however, show receptor transmodulation of the GM-CSF receptor in response to exposure to IL-3 (Walker and Burgess 1985).

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This may be due to the cell lines origin <u>in vivo</u>. It could be derived from a cell which would not normally display tranmodulation <u>in vivo</u>, or alternatively it could be an artifact relating to immortalisation. It could also however, represent a required event in the establishment of the cell line, relieving dependance on CSF. The WEHI 3BD$^+$ line will differentiate in response to G-CSF (Metcalf and Nicola 1982) and so it will be of interest to see if this receptor is affected by exposure to IL-3 or GM-CSF and a direct comparison could be made to the WEHI 3BD$^-$ subline which cannot be induced by G-CSF and has no receptors for G-CSF (Metcalf and Nicola 1982, Nicola and Metcalf 1985).

Lotem and Sachs (1986) have also investigated the mechanisms by which growth factors interact with haematopoietic cells to induce proliferation and differentiation. They have isolated myeloid leukaemic cell lines which proliferate in the absence of exogenous growth factor but will not differentiate unless stimulated by a factor termed MGI-2 which is found in endotoxin serum and the conditioned medium from Krebs ascites cells (Sachs 1982). MGI-2 has no CSF activity itself but it is produced by normal cells (Sachs 1982, Lotem <u>et al</u> 1980). Lotem and Sachs (1982) propose that during normal differentiation the cell will proliferate in response to CSF and produce the differentiation factor MGI-2 which contributes to the maturation of the colony. These cell lines, in conjunction with CSF and MGI-2 activity, have been used to determine the links between proliferation and differentiation and how it may become uncoupled in malignancy (Lotem and Sachs 1977). A factor independent cell line clone 11 was observed not to require exogenous CSF to proliferate (Lotem and Sachs 1982). However , if the differentiation inducing protein MGI-2 was added to the cells, proliferation ceased immediately (Lotem and Sachs 1982). This effect was not due to differentiation as the majority of the cells were still relatively undifferentiated. If, however, exogenous CSF was added to the cultures the cells continued to proliferate and finally differentiated due to the presence of MGI-2 (Lotem and Sachs 1982). It was assumed then that the cells triggered to differentiate by MGI-2 become dependent on exogenous growth factor for their survival and proliferation (Lotem and Sachs 1982).

Subsequently, Lotem and Sachs (1986) demonstrated by a

receptor binding assay that the addition of MG1-2 increases the receptor numbers for GM-CSF on clone-11 from 140 per cell to 4,800 per cell, of which most are occupied by GM-CSF as demonstrated by elution of bound growth factor at low pH prior to the cells receiving any exogenous growth factor. Receptor bound growth factor could explain why these cells remain viable after induction with MG1-2. If the level of autocrine stimulation is not great enough to stimulate proliferation then exogenous factor would have to be added (Lotem and Sachs 1982). The differentiation activity of MG1-2 may be due to its ability to induce cell surface receptors (Lotem and Sachs 1986). Once the CSF receptors are expressed on the cell surface then they would be subject to normal differentiating effects of growth factors. If receptor transmodulation does occur, then it too could take place once the appropriate receptor had been expressed. It is unclear whether the autocrine stimulation of the up-regulated receptors would occur _in vivo_ in response to MG1-2 stimulus. If MG1-2 up-regulated CSF receptors in cells it would make them competent to external stimuli. Bartelmez and Stanley (1985) have described a protein H-1, which has no intrinsic CSF activity. It can however act synergistically with M-CSF on primitive murine bone marrow cells to allow these cells to respond to M-CSF and proliferate (Bartelmez and Stanley 1985). Normally these primitive cells would not respond to M-CSF, so H-1 in the presence of M-CSF, may be increasing the number of M-CSF receptor bearing cells (Bartelmez and Stanley 1985). The synergistic effect observed between H-1 and M-CSF has also been reported for H-1 and IL-3 (Bartelmez _et al_ 1985). In both cases, H-1 permits the CSF to act on a developmentaly earlier cell than the CSF could act on alone (Bartelmez and Stanley 1985 , Bartelmez _et al_ 1985). It is possible that primitive cells cannot respond to CSFs unless they have a receptor for H-1, or H-1 and the CSF. Only after H-1 has bound to its receptor might the rest of the CSF receptors become activated or up-regulated. The protein MG1-2 described by Lotem and Sachs (1986) may have a similar function to H-1 (Bartelmez and Stanley 1985). A protein of this type could perhaps represent a trigger which primes primitive cells to respond to CSFs. The block to differentiation observed in the cell lines used by Lotem and Sachs may represent a failure of these cells to express CSF receptors in an appropriate way, so the differentiating effects of lineage specific

CSFs and receptor transmodulation may not be possible. It is particularly interesting that these cells do not require added growth factor before induction to differentiate, but do seem to require it subsequently. This may be a reflection of the return to "normality" of the cell line once the differentation block has been overcome and for this reason the cause of the differentiation block may shed some light on the process by which growth factors stimulate proliferation and differentiation.

The use of iodinated growth factors to study CSF/receptor interactions has enabled the apparent numbers and cellular distrubutions of several CSFs to be examined (Nicola 1987). These experiments have highlighted a possible mechanism for the regulation of haematopoietic proliferation and differentiation by the transmodulation of CSF receptors by IL-3, GM-CSF, G-CSF or M-CSF (Walker et al 1985). If transmodulation is to be examined in primitive cells, then the use of unfractionated bone marrow cells as a model will present difficulties because of the relatively small percentage of primitive cells present (Metcalf 1984), and the differences in concentration of CSFs required to give biological stimulation in comparison to the binding assay (Park et al 1986b). There are many haematopoietic cell lines with a variety of characteristics which would be amenable to receptor binding studies (Metcalf 1985a, Dexter et al 1980a, Wyke et al 1986). The use of these cells may give some indication of the biological relevance of the dynamic changes observed in receptor numbers in normal bone marrow (Walker et al 1985).

Chapter 1.5.

Possible Mechanisms by Which Growth Factors Elicit Their Effects in Haematopoietic Cells.

Protein Phosphorylation in Biochemical Pathway Control

Growth factors interact with receptor molecules situated on the cell surface, but how does receptor occupancy lead to cellular survival and proliferation? It seems that the cell surface interaction has to be transmitted to the cytoplasmic face of the plasma membrane before it can trigger the appropriate cellular responses via a secondary messenger system (Cohen 1985). One mechanism by which a growth factor can elicit a cellular response is via secondary messengers such as diacylglycerol (DAG), and inositol triphosphate (IP3) (Cohen 1985 , Bell 1986). The proposed chain of events leading to the generation of DAG and IP3 are outlined in Fig1.2. The activated receptor may interact with a closely associated G protein which is allosterically altered when it binds to GTP (Gilman 1984), which may be part of the activation process of the G protein. The G protein is thought to function by activating phospholipase C which in turn produces the secondary messages IP3 and DAG by the hydrolysis of PIP2 (Gilman 1984, Bell 1986) .

The secondary messengers refered to above are then thought to act in the case of IP3 by increasing intracellular levels of Ca^{2+} or, in the case of DAG by activating protein kinase C (PK-C) (Gilman 1984, Nishizuka 1984) . The increased intracellular Ca^{2+} can be complexed to the Ca^{2+} binding protein calmodulin which then regulates Ca-calmodulin dependent enzymes such as some protein kinases (Cohen 1985). The reversible phosphorylation of proteins has been demonstrated to modulate enzyme activity in a number of pathways leading to activation or inactivation of the enzyme (Cohen 1985). In this way rapid changes in phosphorylation states can regulate biochemical pathways.

DAG is the other product of PIP2 hydrolysis and is thought to be a second messenger because of its ability to activate protein kinase C (PK-C) (Bell 1986, Nishizuka 1984). PK-C is a Ca^{2+} dependent protein kinase which in its active form is translocated from the cytoplasm to the cell membrane (Nishizuka 1984). PK-C shows a broad range of substrate specificities, and due to the biochemical relevance of protein phosphorylation, it may play an important role in

Figure 1.2

A scheme illustrating the possible cascade of events following binding of an agonist (growth factor) to its receptor. PI, phosphoinositol; PIP2, phosphoinositol biphosphate; IP3, isositol triphosphate; DG, diacylglycerol.

EXTERIOR | CELL MEMBRANE | INTERIOR

① | ②

Growth Factor ▶▶▶ Receptor ▶▶◀

G-Protein ③

Phosphodiesterase

PI
④ ATP ◀◀◀
PIP$_2$

PIP$_2$ → IP$_3$

DG
▼

Phorbol esters ▶▶▶▶▶▶▶▶▶▶▶▶▶▶▶ Protein Kinase C
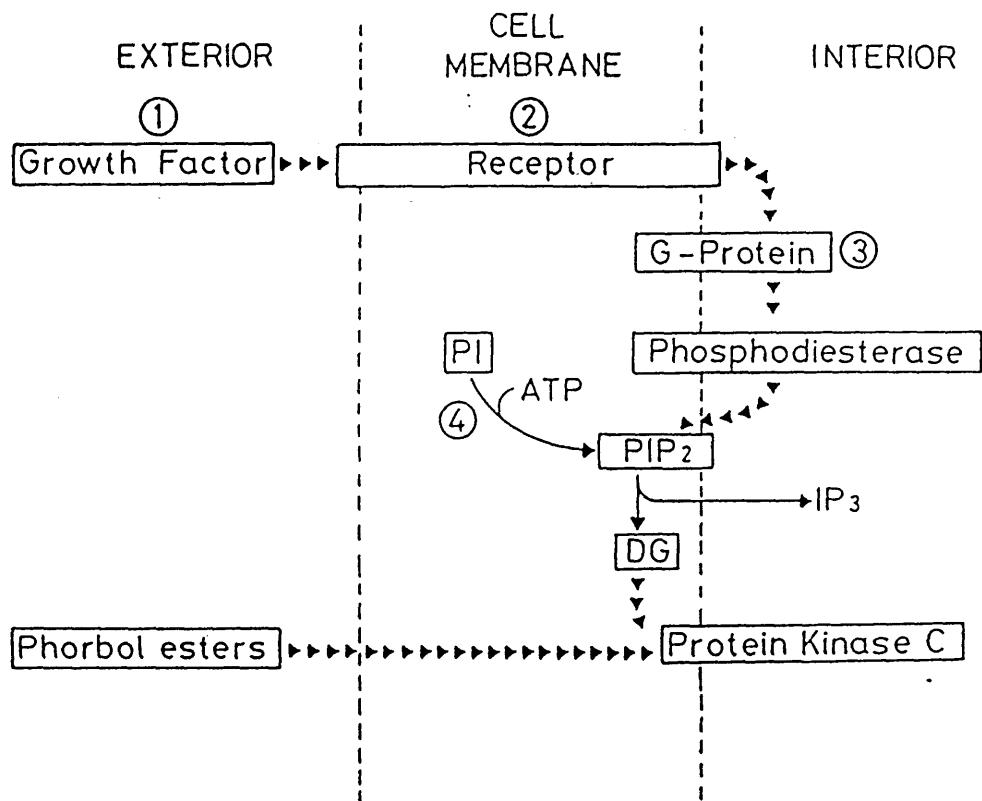
the regulation of receptor mediated responses to growth factors (Cohen 1985, Nishizuka 1984).

How does Growth Factor Receptor Occupancy lead to Cellular Survival and Proliferation.

Whetton et al.(1986α) have used myeloid cell lines dependent on IL-3 for survival and proliferation in order to ascertain the mechanics by which IL-3 elicits a response.   If IL-3 is removed from the IL-3 dependent FPDC-P2 myeloid cell line, the intracellular pools of ATP become depleted and cells die after 12h (Whetton et al.1986α). The addition of IL-3 to FDCP-2 and IL-3 dependent FDCP-MIX1 cell lines results in increased glucose transport across the membrane which promotes ATP production (Whetton et al.1986α).   The production of ATP via glucose transport explains how IL-3 sustains and allows the proliferation of IL-3 dependent cells (Whetton et al.1986α.   The mechanism by which this is regulated in biochemical pathways has been investigated at the level of secondary messengers.

If IL-3 acts through secondary messengers DAG and IP3   to stimulate PK-C and Ca^{2+} mobilisation respectively, then the effects of secondary messengers can be examined both by stimulating PK-C and increasing intracellular Ca^{2+} levels (Whetton et al.1986α.   The phorbol ester TPA activates PK-C, and the calcium ionophore A23187 will increase Ca^{2+} levels in cells (Nishizuka 1984).   Therefore if IL-3 acts through PK-C and Ca^{2+} levels then TPA and A23187 should be able to substitute, at least in part, for IL-3 function (Whetton et al.1986α.   Using the FDCP-MIX1 multipotential IL-3 dependent cell line (Wyke et al 1986), it has been shown that optimal concentrations of TPA and A23187 can relieve the requirement of IL-3 for cell survival and proliferation (Whetton et al.1986α.   A 48h pre-incubation with these components in the absence of IL-3 does not affect the ability of cell lines to form multipotential colonies in semi-solid medium upon appropriate growth factor stimulation (Whetton et al.1986α.   Therefore the viability and proliferative effects of IL-3 are mimicked by TPA plus the calcium ionophore A23187 (Whetton et al.1986α.   Witters et al(1985) have shown that PK-C is capable of phosphorylating the glucose transport protein in erythrocytes.   As previously discussed, protein phosphorylation can alter protein activity (Cohen 1985) and so glucose uptake via a transport protein may be susceptible to this type

of modulation.   The effect of IL-3 on activation of PK-C may explain
how glucose levels are regulated by IL-3 in haematopoietic cells.

Concomitent with PK-C activation, is the translocation of PK-C
from the cytosol to the cell membrane (Nishizuka 1984).   The
association of PK-C with the inner surface of the membrane has been
observed in several factor dependent haematopoietic cell lines.
Stimulation of the IL-2 dependent CT6 line by IL-2 (Farrar and
Anderson 1985), and the FDCP1 IL-3 dependent cell line by IL-3 (Farrar
et al 1985), or IL-3 stimulation of the FDCP-MIX1 cell line (Whetton
et al.1986a, results in the membrane association of PK-C .   It has
also been demonstrated that the translocation event observed in CT6
and FDCP1 cells can be achieved in the absence of growth factors by a
phorbol ester (Farrar and Anderson 1985, Farrar et al1985).   The
activation of PK-C in these cases may change the phosphorylation of
key regulatory molecules involved in cell survival, proliferation and
differentiation.   Evans et al (1987, 1986) have identified a 68kD
protein which is phosphorylated in several factor dependent cell lines
upon stimulation by the growth factors or a synthetic diacylglyceride,
OAG, which will activate PK-C.   Stimulation of the cell lines FDCP1 by
IL-3, NEF60-8 by G-CSF or IL-3, and CT6 cells by IL-2 resulted in
phosphorylation of a common 68kD protein (p68) (as determined by
peptide mapping) (Evans et al 1987).   The NSF60-8 cell line
proliferated in response to GM-CSF as well as IL-3 or G-CSF, but
GM-CSF did not cause the phosphorylation of the common 68kD protein
(Evans et al 1987).   All four of the cell lines show the
phosphorylation of the p68 in response to activation of PK-C by OAG
(Evans et al 1987).

The translocation of PK-C from the cytosol to the membrane in
two cell lines, including FDCP1, in response to growth factors or a
phorbol ester (Farrar and Anderson 1985, Farrar et al1985), and the
phosphorylation of a common protein in several individual cell lines
in response to growth factor or activation of PK-C (Evans et al 1987),
suggests that growth factors may possibly act through PK-C mediated
phosphorylation. For example p68, may be a common regulator protein
involved in anion exchange (Evans et al 1987) The identification of
a common route of action for several factors is of interest in
defining basic regulation of cell survival and proliferation, but does
not explain the differential effects observed upon stimulation of bone

marrow by the various growth factors. In this respect the apparent inability of GM-CSF to stimulate the p68 protein in NSF60-8 cells, even though it stimulates proliferation, is of interest (Evans et al 1987). These results suggest that GM-CSF may act through the phosphorylation of different proteins to IL-3 or G-CSF or indeed through a separate secondary messenger pathway (Evans et al 1987).

It is not known how PK-C is activated in haematopoietic cells in response to growth factors. Certainly a synthetic DAG can activate PK-C, and cause it to phosphorylate the same 68kD protein as binding of the natural ligand (Evans et al 1987, 1986), but this does not mean that the DAG required to activate PK-C is derived from PIP2 hydrolysis nor does it mean the PK-C is necessarily activated by DAG in haematopoietic cells. Whetton et al (1986b). stimulated bone marrow derived macrophages with IL-3 or M-CSF and determined the levels of PIP2 hydrolysis. Treatment of the cells with IL-3 or M-CSF did not alter the levels of PIP2 or IP3 suggesting that proliferation of macrophages in response to these factors is not associated with DAG derived from PIP2 hydrolysis (Whetton et al 1986b). However , there is also some evidence to suggest that DAG can be generated by hydrolysis of phosphatidylcholine (Besterman et al 1986). It is therefore possible that activation of PK-C may be achieved by DAG generation from several pathways each of which could be activated independently .

The phosphorylation of protein by PK-C is an attractive method for controlling the biochemical pathways required for the survival, proliferation and differentiation of haematopoietic cells (Whetton et al 1986a. Kinases can phosphorylate many proteins thus amplifying the initial stimulus and bringing about the activation and inactivation of a diverse number of proteins (Cohen 1985). The role of DAG and IP3 as secondary messengers generated in haematopoietic cells after binding of growth factor has not been clarified. However PK-C has been implicated in post-receptor binding events due to its sub-cellular distribution and its ability to substitute for IL-3 when activated by a phorbol ester on a multipotential haematopoietic cell line (Farrar and Anderson 1985, Farrar et al 1985, Whetton et al 1986a.

Most of the previous studies have concentrated on the role of PK-C in cellular survival and proliferation but have not addressed the

problem of how growth factors cause, or permit, differentiation. As previously discussed, phorbol esters can substitute for growth factors presumably via the activation of PK-C (Whetton et al 1986a). It has been demontrated that in vitro colony growth of bone marrow progeinitor cells can be stimualted by the phorbol ester TPA in the absence of exogenous CSF (Stuart and Hamilton 1980). The resulting colonies are mainly macrophage (Stuart and Hamilton 1980). In this instance TPA could be acting in three ways - it could interact directly with the clonogenic progenitor cell and its progeny, or TPA may cause the haematopoietic cells to release their own growth factors or finally, TPA might sensitise the cells to respond to sub-optimal levels of CSF present in the cultures (Stuart and Hamilton 1980).

Chen and colleagues (1983) have analysed the binding properties of I^{125}-M-CSF to murine macrophages in response to pre-treatment of the cells with TPA. They have shown that TPA down modulates the M-CSF receptor by receptor internalisation rather than an altered affinity for the receptor and its ligand (Chen et al 1983). In contrast, TPA treatment of 3T3 cells and a variety of other lines alters their binding of EGF to its receptor by decreasing the receptor affinity for EGF (Shoyab et al 1979). However, TPA causes a down regulation of EGF receptor in HeLa cells by receptor internalisation (Lee and Weinstein 1978), similar to M-CSF regulation (Chen et al 1983). The M-CSF receptor internalisation observed in macrophage cells after TPA treatment (Chen et al 1983) could be a result of receptor transmodulation (Nicola 1987) as discussed in Chapter1.4. The ability of TPA to stimulate in vitro colonies in the absence of added growth factor (Stuart and Hamilton 1980) could be attributed to internalisation of the vacant M-CSF receptors resulting in colony differentiation as well as proliferation. It would be of interest to see if TPA down regulates any of the other receptors for CSF but this has not been reported thus far.

The down regulation of M-CSF receptors in response to TPA (Chen et al 1983) leads to the interesting question of how this might be achieved. When EGF binds to its receptor, (which has a tyrosine kinase activity), the receptor becomes autophosphorylated at a tyrosine residue (Carpenter and Cohen 1979). EGF receptor down modulation due to decreased affinity for its ligand can be achieved by TPA or PDGF stimulation (Shoyab et al 1979 , Bowen-Pope et al 1983).

These changes in receptor affinity may be caused by PK-C mediated phosphorylation of serine and threonine residues on the EGF receptor molecule (Crochet et al 1984, Schlessinger 1986).

In the case of M-CSF receptor internalisation after TPA treatment (Chen et al 1983), PK-C or a similar molecule may act on the receptor (perhaps to phosphorylate it) causing its internalisation, which would in this instance cause a mitogenic and/or differentiation event. The receptor for M-CSF is the cellular homologue of the viral oncogene v-fms (Sherr et al 1985). The fms gene has tyrosine kinase activity (Sherr et al 1985, Sacca et al 1986) and since its normal cellular function has now been identified as the receptor for M-CSF (Sherr et al 1985), it should be possible to study the manner in which growth factor stimulation is linked to cytosolic proteins and phosphorylation events. Caution must always be placed on interpreting results using TPA since PK-C is normally activated by a very short lived enzyme DAG (Nishizuka 1984). TPA remains active in the cell for longer periods of time and so its stimulating effect on PK-C may not be a true reflection of PK-C normal function. In addition, there may be other controlling events taking place in the cell due to receptor binding that are not mediated via protein phosphorylation. For example, the methylation of cellular proteins has also been demonstrated to alter their acitivities (Pike and Snyderman 1982, Hirata and Axelrod 1980) and so may play a role in determining post-stimulatory effects.

The theories and experimental data concerning the events which occur after the binding of a growth factor to its receptor are still rather preliminary. Secondary messengers have not been studied in any great detail in the haematopoietic system, although as an experimental model it would be ideal since growth factor dependent cell lines are available (Dexter et al 1980a, Metcalf 1985a, Wyke et al 1986). In conjuction with bone marrow assay systems (Pragnell et al 1988) these experiments should enable growth factors to be linked to cellular proliferation and differentiation at the biochemical level.

Chapter 1.6.

The role of Growth Factors in Abnormal Haematopoiesis

There is very little direct evidence to implicate CSFs and their receptors in abnormal haematopoiesis. However due to their _in vitro_ activities (Chap 1.2) it is possible that they may play some important role in the development of myeloid diseases.

Oncogenes such as myc and abl have been implicated in haematopoietic disorders due to their proximity to translocation break points present in cases of Burkitt's lymphoma (Ar-Rushidi et al 1983) and chronic myeloid leukaemia (Shtivelman et al 1985). In addition it has been suggested that deletions in chromosome arm 5q, on which the loci for human FMS and GM-CSF are localised, observed in a small percentage of haematopoietic disorders, might be involved in the development of the disease (Le Beau et al 1986, Nienhuis et al 1985).

Possible mechanisms of growth factor involvement in abnormal haematopoiesis

The human disorders of acute myeloid leukaemia (AML), and chronic myeloid leukaemia (CML), result from the clonal expansion of an abnormal multipotential cell (McCulloch 1979). Eaves et al (1986) have shown that a high percentage of progenitor cells from CML patients remain in cycle when placed in normal LTMC feeder layers. In comparison, normal progenitors go in and out of cycle upon culture feeding. This suggests that continuous cycling gives on the CML progenitors a proliferative advantage since they do not respond to normal regulatory signals (Eaves et al 1986). It is possible that either the CML progenitors do not display receptors for inhibitory molecules or cannot interact with the stromal layer in the correct fashion for regulation to take effect (Eaves et al 1986). Alternatively, these cells may be hyper-responsive to low levels of growth factor or produce, and respond to levels their own growth factors in an autocrine fashion (Eaves et al 1986).

Autocrine stimulation of haematopoietic cells.

Friend Murine Leukaemia Virus (Fr-MLV) infection of LTMC.

Mice infected with replication competent Fr-MLV, produce after a long latency period, a myeloid leukaemic disease (Shibuya and Mak 1982). In order to investigate the steps involved in the progression of the disease, the effect of virus infection of LTMC were

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analysed (Heard et al 1984). The response of non-adherent clonogenic cells to CSF (mainly GM-CSF) in semi-solid media assays was testd at various time intervals after initial infection (Heard et al 1984). During the first 10 weeks post infection, colony forming cells (CFC) were present in the non-adherant fraction at a frequency similar to that in non-infected cultures (Heard et al 1984). Interestingly, infected cultures from 12 to 24 weeks displayed an increase in colony numbers formed in response to CSF and the morphology of the colonies changed until by week 24 they consisted of mostly immature cells (Heard et al 1984). These colonies of immature cells could be re-plated and re-cloned in the presence of GM-CSF and gave rise to factor dependent cell lines (Heard et al 1984). The clonogenic cells present during this period of post-infection events are clearly still dependent on CSF for colony development but exhibit an abnormal response to stimulation and proliferate rather than differentiate (Heard et al 1984). Cell lines can thus be selected for autonomous growth in the absence of exogenous growth factor, after approximately 36 weeks from the initial infection at high frequency (8 out of 20) (Heard et al 1984). The cloning efficiency was greatest in these growth factor independent (FI) cell lines when plated at high cell density suggesting that these lines may be FI due to autocrine stimulation (Heard et al 1984). Similar results were obtained by Heard et al (1983) when mice were infected with Fr-MLV and the resulting myeloblast cell lines were shown to be autocrine stimulated.

The gradual change in response to CSF by the clonogenic cells (Heard et al 1984) in this system provides a mechanism by which cells still remain responsive to CSF and proliferate although some event (e.g lack of receptor transmodulation) may cause the cell to become unable to use this signal to differentiate and so become blocked. Because these cells have a bias towards proliferation there is an outgrowth of these cells which is similar to that observed in some myeloproliferative diseases (McCulloch 1979).

Gisselbrecht et al 1987 have analysed 68 murine myeloblastic cell lines and tumour DNAs for integration of the Fr-MuLV at a chromosomal site termed fim-2. This was originally defined as a site of integration for F-MuLV (Sola et al 1986). They found that 14(20%) of the samples analysed had proviral integration at the fim-2 site , 7 of which were from in vivo F-MLV induced leukaemias and 7 were from

cell lines derived from _in vitro_ infection of LTMC (Gisselbrecht et al 1987). It was shown by sequence analysis that the fim-2 locus spans the 5' end of the c_fms_ gene which codes for the M-CSF receptor (Gisselbrecht et al 1987). Viral integration at this locus resulted in increased levels of normal size c_fms_ message RNA (Gisselbrecht et al 1987). It is possible(but not yet demostrated) that the c_fms_ positive tumours might stimulate their own proliferation by releasing M-CSF (Gisselbrecht et al 1987). These results suggest that the initial abnormal response to exogenous CSF observed in Fr-MLV infected LTMC (Heard et al 1984) may be due to disruption of receptor expression followed by secretion of growth factor. The ability of a haematopoietic cell to respond to proteins secreted by itself would finally relieve its dependence on any other regulatory molecules and result in uncontrolled proliferation.

The viral _fms_ gene differs from its normal c_fms_ counterpart in that it has constitutive tyrosine kinase activity (Sacca et al 1986). The normal membrane bound c_fms_ tyrosine kinase activity is activated upon binding of M-CSF (Sherr et al 1985), thus tyrosine kinase activity is a function of the activated receptor and probably mediates the growth factor response. The introduction of v_fms_ into the IL-3/GM-CSF dependent cell line FDCP1 (Wheeler et al 1987) and the M-CSF depednent cell line BAC-1 (Wheeler et al 1986) converts them to factor independent growth through a non-autocrine method, probably by promiscuous tyrosine kinase activity (Sherr 1987). Both parental lines are non tumorigenic in nude mice prior to induced v_fms_ expression, and induced tumorigenicity is concomitent with growth factor independence (Wheeler et al 1986, 1987) . Thus in this case, cells with a mutation in a receptor gene such that it no longer requires ligand binding to produce a mitogenic response (Sacca et al 1986) require only one mutagenic event to achieve autonomous growth. Cells which proliferate due to autocrine stimulation require two events, the production of the growth factor and the production of receptor. It is possible that one of these may already exist as a normal product of the cell, but in the case of leukaemic cells in which Fr-MLV virus is integrated at the c_fms_ locus (Gisselbrecht et al 1987), the long latency of the disease could be explained by a requirement for at least two events in leukaemogenesis, the first being integration of the virus at the c_fms_ locus, and the second

being the production of growth factor.

It is still unclear however why factors normally involved in differentiation, such as M-CSF (Chap.1.2), do not do so and cause instead proliferation without differentiation. The introduction of the human cfms cDNA into mouse 3T3 cells clearly shows that these cells respond to M-CSF stimulation (Roussel et al 1987) and so in this instance M-CSF acts purely as a mitogen for an inappropriately expressed receptor. In haematopoietic cells a further disruption to the cells internal signal transduction system can be envisaged in order to explain the lack of differentiation observed upon growth factor stimulation.

Abnormal response to CSF by human leukaemic cells

A model can be proposed in which several events could take place resulting in a myeloid leukaemia. One of these events could be the uncoupling of proliferation and differentiation so that the haematopoietic cells no longer differentiate efficiently (Sachs 1982). Another related, or distinct, event could be autocrine secretion of a growth factor which the cells would normally require to be "delivered" to them (Sporn and Roberts 1985). Autocrine stimulation alone would probably not be sufficient for cell transformation but could give a clone of cells a selective advantage (Wong et al 1987). This population of cells could then be more susceptible to further alterations in its genome and so finally give rise to the leukaemia (Wong et al 1987).

Metcalf et al (1974) examined the response of human granulocytic leukaemia cells to monkey lung CSF. Using 150 samples of peripheral blood leukocytes or bone marrow cells, samples from CML patients were found to be slightly less responsive to CSF over all the concentrations of CSF used in comparison to normal cells. Samples from AML patients were slightly more responsive than normal at low CSF concentrations and AML patients in remission showed a normal response. A response like that observed with AML cells could be due to several reasons:-

(i) The cells release some of their own CSF: At low levels of addition exogenous CSF which would be suboptimal for normal colony growth these cells could proliferate if the deficit in CSF was augmented by their own production.

(ii) The clonogenic cells in the AML population are more sensitive to growth factors and so respond by proliferating with lower levels of CSF than normal cells: Hypersensitivity could be achieved by an altered affinity of the receptor for its ligand or an altered sensitivity of the secondary messenger system to ligand binding. In this context, it has been shown that retroviral expression of the normal cmyc gene within the IL-3 dependent FDCP-1 cell line alters its sensitivity to IL-3 (Cory et al 1987). FDCP-1 cells expressing the retroviral cmyc form colonies at lower CSF concentration than control cells (Cory et al 1987). The reason for this is not known but it could reflect changes in the post-receptor signalling pathway.

(iii) The AML clonogenic cells may be part of an expansion of a pre-existing sub-population of progenitor cells which normally exhibit this high sensitivity to CSF. The expansion of this sub-population could be causally related to its CSF sensitiviy (Metcalf et al 1974).

Proliferation of AML cells in the absence of added CSF

Investigators interested in the control of leukaemic cell growth have tried to define which of the presently known growth factors can support AML colony growth in vitro (Griffin and Lowenberg 1986). Both rGM-CSF and rG-CSF can stimulate colony growth (Vellenga et al 1987). Where samples responded to both factors, the colonies were often larger but the total number of colonies formed was always less than the additive compared with numbers formed by the individual factors alone (Vellenga et al 1987). This data implies that G-CSF and GM-CSF can work on overlapping AML clonogenic populations (Vellenga et al 1987). The failure of AML cells to form colonies in semi-solid medium is likely to be due to inadequate growth conditions in vitro (Griffin et al 1986). Bone marrow samples taken from AML patients could have an undefined number of normal progenitor cells present and if these are intrinsic differences between leukaemic progenitor cells and normal cells then the proportion of contaminating normal progenitor cells may contribute to the variation seen between samples. In order to obtain relatively pure populations of such progenitor cells many investigators have used peripheral blood as a source of clonogenic cells (Griffin et al 1986). The peripheral blood normally contains very few clonogenic cells but they can be detected in peripheral blood from patients with leukaemia (Griffin et al 1986).

The growth of haematopoietic colonies in the absence of added CSF has been demonstrated to occur (Moore and Williams 1972). However, the cells responsible supporting this colony formation can be separated from the colony forming cells by fractionation and have been shown to be macrophage and T-cells (Moore and Williams 1972). After removal of these cells colony formation by the normal marrow no longer occurred (Moore and Williams 1972). Thus it was noticed in J. Griffins laboratory that on several occasions AML cells depleted of T cells or both T cells and macrophages show autonomous colony growth in a population of AML cells tested (Griffin _et al_ 1986, Vellenga _et al_ 1987, Young and Griffin 1986, Young _et al_ 1987).

The possible reasons for the autonomous growth of AML cells _in vitro_ was examined in two papers, Young _et al_ (1987), Young and Griffin (1986). Young and Griffin (1986) describe three patients whose progenitor cells show autonomous growth. Conditioned media from these samples could in cases one and two stimulate normal bone marrow progenitors to form granulocyte/macrophage colonies. The cells from case three released no detectable CSF activity. The addition of antisera against GM-CSF to the AML colony assays reduced the autonomous colony numbers formed for cases one and two but not three. It seems likely therefore that the autonomous growth of the cells in cases one and two is due to autocrine stimulation by GM-CSF. Northern blot analysis demonstrated GM-CSF transcripts for cases one and two but not three which supports the proposed autostimulatory role for GM-CSF in these cases.

Young and Griffin (1986) also demonstrate that the addition of rGM-CSF to the cells from cases two and three increased both the colony number and size, whereas colony size increased in case one. This implies that the cells from case two are not maximally stimulated by the autocrine route, nor are cells from case three by whatever route colony growth is achieved. Case one generated 1090 colonies per 10^4 AML cells in the absence of added CSF whereas case two produces 81 colonies per 10^4 AML cells. Case one AML cells secrete 10 - 100 fold more CSF into the growth medium than case two and so this higher level of growth factor release may cause the maximal stimulation in the absence of exogenous stimulation. It is of interest that the differing amounts of GM-CSF released into the media does not correlate with the GM-CSF mRNA levels, and so processing of the GM-CSF protein

may differ in the two cases.

Following the observation that GM-CSF is an effective growth stimulator for AML cells (Griffin et al 1986, Vellenga et al 1987), and that a proportion of AML cells can produce and respond to their own GM-CSF in an autocrine manner (Young and Griffin 1986), Young et al (1987) investigated the expression of GM-CSF in 22 cases of AML. Of the 22 cases, 11 had GM-CSF transcripts detectable by Northern blot analysis and of these 11 cases, cells from 6 of them secrete CSF activity. If antiserum to GM-CSF was added, partial or complete inhibition of colony growth by conditioned media was observed in 5 out of 5 cases tested, all of which showed high levels of both CSF activity and GM-CSF transcripts. Autonomous colony formation in vitro was also observed in 9 out of 22 of the cases, and 5 of these secreted detectable amounts of CSF. Three out of the 9 cases showing autonomus growth have GM-CSF mRNA transcripts. In contrast no GM-CSF transcripts were observed in 4 CMLs nor 11 'common' (pre B cell) acute lymphoblastic leukaemias studied. Normal marrow mononuclear cells and normal monocytes treatment with or without gamma-interferon or phorbol ester did not show GM-CSF transcripts. GM-CSF mRNA expression was not observed in resting T cells but could be detected after phorbol ester treatment (Young et al 1987). In order to rule out the possibility of contamination of AML by T-cells which could be the cause of the observed stimulation and GM-CSF mRNA, Young et al(1987) reprobed their blots with the α chain for the T cell receptor. This gave a negative result for the AML mRNAs (Young et al 1987).

The results described above suggest that GM-CSF expression may have some role to play in the abnormal proliferation of AML cells. It is possible that GM-CSF expression is a normal feature of the progenitor cell component and failure to detect GM-CSF transcripts in normal bone marrow cells (Young et al 1987) may be due to sensitivity as the progenitor cells are relativley rare in normal bone marrow (Metcalf 1984). It seems more likely that GM-CSF is abnormally expressed in some AMLs and that this may contribute the the abnormal proliferation of the cells (Young et al 1987). Of the eleven AML cases which express GM-CSF mRNA, only 6 secrete detectable levels of CSF (Young et al 1987), which may be a reflection of the amount of CSF released and the sensitivity of the detection system (^{3}H incorporation into purified myeloblasts)(Young et al 1987). In addition

it is possible that concentration of the conditioned media (Laker _et al_ 1987), or low pH elution of receptor bound growth factor (Lotem and Sachs 1986) might detect CSF activity in the other cases. It must be noted that a low level of CSF expression may not give the cells any observable proliferative advantage (autonomous growth) _in vitro_ but the situation _in vivo_ may be different.

Not all of the cases studied which showed autonomous colony growth have detectable levels of GM-CSF mRNA or detectable levels of CSF activity (Young _et al_ 1987). This serves to highlight the complexity of this type of analysis. It is possible that either GM-CSF or another factor is responsible for the autonomous colony growth but is undetectable. Alternatively other mutations may have caused a "short circuit" in the growth factor requirements of the cell (Rapp _et al_ 1985).

The retroviral expression of _v-myc_ (Rapp _et al_ 1985) or _v-abl_ (Cook _et al_ 1985) in the factor dependent cell line FDCP-1 relieves it of its strict requirement for IL-3 or GM-CSF in a non-autocrine manner, presumably by acting as a constitutive post receptor signalling event. A situation like this may have occurred in the AML cells that show autonomous growth but no CSF mRNA or CSF activity. A mutation at a receptor growth factor locus would also cause a by-pass in the normal requirement for ligand binding, for example _v-fms_ expression in FDCP-1 (Wheeler _et al_ 1987) cells or M-CSF dependent cell line (Wheeler _et al_ 1986). Although the apparent expression of GM-CSF in AML cells may be involved in a proportion of AML cases it is unlikely to be the only event required to cause the disease.

CSF genes may become activated by physical rearrangement of the gene's normal controlling elements, perhaps mediated by a mutagenic event. For example the WEHI3B myelocytic cell line constitutively produces Il-3 due to insertion of an intercisternal-A virus type particle, 5' to the Il-3 coding region (Ymer _et al_ 1985). Another mode of activation would be indirectly though disturbances in cellular pathways that would normally be controlled by growth factors. Chicken myoblasts and macrophages can be transformed by avian retrovirus carrying the _v-myb_ or _v-myc_ genes (Adkins _et al_ 1984). These cells are still require a source of chicken myelomonocytic growth factor (CMGF), for their survival and proliferation (Adkins _et al_ 1984) . However, upon superinfection of the transformed lines by

retroviruses containing src related genes, cell lines can be derived which show autocrine stimulation by release of CMGF (Adkins et al 1984). The basis for the result of expression of src related genes in these cells is unknown, but these genes have tyrosine kinase activity (Bishop 1985) and may mimic protein phosphorylation normally catalysed by an occupied growth factor receptor (Sherr 1987). If this was the case, and transmembrane signalling had been short circuited, then a non-autocrine mechanism for growth factor independence would seem most likely. However, in this instance (Adkins et al 1984) src gene expression may mimic receptor occupancy for one factor which them induces the release of a second factor, for example CMGF. Such an induction has been observed in endothelial cells stimulated by interleukin 1 which results in the release of human multilineage colony stimulating factor (Zucali et al 1987).

Recently Sakai et al (1987) examined 5 cases of AML for the involvement of IL-1 in tumour cell growth. Using ^{3}H uptake as an indication of cell proliferation, the cells from one case, HN, showed a high spontaneous proliferation rate. The AML cells from HN responded to both IL-1α and Il-1β by increased thymidine uptake. However only addition of anti-Il-1β antiserum and not anti Il-1α inhibited the proliferation of these cells. Il-1 activity was tested for and detected in the conditioned media from HN cells by the mouse thymocyte comitogenic proliferation assay, which was again inhibited by antisera to Il-1β but not Il-1α (Sakai et al 1987) . A molecular probe to Il-1β was used to detect Il-1β specific mRNA in Northern analysis of AN AML cells so confirming its expression. These results indicate that autocrine secretion of Il-1 was involved in the proliferation of these myeloid leukaemic cells. Il-1 has been shown to stimulate the release of GM-CSF from endothelial cells and so it may be having a similar effect in this situation. Sakai et al (1987) also added exogenous GM-CSF to HN cells without observing any increase in proliferation. These results mean that if Il-1 stimulation of HN cells is mediated through autocrine release of a growth factor it is probably not GM-CSF (Sakai et al 1987). CSF activity in the conditioned media of HN cells was not analysed, and so growth factor involvement in this system cannot be ruled out.

A direct approach which assumes that growth factors are part of the multi-step process of leukemogenesis involves the

re-introducion of growth factor genes into haematopoietic cells. Several groups have re-introduced growth factor sequences into either factor dependent cells lines or bone marrow with interesting results. Lang et al(1985) converted the factor dependent FDCP1 cell to factor independent growth by expression of the murine GM-CSF cDNA as has Ostertag et al (1987). These papers will be discussed in more detail later. Wong et al (1987) infected foetal liver cells as well as the factor dependent cells lines FDCP1 and 32D with an IL-3 containing retrovirus. The retrovirus contained the dominant selectable marker neo which allowed them to identify G418 resistant factor independent (FI) foetal liver cells of mixed haemaopoieitic lineages in methocel at a frequency of 35%. These colonies were picked and grown into FI cell lines. 10 of 12 mixed erythroid colonies and 7 out of 7 mast colonies gave cell lines whereas no colonies were derived from GFU-GM and BFU-E colonies (0/4). The examination of 3 cells lines derived from mixed erythroid colonies showed them to be of mast cell phenotype. The conditioned media from these FI lines could stimulate the proliferation of FDCP-1 cells and so were releasing detectable levels of growth factor. Interestingly these cells were not tumorogenic in nude mice when 10 x 10^6 cells were injected. In comparison, the established cell lines FDCP1 and cl-32D became factor independent and tumorogenic when infected with the Il-3 retrovirus (Wong et al 1987). It is reasonable to conclude that the cell lines have undergone some genetic changes already which predispose them to tumour formation upon factor independence. However, factor independence on its own is insufficient to convert normal cells to the malignant state (Wong et al 1987).

It would be interesting to analyse the factor independent mast cell lines derived from foetal liver culture (Wong et al 1987) for spontaneously occuring or induced mutations which would cause them to become tumourigenic. The reconstitution of haematopoiesis in lethally irradiated mice by bone marrow infected with a CSF retrovirus should provide an in vivo answer to whether these cells have a proliferative advantage or not, and what further mutations are requred to generate a leukaemia. Once candidate genes have been identified these too could then be introduced into bone marrow cells or cell lines alone or in conjunction with growth factor sequences.

Summary

Autocrine stimulation of haematopoietic cells by colony stimulating factors is an attractive model whereby cells are released from normal growth restraints, thus creating a proliferative advantage. Myeloid leukaemias, like other cancers, are probably the result of several mutagenic events (Cerruti 1988). If a cell with uncontrolled proliferative capacity is the target for another mutation, for example in a biochemical pathway crucial for differentiation, then this mutated stem cells could become predominant in the population due to its proliferative advantage.

The demonstration by Young et al (1987) that a high proportion of AML cases(11 out of 22) have GM-CSF transcripts suggests a role for GM-CSF autocrine stimulation in the development of AML. In order to establish whether growth factors are involved in abnormal haematopoiesis, further detailed studies such as that carried out by Young et al (1987) will have to be done. There are difficulties with working with such samples as the aeteology of the disease cannot easily be characterised in terms of its molecular pathways and growth factor involvement. The use of an in vivo or in vitro murine system as described by Heard et al (1984) using infection of marrow by FrMLV, or radiation induced leukaemias (Hayata 1983, Azumi and Sachs 1977), are however important in describing the steps invoved in the generation of leukaemia.

Chapter 1.7.

RETROVIRUS VECTORS

Introduction

The activities of growth factors on normal haematopoietic cells and their possible roles in myeloid leukaemia have been discussed in Chapters 1.1 to 1.6 . The molecular cloning of many of the CSF cDNAs and genomic sequences has allowed pure recombinant CSF to be produced and tested for various biological activities (Chap.1.2). The availability of pure growth factors is an important step forward in trying to understand the target cell populations and receptor interactions involved in CSF controlled haematopoiesis (Chap1.4). Another approach that can be used to study gene function is to re-introduce a gene into cells under the control of either its own promoter or a heterologous promoter sequence. This approach is of particular interest in view of the data on autocrine stimulation of haematopoieitic cells (Chap.1.6). Such cells can then be examined for proliferative advantage and lack of response to normal growth signals in comparison to exogenously stimulated cells.

It is possible to transfer genetic information into eukaryotic cells by several methods, the three most commonly considered being transfection by calcium phosphate co-precipitation (Graham and Van Der Ebb 1973) , electroporation (Toneguzzo and Keating 1986) and infection by retrovirus (Bernstein _et al_ 1985). The chosen method must allow a high frequency of transfer into the clonogenic population, which, in the case of bone marrow progenitor or stem cells is less than 0.1% of the total bone marrow population (Metcalf 1984). Stable transfection by calcium phosphate co-precipitation is an inefficient method of DNA transfer since one in 10^4-10^6 fibroblast cells retain and express the integrated transfected sequences (Peterson and McBride 1980 , Debenham _et al_ 1984).

Car _et al_ (1983) and Cline _et al_. (1980) used transfection to transfer dihydrofolate(DHFR)sequences into haematopoietic cells and thus conferring DHFR mediated resistance to methotrexate (MTX). Both groups used genomic DNA from a cell line known to be resistant to MTX as their starting point for DNA transfection into mouse cells which carried a distinct chromosomal marker which allowed bone marrow repopulation to be followed _in vivo_. Cells exposed to DNA from MTX resistant cells were mixed 50:50 with normal bone marrow and then

injected back into recipient mice. Multipotential cells which contained and expressed DHFR were selected in vivo by injection of MTX into the mice for several months. Karyotypic analysis of bone marrow after long term in vivo selection demonstrated that approximately 70% of the bone marrow was made up of donor cells that had been exposed to genomic DNA from MTX resistant cells (Cline et al 1980). From this type of analysis both groups concluded that DHFR sequences had been transferred to bone marrow stem cells by transfection. Neither paper however shows any molecular data that demonstrates copy number or expression of the transfered sequences, and it is possible that the transfected cells undergo amplification of their own DHFR sequences during such a long in vivo selection period. The authors do not however favour this explanation as bone marrow cells tranfected with "wild type" DNA had no selective advantage during MTX administration. Car et al (1983) also exposed fresh bone marrow to DNA from MTX resistant cells and plated them immediately on to semi-solid media to reveal the efficiency of gene transfer. However no MTX resistant colonies were found after screening $8x\ 10^4$ bone marrow cells, and so if transfer of genomic DHFR sequences is occurring it must be at a very low efficiencey.

In contrast, Toneguzzo and Keating (1986) have demonstrated the stable transfer of both the bacterial neo and gpt genes by electroporation into haematopoietic progenitor cells without in vivo selection. Recovery of human bone marrow after electroporation gave an almost 100% recovery of CFU-GM progenitors which were indistinguishable from control colonies. This therefore is ideal as a method of transfer which does not interfere with viability or development. The plasmid pSV7 neogpt can be used to select for G418 resistance or mycophenolic acid (MPA) resistance, after electroporation of bone marrow cells (Toneguzzo and Keating 1986). Resistant colonies grew under selection at a frequency of 0.8 to 2.7% (Toneguzzo and Keating 1986). Gene transfer was further demonstrated by RNA dot blot analysis of pooled MPA resistant colonies. The observation that colonies grown in the presence of MPA were predominantly macrophage wheras G418 resistant colonies were indistinguishable from normal granulocyte/macrophage colonies is of interest.

Although these authors demonstrated a low frequency of gene

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transfer into haematopoietic cells (Toneguzzo and Keating 1986), efficient transfer has only been possible using retroviral vectors (Gilboa 1986).

Retroviral Vectors

The most common method for the transfer of genetic information into eukaryotic cells is calcium phosphate co-precipitation.Stable integration of exogenous DNA into the cellular genome by calcium phosphate co-precipitation (Graham and Van Der Ebb 1973) has several drawbacks:-

i)   It is inefficient with only a small proportion of cells transformed under optimal conditions (Debenham et al 1984).

ii)   Very few established cell lines can be transformed with a high efficiency (Mulligan 1983).

iii) Often the transformed lines contain multiple copies of the newly introduced sequences (up to several hundred) per cell (Mulligan 1983, Wigler et al 1979).

It is therefore difficult to assess how many of these are active and non-mutated (Lebkowski et al 1984).

iv)   The precise mechanism by which cells take up and retain the DNA is unknown (Lebkowski et al 1984).

In comparison, the increasing understanding of the retroviral life cycle has made retrovirus ideal candidates for gene transfer vectors.A number of advantages are outlined below

i)   Infectious virus integrates into the host genome with high efficiency (Mulligan 1983).

ii)   Virus integration results in a predictable proviral structure (e.g. cell-LTR-gene-LTR-cell) (Mulligan 1983, Gilboa 1986).

iii) Low copy numbers (often only one) of proviral sequences occur per cell (Mulligan 1983).

iv)   Gene transfer into a wide variety of cell types is possible (Stocking et al 1986, Cone and Mulligan 1984, Kwok et al 1986)

Retroviral Life Cycle and Genome Structure

A schematic representation of the retrovirus life cycle is shown in Fig.1.3. After absorption of the virus to the cell membrane, the viral RNA genome enters the cell, where the viral RNA genome is reverse transcribed into a double stranded circular DNA molecule which

Figure 1.3

This figure is reproduced from Price (1987).
The retroviral life cycle. This figure is a
schematic diagram of the life cycle of a typical
wild-type retrovirus. The retroviral particle is
adsorbed on to the cell plasma membrane by the
binding of its envelope surface glycoproteins to a
specific surface receptor. Following fusion, the
retroviral genomic RNA passes into the cytoplasm,
and is reverse transcribed into DNA, gains entry
into the nucleus and as a proviral particle
integrates randomly into the host cell chromosomal
DNA. The integrated provirus acts as a typical
chromosomal DNA in that it is inherited by both
daughter cells whenever the cell divides. The
provirus is also transcribed and the retroviral
genes are translated, using the cell's normal
machinery. The assembly of a new retroviral
particle completes the life cycle. The genomic
retroviral RNA transcript comes together with the
retroviral gene products and buds off to form a new
free retroviral particle.

Reverse

DNA

DNA
RNA

Adsorbtion

RNA

DNA

Proviral
circle

Integration

Uncoating

mRNA

mRNA

Assembly

Translation

Budding
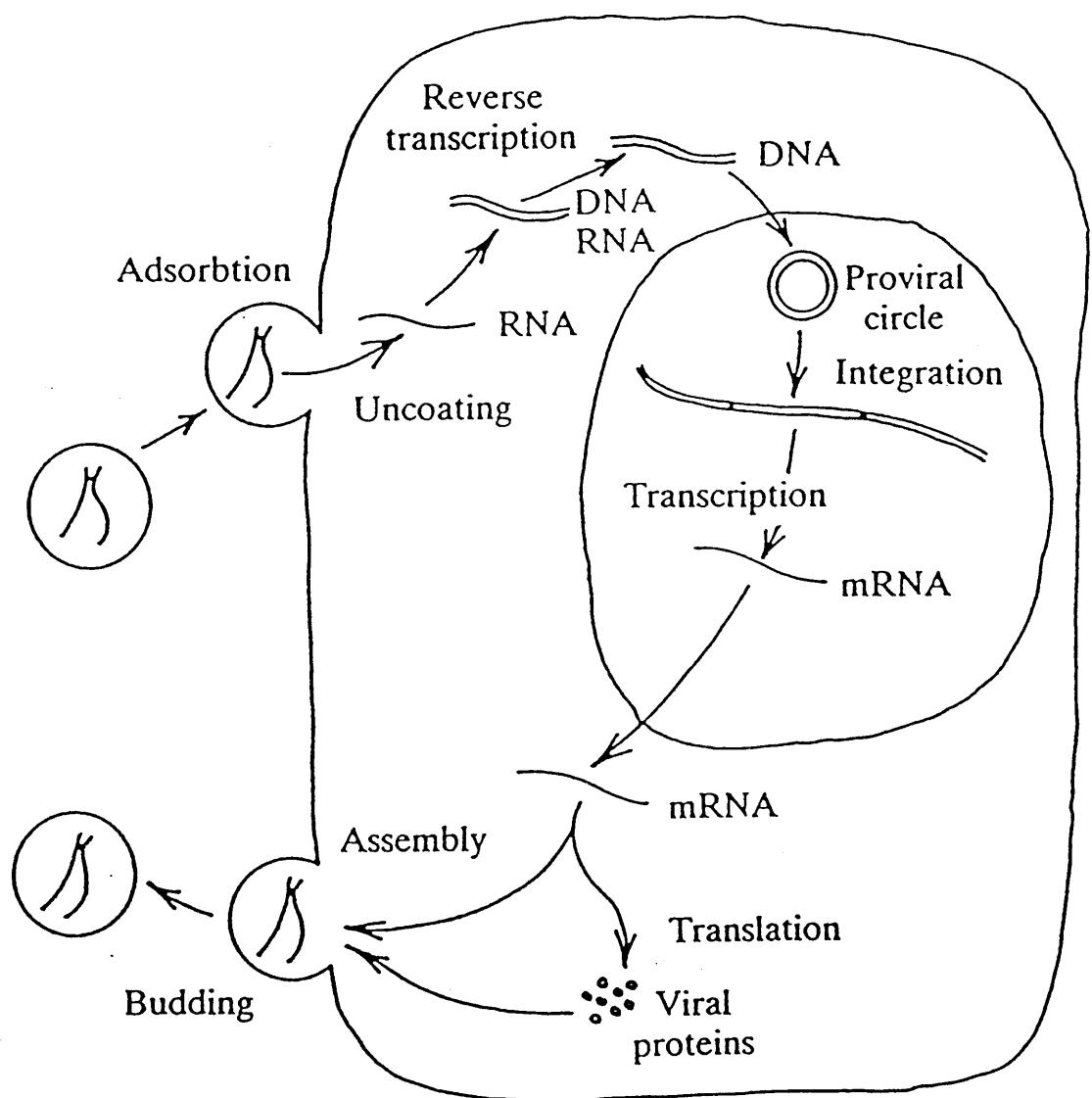
Viral
proteins

integrates into the host genome in a precise manner (Weiss _et_ _al_ 1985). The integrated virus, termed to provirus can be transcribed and packaged by viral and cellular proteins to become an infectious viral particle which is shed from the cell (Weiss _et_ _al_ 1985).

The proviral genome consists of three genes, _gag_, _pol_ and _env_, bounded by two identical stretches of sequence termed the long terminal repeats (LTRs) (Weiss _et_ _al_ 1985). The _gag_ gene encodes a virion structural protein, the _pol_ gene is responsible for the reverse transcriptase activity and the _env_ gene encodes an envelope glycoprotein involved with virus absorption and penetration (Weiss _et_ _al_ 1985). Expression of these proteins is essential for the replicative life cycle of the virus and it is the promoter and enhancer regions found within the LTR which control their expression. The LTRs are always generated upon genome integration and are required for the integration event to take place (Weiss _et_ _al_ 1985). A provirus lacking any _gag_, _pol_ or _env_ sequences cannot complete a replicative cycle on its own but it can utilise these gene products if supplied _in_ _trans_ by a helper virus (Mulligan 1983). By definition a helper virus must be replication competent. Co-infection of a cell with both a helper virus and a replication deficient virus will allow the replication deficient virus to complete its life cycle with the aid of the products supplied _in_ _trans_ by the helper virus (Mulligan 1983).

The provirus generates two polyadenylated messenger RNAs (mRNAs) (Mulligan 1983). A full length proviral transcript initiated on the 5' LTR and terminating on the 3' LTR contains a complete copy of the viral genome whereas a spliced message derived from this, essentially encodes _env_ sequences (Mulligan 1983). It is only the full length genome transcript which is efficiently packaged into infectious viral particles (Mulligan 1983). Packaging of viral RNA into virus particles is in the main directed by sequences at the 5' end of the genome, termed the region (Mulligan 1983 , Mann _et_ _al_ 1983).

Retrovirus Vector Systems

The features of the retrovirus life cycle described above make retroviruses a suitable candidate for a gene transfer system (Mulligan 1983) which can be split into two parts; vector design and

packaging cell lines.   Vectors can be generated by removing varying amounts of viral gag, pol or env information and replacing it with the gene of interest (Cepko et al 1984, Weiss et al 1985). This gene will then be transferred into the target cell as if it were a viral sequence (Mulligan 1983). The vector must retain the sequence found adjacent to the 5' LTR in order to be packaged into infectious virus when the proteins required for replication are supplied in trans (Mann et al 1983). Vector design will be discussed in more detail in a following section.

Packaging Cell Lines

A second part of the vector system is the packaging cell line (Mann et al 1983). These are all fibroblast (3T3) cell lines which harbour retrovirus deleted of a cis region required for viral encapsulation but which express all the viral proteins required for viral replication (Mann et al 1983, Mulligan 1983).

Introduction of retroviral vectors into such packaging cell lines will result in the vector being encapsulated and released into the culture medium (Mann et al 1983). This virus can then be used to infect target cell populations (Cepko et al 1984). The use of helper free virus avoids the possibility of virus spread and any biological effects of the helper virus, a situation which may also arise due to endogenous virus in murine systems (Weiss et al 1985). The spread of helper virus may also render infected cells refractory to subsequent super-infection by retroviral vectors (Weiss et al 1985).

The two most commonly used packaging cell lines are the Ψ 2 (Mann et al 1983) and PA137 lines(Miller and Buttimore 1986) . The Ψ2 line was constructed by Mann et al. (1983) by transfecting a deletion mutant of MoMuLV into 3T3 fibroblasts. This virus had a 350 nucleotide deletion between the putative 5' splice donor site and the AUG that initiates the coding sequence. The reverse transcription assay is a sensitive method for the detection of virus expression (Mann et al 1983). Ψ 2 cells show reverse transcriptase activity whereas viral particles from Ψ2 cells do not. It can therefore be concluded that the defective helper virus mutated in the sequence cannot be efficiently packaged. In order to test whether the Ψ2 line could package replication defective virus (which still retained Ψ sequences), a plasmid containing a MSV derivative was transfected into

it. The MSV construct used contained the gpt dominant selectable marker, and thus if infectious virus was released from the Ψ2 cells then infection of 3T3 fibroblast cells should result in XGPRT resistant colonies. Resistant colonies were observed at a frequency of approximately 5 x 10^3 gpt+ cfu per millilitre of virus suspension.

Retrovirus particles enter cells via a interaction between the virally encoded env protein and cellular receptors(Weiss et al 1985) . The MoMLV helper virus within Ψ2 cells expresses ecotropic viral envelope protein which recognises a receptor only present on mouse and closely related rodent cells (Weiss et al 1985). Therefore Ψ2 cells can only be used to infect a limited number of species. There are however related viruses which, as a consequence of differences in their env genes, can infect a wider variety of species (Weiss et al., 1985) including human cells. The two most common packaging cell lines with expanded host range infectivity are calledΨAm (amphotropic) (Cone and Mulligan 1984) and PA137 cells (Miller and Buttimore 1986). These cell lines were constructed in a similar way to the Ψ2 line (Mann et al 1983), by transfection of packaging defective virus into 3T3 cells and selecting clones which did not release helper virus. Transfection of viral vector into packaging cell lines caused a minority of clones to release helper virus (Miller and Buttimore 1986). This is presumably due to a recombination event between the "endogenous" packaging defective replication competent virus and the packaging competent vector (Miller and Buttimore 1986). In order to reduce the possibility of such an event occurring, Miller and Buttimore (1986) constructed the PA137 cell line by introducing a packaging defective virus into the 3T3 cells which also carried a number of other deletions and replaced the right hand LTR with the SV40 polyadenylation signal . The resultant plasmid has all the necessary information for expression of viral gene products but had significantly less homology to sequences with which the viral vector could recombine with to generate packagable replication competent virus (Miller and Buttimore 1986).

Vector Design

There are three basic vector designs. Each vector may carry a selectable marker gene which enables infected cells to be identified and cloned (Bernstein et al 1985). The first of these vectors is

termed a double expression vector (Gilboa 1986) as described by Cepko et al (1984). As described previously, viral genes are normally expressed from two RNA species, a full length viral genomic transcript and a spliced subgenomic product (Mulligan 1983). Double expression vectors retain the identified sequences for the splice donor and acceptor sites but replace the viral genes with two foreign genes (Gilbo 1986). Thus one gene is expressed from the full length mRNA, while the other is expressed from the sub-genomic message (Gilbo 1986). It is of course possible to express both genes form the full length transcript (Peabody and Berg 1986) but efficient expression of the second gene requres translation of the spliced message (Peabody and Berg 1986). The pZip neo vector designed by Cepko et al (1984) is of this type and before any foreign sequences are cloned into it, there is a one to one ratio of genomic to sub-genomic message. However, ligation of either c-myc (Cory et al., 1987) or murine GM-CSF (Lang et al 1985) into the vector resulted in a decrease of up to 50% of the sub-genomic message in infected cell. It is thus possible that some sequences may inhibit splicing, and so reduce the efficiency of expression of the 3' gene. It is now quite clear that sequences other than those of the splice donor and acceptor sites are required for accumulation of spliced message (Miller and Temin, 1986; Hwang et al., 1986) and the absence of these from double expression vectors could contribute to the poor expression of spliced mRNA in some constructs (Cory et al., 1977 Lang et al 1985). Gene expression in double expression vectors is controlled by the viral LTR and therefore expression is limited to cells in which this LTR is active (Stocking et al 1986). The variation in expression between different promoters will be discussed shortly.

A second basic type of vector expresses one gene from the viral LTR and a second gene form an internal promoter and thus does not rely on splicing to achieve efficient expression of both genes (Gilboa 1986). An example of this type of vector is pV200neo (Episkopou et al 1984). This vector has an internal TK promoter from Herpes simplex virus, in this instance linked to the bacterial neo gene which confers resistance to the antibiotic G418 in eukaryotic cells (Jimenez and Davis 1980). The foreign DNA is expressed from the viral LTR (Gilboa 1986). Such vectors allow a choice in the promoters used to express the transduced gene and therefore more control can be

excercised over gene expression (Episkopou et al 1984). Unfortunately however some promoter sequences may be inhibitory to the virus life cycle and so become deleted in order for the virus to complete replication and infection (Bernstein et al 1985). Emmerman and Temin (1984a, 1986) have also noted that selection for expression of one gene within dual promoter vectors may reduce expression of the second transcriptional unit. This will be discussed in more detail later.

Self inactivating (SIN) vectors are the third basic form of vector (Gilboa 1986). The viral LTRs contain transcriptional enhancer elements which can influence gene activity (Weiss et al 1985). For example the integration of a retrovirus adjacent to a cellular oncogene can disturb its normal expression pattern (Neel et al 1981), therefore if retroviruses are to be used in somatic cell therapy in humans the activation of cellular oncogenes by retroviral enhancer elements must be avoided. In order to study the control sequences of genes, putative control segments can be introduced into recipient cells and their activity monitored (Cone et al 1987). If such sequences are sub-cloned into the vector in the same orientation as the LTRs, then the full length mRNA from the LTR may cause any observed gene activity rather than the putative control sequences under study. One solution is to orientate the sequences under study in the opposite direction to the viral LTRs (Episkopou et al 1984). This approach has been used successfully to study tissue specific gene expression (Episkopou et al 1984) and an inducible promoter (Miller et al 1984). However, viral enhancer elements could still influence some internal promoters irrespective of insert orientation (Yee et al 1987).

The availability of SIN vectors is an important advance in retrovectorology since they were designed in an attempt to circumvent the problems associated with viral enhancers (Gilboa 1987). SIN vectors contain a small deletion in the 3' LRT encompassing either the viral enhancer sequences (Cone et al 1987) or the enhancer/promoter region (Yee et al 1987). These viral transcriptional control elements are situated in the U3 region of the virus (Weiss et al 1985). During viral replication the 3' U3 region becomes duplicated such that the resulting provirus has two U3 regions at either end of the provirus as part of the LTRs (Weiss et al 1985). Any deletion made in the 3' U3

region will therefore be duplicated after replication and integration in the 5' LTR (Weiss et al 1985, Gilboa 1986). Such provirus are transcriptionally silent due to the lack of appropriate control elements (Gilboa 1986, Weiss et al 1985).

The original plasmid vector to be transfected into the packaging cell line only contains a deletion in the 3' U3 region as full length transcripts originating from the 5' LTR are required to generate infectious virus (Gilboa 1986, Weiss et al 1985). One SIN vector, SVXenh-, is deleted of enhancer sequences (Cone et al 1987). It is therefore possible that sequences inserted into this vector might activate the promoter sequences (Cone et al 1987, Yee et al 1987). The pRMH SIN vector (Yee et al 1987) has a deletion which spans both the promoter and enhancer region and is therefore a better candidate for a true self inactivating vector.

Problems Associated with Retroviral Vectors

Vectors containing sequences which may interfere with normal virus replication.

In order for infectious virus particles to be generated, the virus must transcribe a mRNA which extends from the left hand LTR to the right hand LTR. Any sequences inserted into the vector which interferes with this, will result in inefficient or non-existant virus particle formation.

Emerman and Temin (1984 B) constructed retrovirus vectors based on the avian spleen necrosis virus (SNV) which included the herpes simplex virus thymidine kinase (tk) gene, complete with its heterologous promoter. Between the tk selectable marker gene and the left hand LTR, a mouse αglobin gene with(promoter+) or without(promoter-) its heterologous promoter sequences was inserted. These constructs were transfected into tk- chicken fibroblast cells together with helper virus and tk+ cells selected for. Following infection of fibroblast cells with virus supernatant, unintegrated virus was isolated after 3 days and its structure determined by Southern blot analysis. Eleven virus producer clones which shed the αglobin promoter+ construct, released virus of varying sizes of which eight released virus containing deletions in comparison to expected viral size. In comparison the seven α globin promoter- producer

clones shed virus of the expected size.

Two α globin promoter+ clones which shed deleted virus were analysed in more detail and the deletions were mapped. The two deletions were found to be of different sizes but in both cases the entire coding region of the α globin gene was deleted. One deleted virus, (ME107) was cloned and sequenced revealing the loss of both the globin structural gene and the transcriptional control sequences. The deleted ME107 virus was compared to the parental globin promoter + virus in terms of conversion of the tk- fibroblasts to tk+. These results demonstrated that the deleted virus had a higher tk+ transforming abiblity (10 fold higher after normalisation for virus production).

The experiments described above imply that certain sequences may be incompatible with viral replication under certain selective conditions. Emerman and Temin reported "data not shown" to the effect that globin promoter+ vectors which are not selected for tk+ expression do not undergo rearrangements. It is therefore possible that efficient expression of the tk gene in the presence of the globin promoter requires deletions in the globin gene.

The polyadenylation signals of HSV and SV40 have also been associated with vector instability (Joyner and Bernstein, 1983). The insertion of such sequences within retroviral constructs may cause premature polyadenylation of the putative full length viral genomic mRNA. The prematurely terminated mRNA may be unable to complete the retrovirus life cycle and so virus titre would be low. Joyner and Bernstein used vector constructs which contained either the HSVtk gene or the bacterial neomycin gene (neo). The neo gene was flanked by SV40 promoter and polyadenylation signals while the HSVtk gene retained its endogenous control signals. Sogner and Bernstein found that the generation of infectious virus that could confer either the tk+ phenotype or neo resistance was associated with deletions in the polyadenylation sequences of the selectable markers. Thus the presence of these sequences required for post transcriptional modification appears to be incompatible with infective virus.

Spleen necrosis virus (SNV) vectors have been constructed containing the chicken cellular tk gene complete with its own promoter and polyadenylation sequences (Brandyopadhyay and Temein, 1984). Virus production and expression has been shown to differ depending on

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the orientation of the tk gene with respect to the viral LTRs. When the tk gene was in the same orientation (parallel) as the LTRs, virus production was very poor, which was attributed to deletions in the tk gene. In contrast, when the tk gene was in the opposite orientation (antiparallel) to the viral LTRs, viral production was inefficient although the transforming activity was greater than tk in the parallel orientation. The virus generated from the antiparallel construct did not have any gross rearrangements. The lower level of virus release observed in the antiparallel construct in comparison to the parallel construct could perhaps be due to transcriptional interference. Depending on the relative strengths of the SNV LTR promoter and the tk promoter the converging transcripts may interfere with the production of full length transcripts and thus reduce virus production. Antiparallel constructs modified by Bal31 deletion of the tk promoter region resulted in increased virus titres at the expense of tk transforming activity. However Miller et al. (1984) have constructed retrovirus vectors with a rat growth hormone minigene in both transcriptional orientations, both of which give virus producing clones of equivalent titre. It is difficult to generalise as to what sequences will cause vector instability and each vector may have to be considered as unique until its behaviour in terms of virus production and gene expression have been tested.

Epigenetic Suppression of Gene Activity in Retrovirus Vectors

The occurence and problem of gross deletions in retroviral vectors has been discussed in the above section. Distinct from this type of mutation is epigenetic supression. This does not involve mutation at nucleotide level but is related perhaps to changes in chromatin structure or methylation states of genes.

Emerman and Temin (1984) have generated SNV based vectors which contain two selectable markers under control of two promoters. One promoter is always the SNV LTR, the other the tk promoter from HSV (Emerman and Temin 1984). The selectable markers are tk activity (HAT media) and   resistance to G418 (neo^r) (Emerman and Temin 1984). Infected fibroblasts were selected for either tk+ phenotype or neo^r, and individual clones were then re-plated under selection for the "untested" gene. Colony formation was compared to unselected cells or cells selcted for neo^r were replated in HAT (requires

expression of _tk_ gene). Of 12 _neor_ clones treated in this way, 7 failed to re-plate in HAT media, while the other 5 had a range of efficiencies from 1-9%. Similarly clones initially selected for _tk+_ expression failed to re-plate efficiently when transferred to G418. However two clones were identified which did re-plate well in G418. The apparent inability of cells selected to express one gene within the vector to express the second gene was also obvious when growing colonies were marked in the tissue culture plate and subjected to a change of selection conditions. It was observed that after changing the media up to 75% of the clones died.

Southern blot analysis of infected cells did not reveal any gross rearrangements of the provirus and so suppression of the second selectable gene could not be attributed to large deletions or rearrangements of the unselected promoter. Emerman and Temein reasoned that if the suppression was a permanent mutational event, then the rescue of provirus from _tk+_ cell lines that re-plated poorly into G418 media, would, in a subsequent infection give rise to few G418r colonies but many HAT colonies. If, however, the suppression was epigenetic then rescue of the provirus via a helper virus would result in a virus population that could transform cells to a _tk+_ or G418r phenotype at the same frequency as the parental virus stock. In fact, the latter result was obtained and virus rescue was assumed to release the provirus from a particular chromatin configuration which had previously caused the epigenetic suppression. The approach, of rescuing integrated virus in order to prove that an effect is not mutational has one fault however, since that any mutations in the left hand LTR U3 region will not be passed on to the rescued viral transcript. Therefore if suppression was due to point mutations in the left hand LTR U3, the resulting virus stock would not carry this.

It is of interest that the internal _tk_ promoter is less efficient at suppressing activity of the SNV LTR promoter than vice versa. Emerman and Temin suggest that the SNV LTR is a stronger promoter in fibroblast cells than the _tk_ promoter and thus can suppress expression of the _tk_ promoter to a greater extent. By using constructs with various other internal promoters, Emerman and Temin (1986) demonstrated that epigenetic suppression at the biological level could not be observed of the internal unselected promoter was either mouse metallothionein I (MTI) or SV40 early promoter. However,

analysis of viral transcripts did show a reduction in the steady state mRNA from these promoters, concomitant with a reduction in protein. The drop in biochemical levels of the unselected proteins must then still be above a threshold level requred to allow cells survival upon switching of selective conditions.

The observations by Emmerman and Temin (1984α, 1986) have obvious implications for the construction of retrovirus vectors. It would be desirable to prevent epigenetic suppression so that retrovirus vectors can express a nonselected gene efficiently . It is possible that this phenomenon may be restricted to SNV based vectors as there are no other reports of epigenetic suppression from other groups, although this does not mean it is not occurring but merely unobserved under the designs of specific experiments.

Retrovirus Infection and Expression in Haematopoietic Cells

Retrovirus vectors have been used successfully to transfer genetic information into cells from many species including human and mouse (Gruber et al., 1985; Hogge et al., 1987; Williams et al., 1984) A distinction must be made however between virus infection and virus expression. It is possible to infect 100% of a haematopoietic stem cell population (CFU-S) (Lemischka et al., 1986; Dick et al., 1985) and the mature progeny of stem cells as determined by detection of viral sequences by Southern blotting but this reveals nothing about the transcriptional activity of the vector.

Semi-solid assays for progenitor cells have been used to quantitate virus infection as transfer and expression of a dominant selectable marker in such assays will permit colony survival under selective conditions. An early attempt to transfer a neor gene was successful in converting 0.3% of the CFU-GM progenitors to neor (Joyner et al., 1983). This has subsequently been improved upon to yield up to almost 100% neor CFU-GM colonies (Magli et al., 1987).

The detection of gene expression in haematopoietic cells will depend on several factors. If selection is dependent on achieving a particular level of an enzyme to overcome a selective drug, then different optimal levels of expression may be required for different selection systems. Thus the number of methotrexate resistant colonies may differ from the number of G418 colonies after infection by virus containing the DHFR and neo genes respectively (Hock and Miller, 1986;

Kwok et al., 1986). Kwok et al(1986) determined the number of CFU-GM that could produce infective virus after infecting bone marrow with a retrovirus vector in the presence of helper virus. They demonstrated that 10 fold more colonies released virus than could form colonies under selection for methotrexate. Thus viral expression was sufficient to produce viral particles but not sufficient to confer resistance to methotrexate. It is clear from the above examples that "expression" varies with the method of detection.

It is possible that although cells may be permissive for infection, a sub-population may not permit expression, (Anklesaria et al. 1987). Viral integration into the host chromosome is essentially random but may be associated with regions of open chromatin configuration such as the 5' end of active genes (Vijaya et al 1986). However, methylation of DNA is generally associated with gene inacitvity and provirus have been shown to be hypermethylated and transcriptionally inactive in several cells types, such as embryonal carcinoma cell lines and mouse embryos(Jahner and Jaenisch 1985, Stewart et al 1982). It is likely that these early undifferentiated cells preferentially shut off viral transcription by methylating the viral genome (Jahner and Jaenisch 1985, Stewart et al 1982) or trans acting proteins present in the cells repress the viral promoter (Gorman et al 1985). The active repression of virus expression in undifferentiated multipotent cells may be an important consideration in the infection of haematopoietic stem cells.

It is the viral enhancer/promoter region which is responsible for provirus transcriptional activity (Weiss et al 1985). This level of transcription may then be modulated according to chromosomal position effects (Wyke et al., 1987), tissue (Magli et al., 1987; Kwok et al., 1986) or differentiation state differences. Mo-MuLV based vectors have been shown to express dominant selectable markers efficiently in progenitor type colonies formed during in vitro assays (Lim et al., 1987; Magli et al., 1987). However, demonstration of viral expression in CFU-S colonies derived from infected doner marrow has been somewhat elusive. Williams et al. (1986) and McIvor et al. (1987) both transferred retrovirus vectors containing the human ada gene into CFU-S but failed to detect expression of the ada gene at the protein level. Williams et al(1986) demonstrated virus specific mRNA at extremely low levels in CFU-S colonies. Interestingly an SV40

virus promoter included in their retroviral construct is not utilised and only full length viral RNA from the LTR was detectable. Lim et al. (1987) expressed the ada gene from the human phosphoglycerate kinase (PGK) gene promoter within a MoMLV based vector and did show Ada protein activity in mouse CFU-S colonies.

It is becoming clear that the expression of genes within haematopoietic cells is dependent on the promoter used (Magli et al 1987). Magli et al(1987) have screened a number of vectors containing different viral enhancer/promoter elements for expression in haematopoietic cells and shown that although SV40 sequences are efficient in fibroblast cells it is a poor promoter in haematopoietic cells. However the thymidine kinase promoters from HSV was relatively efficient.

The systematic search for promoters which can operate in extremely specialised cells such as haematopoietic stem cells is important. Retrovirus particles are capable of infecting stem cells and integrating into the host genome as discused above, but most retrovirus vectors rely on MoMLV enhancer/promoter sequences for expression (Williams et al. 1986, McIvor et al. 1987). The MoMLV virus quite clearly does not express well in stem cells (Williams et al. 1986, McIvor et al 1987) and so other promoters must be used. To this end both the tk (Magli et al 1987) and pgk promoters (Lim et al 1987) and retroviral enhancers related to MoMLV such as the myeloproliferative sarcoma virus (MPSV) enhancer (Stocking et al 1986), are of interest. Vectors based of MPSV will be discussed in the results section. Also of interest is a viral enhancer sequence related to polyoma virus which is specifically active in haematopoitic cells but not in fibroblasts or epithelial cells (Mosthaf et al 1985).

AIMS OF THIS THESIS

A central aim of this thesis was to study autocrine stimulation by GM-CSF on haematopoieitc cells in terms of factor independent colony formation. In order to do this, two retrovirus vectors containing GM-CSF sequences were used. One of these, mos^{-3}neoGM (kindly provided by Dr. W. Ostertag) was used to subclone GM-CSF sequences in a second retrovirus vector pVneoGM.

The use of these vectors was designed to allow two approaches to the investigation of expression of exogenous sequences in haematopoietic cells, in the hope of overcoming technical difficulties experienced in previous studies (Williams et al 1986; McIvor et al. 1987). An added advantage of this approach was the possibility of making direct comparisons between two vecors differing both in the virus of origin and mode of gene expression.

The question as to how these vectors are expressed of the in fibroblast cells lines was also addressed. Both molecular and cellular techniques were adopted in order to relate molecular expression to growth factor/haematopoietic cell interaction. This work was considered to be of particular importance since it is only once vector expression is established and repeatable in a predictable manner that more complex manipulations, such as infection of primary cells, can be carried out with any conviction that the phenotypic effects observed are due to vector expression.

Finally, optimal conditions for the efficient infection of primary bone marrow cells were examined with specific emphasis of modifications which may effect retroviral-mediated expression of GM-CSF.

MATERIALS AND METHODS

1.   Bacterial Strains

The bacterial strains used in this study were E.coli HB101 and
JM83.   Strains in current use were maintained on Luria agar plates at
4°C.   For long term storage, frozen cultures of each strain were
prepared in nutrient broth containing 30% v/v glycerol and stored at
-80°C.

2.   Media and Antibiotics

L-broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v)
sodium chloride. Ampicillin was included when required at 100μg/ml.

L-agar: L-broth containing 1.5% agar.

Top agar/X-gal: L agar containing 100μg/ml ampicillin and
250μg/ml X-gal.

3.   DNA manipulation, General Techniques

(a)   Handling of DNA

When handling DNA disposable gloves were worn, all buffers,
reagents, glass and plastic ware was sterilised by autoclaving and all
glassware coming into contact with DNA was siliconised. Many of the
techniques used are modified versions of those described in Maniatis
et al. (1982).

(b)   Phenol Extraction

DNA solutions were mixed with an equal volume of phenol,
chloroform, isoamyl alcohol, 8 - hydroxyquinoline (100:100:4:0.4;
w:v:v:w) saturated with 10mM Tris-HCl, pH7.5, 1mM EDTA. The upper
aqueous phase containing the DNA was removed and the phenol layer
re-extracted with an equal volume of 10mM Tris -HCl, pH7.5, 1mM EDTA.
The phenol was AR grade.

(c)   Ethanol precipitation

DNA was precipitated from solution by the addition of 0.1
volume of 3M sodium acetate, pH6.5 or a 0.5 volume 7.5M ammonium
acetate and 2.5 volumes of ethanol. The mixture was chilled for 20min
at -20°C before precipated DNA was pelleted either using a Sorvall HB4
rotor (13,000rpm, 15min, 4°C) or an Eppendorf microfuge for 15min at
maximum speed.   The supernatant was then discarded, the pellet washed
in 70% ethanol and re-centrifuged.   DNA was dried under vacuum and

redissolved as appropriate for further manipulation.

4.   Preparation of plasmid DNA

For solutions and buffers used see table 2.1.

(a)   Rapid plasmid preparation for screening

1.5ml of a culture of bacteria grown overnight in nutrient broth plus the appropriate antibiotic(s) was transferred to an Eppendorf and centrifuged for 3min in an Eppendorf microfuge. Cells were resuspended in 100µl of TEG buffer and incubated at room temperature for 10min. 200µl of alkaline SDS solution was added and the contents of the tube mixed by several sharp inversions, followed by 5min incubation on ice. 150µl of potassium acetate was then added, the solution was gently mixed and left for 5min on ice. The chromosomal DNA was pelleted by centrifugation for 5min at 4°C and the supernatant removed using a Gilson P200 pipette. Plasmid DNA was recovered by phenol extraction and ethanol precipitation. The pellet was washed in 70% ethanol, dried under vacuum and resuspended in 50µl TE buffer. RNA was removed by incubation with Ribonuclease A (20µg/ml) for 30min at 37°C.

(b)   Large scale plasmid preparation (Clewell and Helinski 1970)

A 400ml culture in L-broth containing the appropriate antibiotics was grown to stationary phase at 37°c overnight in a shaking incubator. Cells were harvested and resuspended in 10ml 50mM Tris pH8/25% sucrose and 5ml lysozyme(10mg/ml) added. The mixture was incubated on ice for 20minutes. 5ml of 0.2M EDTA pH7.5 were added, the solution mixed and left for 20 minutes on ice. 0.6ml of 10% NP40 in Tris/sucrose was then added and the tube inverted until lysis was complete. The lysate was then cleared in a Sorvall GSA bottle using a Sorvall SS34 rotor, (15000 rpm, 30mins., 4°C). The supernatant was decanted carefully into a 50ml Falcon tube, an equal volume of phenol added and then mixed for 10 minutes. The solution was then spun at 3000rpm for 20 minutes at room temperature in a bench centrifuge, and the aqueous phase recovered. The aqueous phase was re-extracted with an equal volume chloroform. The aqueous phase was recovered by centrifugation and RNA removed by ribonuclease A treatment (20µg/ml, 30minutes, 37°C). Ribonuclease A activity was removed by phenol/chloroform extraction, and plasmid DNA precipitated by the addition of two volumes ethanol. The pellet was washed in 70% ethanol and resuspended

in 500μl water.

5.   Ligation

Restricted plasmid vector DNA and fragments for ligation were mixed in a 1:3 molar proportion.   Total DNA concentration was usually between 5-50 μg/ml in a 10-20μl total volume. 1/10 volume of 10 times concentrated ligation buffer (table 2.2) and 1μl of T4 DNA ligase were added and the mixture incubated overnight at 15°C.   T4 DNA ligase was obtained from New England Biolabs.

6.   Alkaline Phosphatase treatment of linear, plasmid vector DNA

To minimise recircularisation of plasmid DNA, 5' phosphates were removed from both ends of linear DNA by treatment with calf intestinal phosphatase (Ullrich et al., 1977).   Digested vector DNA was phenol extracted and precipiatated with ethanol before the DNA was resuspended in a minimum volume of 10mM Tris-Cl, pH8.0.   1/10 volume of 10 times concentrated CIP buffer (see table 2.2) and 1μl of calf intestinal phosphatase was added.   The mixture was incubated at 37°C for 30min and the reaction stopped by phenol extraction.

7.   Transformation of bacteria with plasmid DNA (Cohen et al. 1972)

(a)   Preparation of competent cells

Stationary phase cultures of E.coli were diluted in nutrient broth to an A450 of less than 0.01 and grown rapidly with good aeration to mid-exponential phase (A450 = 0.3-0.5).   Cultures were immediately placed on ice before 10ml of these cells were harvested by centrifugation for 3min at 4,000rpm in an M.S.E. chillspin.   Cell pellets were resuspended in 5ml of ice cold 100mM $MgCl_2$, harvested as described above and resuspended in 5ml of ice cold $CaCl_2$.   The cells were kept on ice for 20min before centrifuging as before and resuspending in 600μl of ice cold 100mM $CaCl_2$.   Cells were then kept at 4°C for a minimum of 1h before use and were used within two days.

(b)   Transformation

200μl of the prepared competent cells were incubated on ice for 1h with 10-500ng of plasmid DNA. After a 2min heat shock at 42°C the bacteria were added to 2ml of prewarmed nutrient broth in a 25ml flask without shaking.   Undiluted culture and culture diluted to 10^{-1} and 10^{-2} were spread on nutrient agar containing the necessary

antibiotics to select for plasmid transformed cells.

8.   Agarose Gel Electrophoresis

        Horizontal slab agarose gels (0.5-1% w/v) were prepared using
BRL analytical agarose in TAE or TBE buffer, (table2.3).   Ethidium
bromide was added to both gel and running buffer to a concentration of
0.5μg/ml.   All samples were mixed with 1/6 volume agarose gel sample
buffer before loading (see table 2.3).   Electrophoresis was carried
out with the gel completely submerged in electrophoresis buffer at
25-100V, until the dye front had migrated through 3/4 of the gel.   DNA
was visualised by transillumination with long wave (260nm) UV light.
λ DNA restricted with HindIII was used to supply molecular weight
markers and gave sizes of 23kb, 9.4kb, 6.5kb, 4.4kbh, 2.3kb 2.0kb,
0.5kb and 0.1kb respectively.

9.   Restriction enzyme digestion of DNA

        Digests   were   performed   in   sterile   1.5ml   Eppendorf
microcentrifuge   tubes   according   to   the   enzyme   supplier's
specifications in the recommended buffers.

10.   Agarose gels for Southern blot analysis

        Restriction enzyme digests of genomic DNA (20μg per lane) were
electrophoresed in 0.7% (w/v) agarose gels (19.5 x 14.5 x 0.5cm) with
TAE buffer at 25V overnight.

11.   Agarose gels for Northern blot analysis

        1.4% (w/v) agarose gels (19.5 x 14.5 x 0.5cm) were prepared by
dissolving 2.8g of agar in 145ml of water in a microwave oven.   When
cooled to 60°C, 35.8ml of formaldehyde and 20ml of 10 times MOPS
buffer (table2.3), were added and the gel poured.   RNA samples were
freeze dried, redissolved in 20μl of RNA loading buffer, plus 2μl gel
sample buffer(table2.3),   heated to 65°c/10minutes,   loaded   and
electrophoresed at 2V/cm for 10-16h at 4°C in 1 times MOPS buffer.

12.   Isolation of DNA fragments from agarose gels

        After digestion with the appropriate restriction enzymes, DNA
was electrophoresed in a 1.0% low melting point agarose gel.   Bands
were visualised under long wave UV light and the required band excised

from the gel. 2-3 volumes of H_2O were added to the gel slice and the mixture was heated at $70°C$ until the agarose melted. After rapid cooling on ice, phenol was added, the mixture was vortexed and centrifuged (2,500rpm, 5min). The supernatant was removed and phenol extracted until the interface was clear before DNA was precipitated by the addition of ethanol.

13.   Hybridisation analysis of DNA and RNA (Manniatis et al. 1982)
        Hybridisation analyses were performed essentially as described in the GeneScreen hybridisation instruction manual (NEN, Boston).Solutions used are shown in table 2.4.

(a)   Southern blot transfer of DNA
        DNA samples were electrophoresed and transferred by the Southern (1975) method on to GENEScreen membranes. This involved denaturation of the DNA in the gel in 1.5M NaCl/0.5M NaOH, followed by neutralisation in 3M NaCl/0.5M Tris-HCl pH7.0, then washing briefly with 1 times phosphate buffer. Transfer was performed overnight (r14h) in 1 times phosphate buffer. The membrane was then rinsed in 1 times phosphate buffer and baked at $80°C$ for 2-4h to fix the DNA.

(b).   Northern blot transfer of RNA
        RNA samples were size separated as described in section 11. The gel was soaked in excess 0.1M Tris pH7.5 for 15 minutes and then transfered into excess 0.1M Tris pH7.5 containing 0.5µg/ml ethidium bromide for 15 minutes. The gel was destained for 15 minutes in 1x sodium phosphate buffer (pH6.5). The RNA was then visualised using a U.V. source and a photograph taken. Transfer was performed overnight in 1x phosphate buffer. The membrane was rinsed in 1x phosphate buffer briefly and baked at $80°C$ for 2-4 hours.

14.   Hybridisation
        Solutions used shown in table 2.4
        Membranes were pre-wetted in 1% Triton-X100 then prehybridised in sealed polythene bags in a 10ml volume or hybridisation chambers with a volume of 30-80ml, containing: 50% (v/v) formamide (de-ionised), 5X SSC, 5X Denhardts solutions, 1X phosphate buffer, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS and 100µg/ml of denatured salmon sperm DNA at $42°C$ overnight with agitation. Radioactive

probe was added to a concentration of 5ng/ml and hybridised overnight at 42^0C with agitation. The next day membranes were rinsed in 2X SSC 0.1% SDS several times at room temperature, washed at 60^0C with agitation in 0.1X SSC/0.1% SDS for 1h including three changes of wash solution. Membranes dried on Whatman 3MM paper were sealed in thin polythene bags for autioradiography.

15.   Simultanious Extraction of High Molecular Weight DNA and RNA From Cell Lines (Krieg et al 1983).

Cells were lysed by addition of 10ml phenol equilibrated with 0.3M Na Acetate, and 10ml of 0.3M Na acetate, 0.5% SDS, 5mM EDTA. Adherant cell lines were lysed in situ after removal of the culture media from 75cm^2 or 175cm^2 flasks containing approximately 10^7 cells or more, and the lysis solution transfered to a 50ml Falcon tube after gentle shaking for 5 minutes. Non-adherent cell lines were pelleted in 50ml Falcon tubes prior to lysis. After gentle shaking for 5 minutes, 10ml chloroform were added, and the mixture shaken for another 5 minutes. The aqueous and organic phases were then separated by centrifugation (3500rpm, 20 minutes, 4^0C).

The aqueous phase was collected and re-extracted with 10ml chloroform as descibed above, prior to the addition of 2 volumes of ethanol in order to precipitate the nucleic acids, (at least 1 hour at -20^0C).

The ethanol precipitated nucleic acids were pelleted by centrifugation, and resuspended in 2ml autoclaved water. The RNA was selectively precipitated by addition of an equal volume of 4M LiCl at 4^0C overnight. The precipitate was pelleted in a Sorval centrifuge (SS34 rotor, 15000rpm, 4^0C), and the RNA resuspended in sterile water. Contaminating DNA was removed by the addition of $MgCl_2$ (5mM final concentration), together with 20µg/ml DNAase1, (RNAase free, Boeringer Mannheim). After a 20 minute incubation at room temperature, the reaction was terminated by freezing at -20^0C or ethanol precipitation. The RNA was stored at -20^0C. The DNA which was contained in the LiCl supernatant, was recovered ethanol precipitation and resuspended in sterile water.

16.   Preparation of Radioactively-Labelled DNA Fragments.
Random Priming Method.

Approximately 50-100ng of the probe DNA insert is added to

$32\mu l$ water and boiled for 7 minutes to denature the DNA. Add $10\mu l$ oligo labelling buffer (OLB-table2.5), $2\mu l$ bovine serum albumen (BSA 10mg/ml), $5\mu l$ alpha ^{32}P radioatively labelled dCTP (Amersham code PB10205) and $0.5\mu l$ Klenow. The above mixture was mixed gently and incubated for 2-4hr at room temperature. The reaction was stopped by the addition of $100\mu l$ 0.5M EDTA.

Unincorporated nucleotides were removed by gel filtration through a Sephadex G50 column. The specific activity of the labelled probe calculated (usually $1-2 \times 10^8$ cpm/μg) and the appropriate amount used in the hybridisation. The probe was always boiled for 5 minutes and chilled quickly on ice before use.

Tissue Culture

All solutions used are shown in table 2.6

17.   Maintenance of Cell Lines

All cell lines were maintained in SLM (table2.6). During passage of the FDCP1 cell line, 10% WEHI conditioned media was included. 3T3 fibroblasts (Todaro and Green 1963) and packaging cell lines were passaged twice a week by aspirating off the old media washing the cells once in PBS and adding 2ml 0.125% trypsin/25cm flask (table2.6). Detached cells were diluted 1:3 for passage. The non-adherent FDCP1 cell line (Dexter et al. 1980a was also passaged twice a week by removing two thirds of the culture media and replacing this with fresh media. In any experiment which required the FDCP1 to be replated in semi-solid media, the cells were washed twice in PBS to remove growth factors prior to replating.

18.   Freezing of Cells

Cells to be frozen under liquid nitrogen were resuspended in SLM plus 10% glycerol and aliquoted into Nunc cryotubes (0.75ml/tube). The tubes were then stored at -70^0C for between 12hours and several weeks, after which they were stored under liquid nitrogen.

19.   Transient Transfection of Virus Packaging Cell Line (Sompayrac and Danna 1981)

A 2.5mg/ml solution of DEAE-dextran was made by dissolving 1.25g DEAE-dextran in 250ml 1M Tris and the pH adjusted to 7.3. 250ml of sterile water was added to make 500ml of a 2.5mg/ml solution which was filter sterilised. Prior to use the stock solution of DEAE-dextran was diluted 1:9 with SLM, 0% FCS, and sterilised.

Cells to be transfected were seeded out 24hr previously at $5x10^5$ cells/25cm flask in SLM/10% FCS. Media was removed from the cells and replaced with 5ml DEAE-dextran/SLM/0% FCS and 5-25µg plasmid DNA and incubated for 1 hour at 37°C. The cells were then washed twice in SLM/0% FCS before incubating the cells in 5ml SLM/10% FCS for 48 hours. After 48 hours the virus containing culture media was removed, filtered through a 0.45µ disposable filter and either stored at -20°C for up to 1 month or used immediately.

20.   Ca Po$_4$ Co-Precipitation Method For Stable Transfection. (Wigler et al. 1979; Graham and Van Der Ebb 1973)

One day before transfection, the cells were reseeded at a density of $5x10^5$/75cm flask. The precipitate was formed as follows: 60µl of CaCl$_2$ was added to 540µl water containing 0.5-3µg plasmid DNA and 20µg sheared genomic carrier DNA. 600µl of 2xHBS (table2.6) was added slowly dropwise while vortexing. A precipitate was allowed to form at room temperature for 30 minutes. The DNA/CaPo$_4$ precipitate was then pipetted onto the cells and left overnight. The next day the cells were washed with PBS and incubated in SLM for a further 48 hours. The cells were then trypsinised and reseeded at $5x10^5$ cells/10cm tissue culture petri dish.

21.   G418 Selection (Colbere-Garapin et al. 1981)

Aliquots of sterile stock G418 (Gibco, 80mg/ml in water) were frozen at -20°C and was added to cultures at a range of concentrations from 0.4mg/ml to 2mg/ml. G418 resistant fibroblast colonies were scored after 14 days by the addition of Giemsa stain to a final concentration of 10% for 2 minutes. The cells were washed gently in water and air dried.

22.   Virus Infections. (Magli et al. 1987; Mulligan 1983; Laker et al. 1987)

Virus containing media was harvested from virus producing cell lines reseeded 24hr previously at $5x10^5$ cells/25cm flask, passed through a 0.45µ filter and either used immediately or stored at -20°C for 1 month.

Fibroblasts to be infected were seeded out at $5x10^5$ cells/25cm flask 1 day before infection. Immediately before virus infection the

culture media was changed. The fibroblasts were exposed to an aliquot of virus supernatant, (usually 50µl), for 24hr in the presence of 6µg/ml polybrene, (Toyoshima and Vogt 1969) after which the cells were washed and incubated for a further 48hr, The cells were then trypsinised, counted in a Coulter counter and replated at 5×10^5 cells/10cm petri dish (10ml media) . A minimum of 4 dishes were used per experiment and G418 (see21) was added at a concentration of 800µg/ml. For each experiment two 10cm petri dishes containing 200 cells were plated out in non-selective media in order to determine replating efficiency.   Infected cells were incubated for 2 weeks at which time well isolated colonies were picked after removal of the culture media with a Gilson p200 pipette.   Colonies were also counted by the addition of Giemsa stain (10% v/v) to the media for 2 minutes, followed by a brief wash in water.

Bone marrow cells harvested as described in (23) and FDCP1 cells were exposed to virus by co-cultivation with virus producing cells seeded out 24hr previously at 5×10^5 cells/25cm flask . Polybrene was added at 6µg/ml and WEHI conditioned media to 10% (FDCP1 cells), or 2% (bone marrow cells).   Co-cultivation was allowed to take place for 24hr after which non-adherent cells were recovered. The non-adherent cells were washed twice in PBS to remove polybrene and growth factors prior to replating. Details of individual infections are described in the results chapter 6.

23.   <u>Harvesting of Bone Marrow.</u>

Normal bone marrow was obtained from the femurs of 8-12 week old female NIH mice (obtained through Olac directly, or bred at the Beatson). A minimum of two animals were used per experiment. The femurs were cleaned of surrounding tissue and both ends of the femur cut off using scissors. The core of bone marrow in the femur cavity was flushed out with 1ml SLM by inserting a 21 gauge needle into one end of the femur. The cells were dispersed into a single cell suspension with a 10ml pipette and nucleated cells counted using a haemocytometer by diluting a sample of cells 1 in 10 with 0.3% methylene blue in 90% ethanol. Approximately 10^7 nucleated cells are recovered per femur.

24. <u>Conditioned Media (Pragnell et al. 1988)</u>

The WEHI 3B (Metcalf and Nicola 1982; Ihle <u>et al.</u> 1982), AF1

(Pragnell et al. 1988) and L-929 (Stanley and Heard 1977) cell lines were grown in roller bottles with SLM/10% FCS to half confluence. Spent media was then removed, replaced with fresh media and the cultures allowed to grow for a further 3 days. The conditioned media was removed, passed through 0.45 and 0.22μ filters and stored at -20°C.

25. Clonogenic Assays

1. CFU-S (Till et al 1961)

CFU-S were assayed by injecting 0.2ml aliquots containing $5-8x10^4$ nucleated bone marrow cells (harvested as described in 23) into female NIH mice exposed 24 hours previosly to 8.2 Gy X-irradiation (10 per group). Cells to be injected were resuspended in SLM containing 0.2% serum. Spleen colonies were counted 10 days later after fixation of the whole spleen in Bouins fixative.

2. CFU-C Progenitor Assay (Pragnell et al 1988)

CFU-C were assayed by culturing $7-5x10^4$ cells per 3cm petri dish containing 0.3% agar or 0.9% methocellulose in supplemented alpha-modified MEM containing 25% FCS (Table2.6). Conditioned media from the AF1 or WEHI 3B cell lines were used as sources of colony stimulating factor. The 1ml cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air and colonies were counted on day 7 using an inverted microscope.

3. CFU-A Primitive Progenitors (Pragnell et al 1988)

For the detection of CFU-A, 10^4 cells in 4ml supplemented alpha-modified MEM (Table 2.6) containing 25% FCS and 0.3% agar or 0.9% methocellulose were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929 conditioned media and AF1-19T conditioned media in a 6cm petri dish. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2 , 5% O_2, 85% N_2 for 11 days. Colonies were stained overnight with INT (2-(4 Iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride) overnight (Boll et al 1977) and those with a diameter of greater than 2mm were scored.

4.   <u>FDCP1 Clonogenic Assay</u>

Details of individual experiments are given in the results.
FDCP1 cells were washed twice in PBS to remove residual growth factors
before being added to 0.3% agar or 0.9% methocellulose in supplemented
alpha-modified MEM (Table 2.6) containing 25% FCS at the desired cell
density.

26.   <u>Microwell Assays (Metcalf et al. 1986; Lang et al. 1985)</u>

Microwell assays for GM-CSF activity in conditioned media were
performed using Falcon Terasaki 60 well plates containing 500 FDCP1
cells in 10μl SLM/10% FCS.   Viable cell counts were performed after
48hr of incubation at 37°C.

27.   <u>5-Fluoruracil Treatment</u> (Pragnell <u>et al</u> 1988)

Groups   of   5 NIH mice were given a single   dose   of
5-Fluorouracil (5FU) at 150mg/kg.   Bone marrow cells were obtained at
different times after treatment as described in section 23.

Table 2.1 Plasmid Preparation.

TEG buffer
50mM glucose
25mM Tris HCl pH8
10mM EDTA
4mg/ml lysozyme

Alkaline/SDS
0.2N NaOH
1% SDS

Potassium Acetate 5M

Table 2.2 Ligation of DNA

Ligation buffer x10

Tris/CL pH7.5   500mM
$MgCl_2$ 100mM
ATP   1mM
BRL nuclease free BSA 1% w/v

Alkaline Phosphotase (CIP) buffer x10

Tris/HCL pH9   500mM
$MgCl_2$ 10mM
$ZnCl_2$ 1mM
spermidine   10mM

Table 2.3 Agarose Gel Electrophoresis

TEA buffer pH8

Tris base	40mM
EDTA	2mM
NaCl	20mM
NaAc	20mM

TBE buffer pH8

Tris borate	89mM
Boric acid	89mM
EDTA	2.5mM

MOPS buffer x10 pH7

MOPS(Sigma)	0.4M
NaAc	0.1M
EDTA	0.01M

Gel. Sample Buffer

Tris/CL pH7	125mM
Glycerol	20% w/v
EDTA	5mM
Bromophenol blue	0.25% w/v
xylene cyanol	0.25% w/v

RNA loading buffer

MOPS buffer	1x
Formamide	50% v/v
Formaldehyde	2.2M

Table 2.4 Transfer and hybridisation buffers.

20x SSC   sodium chloride   3M
                           sodium citrate   0.3M

20x Phosphate buffer pH6.5 Na_2HPO_4 0.5M
                              NaH_2PO_4 0.5M

50x Denhardt's solution
Ficoll-400   1% w/v
polyvinylpyrrolidone   1% w/v
BSA (Pentax fraction V)   1% w/v

Table 2.5 Random Priming Labelling Buffer.

OLB-250µl   1M Tris pH7.4   62µl
                           1M $MgCl_2$ 6µl
                           2-mercaptoethanol   1µl
                           15mM dATP   2µl
                           15mM dGTP   2µl
                           15mM dTTP   2µl
                           2M Hepes pH6.6   125µl
                           150 OD units/ml
                           calf thymus hexanucleotides
                           (Pharmacia)   50µl

Store at $-20^{\circ}C$

Table 2.6 Tissue Culture

Culture Medium (SLM)

Supplemented modified Eagles medium (SLM , Gibco cat. no.
                              043-01136M)
10% foetal calf serum (FCS, Gibco)
2mM Glutamine

Dulbecco's phosphate-buffered saline, solution A (PBS)

KCl   2.68mM
KH_2PO_4 1.47mM
NaCl   0.137M
$Na_2HPO_47H_2O$ 8.06mM

Trypsin dilutant, PBS/EDTA.

PBS
1mM EDTA

Trypsin

0.15% trypsin in PBS/EDTA

2x Hepes buffered saline

NaCl   280mM
Na_2HPO_4 1.5mM
Hepes pH7.4   50mM

Adjusted to pH7.12 with HCl

MEM Alpha Stock (Gibco)

Disolve 5 Litre Pack (Gibco) in 1400ml water, add 50ml MEM
x100 vitamins (Gibco), 10ml Phenol red and 100mg gentamycin
sulphate. Filter sterilise.

MEM Alpha x2

For 100ml

Alpha stock   21ml
200mM glutamine   1ml
7.5% $NaHCO_3$ 3ml
serum   25ml

Add equal volumes of MEM Alpha x2 and agar or methocellulose
at the required viscosity to obtain MEM Alpha x1.

Table 2.7 Sources of Chemicals

Unless otherwise stated, all chemicals were obtained from BDH Chemicals, Poole, Dorset, UK, or Sigma Chemical Co, St Louis, Missourri, USA.

Sephadex G50   Pharmacia, Uppsala, Sweden.
polnucleotide kinase

ampicillin   Vestric, Glasgow, Scotland.

Methocel MC4000   Fluka, Buchs, Switzerland.

Bacto Agar   Difco, Detroit, USA.

Agarose   BRL, Rockville, Marryland, USA.
low melting point agarose
restriction endonucleases
bacterial alkaline phosphatase
Klenow large fragment
T4 DNA polymerase

Special liquid medium   Gibco Ltd, Paisly, Scotland.
Foetal bovine serum
glutamine
G418

CHAPTER THREE

3.1   Subcloning of GMΔ7 into pV200neo

The retroviral vector pV200neo (Episkopou al., 1984) is based on MoMLV and is a double expression type of vector (Chap.1.7). Selection of this vector can be achieved in eukaryotic cells by virtue of the neomycin (neo) gene under the control of the herpes simplex virus thymidine kinase (HSVtk) promoter (see Fig. 3.1, 3.2A). The vector pV200neo (pVneo) has a unique BamH1 restriction site, 5' to the tkneo selectable marker into which fragments of DNA can be subcloned (Fig.3.1). Subcloning of GMΔ7 into the BamH1 site requires the 5' LTR for its expression and so the insert must be orientated parallel with the viral LTRs.

Although a full length GM-CSF cDNA has been cloned (Gough et al., 1985), it has been shown that deletion of 5' non coding sequence increased growth factor production from cos cells transfected with an expresssion vector carrying the deleted GM-CSF cDNA (Gough et al., 1985). The 5'deleted cDNA was termed GMΔ7 (Fig.3.2B, Gough et al., 1985). In order to express GM-CSF in haematopoietic cells, two vectors were used. Dr W. Ostertag kindly provided mos^{-3}neoGM which relies on viral splice signals for efficient expresion of GM-CSF, (Laker et al 1987, Ostertag pers. comm.). However, due to the problems associated with splice type vectors discused in chapter 1.7, it was decided to subclone GMΔ7 from mos^{-3}neoGM into the double expression vector, pVneo. Thus, both vectors contain identical GM-CSF inserts , and can be used in parallel to compare vector mediated gene expression. To subclone the approximately 920 base pair GMΔ7 BamH1 fragment was removed from mos^{-3}neoGM (Fig. 3.2B, 4.1), ligated to BamH1 digested pV200neo (pVneo) and transformed into E.coli strain HB101 as described in Materials and Methods. Transformants were selected on the basis of ampicillin resistance. DNA from twenty clones were examined by restriction analysis with BamH1, (Fig. 3.3). Clones 3, 11, 14 and 20 appeared to have inserts, (Fig.3.3).

In order to establish the orientation of the inserts within the retroviral vector, a diagnostic Pst1 digest was carried out. The predicted fragment sizes are shown in Fig 3.4, 3.1, 3.2.

Fig.3.5A shows Pst1 digests of samples 3, 11, 14 and 20 and reveals that only clone 3 contains the GMΔ7 fragment in the correct

Fig. 3.1

A <u>pV200neo (pVneo)</u>

Redrawn with modifications from Episkopou <u>et</u> <u>al</u>.,
(1984)

LTR - long terminal repeat

pBR - pBR322 sequences

TK - sequences from herpes simplex virus thymidine
kinase promoter

NEO - neomycin coding sequences

s.d. - splice donor sequence (viral encoded)

B   pVneoGM

Derived from pVneo by subcloning GMΔ7 into the
<u>BamH</u>1 cloning site as described in 3.1.

All sizes are in base pairs.

A_pV200 neo

Hind III
Eco RI
Sst I

pBR

1230

Hind III
Sst I
s.d.

594

L.T.R.

417

Bam HI
Pst I
Bgl II
Pst I

270

pBR

138

TK NEO

1166

Pst I

3800

621

594

L.T.R.
Sst I

670

EcoRI

pBR322

B_pV neo GM

Hind III
Eco RI
Sst I

Hind III

Sst I
s.d.

Bam HI
Pst I

GM Δ7

920

Bam HI
Pst I
Bgl II
Pst I

Pst I

Sst I

EcoRI
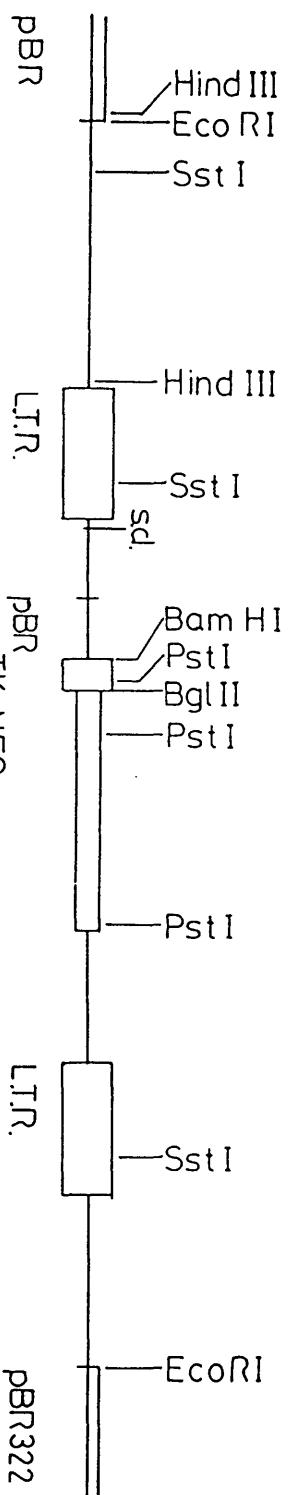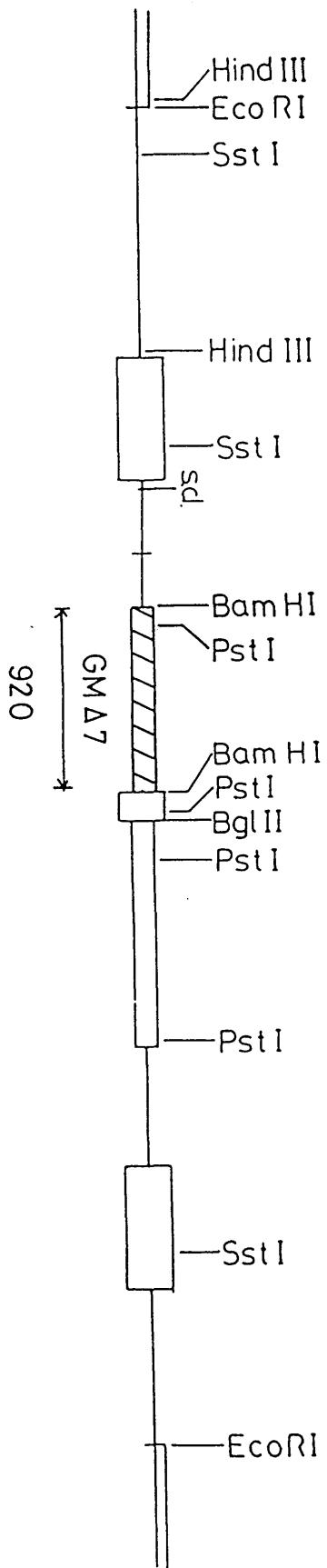
Figure 3.2

A   Diagram to show Pst1 sites in the TKNEO region
of pVneo(pVneo).

B   Relationship of GMⱯ to GM–CSF 3.2 including
Pst1 sites.

All sizes are in base pairs.

A pVNEO   TK NEO fragment

TK   NEO

6123
to PstI   Bam PstI PstI
in pBR 322 HI

I03I 24 9

925

PstI

2663 to PstI in pBR 322

B

i) GM 3.2

EcoRI

1035

PstI

EcoRI

ii) GM Δ7

BamHI PstI

920

828

92

BamHI
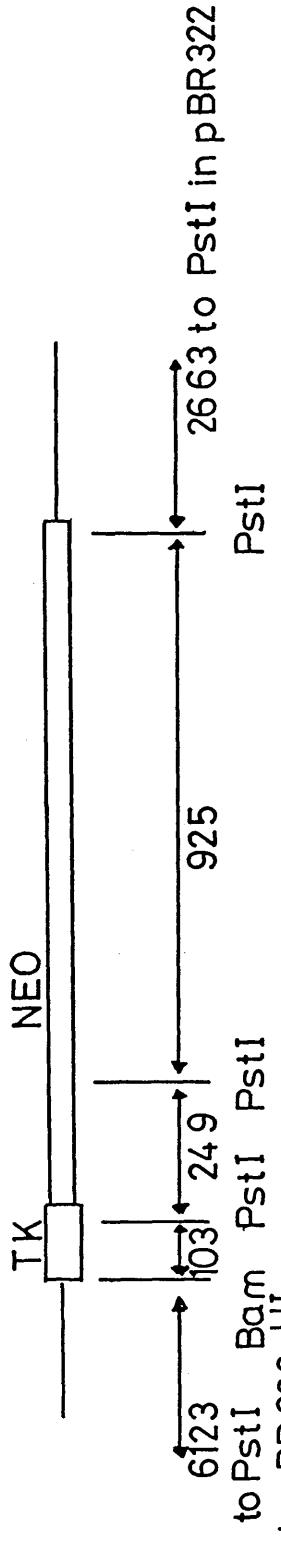
Figure 3.3

Construction of pVneoGM

BamH1 cut pVneo ligated with GM 7. Miniprep DNA from 20 transformants cut with BamH1.

Samples 3, 11, 14 and 20 had inserts released by BamH1.

M - EcoR1 λ markers.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14   M 15 16 17 18 19 20
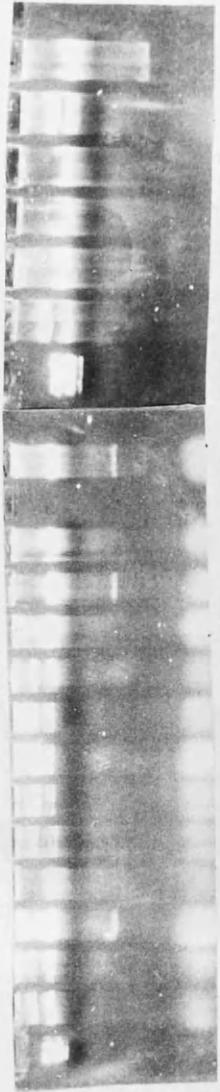
orientation with a fragment of approximately 6215 base pairs similar to control cut pV200neo (Fig 3.5B). Samples 11, 14 and 20 contain the larger 6951 base pair fragment. The correct orientation of GMΔ7 was confirmed by the experiment shown in Fig. 3.5C. In comparison to Pst1 cut pV200neo, sample 3 contains a 931bp fragment which would not be present if the insert was antiparallel to the viral LTRs. Sample 3 was therefore confirmed to be the desired transformant and was renamed pVneoGM.

3.2   Subcloning of probes into Bluescribe Vector

The Bluescribe vector (Vector Cloning Systems) contains a multiple cloning site identical to that found in pUC19 (Pharmacia) but flanked by the RNA polymerase promoters T3 and T7. The bluescribe vector has the advantage of permitting colour selection for inserts due to the presence of the beta galactosidase gene in the appropriate host/inducer system (see Materials and Methods). Once an insert has been cloned into the vector and i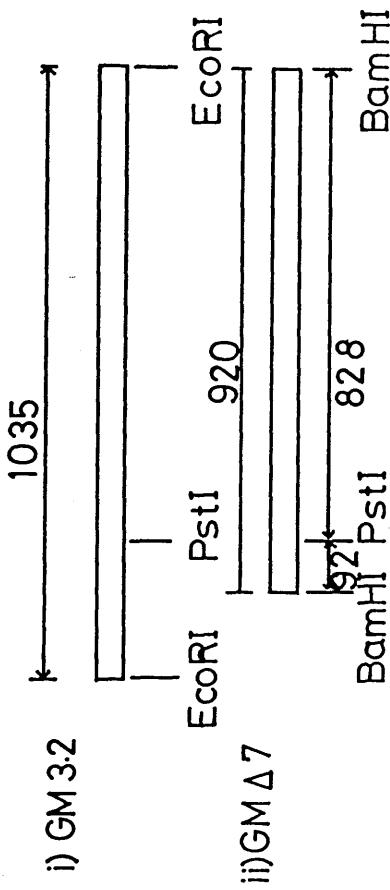ts orientation deduced, sense or antisense RNA probes can be generated using the T3 or T7 promoters (Melton et al 1984). In addition the inserts can be labelled by random priming (See Materials and Methods).

In order to carry out molecular analysis on RNA and DNA from transfected or infected eukaryotic cell lines the following probes were subcloned (Fig.3.6):-

i)   tk promoter

ii)   neo gene

iii)   GMΔ7

iv)   promoter containing fragment of MoMLV LTR

The tk promoter and neo gene were subcloned from plasmid p61 Cl 24 kindly donated by Dr J Lang (Fig 3.6), GmΔ7 from mos^{-3}neoGM (Fig 4.1) and the MoMLV LTR sequences from pV200neo (Fig. 3.1 Episkopou et al 1984).

96

Figure 3.5

Subcloning of GMΔ7 into pVneo: determination of insert orientation.

A   All samples were cut with Pst1 and run on a 1% TBE agarose gel. Lane 1, pVneo; lane 2, sample 3; lane 3, sample 11, lane 4, sample 14, lane 5 sample 20.

Markers are EcoRI cut λ and EcoRI/HindIII cut λ .

B   Lane 1, pVneo; lane 2 sample 3.   Both samples were cut with PstI and run on a 1% TEA agarose gel. Markers are EcoRI/HindIII cut λ and HindIII cut λ .

C   Both samples are cut with Pst1 and run on a 4% acrylamide gel. Lane 1 pVneo; lane 2 sample 3. Markers are HpaII cut pBR322, HaeII cut pBR322 and EcoRI/HindIII cut λ .

A

7·55
5·93

21·8
5·24
3·38
1·96

1 2 3 4 5

B

6.67
4.26
2.25

1 2

C

930
880
590
450/430
370
2 27

1 2
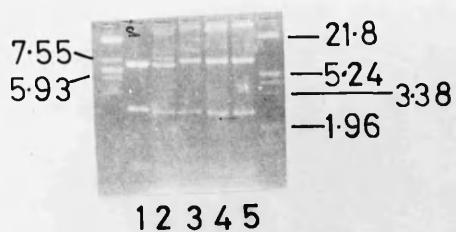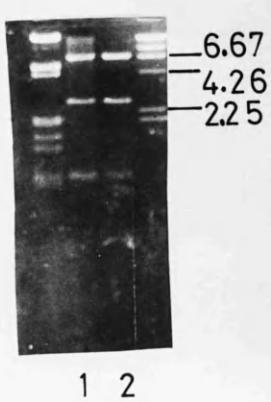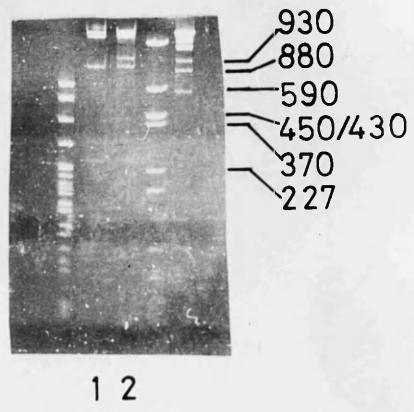
<u>Figure 3.6</u>

Bluescribe vector was cut and ligated to insert DNA as in
Materials and Methods. The table below sumarises the
restriction digests used. After ligation, JM83 bacteria were
transformed and white colonies picked for analysis.
Plasmids containing the correct inserts were mapped for
insert orientation. In all the figures , orientation of the
insert within the polylinker is shown by an arrow. For
simplicity only the polylinker region is shown.
T3 and T7 are the promoter sequences for RNA polymerases T3
and T7 respectively

<u>Figure</u>	<u>Bluescribe digest</u>	<u>Insert</u>	<u>New Plasmid Name</u>
3.6C	<u>Bam</u>H1/<u>Hinc</u>11	<u>Bgl</u>11/<u>Sma</u>1 1kb neo fragment from p61cl-24	BS-NEO
3.6C	<u>Bam</u>H1	<u>Bam</u> H1/<u>Bgl</u>11 250bp TK promoter fragment from p61cl-24	BS-TK
3.6B	<u>Bam</u>H1	<u>Bam</u>H1 920bp fragment from mos^{-3}neoGM	BS-GM
3.6A	<u>Eco</u>R1/<u>Sma</u>1	<u>Eco</u>R1/<u>Sma</u>1 1.8kb fragment from pV200neo	BS-pV200

A
pV200neo 5'LTR

LTR →

EcoRI   SmaI

EcoRI / SmaI

1800 bp

BS-pV200

T 7   T 3

EcoRI   SmaI   HindIII

B

GM∆7

BamHI PstI   BamHI

T 7   T 3

SstI   Bam   PstI   BamHI   HindIII
        HI

BS-GM
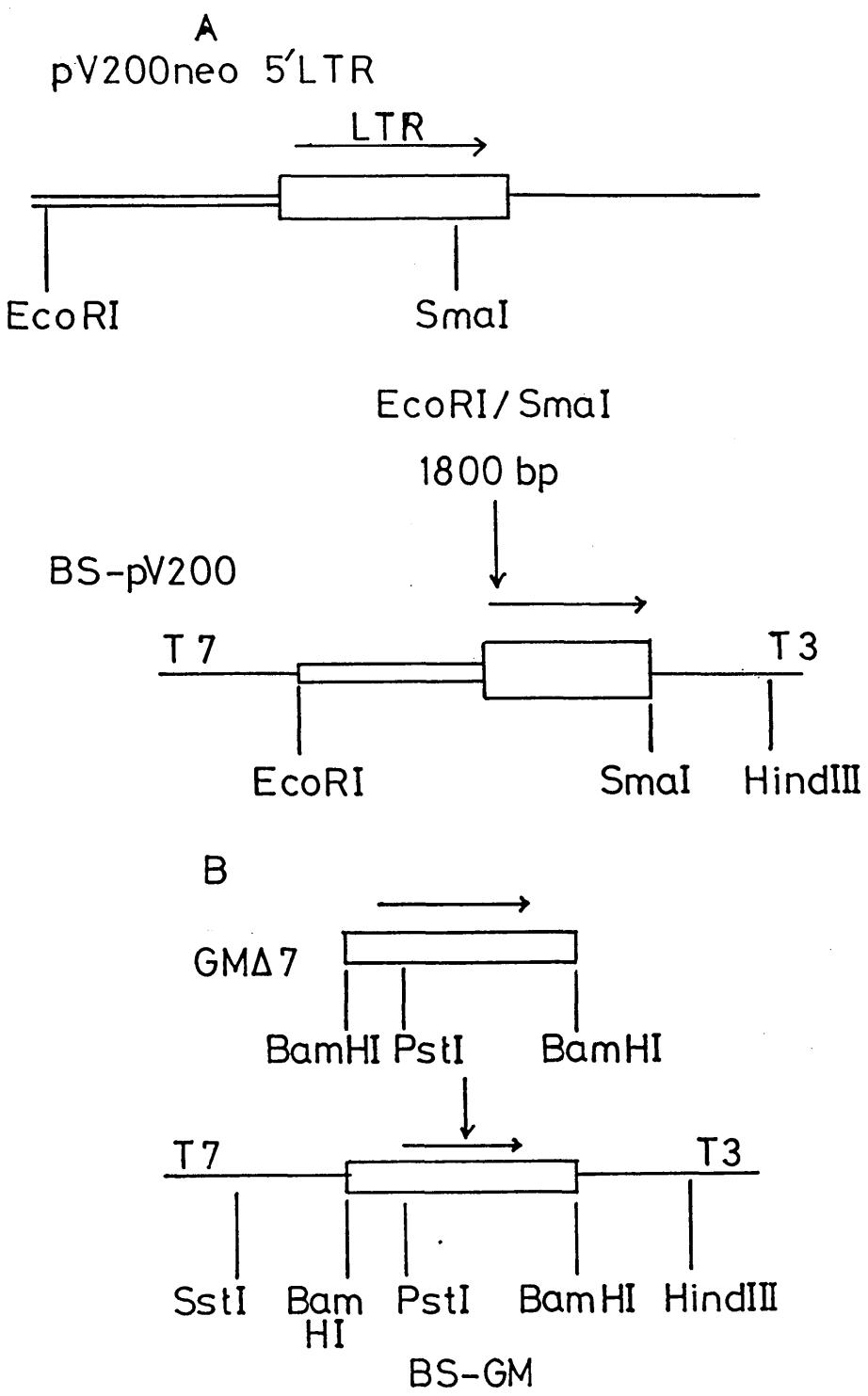
C.
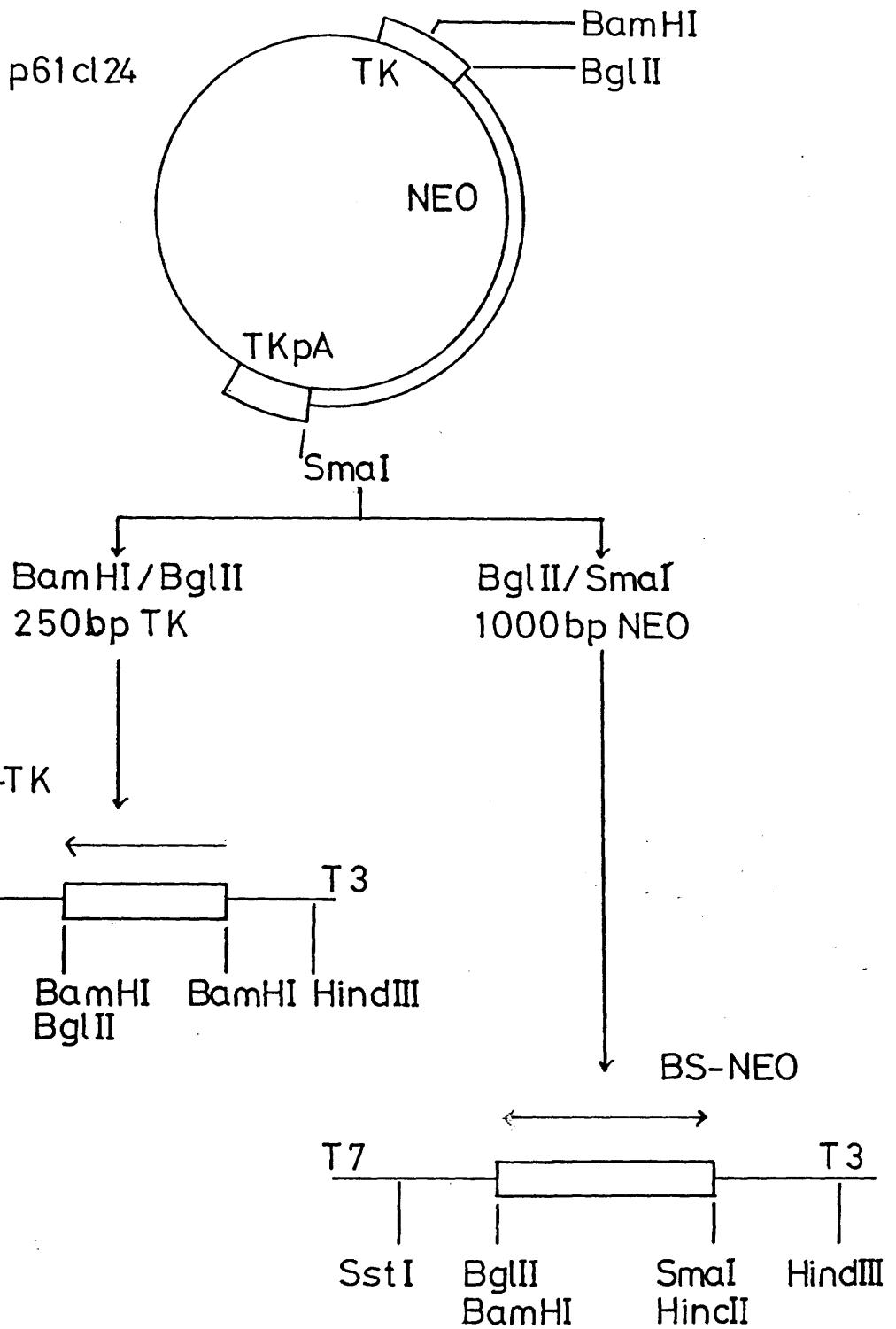
CHAPTER FOUR

GENERATION AND ANALYSIS OF VIRUS PRODUCING CELL LINES

Introduction

In order to generate virus producing clones, retroviral constructs must be introduced into a packaging cell line(Mulligan 1983). This can be achieved in two ways. The vector can either be transfected by calcium phosphate co-precipitation into the packaging cell line, or a transient transfection of the Ψ Am (amphotropic) packaging cell line can be carried out and rescue virus particles rescued 48 hours after transfection (See Materials and Methods, Miller et al 1986). The amphotropic virus produced during the transient transfection is then used to generate stable virus producing cell lines by infecting ecotropic Ψ2 cells (Miller et al., 1986). In either case, stable integrants can be selected by resistance to antibiotic. The latter method relies on the availability of two packaging cell lines which release virus particles capable of recognising different cell receptors (Miller et al., 1986). Ψ2 cells produce ecotropic envelope protein and so will be refractory to superinfection by an ecotropic virus (Miller et al., 1986. Weiss et al., 1985). However by packaging the vector in a virus particle comprising of amphotropic envelope molecules this block can be overcome (Miller et al., 1986).

The use of the infective virus to generate virus producing cell lines has several advantages over stable transfection. Calcium phosphate co-precipitation is relatively inefficient generating only about one transfectant per 10^4 fibroblast cells (Debenham et al. 1984). The integrated DNA is often rearranged and is present in multiple copies thus the virus produced from such cell lines may be a heterogeneous mixture of normal and re-arranged virus, (Mulligan 1983, Lebkowski et al. 1984)

Virus infection is on the other hand a very efficient process, capable of generating up to 10^6 drug resistant 3T3 cells per millilitre of virus supernatant (Miller et al., 1986). Infection of retrovirus packaging cell lines should generate virus producing cell lines which harbour only one or at most a few copies of the retrovirus and so virus production should be relatively homogeneous (Miller et al., 1986). Rearrangement of provirus within the packaging cell line should not be common, as the cell line is generated by a biologically

active retrovirus which integrates in the defined orintation of cellular DNA-LTR-viral sequences-LTR-cellular DNA (Miller et al., 1986, Bernstein et al 1985). It has also been demonstrated that genes introduced into cells by viral infection are more efficiently expressed than genes introduced by DNA transfection (Hwang and Gilboa, 1984). Thus it should be possible to generate virus producing cell lines with higher virus titres by this approach (Miller et al., 1986).

4.1 Retrovirus vectors used in this study

The four retroviral vectors that have been introduced into the Ψ2 packaging cell line are shown in Fig. 4.1. These are pVneo (Episkopou et al., 1984) and its GM-CSF derivative pVneoGM, (Fig.3.1B), pmos^{-3}neo and its GM-CSF derivative, pmos^{-3}neoGM (Laker et al, 1987; Oestertag pers. comm.). The GM-CSF insert is GMΔ7 in both cases. The pVneo vector of Episkopou is based on MoMLV and contains the bacterial neomycin gene (neo) which confers resistance to the antibiotic G418 (Santerre et al.1984). The neo gene is transcriptionally controlled by the herpes simplex virus thymidine kinase promoter. pVneoGM relies on a full length message starting from the 5' LTR and terminating in the 3' LTR for expression of the GM-CSF sequences. The predicted transcripts form both pVneo and pVneoGM are shown in Fig. 4.2.

In vivo, MoMLV is associated with long latency lympoid disease (Weiss et al 1985) and the related virus, Moloney murine sarcoma virus (MoMSV) which carries the mos oncogene (Weiss et al 1985), will cause sarcomas in vivo and transform fibroblasts in vitro (Ostertag et al. 1980). The vector pmos^{-3}neo is based on myeloproliferative sarcoma virus (MPSV) (Ostertag et al. 1980,Laker et al.1987, Ostertag pers. comm.). MPSV, like MoMSV carries the mos oncogene (Stocking et al. 1985) but not only will MPSV transform fibroblasts in vitro and cause sarcomas in vivo but it will also induce proliferation of both early myeloid and erythroid progenitor cells and CFU-S (Ostertag et al. 1980, Klein et al. 1981). The LTRs of MPSV have been shown to be derived from MoMLV and that the expanded host cell range of MPSV is due to point mutations in the U3 region of the LTR (Stocking et al., 1985,1986).

pmos^{-3}neo, like pVneo carries the neomycin gene but it is the viral LTR which exerts control over transcription rather than a

Figure 4.1

Vectors

The four vectors used in this study are shown in this figure. Relevant restriction sites are marked. pVneo (Episkopou et al., 1984) and pVneoGM are based on MoMLV. pmos^{-3}neo (Laker et al., 1987; Ostertag, pers. comm.) and pmos^{-3}neoGM (Ostertag pers. comm) are based on myeloproliferative sarcoma virus. All sizes are in base pairs and the vectors are pBR322 based.

s.d. - viral splice donor site

s.a. - viral splice acceptor site

pBR - pBR322 plasmid sequences.

pV200 neo

Hind III | EcoR1 | Sst1 | Hind III | Sst1 | sd. | Bam H1 | Pst1 | Bgl II | Pst1 | Pst1 | Sst1 | EcoR1

pBR   L.T.R.   pBR   TK NEO   L.T.R.   pBR322

1230   594   417   270   138   1166   621   594   670

3800

pV neo GM

Hind III | EcoR1 | Sst1 | Hind III | Sst1 | sd. | Bam H1 | Pst1 | Bam H1 | Pst1 | Bgl II | Pst1 | Pst1 | Sst1 | EcoR1

GM Δ7

920

pMOS⁻3 neo

EcoR1 | Pst1 | Sst1 | Pst1 | Xho1 | Pst1 | Pst1 | BamH1/Bgl II | Sma1 | BamH1 | Xba1 | Pst1 | Sph1 | Hind III | Kpn1 | Sst1 | Bgl II/BamH1

pBR   LTR   sd.   NEO   sa   LTR   pBR

594   1796   1480   1080   594

5540

pMOS⁻3 neoGM

EcoR1 | Pst1 | Sst1 | Pst1 | Xho1 | Pst1 | Pst1 | BamH1/Bgl II | Kpn1 | Bam H1 | Pst1 | Bam H1 | Sst1 | Bgl II/BamH1

sd.   sa   GM Δ7

920

Probes used in Southern and Northern blotting. The
figure shows diagramatically the predicted proviral
structures and transcripts for each of the four
vectors. Predicted transcript sizes are in
kilobases.

The positions of the DNA sequences recognised by
the restriction enzymes SstI and BamHI are shown.
Digestion of genomic DNA with SstI should release a
DNA fragment containing almost the complete
provirus which can be used to examine provirus
integrity. The distance between the SstI sites of
the viral LTRs is approximately the same as the
size in kilobases given for the full length viral
transcripts shown under each provirus. Digestion
of genomic DNA with BamHI, which cleaves the
proviral structure internally, when probed with
neo gives some indication of the number of viral
copies per genome. The probes used in all blots
are shown as solid blocks under the region of the
provirus to which they correspond. The 1kb neo
fragment used was a BglII/SmaI digest from plasmid
p61 cl-24 (see Fig.3.6c). The 381bp GM-CSF
fragment was a PstI/EcoRV digest from plasmid
pGM3.2. All probes were labelled by random priming
as described in Materials and Methods.
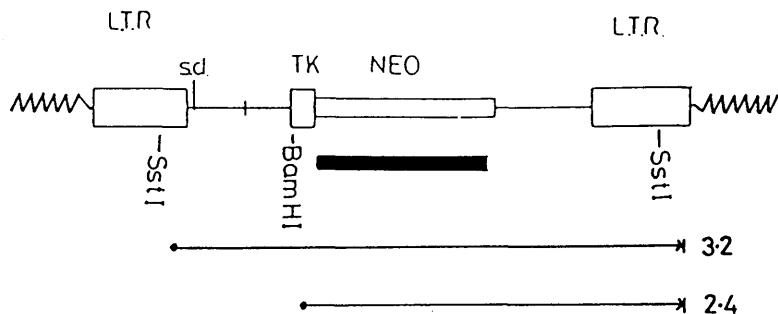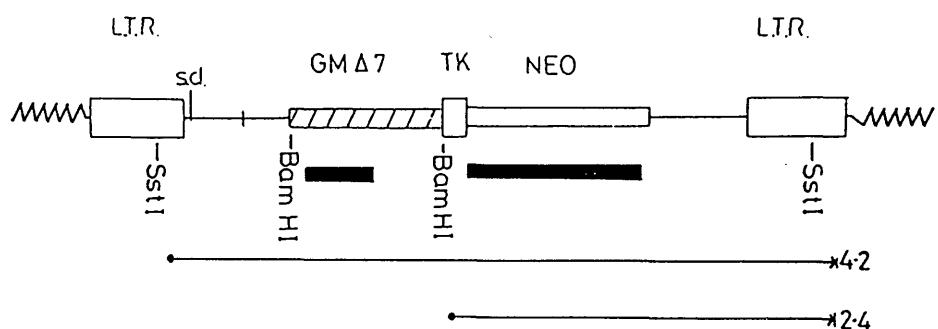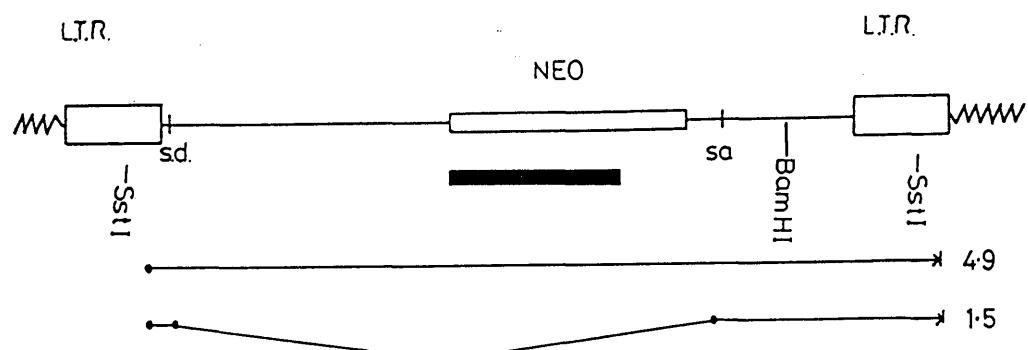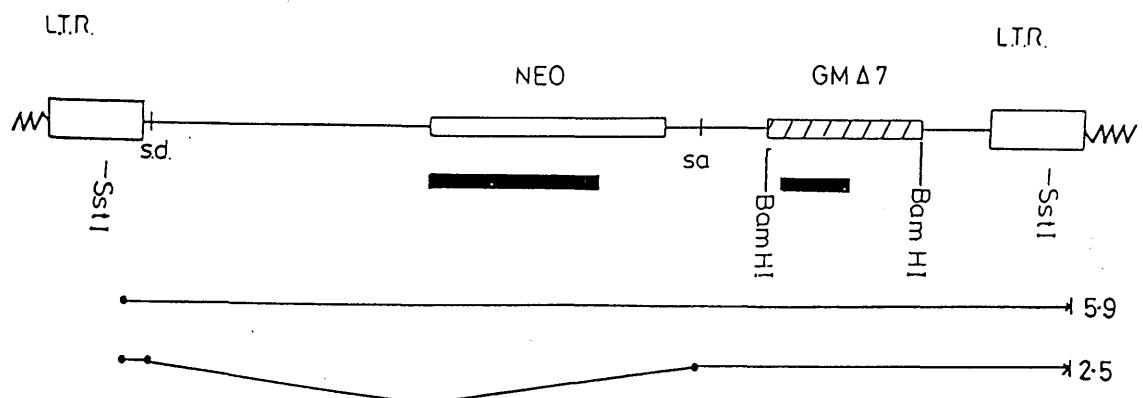
thymidine kinase promoter. The GMΔ7 sequences in pmos⁻³neoGM also rely on the viral LTR for their expression as this is a splice type vector (see1.7). Efficient expression of GMΔ7 in pmos⁻³neoGM relies on the viral splice donor and acceptor sites being utilised to generate sub-genomic mRNA (Laker et al 1987, Ostertag pers. comm.) The predicted mRNAs derived from pmos⁻³neo and pmos⁻³neoGM are shown in Fig. 4.2.

4.2 Generation of Virus Producing Cell lines and Infection of 3T3 fibroblasts

Two methods were used to generate virus producing Ψ2 clones, details of which are in Materials and Methods:-

1) Stable transfectants using calcium phosphate co-precipitation: One microgram of each plasmid to be transfected was added to sheared genomic carrier DNA and precipitated onto Ψ2 cells seeded out 24 hours previously at 5×10^5 cells per 75cm² flask. The transfected cells were then left for 24 hours, at which time the medium was replaced with 20ml of fresh medium. After a further 48 hours , the cells were trypsinised, counted and seeded at a density of 5×10^5 cells per 10cm plate in the presence of 800 μg/ml of G418. All the vectors used in these studies carry the neomycin gene which confers resistance to G418. The plates were incubated for two weeks and distinct G418 resistant colonies were cloned and expanded under selective conditions (800 μg/ml). Control plates had no colony growth in the presence of 800μg/ml G418.

2) Transient transfection into ΨAm (Amphotrophic) packaging cell line, followed by virus rescue after 48 hours and infection of Ψ2 cells (Details in Materials and Methods). ΨAm cells had been seeded out 24 hours previously at a concentration of 5×10^5 cells per 75cm² flask and plasmid was transiently transfected using DEAE dextran . The media was changed 1 hour after the transfection and virus was harvested after a further 48 hours. The media containing virus was passed through a 0.45μ filter and used immediately to infect Ψ2 cells which had been seeded out 24 hours previously at 5×10^5 cells per 25cm² flask. Media was removed from the Ψ2 cells and 5ml of virus containing media was added to the 25cm² flask together with polybrene (6 μg/ml) to aid virus absorption. The cells were then incubated for a further 24 hours then washed with PBS and 5ml of fresh media added.

A 48 hour period to allow virus expression then followed, after which the cells were trypsinised counted and seeded into 10cm dishes at a density of 5×10^5 cells per dish in the presence of 800 µg/ml G418. After two weeks G418 resistant colonies were cloned and expanded in the presence of 800µg/ml G418.

Table 4.3 shows 13 Ψ2 virus producing clones that were isolated either by stable transfection (T) or transient transfection followed by infection (I). The vector introduced into each clone (and therefore the virus released) is also shown.

The virus titre released form each clone was assayed as follows:- Ψ2 clones were plated out at 5×10^5 cells per 25cm^2 plate with 5ml of media without G418. After 24 hours the media was removed and used immediately for virus assay. A 50 µl aliquot of virus supernatant was added to 3T3 fibroblasts seeded out 24 hours previously at 5×10^5 in a 25cm^2 flask. Virus absorption to the cells was aided by the addition of poybrene(6 µg/ml) and left on the 3T3 cells for 24 hours, before the cells were washed in PBS and 5ml fresh media added. After a 48 hour expression period the 3T3 fibroblasts were trypsinised, counted and seeded at 5×10^5 cells per 10cm dish in the presence of 800 µg/ml G418. The plates were incubated for 2 weeks before the frequency of G418 resistant colonies was calculated. The number of G418 resistant colonies was adjusted for re-plating efficiency, the fraction of infected cells plated out and virus volume to give a value for the number of G418 resistant 3T3 cells generated per millilitre of virus supernatant (G418 colonies per ml). Table 4.3 shows the virus titres of the 13 Ψ2 clones on up to three separate experiments.

Titre is determined by how efficiently the viral genome is encapsidated and the degree of selectable gene expression in any given cell type. Although the virus titres vary between clones from about 2×10^3 cfu/ml to 5×10^5 cfu/ml these figures are similar to reported titres for the Ψ2 and amphotropic packaging cell lines (Eglitus et al.,1985; Chang et al., 1987; Kwok et al., 1986; Dick et al., 1985; Miller and Buttimore 1986; Mann et al., 1983). There is also variation between independent titring of the same clone, for example in Fig4.3, the virus titre of clone 11 was measured three times and showed up to a ten fold difference in virus titre. The reasons for such differences within a clone may be a reflection of the degree of

Figure 4.3

Generation of virus producing clones and their viral titres in 3T3 fibroblasts.

Virus producing cell lines were generated in two ways; either by transfection using calcium phosphate co-precipitation (T) or alternatively, an amphotropic virus packaging cell line (ΨAm) was transfected by DEAE Dextran and transiently released virus was harvested after 48h. The virus containing media ws then used to infect Ψ2 cells (I).

Transfected (T) or infected (I) Ψ2 cells were placed under G418 (800μg/ml) selection for two weeks. Well separated colonies were then expanded. In order to titre virus release from Ψ2 clones, virus containing media (50μl) was used to infect 3T3 fibroblasts. The number of 800μg/ml G418 resistant colonies was determined after two weeks and adjusted for plating efficiency, proportion of cells replated and volume of virus used. This gives a figure for colony forming units per ml virus supernatant (colonies/ml). The titre on 3T3 fibroblasts was performed on up to three independent occassions for each clone.

VIRUS TITRES OF INDIVIDUAL Ψ2 CLONES

CLONE	GENERATED BY TRANSFECTION(T) OR INFECTION(I)	VECTOR	TITRE ON 3T3 CELLS $G418^R$ COLONIES/ML		
			1	2	3
1	T	$mos^{-3}neo$	2.3×10^5	2×10^5	
2	T	$mos^{-3}neo$	$>10^5$	3.5×10^3	
7	T	$mos^{-3}neo$	1.6×10^4	1.7×10^4	
41	I	$mos^{-3}neo$	2.5×10^5	5.3×10^5	
31	I	$mos^{-3}neoGM$	4.1×10^4	5.3×10^4	
34	I	pVneo	1.4×10^4	2×10^3	
35	I	pVneo	5.8×10^5	$>10^5$	
36	I	pVneo	5×10^3	5.9×10^3	
37	I	pVneo	7.6×10^4	$>10^5$	
38	T	pVneo	2.3×10^4		
11	T	pVneoGM	2.8×10^4	3.3×10^5	6.0×10^4
13	T	pVneoGM	1.2×10^5		
15	T	pVneoGM	5.3×10^4	$>10^5$	

confluence at the time of virus harvest, or it may also reflect experimental error in cell counting and incubator variation. From these experiments there does not appear to be any gross differences in virus titres on 3T3 fibroblasts from virus producer lines generated by transfection or infection as suggested by Miller et al. 1986, nor are there any apparent differences in titre between the four vectors. It is possible that real differences in titre do exist but the experimental method used to titre virus here did not show it. By taking a range of dilutions of virus rather than any one value then comparative differences in virus titre may be observable.

4.3 <u>Molecular analysis of Ψ2 virus producer clones and 3T3 cell lines derived by infection.</u>

Although the data in table 4.3 demonstrates that virus producing clones can be made, it is important that the virus released is unrearranged and expresses its transcriptional units as predicted (Fig.4.2). It is also desirable that infected cells only contain one copy of the virus so that any phenotypic effect may be attributed to that single provirus copy. If cell lines contain multiple copies of a gene then the analysis of its integrity in all copies and their transcriptional activity is complex.

Southern Analysis

All four of the retroviral vectors used in this study (fig. 4.1 and 4.2) generate a DNA fragment corresponding to the full length of the virus genome when digested with <u>Sst</u>1 (Fig.4.2). Since the restriction enzyme <u>BamH</u>1 cuts the retrovirus vectors internally, gross rearrangements can be detected by <u>Sst</u>1 digest and vector copy number can be evaluated by <u>BamH</u>1 digest. The <u>neo</u> probe shown in Fig. 4.2 was used in the experiments described below. The fragment sizes detected in the Southern blot in Fig.4.4, lanes 1 to 4 reflect the integrity of each of the four basic vector types in these cell lines and their relative sizes. It can be seen that the viral genome sizes are 4.4kb for pVneo (lane 1), 5.75KB for pVneoGM (LANE 2), 6.0kb for mos^{-3}neo (lane 3) and 7.0kb for mos^{-3} neoGM (lane 4). Although these sizes differ from the predicted genome sizes for the virus shown in Fig. 4.2 and 4.1, the relative sizes are correct. In separate experiments, DNA from clone 35 cut with <u>Sst</u>1 results in a <u>neo</u> hybridising fragment of 3.3kb (Fig.4.5 lane 1; Fig 4.6 lane 1). Thus

Southern analysis of virus producer clones.

Samples were run on a 0.7% TEA agarose gel and 20µg
of DNA was loaded per lane. Size markers are
HindIII cut λ. The blot was probed with neo.

Lane	Clone	Vector	Digest
1	cl-35	pVneo	SstI
2	cl-11	pVneoGM	SstI
3	cl-41	$mos^{-3}neo$	SstI
4	cl-31	$mos^{-3}neoGM$	SstI
5	cl-35	pVneo	BamHI
6	cl-11	pVneoGM	BamHI
7	cl-41	$mos^{-3}neo$	BamHI
8	cl-31	$mos^{-3}neoGM$	BamHI
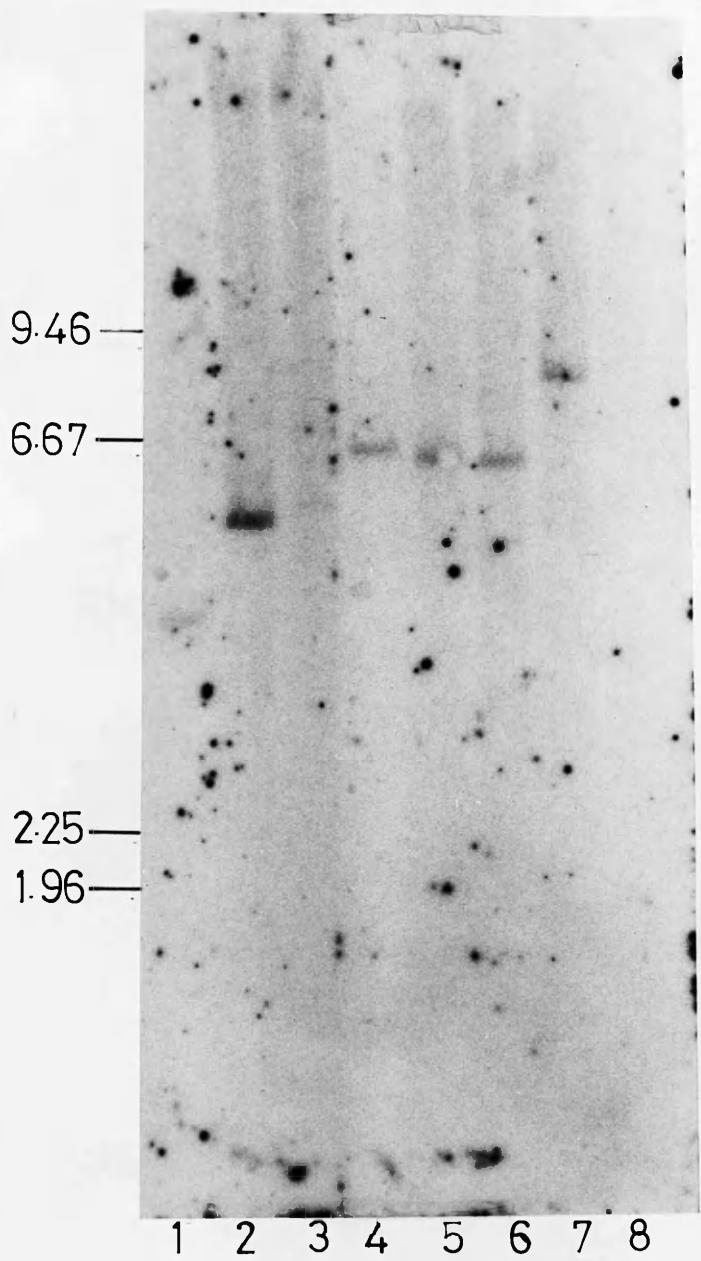
Figure 4.5

Southern analysis of virus producer cell lines and
infected 3T3 fibroblasts.

The cell lines 3T3 11-5 and 3T3 11-6 were derived
by infection of 3T3 fibroblasts with virus from
cl-35. Cell lines 3T3 24-4 and 3T3 24-10 were
derived by infection of 3T3 fibroblasts by virus
from cl-1. Infections carried out as described in
Material and Methods.

Lane	Clone	Vector	Digest
1	cl-35	pVneo	SstI
2	3T3 11-5	"	"
3	3T3 11-6	"	"
4	cl-35	"	BamHI
5	3T3 11-5	"	"
6	3T3 11-6	"	"
7	cl-1	$mos^{-3}neo$	SstI
8	3T3 24-4	"	"
9	3T3 24-10	"	"
10	cl-1	"	BamHI
11	3T3 24-4	"	"
12	3T3 24-10	"	"

9·5 —
6·7 —
5·3 —
4·2 —
3·3 —

2·3 —
1·9 —

1 2 3 4 5 6 7 8 9 10 11 12
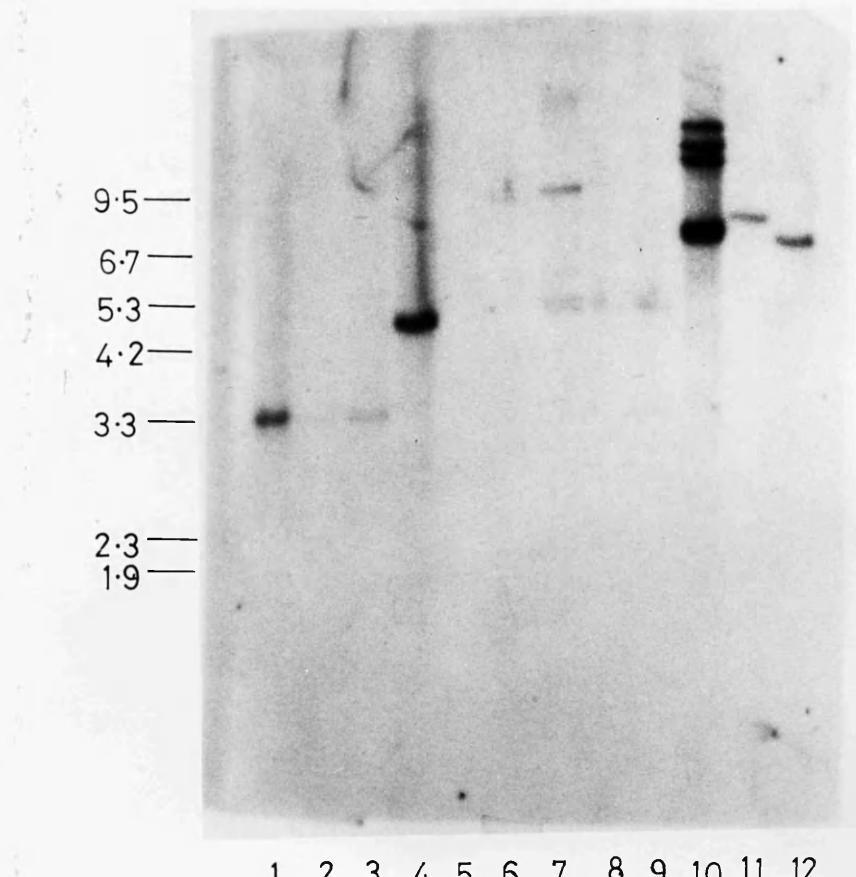
differences observed in sizes of hybridising bands for any identical DNA sample cut with the same enzyme on different occassions may be due to the differances in the running of the markers. However it is clear from the relative sizes of the hybridising fragments in Fig4.4 lanes 1-4 that the vectors are not grossly rearranged in these Ψ2 clones.

The three Ψ2 clones - Cl-35, Cl-41, and Cl-31 were generated by infection, where as Cl-11 was generated by transfection, (Fig.4.3). BamH1 digests of genomic DNA from these clones (Fig 4.4 lanes 5-8) demonstrate that all four clones probably contain only one copy of the vector as only one hybridising fragment is observed in each cell line. This is expected for clones generated by viral infection which usually only leads to integration of a few copies of the virus per genome (Mulligan 1983).

The Ψ2 virus producer clone, Cl-1 was generated by DNA transfection. Cleavage of genomic DNA from Cl-1 by BamH1 reveals a complex pattern of many neo hybridising fragments (Fig.4.5 lane 10) indicating multiple copies of the vector. Digestion of this same DNA with Sst1 (Fig 4.5 lane 7) also reveals multiple bands hybridising to neo which suggests that during transfection the multiple copies of the plasmid have become rearranged. Interestingly, two 3T3 cell lines (3T3 24-4 and 3T3 24-10) derived by infecting 3T3 fibroblasts with virus from Cl-1 contain only one copy of virus each (Fig 4.5 lanes 11 and 12) which appear to be of the correct size (4.9kb) and unrearranged (Fig. 4.5 lanes 8 and 9). Clone 1 was kindly assayed for helper virus release by Dr D. Onions and found to be positive. It is therefore possible that vector sequences in this clone have undergone recombination with one of the endogenous packaging defective helper viruses. Although clone-1 contains many copies of the vector, the virus it releases is of the predicted size. It cannot be assumed however that this is a homogeneous population of viral particles due to the multiple vector copies in Cl-1.

One virus producer cell line, Cl-35, which contained only one copy of pVneo per genome (Fig 4.4 lane 5; Fig. 4.5 lane 4 and FIg. 4.6 lane 2) and did not show any gross rearrangements (Fig4.4 lane 1; Fig4.6 lane 1) was used to infect 3T3 fibroblasts which were then selected for G418 resistance (G418r). Four G418 resistant 3T3 cell lines that had been generated from this infection were analysed for the integrity of the viral genomes present. In Fig. 4.6 lane 1 an

Sst1 digest of the parental virus producing cell line Cl-35 reveals a 3.3kb neo hybridising fragment. Lanes 3,5,7,9 show DNA from four independent 3T3 cell lines generated by infection with virus from Cl-35. All four cell lines have genomes of identical size to the parental Ψ2 clone and therefore no gross rearrangements have occurred. Unfortunately the copy number of virus per genome can only be ascertained in one 3T3 line, 3T3 11-6 (Fig. 4.6 lane 8).

A similar analysis of three independent G418r 3T3 cell lines derived by infection from virus from clone 11 (Fig.4.7), also demonstrated intact proviral sequences identical to that in the parental virus producing line (Fig.4.7 lanes 1-4). Viral copy number per genome was only estimated for the parental virus producing clone, Cl-11 (Fig4.7 lane 5) and one 3T3 cell line (Fig.4.7 lane 8) In both cases there was only one virus copy per genome.

Northern Analysis

RNA was extracted from the Ψ2 virus producing cell lines and virus containing 3T3 cell lines by phenol extraction and precipitation of the RNA with lithium chloride (Details in Materials and Methods). Although it was known the vectors used can generate G418r clones (Fig4.3) and therefore express the neo gene, it was important to establish the molecular basis for expression of this and other transcripts arising within the vectors. (Fig.4.2). Northern analysis was used to study steady state levels of viral encoded mRNA species, however, as with all the transcription studies RNase protection experiments (Melton et al 1984) with the appropriate probes (Chap.3.2) would be required to confirm the predicted transcriptional units,(Fig.4.2).

RNA from virus producing cell lines was isolated as described in Materials and Methods before separating on a 1.4% denaturing formaldehyde agarose gel. The gel stained with ethidium bromide prior to blotting is shown in Fig. 4.9C. The 28S and 18S ribosomal bands are clearly visible in the ethidium bromide stained gel indicating the RNA is not degraded. After blotting the filter was hybridised with neo (Fig 4.8A), exposed to film, stripped of neo probe and re-probed with GM-CSF (Fig 4.8B).

In cell lines containing the vectors pVneo or pVneoGM, two transcripts should be detectable if the neo probe is used (Fig4.2).

Figure 4.6

Southern analysis of virus producing clone 35 and
3T3 fibroblast cell lines derived by infection from
virus released from clone 35.

Lane	Clone	Vector	Digest
1	cl-35	pVneo	SstI
2	"	"	BamHI
3	3T3 11-8	"	SstI
4	"	"	BamHI
5	3T3 11-7	"	SstI
6	"	"	BamHI
7	3T3 11-6	"	SstI
8	"	"	BamHI
9	3T3 11-5	"	SstI
10	"	"	BamHI

Samples were run on a 0.7% TEA agarose gel and the
filter was probed with neo. The G418 resistant cell
lines were generated by infecting 3T3 cells with
virus from cl-35.

22 —

5 —
4·2 —
3·3 —

1·9 —

1 2 3 4 5 6 7 8 9 10
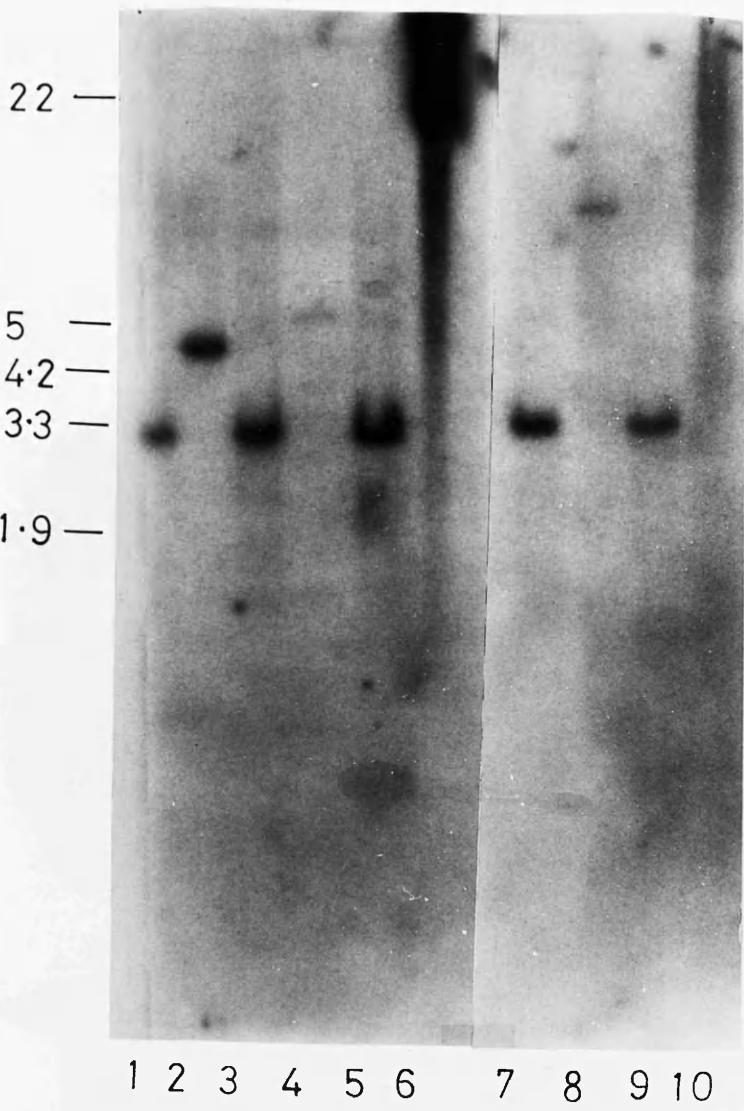
<u>Figure 4.7</u>

Southern analysis of virus producer clone 11 and 3T3 fibroblast cell lines derived by infection from virus from clone 11.

Lane	Clone	Vector	Digest
1	cl-11	pVneoGM	<u>Sst</u>I
2	3T3 25-6	"	"
3	3T3 25-8	"	"
4	3T3 25-9	"	"
5	cl-11	"	<u>BamH</u>1
6	3T3 25-6	"	"
7	3T3 25-8	"	"
8	3T3 25-9	"	"

Samples were run on a 0.7% TEA agarose gel and the filter was probed with <u>neo</u>. The G418 resistant 3T3 fibroblast cell lines 3T3 25-6, 3T3 25-8 and 3T3 25-9 were generated by infecting 3T3 cells with virus from cl-11 as described in Materials and Methods.
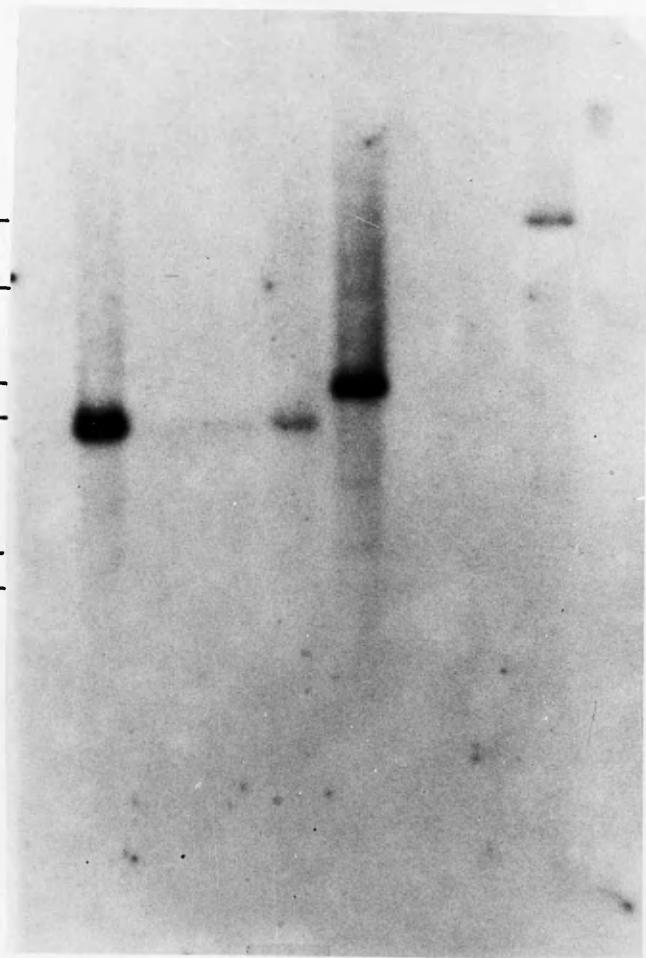
Figure 4.8

Northern analysis of virus producing clones.

Lane	Clone	Vector
1	Ψ2	/
2	cl-35	pVneo
3	cl-35	pVneo
4	cl-11	pVneoGM
5	cl-15	pVneoGM
6	cl-41	$mos^{-3}neo$
7	cl-41	$mos^{-3}neo$
8	cl-31	$mos^{-3}neoGM$
9	"	"
10	"	"
11	cl-1	$mos^{-3}neo$
12	AFI[*]	/

Each sample represents an independent isolation of
RNA. The samples were run on a 1.4% formaldehyde
denaturing gel. AFI are normal rat kidney cells
infected with AFI virus (Pragnell et al.1988).

A   probed with neo

B   The same filter re-probed with GM-CSF.

C   The Northern gel stained with ethidium bromide
prior to blotting.   The markers are 28S ribosomal
RNA - 4.7kb,   18S ribosomal RNA - 1.8kb.

D Lane	Clone	Vector
1	cl-35	pVneo
2	Ψ2pV-4	"
3	cl-11	pVneoGM
4	cl-15	"

The samples were run on a 1.4% denaturing gel and
the filter was probed with neo.

In some mRNA samples an apparent hybridisation to a
mRNA species of molecular size similar to the 18S
ribosomal RNA is observed and is likely to be
related to the running of the ribosomal RNA.

C

28S

18S

1 2 3 4 5 6 7 8 9 10 11 12

D

28S

18S
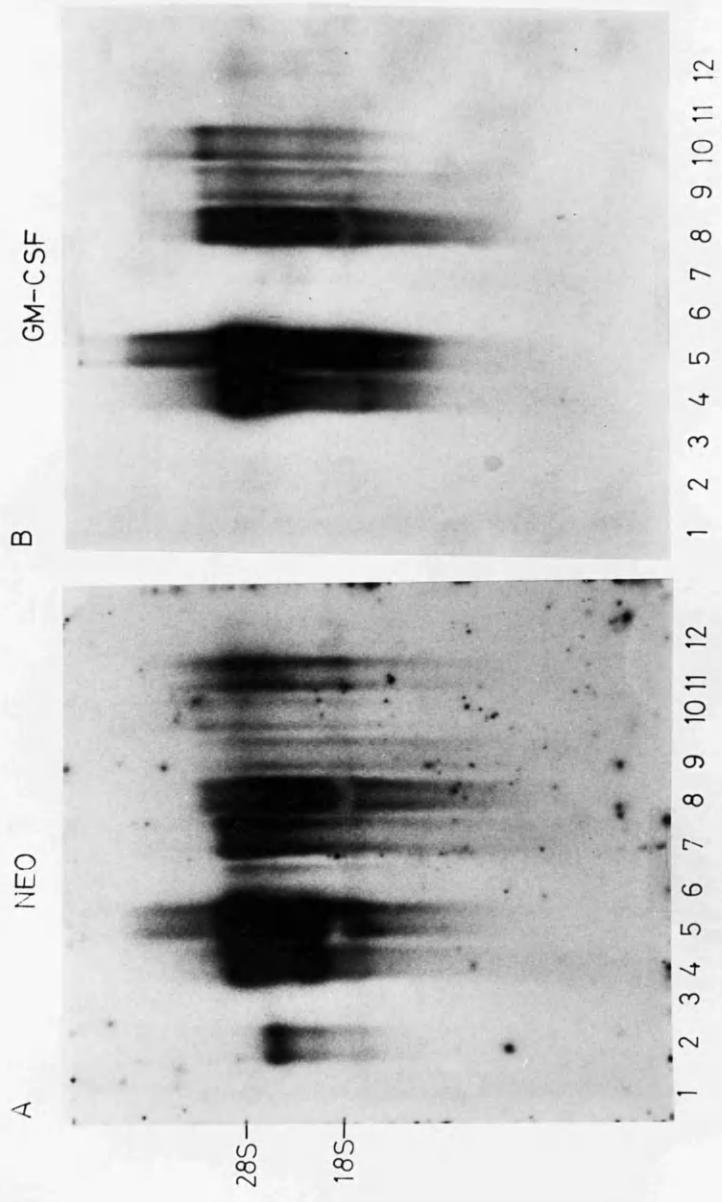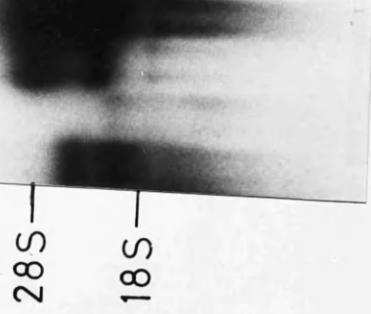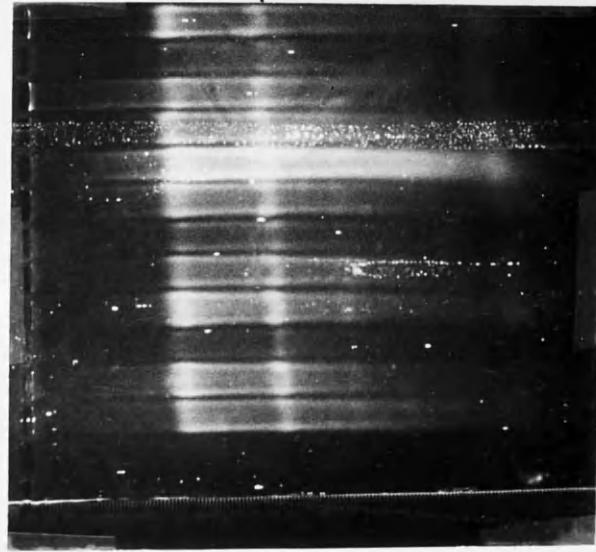
1 2 3 4

Figure 4.9

Northern analysis of virus infected 3T3 fibroblasts and their respective virus producing cell lines.

A

Lane	Clone	Vector
1	cl-11	pVneoGM
2	3T3 25-6	"
3	3T3 25-8	"
4	3T3 25-9	"

B

Lane	Clone	Vector
1	cl-35	pVneo
2	3T3 11-3	"
3	3T3 11-6	"

Samples were run on 1.4% formaldehyde denaturing gels and the filters were probed with neo. The markers were 28S = 4.7kb; 18S = 1.8kb. The 3T3 cell lines 3T3 25-6, 25-8 and 25-9 were derived by infection of 3T3 fibroblasts by virus from cl-11.; The 3T3 cell lines 3T3 11-3 and 11-6 were derived by infection of 3T3 fibroblasts by virus from cl-35.
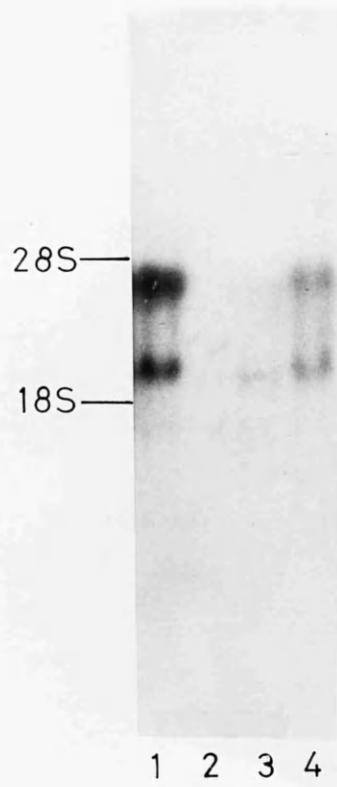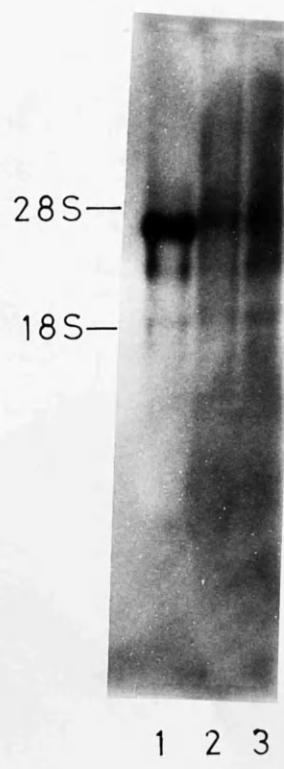
Cell lines containing pVneo should have transcripts of sizes 3.2kb and 2.4kb and pVneoGM containing cells should also have the 2.4kb transcript but due to the insertion of GM Δ7 the full length transcript should increase in size to 4.2kb. Fig 4.8A lane 2 has RNA from Cl-35(pVneo) and two RNA species which hybridise to <u>neo</u> can be observed. Hybridisation to the smaller species is faint suggesting that at the time of cell harvest the tk promoter is not functioning efficiently since the smaller 2.4kb <u>neo</u> specific RNA is transcribed form the tk promoter (Fig 4.2). However a previous preparation of mRNA from Cl-35 cells (Fig 4.8 D lane 1) did show equal representations of both the 3.2kb and the 2.4kb RNAs. This observation suggests that cell lines change during passage either to unstable gene expression, or selection of cell line varients. If expression of a gene has some phenotypic effect then alteration of its expression during cell passage may have to be taken into account.

The cell lines Cl-11 and Cl-15 both contain the pVneoGM vector(Fig4.3). Fig.4.8A lanes 4 and 5 shows total mRNA form these cells hybridised to <u>neo</u>. Two species of mRNA hybridise to the <u>neo</u> probe, the larger of which corresponds to the predicted size of the full length viral transcript which encodes both GMΔ7 and <u>neo</u>, and the smaller transcript is the <u>tkneo</u> specific transcript. Indeed when reprobing this filter with a GM-CSF probe (Fig4.8B lanes 2 to 5) only the larger message in clones 11 and 15 was detected (lanes 4 and 5), suggesting that the predicted transcriptional units drawn schematically in Fig 4.2 do exist and are present in fibroblast RNA.

RNAs from virus producer cell lines containing mos^{-3} neo and $mos^{-3}neoGM$ were also included on the filter shown in Fig. 4.8. Hybridisation with a <u>neo</u> probe should reveal full length viral transcripts of 4.9kb and 5.9kb in cells containing the $mos^{-3}neo$ vector and the $mos^{-3}neoGM$ vectors respectively (Fig.4.2). Lanes 6-11 of Fig.4.8A contain RNA from $mos^{-3}neo$ and $mos^{-3}neoGM$ containing ψ2 cell lines. All these samples show a considerable smear of hybridisation without any clear bands despite the integrity of the RNA (Fig 4.8C). These RNAs were isolated from three independent cell lines - Cl-1, Cl-31 and Cl-41 and in the cases of CL -31 and Cl-41, more than one independent RNA preparations were made from the same cell line (Fig4.8 lanes 6 and 7, 8-10). It is possible that the smear of hybridisation which originates from the largest predictable transcript size

(Fig4.2), is perhaps a feature of this vector. Hybridisation to GM-CSF only occurs in mRNA from cell lines containing $mos^{-3}neoGM$ vector (Fig. 4.8 B lanes 8-10). From the position of the GMΔ7 insert within the vector (Fig.4.2) the GM-CSF probe should hybridise to the full length viral genome transcript and a smaller 2.5kb sub-genomic transcript generated by splicing out the neo containing part of the virus. No distinct bands are observed with GM-CSF in lanes 8-10 Fig. 4.8B except for the upper size limit of the hybridising RNA. As with the neo probe it is difficult to assess the transcriptional units within the mos^{-3} neo based vectors. The observation that a GM-CSF probe hybridises to RNA of a size greater that 2.5kb (i.e the size of the predicted sub-genomic message in mos^{-3} neoGM) suggests that sub-genomic splicing does not occur efficiently in this vector and therefore translation of the GMΔ7 transcript may be inefficient as the second protein of a bicistronic full length mRNA. Laker et al. (1987) also report that sub-genomic mRNA was not observed in fibroblasts using a similar vector.

Expression of pVneo and pVneo GM in 3T3 fibroblast cell lines derived by Virus infection.

The virus producing cell line Cl-11 contains one copy of the pVneoGM vector per genome (Fig.4.4 lane 5; Fig 4.7 lane 5) which has not been rearranged (Fig.4.4 lane 2; Fig 4.7 lane 1). The 3T3 cell lines 3T3 25-6, 3T3 25-8 and 3T3 25-9 were selected for G418r after infection by virus released from Cl-11 and all three cell lines contain intact retroviruses (Fig. 4.7 lanes 2,3,4). Moreover it is likely that 3T3 25-9 contains only one provirus copy per genome as assessed by Southern analysis (Fig4.7 lane 8). mRNA was extracted from the Cl-11 and the three cell lines derived by infection with virus from Cl-11, in order to examine the expression of pVneoGM in several related cell lines. Fig. 4.9 A lanes 3 and 4 shows that when the mRNA extracted from Cl-11 and 3T325-9 was hybridised to neo, the two characteristic mRNA species associated with pVneoGM expression (Fig4.2) were observed. It can be concluded that the viral mRNA observed for Cl-11 and 3T3 25-9 are transcribed form a single virus within the cell lines. In the case of Cl-11 this is a single transfected vector (Fig.4.3), while in 3T325-9 expression is from a single provirus.

A similar analysis was carried out on mRNA extracted from

Cl-35 and infected cell lines 3T311-3, 3T311-6 (Fig 4.9B). Cl-35 and 3T311-6 were generated by viral infection and contain one copy of unrearranged provirus each, (Fig 4.4 lanes 1 and 5; Fig. 4.5 lanes 1,4,3 and 6, Fig4.6 lanes 1, 2, 7 and 8). The parental and 3T3 fibroblast cell lines derived by viral infection all show the characteristic hybridisation to two mRNA species (Fig4.9B) which in the cases of Cl-35 and 3T311-6 results from transcription of a single proviral copy per genome.

4.4 Growth Factor release from Ψ2 virus producing cells and infected 3T3 fibroblasts

The previous sections have dealt with the molecular analysis of Ψ2 clones which produce virus and 3T3 fibroblast cell lines derived by virus infection. It is possible that if a particular cell line harboured a GMΔ7 retrovirus and the mRNA was efficiently translated and the protein transported to the cell membrane, that GM-CSF activity should be detectable in the culture medium. The level of GM-CSF activity in the culture medium may then reflect the molecular expression of the vectors. In order to investigate this possibility the amount of GM-CSF present in culture medium conditioned by various cell lines was measured (Fig. 4.10 and 4.12). The culture media from semi-confluent Ψ2 or 3T3 fibroblast clones was removed after 3 days,(3 day conditioned media) and stored at -20^{0}C. Conditioned media from virus producing clones was heated to 56^{0}C prior to use inporder to inactivate virus in the media. Growth factor activity in the conditioned media was tested in the CFU-C progenitor assay (as described in Materials and Methods). Briefly, 100 µl of the test media was added to the bottom of a 3cm dish either in the presence or absence of antiserum to GM-CSF. Antiserum to GM-CSF (Delamarter et al., 1985) was used to define the specificity of the colony stimulating factor activity. In experiments using antiserum to GM-CSF the test media was incubated for 30min at 37^{0}C prior to addition to the culture dish. Bone marrow cells from the femurs of NIH mice were added as a suspension in 0.3% agar at a cell density of 7.5x10^4 cells/ml (1ml per 3cm dish). The dishes were incubated in a humidified 5% CO_2 incubator for 7 days and colonies counted using a dissection microscope.

Conditioned media from Ψ2 cell lines which release either pVneo or mos^{-3}neo virus do not exibit colony stimulating activity (Fig. 4.10 expts. 1,5 and 6). The Ψ2 cell lines which harbour either pVneoGM or mos^{-3} neoGM gave variable results. Clones 11 and 13 release pVneoGM virus and conditioned media from these cells stimulates a frequency of colony growth from bone marrow progenitors (Fig. 4.10 expts 2 and 3) comparable to control stimulation by pure recombinant GM-CSF protein (Fig. 4.10 expt 8). The colony stimulating activity released form these cell lines is GM-CSF since addition of antiserum to GM-CSF to the cultures reduces colony stimulation to zero (Fig. 4.10 expts. 2 and 3).

Clone 15 also releases pVneoGM virus and conditioned media from this cell line stimulates colony growth in the progenitor assay (Fig. 4.10 expt 4). However this activity was only partially reduced by antiserum to GM-CSF. It is possible that the titre of antiserum is not high enough to completely neutralise the GM-CSF protein present. However the same batch of antisera completely neutralised GM-CSF activity form Cl-11 conditioned media (Fig. 4.10 expt 2), which in the absence of antisera stimulated 52 colonies per 10^5cells. Therefore it is more likely that some of the activity observed in the conditioned media from Cl-15 is due to some other growth factor (or factors) other than GM-CSF.

The conditioned media from mos^{-3}neoGM virus releasing clone-31 has no colony stimulating activity (Fig. 4.10 expt. 7). As shown in chapter 4.3 and in Fig 4.8B, clone 31 does harbour mos^{-3} neoM viral sequences and expresses both neo and GMΔ7 at the transcriptional level. However as discussed in the previous section it is apparent that translation of GMΔ7 in this cell line is from either in inefficiently spliced sub-genomic mRNA or from the second cistron of a bicistronic, full length viral mRNA. The virtual absence of any CSF activity in the conditioned medium from Cl-31 may be a reflection of the inefficient expression of the GMΔ7 part of the viral genome.

CSF activity in conditioned media from virus infected 3T3 fibroblasts

A variety of 3T3 fibroblast cell lines which had been established by virus infection and G418 selection (see Materials and Methods) were tested for release of growth factor into the culture

<u>Figure 4.10</u>

Growth factor release from Ψ2 cells.

The 3 day conditioned media (CM) from Ψ2 virus producer clones (Fig. 4.3) was assayed for growth factor activity in the CFU-C progenitor assay. 100μl of the test CM was added to 3cm petri dishes. 1ml of 0.3% soft agar containing $7.5x10^4$ cells was then overlayed. For experiments involving antiserum to GM-CSF, the test CM was incubated for 30min at 37^{0C} with the antiserum prior to addition to the culture dish. Colonies were counted after 7 days with the aid of an inverted microscope and expressed as colony number per 10^5 bone marrow cells plated . Experiments 1,2,6,8,9 and 10 were repeated twice, initially in duplicate plates, then triplicate. All other experiments were performed once in triplicate. Recombinant GM-CSF (rGM-CSF) at 875pg/ml was used as a control stimulus.

GROWTH FACTOR RELEASE FROM Ψ 2 CELLS

EXPT.	CONDITIONED MEDIA	VECTOR	ANTISERA TO GM-CSF	COLONIES/10^5 CELLS
1	cl-35	pVneo	–	0
2	cl-11	pVneoGM	–	52
			+	0
3	cl-13	pVneoGM	–	34
			+	0
4	cl-15	pVneoGM	–	66
			+	21
5	cl-41	$mos^{-3}neo$	–	0
6	cl-1	$mos^{-3}neo$	–	0
7	cl-31	$mos^{-3}neoGM$	–	2
			+	0
8	rGM-CSF		–	34
			+	0

Figure 4.11

Growth factor release by 3T3 fibroblasts infected by retrovirus vectors.

13 3T3 fibroblast cell lines established after viral infection were analysed for growth factor release into the culture medium. The CFU-C progenitor assay was used to evaluate stimulatory activity in the conditioned media from the 3T3 cell lines. 100µl of conditioned media (CM) was added to 3cm petri dishes which were overlayed with 1ml of 0.3% soft agar containing 7.5×10^4 bone marrow cells. Colonies were counted 7 days later with the aid of an inverted microscope and the number of colonies expressed per 10^5 input bone marrow cells. Controls were CM from uninfected 3T3 cells, special liquid medium (SLM), AFI CM (Pragnell et al., 1988 and no added medium. In each case 100µl were added per 3cm dish. Experiments 10 and 11 were repeated 3 times. Experiments 11, 2, 3, 4, 9, 12, 13, and 17 were repeated twice. Experiments 5, 6, 7, 8, 14, 15, and 16 once. Duplicate plates were counted for each experiment.

GROWTH FACTOR RELEASE BY 3T3 CELLS

EXPT.	CONDITIONED MEDIA	VECTOR	COLONIES/10^5 CELLS
1	11-3	pVneo	118
2	11-5	pVneo	102
3	11-6	pVneo	120
4	11-7	pVneo	110
5	11-8	pVneo	57
6	25-6	pVneoGM	103
7	25-8	pVneoGM	94
8	25-9	pVneoGM	79
9	24-4	mos^{-3}neo	77
10	10-1	mos^{-3}neoGM	116
11	10-7	mos^{-3}neoGM	128
12	10-8	mos^{-3}neoGM	88
13	10-9	mos^{-3}neoGM	105
14	3T3	Uninfected	0
15	S.L.M.		0
16	/		0
17	AFI		61

medium. Figure 4.11 summarises the results of 13 3T3 cell lines which harbour the virus vectors shown. It is immediately obvious that conditioned media from all of these cell lines have CSF activity irrespective of the vector with which they were infected. For example, although the cell lines in Fig. 4.11 Expts. 6,7,8,10,11,12 and 13 should be capable of producing GM-CSF activity, as they were infected with either pVneoGM or $mos^{-3}neoGM$. The cell lines in experiments 1-5 and 9 were derived from infection by pVneo or $mos^{-3}neo$ and should not produce CSF activity. However, in comparison to uninfected 3T3 fibroblasts (Fig 4.11 expt. 14) the conditioned media had a higher titre of CSF activity than unstimulated controls.

This result is not entirely unexpected as Koury and Pragnell (1982) have shown that many fibroblast lines including NIH3T3 cells can be induced to release granulocyte/macrophage type stimulating activity after infection with retrovirus.

Discussion

Virus producing cell lines have been established which release virus of the four vectors required in this study (Fig4.3). Analysis of Ψ2 virus producing cell lines by Southern blotting revealed that in most instances the vector genome was not gross.ly rearranged, and was often present in only one copy per cell genome (Fig4.4). The virus particles released from the cell lines can therefore be assumed to be homogeneous. Southern analysis of 3T3 fibroblasts infected with virus, confirmed that the same sized virus was present in parental virus producing cells and 3T3 cell lines derived by infection with virus from the parental line (Figs. 4.6 and 4.7). Of the two methods used to generate the virus producing clones (transfection and infection) those produced by infection showed fewer abnormalities in virus expression. One clone, Cl-1 derived by transfection (Fig. 4.3), contained many copies of the viral vector some of which were probably rearranged (Fig. 4.5). Although virus of the correct size was released from this cell line, (Fig.4.8) it cannot be ruled out that a heterogeneous population of virus particles is released. In addition, this clone released helper virus (helper virus assay kindly performed by Dr D. Onions) and so is unsuitable for some experiments.

Another clone, Ψ2pV4 which was generated by transfection of pVneo into Ψ2 cells , failed to show any detectable virus production

(data not shown) but the Ψ2 clone was resistant to G418.   Only one
mRNA species was detectable by hybridisation to neo ,(Fig. 4.8 D lane
1), and it corresponded to the 2.4kb tkneo transcript predicted to be
present in cells containing this construct (Fig4.2).   The failure to
detect hybridisation of a mRNA species corresponding to the full
length genomic viral mRNA, suggests that full length viral mRNA is not
being produced.   It is therefore possible that the lack of detectable
virus production form Ψ2pV4 is due to an rearranged vector which is
still capable of producing neo specific mRNA but which does not
produce packagable full length mRNA.

In the light of the results obtained with clone 1 and Ψ 2pV4
it is perhaps better to generate virus producing cell lines by
transient transfection followed by infection rather than stable
transfection.   This approach would select for vector molecules that
can complete one round of virus replication and retain a selectable
marker.   Vectors introduced into packaging cell lines in this way may
avoid random DNA breaks in the transfected plasmid which may occur
prior to plasmid integration into the host genome

Once the vector had been introduced into the packaging cell
lines and these had been tested for virus release, Northern analysis
of pVneo or pVneoGM containing cells was used to demonstrate that the
predicted viral transcriptional units (Fig4.2) were utilised (Fig. 4.8
A, B, D), although RNase protection studies (Melton et al 1984) would
be required to formally demonstrate this.   The efficient use of
MoMLV LTR promoter in the fibroblast cells (Fig4.9A, B) resulted in
detectable growth factor release from Ψ2 cells harbouring the pVneoGM
vector which could, in most instances be neutralised by antiserum
against GM-CSF (Fig. 4.10).

At the molecular level, cells harbouring the vectors $mos^{-3}neo$
and $mos^{-3}neoGM$ were more difficult to analyse due to the lack of
discernable discrete neo or GM-CSF hybridising mRNA species (Fig. 4.8)
A predicted 2.5kb sub-genomic mRNA that should be generated from cells
containing the $mos^{-3}neoGM$ vector and detected by the GM-CSF probe
(Fig. 4.2) was not observed (Fig. 4.8 B).   However the GM-CSF probe
did hybridise to mRNA of higher molecular weight, suggesting that the
viral splice donor and acceptor sites were not used.   This would
result in a polycistronic mRNA from which GM-CSF would have to be
translated as the second protein after the neo product (Fig 4.2) a

process which is likely to be a rather inefficient. The lack of detectable colony stimulatory activity in the conditioned media from Cl-31 (Fig 4.10) which harbours a $mos^{-3}neoGM$ vector, suggests that translation of the mRNA species that hybridises to GM-CSF in this clone (Fig4.8 B) is inefficient as suspected.

CHAPTER FIVE

Retroviral Infection of FDCP1 Cell Line

Introduction

One aim of this project was to study the growth characteristics of haematopoietic cell lines which express murine GM-CSF cDNA from a constitutive promoter. If a cell line requires GM-CSF for its survival and proliferation in culture, autocrine stimulation of the cell line can be compared to growth response to exogenously added GM-CSF. In turn, the growth properties of haematopoietic cells harbouring a GM-CSF retroviral vector should be a reflection of the molecular expression of the vector. By studying both the cellular behaviour and the molecular expression of the vector, it should be possible to determine whether retroviral vectors can be used to generate predictable and repeatable effects, which is important since it is only in the confidence that the phenotypic effect observed in cultures is due to retroviral expression, that the more complex situation of retroviral mediated gene transfer into primary haematopoietic cells can be addressed.

Retroviral expression could be studied exclusively in fibroblast cell lines, but haematopoietic and fibroblast cells are clearly specialised for different purposes and gene expression may vary between different cell lineages (Magli et al., 1987; Kwoik et al., 1986; Williams et al., 1986; McIvor et al., 1987). It is also conceivable that cells at different stages of differentiation within any lineage may, on infection, permit varying degrees of viral expression (Gorman et al., 1985; Franz et al., 1986; Rubenstein et al., 1984; Williams et al., 1986).

In order to study retroviral expression in haematopoietic cells, gene transfer can be carried out in both haematopoietic cell lines and primary bone marrow derived haematopoietic cells.

The FDCP1 cell line is a non-adherent murine haematopoietic cell line derived from a long term marrow culture, which displays primitive granulocytic morphology but does not form CFU-S foci in spleens of irradiated mice (Dexter et al1980a). It is ideal as a recipient cell line for GM-CSF vectors as it is dependant for its survival in culture on exogenously added GM-CSF (or Il-3) (Dexter et al 1980a, Hapel et al 1984). Thus once the GM-CSF vector has been transferred into the factor dependent FDCP1 cell line, the phenotypic

properties should be a result of autocrine stimulation due to expression of vector encoded GM-CSF.

Autonomous growth of tissue culture cells and tumours

Malignant cells in tissue culture require fewer exogenously added growth factors than normal cells to trigger proliferation to allow their survival (Holley et al., 1975). The difference between "normal" and "malignant" cells could reside in the fact that malignant transformed cells produce and respond to growth factors which normally have to be supplied (Sporn and Roberts, 1985). Such cells are described as being autocrine stimulated (Sporn and Roberts, 1985). The reduced requirement for growth factors may also be due to an increased sensitivity to low levels of exogenous factor suboptimal for normal cell growth (Cory et al., 1987).

In support of the autocrine hypothesis many small cell lung cancer (SCLC) cell lines have been shown to secrete the growth stimulator bombesin and bombesin like peptides (Cuttitta et al., 1985). These SCLC cell lines will grow clonally in vitro and as xenographs in vivo, but their growth can be inhibited by antiserum specific to bombesin (Cuttitta et al., 1985. It has also been observed that murine cells transformed with murine sarcoma virus become less dependent on serum growth factors and secrete autostimulatory polypeptides referred to as transforming growth factors.It is not clear however, if in all cases of autocrine stimulation the growth factor must necessarily be released from the cell to elicit a response (Robbins et al. 1985, Betsholtz et al. 1984)

Fibroblasts infected with simian sarcoma virus (SSV) produce a virally encoded protein which has extensive homology to the beta chain of human platelet derived growth factor (PDGF), (Waterfield et al., 1983; Doolittle et al., 1983). Fibroblasts which have receptors for PDGF and are infected with SSV, proliferate in response to the virally encoded $p28^{v-sis}$(Huang et al., 1984; Johnson et al., 1985). Betsholtz et al. (1984) reported expression of PDGF like molecules from a human osteosarcoma cell line with constitutively activated PDGF receptors, however antibodies raised against PDGF failed to inhibit the proliferation of the cell line and so in this instance PDGF may not

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effect the phenotype of the cell. Therefore, alternatively the growth factor may not have to be released (and so be available for antibody neutralisation) in order to activate their receptors and it is of interest that Robbins et al. (1985) report that the majority of PDGF in SSV transformed fibroblasts remains associated with the cell membrane.

Autocrine stimulation of haematopoietic cells

Two groups have reported retroviral mediated transfer of GM-CSF into haematopoietic cells resulting in autocrine stimulation of the recipient cell line (Laker et al., 1987; Lang et al., 1985). However, despite the fact that both groups used the same recipient cell line (GM-CSF dependent FDCP1) slightly different results were reported. Lang et al. used a MoMLV based vector termed GMV, which after infection of the FDCP1 cell line resulted in cell lines which grew in the absence of exogenously added GM-CSF and released GM-CSF into the culture medium. Although the GM-CSF activity in conditioned medium from GMV infected cells could be neutralised by antiserum raised against GM-CSF, direct addition of antiserum to microwell cultures of factor independent GMV infected FDCP1 cells did not inhibit their proliferation. Thus the released GM-CSF may not be responsible for the observed factor independence. However, the GM-CSF vector appears to be responsible for the factor independent phenotype of the infected FDCP1 cells since infection with parental virus does not result in factor independence , although high affinity growth factor/receptor interaction may occur very quickly at the cell surface, or even internally, so excluding antiserum interference. Indeed, the possibility of a membrane bound form of GM-CSF has been proposed by Gough et al (1985). Such GM-CSF could possibly interact with its cognate receptor without being released from the cell surface. A similar situation may occur in the osteosarcoma cell line described above which releases PDGF (Betsholtz et al., 1984).

Laker et al. (1987) have infected the FDCP1 cell line with GM-CSF retroviruses based on MPSV and MoMLV based Z-GMV. In contrast to the above observations by Lang et al. (1985), cell lines derived from GM-CSF virus infections apparently still required release of endogenously produced GM-CSF as part of the autocrine stimulation pathway. However, after repeated cell passage the requirement for

released growth factor reduced and the factor independent cell lines became more similar in phenotype to those described by Lang _et al_. (1985). Laker _et al_. (1987) propose that during cell passage further mutations may occur which result in the eventual loss of requirement for external stimulation by growth factor. It is possible therefore that the phenotype of the cell lines described by Lang _et al_. is either due to prolonged passage of factor independent clones in tissue culture or a reflection of initial cell cloning conditions such as cell density during selection for factor independence (Metcalf _et al_., 1987c).

5.1   Retroviral infection of the FDCP1 cell line

The FDCP1 cell line has been infected with a variety of neomycin containing retrovirus resulting in up to 20% of the clonogenic population becoming resistant to G418 (Lang _et al_., 1985; Cory _et al_., 1987; Metcalf _et al_., 1987c). Laker _et al_. (1987) directly compared the ability of MPSV vectors to generate either G418 resistant fibroblast or FDCP1 haematopoietic colonies, and established that both fibroblasts and haematopoietic cells were infected with equal efficiency. This is in contrast to the low efficiency of infection of haematopoietic cells observed by Lang _et al_. (1985) using MoMLV based vectors.

In this study, infection of FDCP1 cells was carried out by co-cultivating up to 2×10^6 cells for 24h with the $\Psi 2$ virus producer clones seeded out 24h previously at 5×10^5 cells per 25cm^2 flask. Co-cultivation of the virus producing cell line with the target cell population has been demonstrated to be more efficient than virus supernatant for infection of haematopoietic cells, (Lang _et al_. (1985; Eglitis _et al_., 1985). The FDCP1 cell line is normally passaged using 10% WEHI 3B CM as a source of growth factor (Dexter _et al_ 1980a), therefore conditioned media (CM) from the WEHI 3B cell line was added to a final concentration of 10%. Polybrene at 6 µg/ml was added to aid virus absorption. After 24h non-adherent cells were removed, washed twice in PBS to remove growth factors and resuspended in medium which contained no added growth factor. The FDCP1 cells were counted using a Coulter counter and left on ice. The cells were then mixed with semi-solid media (0.3% agar or0.9% methocellulose) as a single cell suspension at the appropriate cell density and dispensed

into 3cm dishes in 1ml aliquots. The 3cm dishes were prepared by adding the required growth or antibiotic supplements prior to the addition of cells. Thus the appropriate concentration of G418 or growth factor could be varied between plates.

After each infection the cells were plated out in semi-solid medium under the following conditions (Fig 5.1) :-

i) normal growth conditions + 10% WEHI 3B CM, - G418. This gives the replating efficiency of the cells.

ii) Selection for <u>neo</u> expression 10% WEHI 3B CM + 1mg/ml G418.

iii) Selection for GM-CSF expression, -WEHI 3B CM, - G418.

The cells were grown under selective conditions for one week and colonies counted using an inverted microscope. If infected parental FDCP1 (FD cells) were incubated without added growth factor, no colony formation occured (Fig 5.7). Similarly in the presence of 10% WEHI 3B CM and 1mg/ml G418, no colony formation occured (Fig. 5.7). The relative plating efficiency is the ratio of colony formation under selective conditions to colony formation under non-selective conditions (+ 10% WEHI 3B CM, no G418) expressed as a percentage.

Prior to the addition of FD cells to the virus producer cells, the 24h old virus medium was removed and stored at -20°C for up to one month. This medium could then be assayed for the presence of virus on 3T3 fibroblast cells, thus giving as indication of the viral titre of the cell line immediately prior to the addition of FDCP1 cells. The virus titre on 3T3 fibroblasts is also shown in Fig. 5.1.

<u>G418 resistance in FDCP1 cells</u>

All four of the viruses used in this study (Fig 4.2) can be selected for G418 resistance (neomycin selection). Following infection of the FD cell line and selection for <u>neo</u> expression (G418, + WEHI 3B in Fig. 5.1), varying numbers of G418 resistant colonies grew in each experiment, demonstrating that viral infection and expression could be achieved using all four vectors. There is a slight indication that mos^{-3}neo virus may be more efficient than pVneo in confering G418 resistance to FD cells (Fig. 5.1 experiments A and B) however, in one other paired experiment using clones which release pVneo or mos^{-3}neo virus, the mos^{-3} neo producer clone was very inefficient in conferring G418 resistance to FD cells (Fig 5.1 expt.

Figure 5.1

Retrovial infection of FDCP1 cell line

Infection of FDCP1 (FD) cells was carried out by co-cultivation of up to 2×10^6 FD cells with virus producing cells seeded out 24h previously at 5 x 10^5 cells per 25cm^2 flask. After 24h, non-adherent cells were removed. 3cm petri dishes were prepared by addition of growth supplement (10% WEHI) and antibiotic (1mg/ml G418) and overlayed with 0.9% methylcellulose (1ml per 3cm dish) containing a range of cell densities from 10^2 to 10^5 cells/ml. At least 3 cell densities wre used in each experiment, with 3 or 5 plates per point. The infected cells were plated out under these conditions:-

i) 10% WEHI CM, this us the replating efficincy of the cells under normal growth conditions.

ii) + 1mg/ml G418, + 10% WEHI CM selection for neo expression.

iii) -G418, -WEHI CM, this selects for growth factor independent growth.

The relative plating efficiency is the ratio of colonies which develop under the selective conditions to colony formation under normal conditions expressed as a percentage.

If no colony formation occurred, the frequency of one "imaginary" colony out of the total number of cells plated was calculated and the frequency of colony formation expressed as less than this.

Also included is the virus titre on 3T3 fibroblasts of the clones immediately prior to the addition of FD cells. Colony formation is expressed as G418 colony forming units per ml on 3T3 fibroblasts.

RETROVIRAL INFECTION OF FDCP1 CELL LINE

EXPT.	CLONE	VECTOR	TITRE ON 3T3'S	REPLATING EFFICIENCY +WEHI %	RELATIVE PLATING EFFICIENCY %	
					+G418 +WEHI	−G418 −WEHI
A	37	pVneo	$>10^5$	32	8	$<3.6 \times 10^{-2}$
	41	$\text{mos}^{-3}\text{neo}$	5.3×10^5	39	60	$<2.6 \times 10^{-2}$
B	34	pVneo	2×10^3	44	1	$<2.3 \times 10^{-2}$
	7	$\text{mos}^{-3}\text{neo}$	1.7×10^4	40	6	$<2.5 \times 10^{-2}$
C	36	pVneo	5.9×10^3	127	3	$<7.9 \times 10^{-3}$
	2	$\text{mos}^{-3}\text{neo}$	3.5×10^3	156	0.9	$<6.6 \times 10^{-3}$
D	35	pVneo	$>10^5$	4	4	$<3 \times 10^{-2}$
	11	pVneoGM	2.8×10^4	10	2.5	1.8
E	11	pVneoGM	6.9×10^4	26	3	1.6
	15	pVneoGM	5.3×10^4	16	6.5	2.4
F	1	$\text{mos}^{-3}\text{neo}$	2×10^5	20	49	$<5.6 \times 10^{-3}$
	31	$\text{mos}^{-3}\text{neoGM}$	5.3×10^4	33	5	0.1

C).   The percentage of clonogenic FD cells which can be infected and express the neomycin gene ranges from 0.9% to 60% and is therefore as efficient as previously published infections of FD cells (Lang et al., 1985; Cory et al., 1987; Metcalf et al., 1987).

Virus expression of GM-CSF in the FDCP1 cell line

By plating out virus infected FD cells in methocell without WEHI 3B CM or G418, it should be possible to generate factor independent (FI) colonies, if the retrovirus used in the infection contains and expresses GM-CSF sequences (Lang et al., 1985; Laker et al., 1987).   Fig 5.1 shows that all infections with either pVneoGM or mos^{-3}neoGM generated growth factor independent colonies.   In the case of pVneoGM infected FD cells between 1.6% and 2.4% of the clonogenic cells infected developed into factor independent colonies whereas only 0.1% of the clonogenic cells infected by mos^{-3}neoGM resulted in factor independent colony growth.   The lower conversion of mos^{-3}neoGM infected cells to factor in dependence could be due to a lower percentage of infected cells than observed in the experiments using pVneoGM, but mos^{-3}neoGM virus converts 5% of FD clonogenic cells to neomycin resistance which is comparable to pVneoGM virus (Fig 5.1). It can therefore be concluded that mos^{-3}neoGM virus from clone 31 efficiently infects FD cells but when the GM-CSF unit of the vector is assayed for expression, it is less efficient in converting FD cells to factor independence by a factor of ten, than the pVneoGM vector.   In viral infections using either pVneo or mos^{-3}neo, if no factor independent colonies were observed, a frequency for one imaginary factor independent colony out of the total number of cells plated out can be calculated, and the conversion to factor independence expressed as less than this.   Therefore although the conversion of FD cells to factor independence is lower using mos^{-3}neoGM virus producer cells, than Ψ2 clones releasing pVneoGM, the number of factor independent colonies is at least 100 fold greater than any spontaneous background of factor independent colonies (Fig 5.1).   Only in one infection with a mos^{-3}neo virus were sufficient numbers of FDCP1 cells plated out (5.55x10^{5}cells) which demonstrated spontaneous colony formation at a frequency of 1 in 7.9 x 10^{4}cells plated out (1.26 x 10^{-3} %) (data not shown).

5.2   Establishment of virally infected FDCP1 cell lines

A number of cell lines were established from the virally infected FD cells to enable further studies on vector expression to be carried out.   In order to generate cell lines, infected FD cells were selected for either resistance to G418 or for growth factor independent colony formation.   These colonies were picked in a volume of 50 µl using a Pippetteman and transferred into liquid medium which maintained the initial selection. Fig 5.2 shows the number of successfully established cell lines, the viruses they contain and the conditions under which they were selected.   All colonies selected in +WEHI, +G418 conditions, could be immediately transferred into 5ml of growth media in 25cm^2 flasks.   However colonies which were selected for growth factor independence (-WEHI, - G418) had to be transferred initially in 100 µl of medium in 96 well dishes, and allowed to reach a high cell density, before transfer into 1ml of culture medium in 6 well dishes (Costar).   Again, these cells were left until a high cell density was reached, and then transferred to 5ml of medium in 25cm^2 flasks.   Selection pressure was maintained at all times.   The factor independent cell lines (-G418, -WEHI) were maintained and passaged at high cell density as it was observed that cell growth did not occur if the cells were seeded out too sparsely.   It should be noted that of 89 pVneoGM factor independent colonies transfered only 18 generated cell lines (Fig. 5.2).   In comparison relatively few colonies had to be picked in order to establish all the other cells lines (Fig 5.4).

Once the cell lines were established, experiments were designed to examine expression of the retroviral vectors in the FD cells under different selective conditions.   Thus the expression of both selected and non-selected genes could be related to cell line phenotype.   For example cells grown in +G418, +WEHI conditions are dependent only on expression of neo for their survival. However if the cell line harbours a GM-CSF retrovirus and the GM-CSF unit is transcriptionally active then these cells should replate under factor independent growth conditions (-G418, -WEHI).   It is possible that the previously discussed phenomenon of epigenetic suppression (Emmerman and Temin 1984, Chap.1.7) may act on these vectors to silence non-selected genes which would result in their inability to replate into previously nonselected conditions.

Figure 5.2

Establishment of cell lines from virus infected
FDCP1 cells.

A number of cell lines were established after
infection of the FD line by the four retroviruses
used in this study (Fig. 4.1). Varying numbers of
well isolated colonies were picked after one week
of culture and maintained under the same growth
conditions in which they were originally selected.
All clones which were selected in +G418, +WEHI were
transferred directly from methylcellulose into
$25cm^2$ flasks containing 5ml media including the
appropriate growth factor and antibiotic
supplements necessary for the maintainance of
selction.

All factor independent colonies (-1mg/ml G418 -10%
WEHI were transferred from methylcellulose to 96
well dishes (100µl) then to 6 well dishes (1ml
medium) and to $25cm^2$ flasks (5ml). Selection was
maintained at all times and the cells were allowed
to attain a high density before transfer into
increasing volumes of media.

ESTABLISHMENT OF CELL LINES FROM VIRUS INFECTED FDCP1 CELLS

VIRUS	SELECTION	NO. OF COLONIES PICKED	NO. OF CELL LINES ESTABLISHED
pVneo	+G418 +WEHI	24	11
pVneoGM	+G418 +WEHI	24	11
pVneoGM	−G418 −WEHI	89	18
$mos^{-3}neo$	+G418 +WEHI	24	24
$mos^{-3}neoGM$	+G418 +WEHI	24	24
$mos^{-3}neoGM$	−G418 −WEHI	12	7

5.3   Replating of individual FDCP1 cell lines harbouring either pVneo or pVneoGM initially selected in +G418, +WEHI.

The twelve clones shown in Fig. 5.3 contain either pVneo or pVneoGM and were selected and maintained in media selective for neo expression. The cell lines which contain pVneoGM can be used to study the expression of the non-selected GM-CSF gene by replating into factor independent conditions in methocell. Fig. 5.3 shows the clones, the virus they contain, their replating efficiency in methocell under normal conditions for the parental FD cell line (+10% WEHI CM) and their relative plating efficiencies under selective conditions for either neo expression (+ G418, +WEHI), GM-CSF expression, (-G418, -WEHI) or double selection for neo and GM-CSF (+G418, -WEHI). Cells were plated out in triplicate at 10^3, 10^4, 10^5 cells per ml but only plates at 10^3 cells per ml were informative.

All twelve clones replate with varying but high (greater than 70%) efficiency into methocell containing +G418, +WEHI. This efficiency is to be expected as all these cell lines were maintained in liquid culture in +G418, +WEHI. Also as expected the six clones B4, B5, A3, B8, A9, and A11 which harbour pVneo do not generate factor independent colonies when replated under the appropriate conditions (-G418, - WEHI) at 10^3, 10^4, and 10^5 cells per millilitre (10^3 cells only shown in table 5.3).

The clones E8, E5, D5, D4, E4 and E11 contain pVneoGM and were selected for neo expression (Fig. 5.3). These six clones can therefore be used to investigate GM-CSF expression from the non-selected transcriptional unit of pVneoGM by replating the cells under conditions which select for factor independent colony growth (-G418, -WEHI). When this was carried out the six clones showed variable plating efficiency in -G418 -WEHI conditions in relation to +WEHI, ranging from 0 to 60% (Fig. 5.3). The low plating efficiency of five of these clones (E5, D5, D4, E4, E11) in the absence of added growth factor in comparison to colony growth stimulated by WEHI CM implies that autocrine stimulation of these clones by GM-CSF is not so efficient as exogenous stimulation by WEHI CM. This may be due to differences in efficiency of stimulation by GM-CSF and WEHI CM, however addition of recombinant GM-CSF to parental FDCP1 cells results in a replating efficiency almost as high as WEHI CM (Fig. 5.10). The lower plating efficiency of the clones in the absence of growth factor

<u>Figure 5.3</u>

Replating of individual FDCP1 clones selected for
G418 resistance (+G418, +WEHI).

12 clones selected for G418 resistance (+1mg/ml
G418, +10% WEHI) were plated out under several
growth conditions. This figure shows these clones
and the virus they harbour. The cells were plated
out at 10^3, 10^4 and 10^5 cells per ml (3cm plates,
1ml per plate in triplicate) and colonies counted 7
days later. Only the data for cells plated out at
10^3 cells/ml is shown. The relative plating
efficiency is the ratio of colony formation under
selective conditions to colony formation under
non-selective conditions (+ 10%WEHI) expressed as a
percentage.

n.d. = not done

REPLATING OF INDIVIDUAL FDCP1 CLONES SELECTED +G418 +WEHI

CLONE	VIRUS	REPLATING EFFICIENCY % +WEHI	RELATIVE PLATING EFFICIENCY %		
			+G418 +WEHI	−G418 −WEHI	+G418 −WEHI
B4	pVneo	25	124	0	n.d.
B5	pVneo	10	50	0	n.d.
A3	pVneo	5	114	0	n.d.
B8	pVneo	15	98	0	n.d.
A9	pVneo	4	200	0	0
A11	pVneo	2	160	0	0
E8	pVneoGM	33	77	60	n.d.
E5	pVneoGM	15	20	7.5	n.d.
D5	pVneoGM	17	72	15	n.d.
D4	pVneoGM	7	107	10	28
E4	pVneoGM	22	58	4	4
E11	pVneoGM	7	70	0	n.d.

is perhaps due to inefficient production of retrovirally encoded GM-CSF by the FDCP1 cells themselves. The clones E8, E5, D5, D4, E4 and E11 were initially selected for neo expression and therefore if epigenetic suppression was ocurring (Emmerman and Temin, 1984) then selection for G418 resistance may have resulted in the inability of the integrated vectors to express the second transcriptional unit (the GM-CSF sequences) efficiently. Only one clone, E11, did not form any colonies under selection for factor independence (Fig 5.3) and so in five out of six clones if epigenetic suppression of viral sequences is occurring then it is not absolute and some GM-CSF is produced to which the cells can respond in an autocrine fashion. Two clones, D4 and E4 which were replated under double selection for neo and GM-CSF expression (Fig 5.3) were capable of colony formation. Thus even if initial selection for neo does cause suppression of GM-CSF expression both genes are still capable of expression to a level which allows colony growth during double selection.

5.4   Molecular Analysis of Clone E11

One FDCP1 derived clone, E11, which contains pVneoGM and was selected initially for neo expression was unable to replate into methocell without added growth factor (Fig 5.3). It was decided to analyse this clone in more detail at the molecular level in order to determine why clone E11 showed this phenotype. Northern ananlysis of total RNA extracted from FD clones derived from infections with pVneo or pVneoGM probed with neo (Fig 5.4) confirms that the two predicted mRNA species (Fig 4.2) observed in fibroblasts are present. The one exception to this is clone E11. Hybridisation of mRNA from E11 to neo should detect two RNA species of identical molecular weight to those detected in Fig. 5.4A lanes 5,6 and 8. However the larger full length viral mRNA which encodes the GM-CSF protein (Fig 4.2) is not detected (Fig 5.4 lane 7). The smaller 2.4kb mRNA which may be directed from the tk promoter adjacent to the neo gene (Fig. 4.2) is present (Fig 5.4A lane 7). The possibility therefore exists that selection for neo expression from the tk promoter has resulted in epigenetic suppression of the MoMLV promoter sequences responsible for expression of full length viral transcripts. An alternative explanation to the unexpected transcriptional pattern in clone E11 is that the vector has become mutated or rearranged such that tkneo transcription is still

possible, but full length message production has become disrupted. In an attempt to visualise gross rearrangements of the virus, genomic DNA from several FD cell lines was digested with Sst1 which cleaves once within each of the viral LTRs (Fig. 4.2). On probing a Southern filter which contained DNA from six FD clones including E11 with neo, five of the six clones contained viral fragments of the predicted sizes (Fig. 5.5, Fig. 4.2, Fig. 4.1). The genomic DNA from clone E11 cut with Sst1 hybridised to a 15kb fragment on probing with neo. If the virus was totally intact in clone E11, a predicted 4.2kb hybridising fragment should be present (Fig 5.5 lanes 5 and 6) therefore the provirus has undergone some rearrangement in this cell line which is most probably the reason for the lack of full length viral transcript encoding the GM-CSF sequences. It can be concluded from this that the inability of clone E11 to replate under selective conditions for factor independent growth is probably due to viral rearragement and not epigenetic suppression.

5.5   Growth factor independent colony formation by FD clones containing pVneoGM

The possibility exists that FD cells containing pVneoGM do not produce high enough levels of GM-CSF to support maximal colony growth in an autocrine fashion (section 5.3). The reason for this is not clear. The clones studied in section 5.3 were initially selected in the presence of added growth factor and therefore it was decided to examine the replating characteristics of pVneoGM containing FD cells which were initially selected for growth factor independent colony growth in an attempt to obtain further insight into the expression of GM-CSF.

All the FD clones in Fig. 5.6 contain pVneoGM and were selected for growth factor independent colony growth (-G418, -WEHI). Although the initial selection procedure did not require expression of the neo gene when these clones were replated into G418 (Fig 5.6) (+G418, +WEHI) all replated with high but variable efficiency. Therefore the neo gene is not adversely affected in terms of expression in the eight clones initially selected for viral GM-CSF expression.

As expected the three factor independent clones R5, R6 and R9 generated factor independent colonies when plated in methocell without

<u>Figure 5.4</u>

Northern blot analysis of FDCP1 clones infected
with retroviruses.

A   autoradiograph of Northern blot

Lane	Clone	Virus	Selection	
1	FDCP1	/	/	
2	A3	pVneo	+G418,	+WEHI
3	B4	"	"	"
4	B5	"	"	"
5	E5	pVneoGM	"	"
6	E8	"	"	"
7	E11	"	"	"
8	T11	"	-G418,	-WEHI

B   Ethidium bromide stained gel prior to blotting
Total RNA was extracted from FD cell lines as
described in Materials and Methods.   Approximately
20μg of total RNA was loaded per lane fractionated
on a denaturing gel and analysed by Northern
Blotting using a <u>neo</u> probe (Fig. 4.2)
Size markers were 28S - 4.7kb, 18S 1.8kb.

A

28 S-

18S-
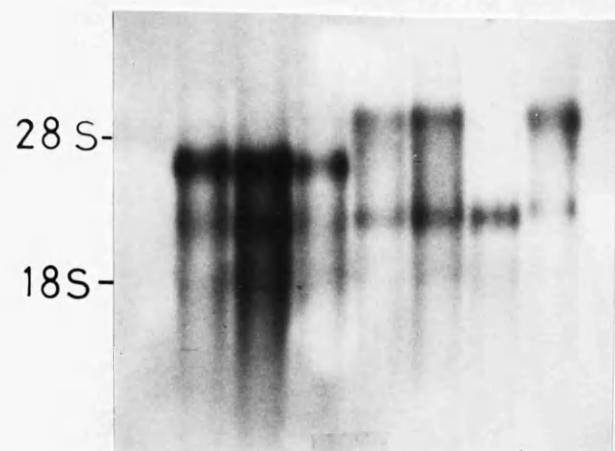
1 2 3 4 5 6 7 8

B
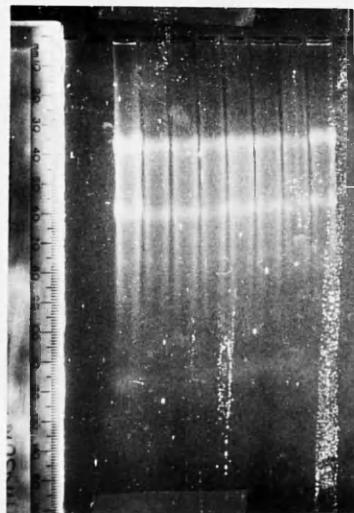
1 2 3 4 5 6 7 8

<u>Figure 5.5</u>

Southern analysis of virus infected FDCP1 clones.

Lane	Clone	Virus
1	FDCP1	/
2	A3	pVneo
3	B4	"
4	B5	"
5	E5	pVneoGM
6	E8	"
7	E11	"

All genomic DNA was digested with <u>Sst</u>I and probed with <u>neo</u> to assess viral integrity (Fig4.2). The clones A3, B4, B5, E5, E8 and E11 were all initially selected for neomycin expression. Cells were always maintained in selctive media during cell culture. Markers are <u>Hind</u>III digested λ . Sizes are in kilobases.

15 —

9·5 —

6·7 —

4·2 —

3·2 —

2·3 —

1·9 —

1 2 3 4 5 6 7
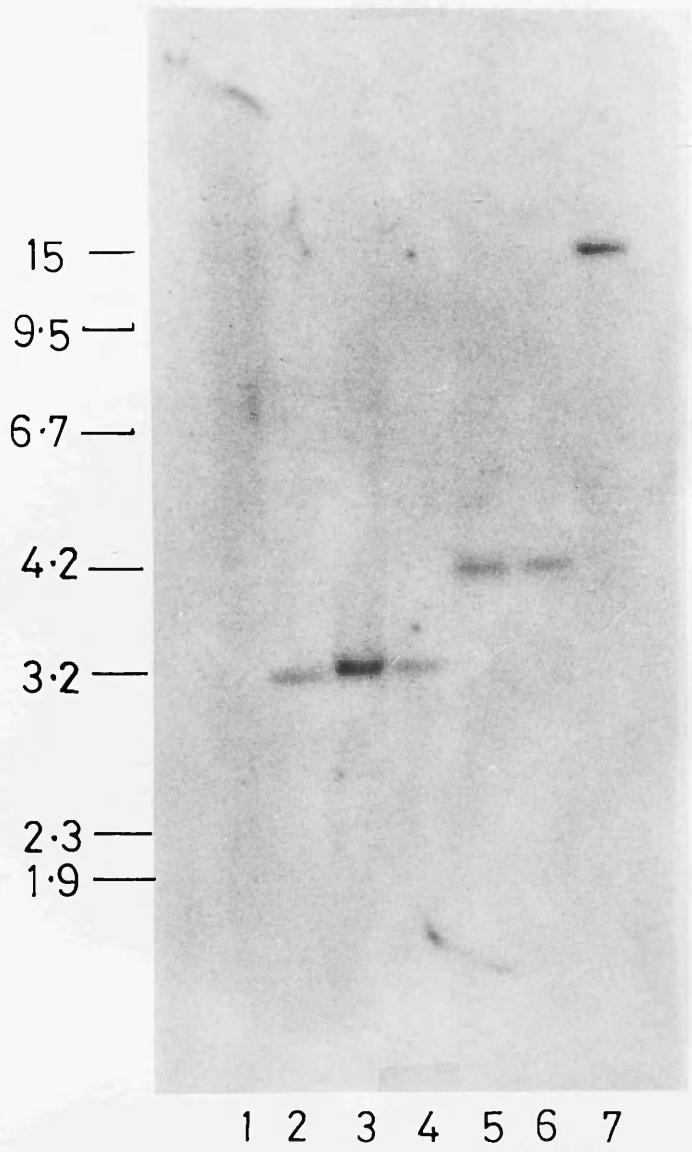
growth factor (-G418, -WEHI) at a cell density of 5 x 10^4 cells/ml, however this cell density resulted in colony numbers per plate which were too high to count accurately (Fig 5.6). In order to obtain quantitative replating data for the factor independent clones, 8 clones including R5, R6 and R9 were plated out at low cell density (10^3 cells /ml). The results shown in Fig 5.6 demonstrate that only one factor independent clone (S2) generated colonies at this cell density. This is surprising as these clones were selected for factor independent growth and are capable of colony formation at higher cell density (5 x 10^4 cells/ml). In addition at low cell density (10^3 cells/ml) they are capable of forming colonies in the presence of WEHI or G418 (Fig. 5.6). It is therefore possible that these apparently factor independent colonies did not produce sufficient growth factor to allow efficient colony growth at low cell density (10^3 cells/ml) despite their initial selection. If this is the case, the previous data regarding FD (pVneoGM) clones selected for neo expression which display a poor cloning efficiency in factor independent growth conditions (-G418, -WEHI) in comparison to exogenous stimulation by WEHI or selection in +WEHI, +G418 (Fig. 5.3) may be interpreted as inherently poor expression of viral encoded GM-CSF in FD cells rather than epigenetic suppression. Thus, although the colonies selected in -G418, -WEHI may appear to be completely factor independent during cloning their replating characteristics indicate a requirement for high cell density which presumably maintains the overall concentration of autocrine growth factor at a level compatible with colony formation.

If the factor independent pneoGM FD cells are producing low levels of GM-CSF and this determines these cell lines requirement for high cell density in order to form factor independent colonies, then it should be possible to replace the high density dependent colony growth by the addition of exogenous growth factor. Thus levels of growth factor sub-optimal for the proliferation of parental FDCP1 cells could supplement the low level of autocrine produced GM-CSF from factor independent clones resulting in colony growth.

The experiment shown in Fig. 5.7 was designed to examine any differential response of the parental FDCP1 cell line and two factor independent cell lines, R5 and R6, to exogenously added recombinant GM-CSF, (rGM-CSF) which was added to a final concentration of 0.1%

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(9pg/ml). Fig 5.7 shows that the parental FD line was not stimulated to form colonies by 0.1% rGM-CSF (-G418, + 0.1% rGM) and as expected no colonies formed when growth factor was omitted (-G418, -WEHI). In contrast clone R5 had a relative plating efficiency of 13% in the absence of growth factor (-G418, -WEHI) which increased to 46% with the addition of 0.1% rGM-CSF. Similarly, the factor independent clone R6 showed the relative replating efficiencies of 17% and 75% in the absence of growth factor and with the addition of 0.1% rGM-CSF respectively. The above data indicates that the cell lines containing the pVneoGM vector have a proliferative advantage over the parental FD cell line which may be due to a low level of virally encoded GM-CSF. This conclusion was confirmed by the data shown in Fig. 5.8 which relates colony formation to growth factor titration for clones R5, R6 and the parental FD cell line, as it clearly shows that the two factor independent cell lines R5 and R6 do have a growth advantage over the parental FD cell line over a range of rGM-CSF concentrations from 0 to 27pg/ml and confirms the two factor independent cell lines R5 and R6 respond to exogenous growth factor by increasing their efficiency of colony formation under conditions sub-optimal for parental FD colony development (Figs. 5.7 and 5.8).

The data discussed above suggests that pVneoGM containing FD cell lines produce levels of virally encoded GM-CSF which at low cell density is insufficient for maximal autocrine stimulated colony development. In an attempt to prove finally that GM-CSF is released from pVneoGM containing factor independent cell lines, growth media from three lines R6, R5 and R9 was removed after three days (3 day conditioned medium, CM) passed through a 0.2μ filter and then tested for growth factor activity. The CM was added to 500 parental FDCP1 cells in a final volume of 10μl in Terasaki dishes, as 10% of the total volume, either in the presence or absence of antisera raised against rGM-CSF (DeLamarter et al., 1985). As the FDCP1 cell line responds to GM-CSF, if sufficient levels of growth factor are present in the test CM, then the FD cells should proliferate in the absence of antiserum against GM-CSF, but not in its presence. The use of Terasaki dishes and FDCP1 indicator cells is a very sensitive method for detecting growth factor activity and will detect down to approximately 1-2 pg/ml GM-CSF (Metcalf et al., 1986a). Duplicate wells are scored after 48h for evidence of proliferation of the

Figure 5.6

Replating of individual factor independent (FI) clones infected by pVneoGM.

Factor independent cell lines which were derived by infection with pVneoGM and initially selected under -G418, -WEHI growth conditions were analysed for colony formation under various growth conditions and at two cell densities (10^3 cells /ml and 5×10^4 cells/ml). Triplicate 3cm dishes were used containing 1ml of methylcellulose per dish. WEHI CM was added to a final concentration of 1mg/ml and G418 was added to a concentration of 10% if required. The relative plating efficiency is the ratio of colony formation under selective conditions to colony formation under non-selective conditions (+10% WEHI) expressed as a percentage.

n.d. not done

TNTC colony numbers too numerous to count.

REPLATING OF INDIVIDUAL F.I CLONES INFECTED BY
pVneoGM IN −G418, −WEHI

CLONE	CELL DENSITY (ml)	REPLATING EFFICIENCY % +WEHI	RELATIVE PLATING EFFICIENCY %		
			+G418 +WEHI	−G418 −WEHI	+G418 −WEHI
R5	5×10^4	>1	T.N.T.C.	T.N.T.C.	n.d.
R6	5×10^4	>1	T.N.T.C.	T.N.T.C.	n.d.
R9	5×10^4	>1	T.N.T.C.	T.N.T.C.	n.d.
R5	10^3	5	42	0	0
R6	10^3	0.5	40	0	0
R9	10^3	1	146	0	0
R12	10^3	7	76	0	0
S1	10^3	1	66	0	0
S2	10^3	3	93	10	30
S9	10^3	1.4	237	0	0
T11	10^3	2	57	0	0

Figure 5.7

Response of factor independent (FI) clones to
exogenous growth factor.

Clones R5 and R6 which were initially identified as
colonies which grew in the absence of added growth
factor after infection with pVneoGM. The relative
plating efficiencies of these two clones in
response to low levels of exogenously added growth
factor 0.1% recombinant GM-CSF (9pg/ml) was
compared to the response of the parental FDCP1 (FD)
line. Triplicate 3cm dishes containing 1ml
methocell were used per point. G418 was used at
1mg/ml. WEHI CM was included to a final
concentration of 10% when required. The relative
plating efficiency is the ratio of colony formation
under selective conditions to colony formation
under non-selective conditions expressed as a
percentage. Colonies of more than 20 cells were
counted on day 7.

RESPONSE OF F. I. CLONES TO EXOGENOUS GROWTH FACTOR

CLONE	CELL DENSITY (ml)	REPLATING EFFICIENCY % +WEHI	RELATIVE REPLATING EFFICIENCY %		
			+G418 +WEHI	−G418 −WEHI	−G418 +0.1%rGM
FD	5×10^4	>1	0	0	0
R5	5×10^3	4.5	130	13	46
R6	5×10^3	0.5	158	17	75

Titration of recombinant GM-CSF (rGM-CSF) against colony formation.

Colony formation of factor independent cell lines R5 (o) and R6 (x) and the parental FDCP1 cell line (●) in response to a titration of rGM-CSF. The rGM-CSF was diluted in PBS and added to the petri dishes in a volume of 100µl. All points were scored in triplicate using 3cm dishes containing 5 x 10^3 cells in 1ml methocellulose. Colonies of more than 20 cells were counted after 7 days incubation. Colony numbers are expressed as a ratio of the number of colonies formed at any concentration of rGM-CSF to the colony numbers formed on stimulation of the parental FDCP1 cell line with 875pg/ml rGM-CSF.

pg/ml rGM-CSF

0   7   14   27   55   109   219   437

0   10   20   30   40   50   60   70   80   90   100   110   120
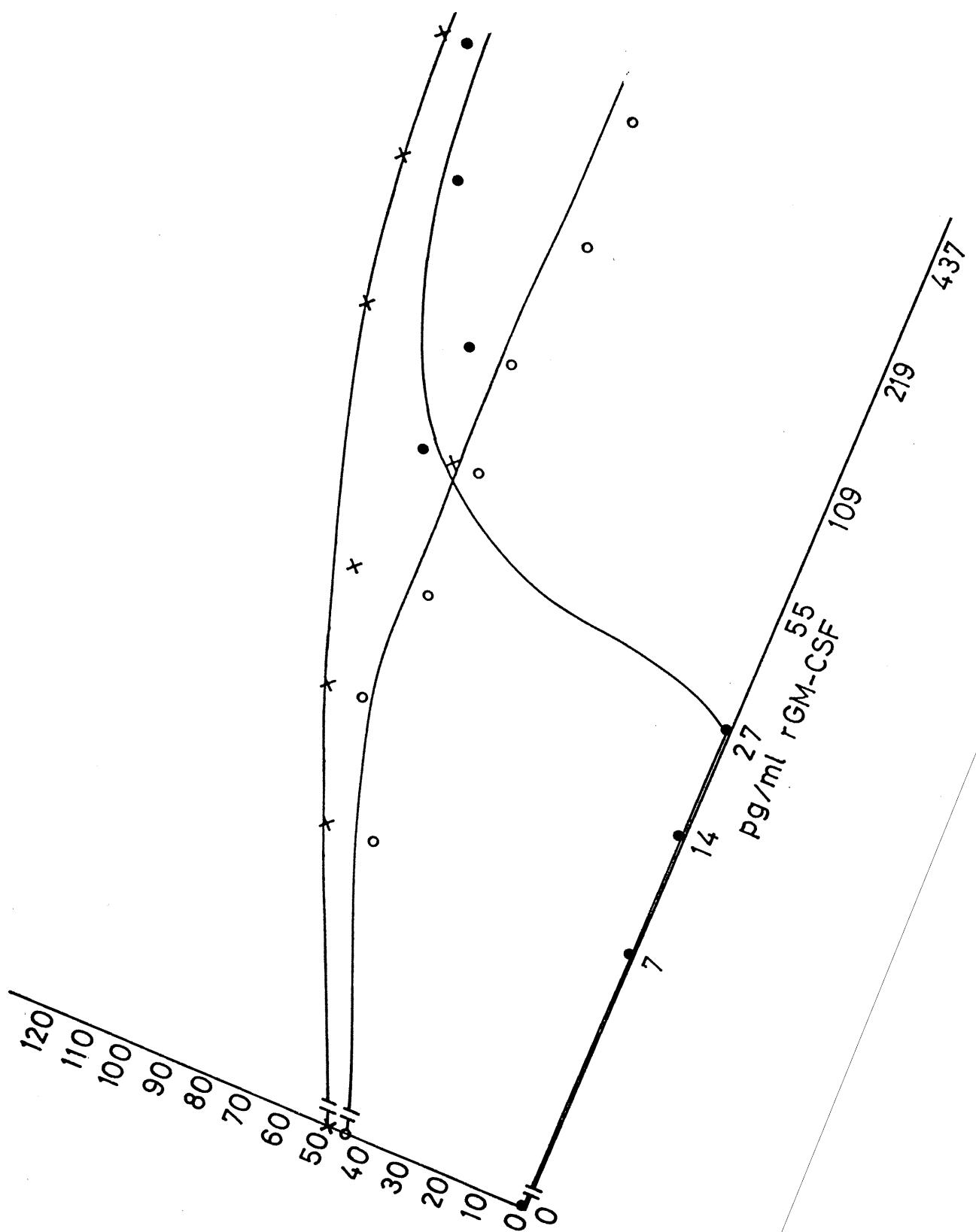
indicator FDCP1 cell line by comparing the area of the well bottom covered by cells in comparison to exogenously added rGM-CSF (875 pg/ml), fresh WEHI CM and spent WEHI CM. Spent WEHI CM was obtained by removing the growth medium from parental FD cells after 5 days of culture.

The results of this experiment are shown in Fig. 5.9. As expected the FD cells proliferate in response to rGM-CSF and WEHI CM, but antiserum against rGM-CSF only inhibits FD proliferation when rGM-CSF is added but not WEHI CM (the active factor in WEHI CM is likely to be Il-3 to which the FDCP1 cell line also responds). The conditioned medium from the FDCP1 cell lines do not stimulate proliferation of the FD cells when added at a final concentration of 10%. This is perhaps not unexpected as these cell lines must be passaged at high cell density in liquid cultures and as described above probably only release low levels of GM-CSF. The autocrine produced GM-CSF is probably consumed by the factor independent cell lines almost instantly, due to its rather sub-optimal production. The observation that the conditioned medium from the parental FDCP1 stock which is maintained in 10% WEHI CM was also incapable of stimulating FDCP1 proliferation suggests that the requirement of FD cells for growth factor for their survival depletes growth factor from the culture medium, reducing it to a level below that which is detected by this assay.

5.6 Replating efficiency of FD clones containing mos^{-3} neo and mos^{-3}neoGM

The generation of FD clones containing mos^{-3}neo and mos^{-3}neoGM is described in section 5.1. As with pVneo/pVneoGM FD clones, mos^{-3}neo/mos^{-3}neoGM clones were plated under different selection conditions in order to assess the expression of the viral constructs they harbour. All the mos^{-3}neo clones shown in Fig. 5.11 were initially selected for resistance to G418 and as expected replated efficiently into methocell under similar conditions to those in which they were selected in, (+G418, +WEHI). Also as expected all clones which only contain the mos^{-3}neo vector do not form colonies under selection for factor indepedent growth (Fig. 5.11, clones I 12, I11, I2 and I10). The three clones L1, L11 and L12 all contain mos^{-3}neoGM and should therefore have the potential to express GM-CSF. However no

Figure 5.9

Assay for growth factor release by factor independent (FI) clones.

Three FI clones R5, R6 and R9 were tested for production of growth factor. Conditioned medium from the cell lines was prepared as in Materials and Methods and added to 500 FDCP1 cells as 10% of the final volume using Terasaki dishes (60 wells per dish). Each well contained a final volume of 10µl. Antiserum to GM-CSF was added at 10% final volume, when required. As controls fresh WEHI CM was added to 10% final volume, recombinant GM-CSF (rGM-CSF) was added to 875pg/ml). The conditioned medium from the parental FDCP1 cell line 5 days after removal of growth medium (which contains 10% WEHI CM) was also included (spent WEHI). Duplicate wells were scored after 48h for proliferation of the indicator FDCP1 cells in comparison to control conditioned medium .

CONDITIONED MEDIA FROM F.I. CLONES

EXPT.	CONDITIONED MEDIA	ANTISERA TO GM-CSF	CELL PROLIFERATION
1	Fresh WEHI	−	+
		+	+
2	rGM-CSF	−	+
		+	−
3	R5	−	−
		+	−
4	R6	−	−
		+	−
5	R9	−	−
		+	−
5	Spent WEHI	−	−
		−	−

Figure 5.10

Stimulation of FDCP1 colonies by recombinant GM-CSF (rGM-CSF) and WEHI conditioned medium (WEHI CM). Triplicate 3cm plates containing 5×10^3 FDCP1 cells per ml of methocellulose (1ml per plate) were stimulated to form colonies by either the addition of 10% WEHI CM or 875pg/ml rGM-CSRF. Colonies were scored after 7 days of incubation and the ratio of the number of colonies formed to number of cells plated out expressed as a percentage. This is defined as the replating efficiency.

STIMULATION OF FDCPI COLONY FORMATION BY
WEHI 3B CONDITIONED MEDIUM AND rGM-CSF

SOURCE OF STIMULATION	REPLATING EFFICIENCY %
10% WEHI CM	20
875 pg/ml rGM-CSF	16

Figure 5.11

This figure shows 7 individual FDCP1 clones which contain either mos^{-3}neo or mos^{-3}neoGM and were initially selected for G418 resistance (+1mg/ml G418, +10% WEHI CM). These clones were replated at the appropriate cell density into 3cm petri dishes under the appropriate selective growth conditions. All points were scored in triplicate. Colonies were counted after 7 days and the relative plating efficiency expressed as the ratio of colony formation under selective conditions to colony formation under non-selective conditions (+10% WEHI expressed as a percentage.

TNTC - Colony number per plate was too numerous to count.

n.d. not done

REPLATING OF INDIVIDUAL FDCP1 CLONES SELECTED +G418 +WEHI

CLONE	VIRUS	CELL DENSITY(ml)	REPLATING EFFICIENCY % +WEHI	RELATIVE REPLATING EFFICIENCY %		
				+G418 +WEHI	−G418 −WEHI	+G418 −WEHI
I12	$mos^{-3}neo$	10^2	44	100	0	n.d.
I11	$mos^{-3}neo$	10^2	7	250	0	n.d.
I2	$mos^{-3}neo$	10^3	5	180	0	0
I10	$mos^{-3}neo$	10^3	11	100	0	0
I12	$mos^{-3}neo$	10^4	44	T.N.T.C.	0	n.d.
I11	$mos^{-3}neo$	10^4	7	T.N.T.C.	0	n.d.
L12	$mos^{-3}neoGM$	10^2	41	100	0	n.d.
L11	$mos^{-3}neoGM$	10^2	6	51	0	n.d.
L1	$mos^{-3}neoGM$	10^3	5	93	0	0
L12	$mos^{-3}neoGM$	10^4	41	T.N.T.C.	0	n.d.
L11	$mos^{-3}neoGM$	10^4	6	T.N.T.C.	0	n.d.

Figure 5.12

Replating of factor independent clones generated by infection with mos^{-3}neoGM virus.

This figure shows the replating in methocellulose of four cell lines derived from colonies which displayed factor independence after co-cultivation with a mos^{-3}neoGM producing cell line. The relative replating efficiency is the ratio of colony formation under selective conditions to colony formation under non-selective conditions (+10% WEHI) expressed as a percentage. The appropriate density of cells in methocellulose were added to 3cm petri dishes (1ml per dish) under to appropriate selective conditions. All points were performed in triplicate.

n.d. - not done.

REPLATING OF INDIVIDUAL F.I. CLONES INFECTED BY mos^{-3}neoGM
AND SELECTED IN -G418, -WEHI

CLONE	CELL DENSITY (ml)	REPLATING EFFICIENCY % +WEHI	RELATIVE REPLATING EFFICIENCY %		
			+G418 +WEHI	-G418 -WEHI	+G418 -WEHI
N11	10^4	0.43	230	5	n.d.
N12	10^4	2.4	158	36	n.d.
N1	10^3	6	0	0	0
N2	10^3	3	0	0	0

factor independent colonies (-G418, -WEHI) were observed even when clones L12 and L11 were plated out at 10^4 cells/ml (Fig. 5.11).

Four cell lines N11, N12, N1 and N2 (Fig. 5.12) were initially selected for their growth factor independent phenotype (-G418, -WEHI) after infection with mos^{-3}neoGM. Both N11 and N12 still express the neomycin gene as demonstrated by replating the clones in +G418, +WEHI when both clones formed colonies in methocell under selection for factor independent growth (-G418, -WEHI). As with pVneoGM containing clones (see sections 5.3, 5.5) autocrine stimulation by mos^{-3}neoGM encoded GM-CSF is less efficient than exogenous stimulation by WEHI relative replating efficiency (Fig. 5.12). The lack of colony formation observed by mos^{-3}neoGM containing clones under factor independent growth conditions (-WEHI, -G418) in Fig. 5.11 is probably a reflection of the poor expression of virally encoded GM-CSF in three clones. The mos^{-3}neoGM clones in Fig. 5.11 were initially selected for neo expression which is an extremely efficient method for the generation of virus containing clones (Fig. 5.1). Therefore if virally encoded GM-CSF expression is low but variable, many clones may have to be screened before rare clones which can produce enough viral GM-CSF to replate under factor independant conditions to be observed. It is possible that the two clones N11 and N12 which were initially selected for factor independence and do generate colonies in factor independent semi-solid growth medium (Fig. 5.12) are examples of such a sub-population of clones theoretically present within the neo selected population of clones. Clones N1 and N2 do not form colonies when replated into G418 (Fig. 5.12). The four clones N11, N12 N1 and N2 were derived from an infection in which the number of factor independent clones generated by infection was approximately only twice the spontaneous background (data not shown) and so the inability of N1 and N2 to replate into G418 may mean that these are spontaneous factor independent mutants.

Discussion

A number of virus containing FDCP1 cell lines have been generated in an attempt to investigate retroviral expression in haematopoietic cell lines. Specifically, it was of interest to analyse viral expression of GM-CSF in relation to autocrine stimulated proliferation of the FD cell line. In view of the results presented

in section 4 which documents growth factor release from virally infected fibroblasts, irrespective of the genes carried by the vector (Koury and Pragnell, 1982) it was important to establish whether viral infection of the factor independent FDCP1 cell line with either pVneo or $mos^{-3}neo$ resulted in the generation of the growth factor independent colonies. As can be seen from Fig. 5.1, factor independent colonies were not observed in any virus infection involving pVneo or $mos^{-3}neo$. It can therefore be assumed that the colony growth observed after infection of the FD line with pVneoGM (Fig. 5.1) or $mos^{-3}neo$ (Fig. 5.1) is due to retrovirally expressed GM-CSF.

The frequency with which factor independent colonies are generated by both viruses (pVneoGM and $mos^{-3}neoGM$) is not however the same. The virus pVneoGM appears to be capable of producing approximately 10 fold more factor independent colonies than $mos^{-3}neoGM$ virus, (Fig. 5.1). This latter observation may be related to earlier observations on virally directed expression in fibroblast cells (section 4). In section 4 it was noted that $\psi 2$ cells which produced pVneoGM virus could be shown to have GM-CSF activity in their culture medium, whereas $mos^{-3}neoGM$ did not. The lack of detectable growth factor from Cl-31 cannot be ascribed to the clone containing a deleted virus which no longer includes the GM-CSF sequences as viral transcripts which hybridise to a GM-CSF probe can be detected. Also virus from Cl-31 does generate factor independent FD clones after infection at a frequency of at least 20 fold greater than background. Possibly, the reduced abililty of $mos^{-3}neoGM$ virus to generate factor independent colonies in comparison to pVneoGM virus is due to poor processing of the full legth genomic mRNA to generate the GM-CSF specific sub-genomic message. FD cells containing pVneo or pVneoGM do show the two predicted neo hybridising mRNA species (Fig. 5.4) predicted from the vector design (Fig. 4.2) however mRNA from FD cells containing $mos^{-3}neoGM$ virus was not analysed and so the above explanation is based on the viral transcripts observed in fibroblast cells. Both Laker et al. (1987) and Lang et al. (1985) discuss vectors which rely on viral splice signals for expression of sub-genomic message with the conclusion that splicing may not be efficient. It may be that the poor expression of GM-CSF from mos neoGM containing cells is related to this problem.

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FD cell lines which contain pVneoGM generally display poor cloning efficiencies at low cell densities (10^3 cells/ml) in the absence of added growth factor (Figs. 5.3, 5.6) despite having been cloned due to their factor independent phenotype. However at high cell density it was observed that colony formation did occur (Fig 5.6). The possibility that these cell lines produced low levels of GM-CSF which required them to be maintained at high cell density in order to raise the level of GM-CSF above a threshold level for survival and proliferation was tested. The addition of low levels of exogenous rGM-CSF increased the cloning efficiency of two factor independent cell lines R5 and R6, without stimulating the parental cell line (Fig. 5.7, 5.8). Thus, it can be assumed that the low level of GM-CSF produced by the factor independent cells is "optimised" by the addition of exogenous growth factor and cloning efficiency in methocell rises accordingly.

Although the mos^{-3}neo and mos^{-3}neoGM containing clones were not studied in so much detail, the mos^{-3}neoGM clones showed a poor cloning efficiency in factor independent conditions similar to pVneoGM containing clones (Figs. 5.11 and 5.12). Therefore it is possible that such clones also produce very low levels of growth factor which is insufficient to promote efficient colony formation in semi-solid medium.

CHAPTER SIX

Retroviral Infection of Murine Bone Marrow Cells

Introduction

    The results in chapter five which examined the efficiency of retroviral gene expression in FDCP1 derived cell lines, were very encouraging. The cell lines displayed variation in plating efficiencies in WEHI CM and relative plating efficiencies in +G418, +WEHI or -G418, -WEHI, but expression of the neomycin gene was apparently efficient, whereas expression of GM-CSF was sub-optimal for efficient clonal development at low cell density. With the assurance that these vectors could infect a GM-CSF dependent haematopoietic cell line and alter its phenotype by expression of virally encoded GM-CSF, bone marrow infections were attempted.

    The success of earlier attempts to infect bone marrow stem and progenitor cells was disscussed in chapter 1.7. These experiments used a number of related protocols to infect bone marrow cells and these will now be discussed.

    Optimal conditions for infection of bone marrow progenitor and stem cells appear to be attained by the co-cultivation of the target cell population for 24h with the virus producing cell line. This is prefered to the addition of viral supernatants removed form the ψ2 cells to the target cells (Hogge et al., 1987; Eglitus et al., 1985), since presumably, bone marrow cells are constantly exposed to fresh virus particles throughout the 24h incubation period. Moreover the efficiency of infection is greatest when high titre virus producer clones are used as a source of virus (Hogge et al., 1987; Eglitus et al., 1985). One problem which may be encountered during co-culivation of virus producer cell lines and bone marrow is the contamination of bone marrow cells with ψ2 fibroblasts which might lead to fibroblast colonies forming in the semi-solid medium. This problem can however be circumvented by irradiating the ψ2 cells prior to co-cultivation, a procedure which does not appear to affect their ability to produce virus (Hogge et al., 1987; Magli et al., 1987; Dick et al., 1985; Keller et al., 1985).

    The reliance of progenitor and stem cells on a source of growth factor for their short term survival in tissue culture has led to a source of Il-3 being included during the 24h co-cultivation of bone marrow (Lemischka et al., 1986; Magli et al., 1987 Keller et al.,

1985; Dick et al., 1985). The use of growth factor during viral infection may also trigger quiescent stem cells into cell cycle. An important characteristic, since cell replication may be required for efficient viral integration (Weiss et al., 1982). Both Eglitus et al. (1985) and Lemischka et al. (1986) have observed increased proviral integration into CFU-S if a source of Il-3 is included during the exposure of bone marrow to virus. However Eglitus et al. (1985) state that this increase is in fact very slight, and the average number of virally infected CFU-S per spleen rises only from 8.4 to 9.6.

In an attempt to increase the proportion of multipotential cells and so enrich the target cell population for virus infection, bone marrow from 5-fluorouracil (5FU) treated mice is often used (Bradley and Hodgson 1979). These workers observed that after a single intravenous dose of 5FU, a primitive cell population, with a high proliferative capacity (HPP-CFC), demonstrated a characteristic recovery pattern over a period of days. Thus bone marrow recovered at the appropriate time point post 5FU treatment is enriched for HPP-CFCs. Several investigators have used bone marrow recovered either 2 or 5 days post 5FU treatment (Keller et al., 1985; Dick et al., 1985; Williams et al., 1986; Lemischka et al., 1986) in infection procedures. However there is little direct evidence to substantiate the use of 5FU treated bone marrow in viral infections. Lemischka et al. (1985) compared infection efficiency between 1 and 2 day post 5FU bone marrow but did not include normal bone marrow in this study, therefore although it appeared that CFU-S in 2 day post 5FU marrow could be infected more efficiently than CFU-S in 1 day post 5FU marrow, no proper control was included. Treatment of bone marrow with 5FU may therefore not necessarily be advantageous (Magli et al., 1987).

A more systematic approach which has experimentally been shown to increase the frequency of infection of colony forming cells, is to include after co-cultivation, and prior to selection in semisolid media, a 48h liquid culture pre-selection step in the presence of 2mg/ml G418. Keller et al. (1985) demonstrated that pre-selection in G418 for 48h reduced the colony forming cells (CFC) to 15-50% of the input number but a high percentage of these were infected (60-90%). In comparison, 50-95% of CFC survived the 24h co-cultivation period but without 48h pre-selection in 2mg/ml G418

only 10-20% were infected. Magli et al. (1987) also show that pre-selection in high levels of G418 leads to a 3 to 5 fold increase in infected CFU-GM.

The tentative identification of steps which may be altered to improve efficiency of retroviral infection of haematopoietic stem and progenitor cells, so far achieved, demonstrates that conditions for efficient infection frequencies are not yet fully established.   While some paramenters such as virus titre and pre-selection do appear to have some significant effect on increasing the efficiency   of infection, others, such as the inclusion of Il-3 during the infection step, or the use of 5FU marrow, have less proven effects. Since some of these alternatives are labourious, it was considered important to more fully examine the effects of pre-selection, or 5FU marrow.

6.1   Preliminary experiments on bone marrow cells prior to viral infections

To infect bone marrow cells, cells must be removed from the femur of mice and co-cultivated with the virus producing cell line for 24h (Eglitus et al., 1985). After this period, non-adherent cells are removed and added to semi-solid media as a single cell suspension. Obviously the number of clonogenic cells per millilitre of semi-solid medium will determine colony density after incubation.   Therefore if different assays such as the CFU-A stem cell assay (Pragnell et al., 1988) and the CFU-GM progenitor assay require different cell densities, any effective loss or gain of clonogneic cells during co-cultivation may affect the assay conditions.   Also, if too many cells are plated out in any dish, cross-feeding between cells of autocrine produced factors may occur.   In either case it is difficult to draw meaningful conclusions.

It was therefore of interest to investigate any effective deviations from the normal frequency of colony forming units per 10^5 bone marrow cells after a 24h incubation period. Bone marrow from the femurs of NIH Beatson mice was added to $25cm^2$ tissue culture flasks at a density of 1-2 x 10^7 nucleated cells per flask (10mls media), in the presence of 2% WEHI CM, in order to maintain primitive cells (Koike et al., 1986). Polybrene was also added to the culture medium at 6 μg/ml, a substance normally added during virus infections to enhance viral absorption to the cell membrane.   The final volume of

the medium was 10ml per flask. No virus, or virus producing cells were present during this experiment. After a 24h incubation period, non-adherent cells were removed by gently standing the flasks upright and allowing the medium to drain to the bottom. The medium was then removed using a 10ml pipette, and the media used to wash the adherent cells once, after which it was spun at 1.5K for 10min in a bench top centrifuge and resuspended in fresh medium (approximately 10^6cells/ml). The cells were kept on ice until used. At this point the nucleated cells were counted and used in three assays as described in Materials and Methods:-

i)   The CFU-S _in vivo_ stem cell assay.

ii)   The CFU-A _in vitro_ stem cell assay.

iii) The CFU-C progenitor assay.

The numbers of progenitors and stem cells present per 10^5 cells after the 24h incubation period were compared to their frequency in fresh unincubated bone marrow which had been used directly in all three assays. The results are shown in Fig. 6.1. Fig. 6.1 shows that there is an effective drop in the frequency of progenitor CFU-C of approximately one third. However, if stem cell frequency is assayed _in vitro_ (CFU-A), or _in vivo_ (CFU-S), then almost two thirds of the assayable stem cells are lost.

The rather large loss of non-adherent stem cells considerably reduces the target cell population for virus infection, therefore it was considered important to establish the reasons for this loss.

It was observed that over the 24h incubation period, bone marrow derived cells adhered to the bottom of the tissue culture dish. This is presumably one event which occurs during the initial stages of long term marrow culture. Two groups, Mauch et al. (1980) and Schofield and Dexter (1985) have documented the presence of CFU-S in the adherent layer of the long term marrow cultures (LTMC). Interestingly, the latter have greater self-renewal properties than the non-adherent CFU-S , and may be more primitive. Although this data refers to LTMC, it is possible that some of the stem cell losses observed in Fig. 6.1 may be due to CFU-A and CFU-S adhering to the tissue culture flask directly, or through other adherent cells. The observations by Schofield and Dexter (1985) and Mauch et al. (1980) on the qualitative differences between adherent and non-adherent stem cells may be of importance if the same discrimination is present

during short term culture. In the short term culture the only bone marrow derived cells recovered after co-cultivation with virus producers are non-adherent cells. Also of interest is the report by McCulloch and Till (1971) which documents quantitative and qualitative changes in suspension bone marrow and progenitor stem cells cultivated with or without a source of growth factor over a period of two days.

In order to assess the proportion of stem cells partitioned between the non-adherent and adherent fraction of cells after a period of culture, bone marrow was incubated for either 24h or 48h after which both fractions were assayed for stem cells using the in vitro assay of Pragnell et al. (1988). In total, twelve $25cm^2$ (10ml volume) tissue culture flasks were inoculated with 10^7 freshly isolated nucleated bone marrow cells. Pairs of flasks were supplemented with either 0, 1, 2, 3, 4, or 5% WEHI CM and one member of each pair was harvested after 24h of incubation, the other after 48h. By varying the concentration of WEHI CM present during the incubation, any gross effects on stem cells numbers should be observed. Non-adherent cells were harvested as described above and the remaining adherent cells were scraped from the flask bottom in 1ml of medium using a rubber policeman. The adherent cells were then passed through a 19 gauge needle in order to disperse them into single cells. The cells from both fractions were spun at 1.5K for 5min, washed once in medium, respun, and the pellet resuspended in fresh medium. The nucleated cells were then counted and assayed in the CFU-A assay to determine the number of in vitro stem cells present. Figure 6.2 shows the number of CFU-A type colonies present in the adherent (hatched columns) and non-adherent (open columns) fractions, after 24h (6.2A) and 48h (6.2B) in the presence of increasing amounts of WEHI CM. The number on the vertical axis is the ratio, expressed as a percentage, of the CFU-A colonies per 10^5 cells in any given experimental condition, to the number of CFU-A type colonies per 10^5 cells found in the starting bone marrow material prior to incubation. It can be seen from Fig. 6.2 that a varying percentage of the total recoverable CFU-A type colonies under any experimental condition are found on the adherent layer of the tissue culture flask. For example, after a 24h incubation in the presence of 5% WEHI CM, almost 50% of the total recoverable CFU-A from both the adherent and non-adherent layers are in the adherent layer. Therefore

Figure 6.1

24 hour incubation of bone marrow.

Freshly isolated bone marrow cells were incubated for 24h at a density of 1-2 x 10^7 nucleated cells per 25cm^2 flask (10ml) in the presence of 2% WEHI CM. The non-adherent cells were then gently harvested and assayed in vitro for CFU-C progenitor cells and CFU-A stem cells and in vivo for CFU-S stem cells. The number of colonies per 10^5 cells in each assay was compared to the frequency of CFU-C, CFU-A and CFU-S in the starting material before incubation.

CFU-C assay Five 3cm petri dishes containing 1ml of methocellulose and 7.5 x 10^4 bone marrow cells were used per plate.

CFU-A assay Five 6cm petri dishes containing 4ml of methocellulose and 5 x 10^3 cells for control incubated marrow and 2 x10^4 cells for 24h incubated marrow were used per point.

Both the CFU-C and CFU-A assays were performed in 5 separate ocassions. The values shown represent the average colony number per 10^5 cells for the 5 experiments. The standard deviation is shown in brackets.

CFU-S assay CFU-S were assayed by injecting 0.2ml aliquots of 5 x 10^4 unincubated fresh marrow or 10^5 24h incubated marrow into 8.2 Gy X- irradiated mice. Spleen foci from 4 mice were counted for the injection of normal marrow, 7 mice for incubated marrow. The average number of foci per 10^5 cells injected is shown. The standard deviation is in brackets.

24 HOUR INCUBATION OF BONE MARROW CELLS

| ASSAY | COLONIES/10^5 CELLS | | % |
	BEFORE	AFTER	LOSS
CFU-C	72 (35)	45 (17)	37
CFU-A	93 (25)	23 (10)	75
CFU-S	34.5 (7)	10.6 (3.4)	70

Figure 6.2

Presence of CFU-A stem cells in adherent versus non-adherent bone marrow cells.

$1-2 \times 10^7$ nucleated bone marrow cells were incubated for either 24 or 48h in the presence of 0 - 5 % WEHI. All incubations were in 25cm^2 tissue culture flasks in a final volume of 10ml medium. After either 24 of 48h the non-adherent cells were removed by gentle pippetting and the cells which had adhered to the tissue culture flask were removed by scraping with a rubber policeman, followed by cell disaggregation through a 19 gauge syringe needle. The nucleated cells were counted and assayed for CFU-A stem cells. The frequency of adherent (hatched columns) and non-adherent (open columns) is expressed as a percentage of the frequency of CFU-A present in the starting material. 3x6cm petri dishes were used per point, containing 4×10^3 cells per ml (4ml soft agar per plate).
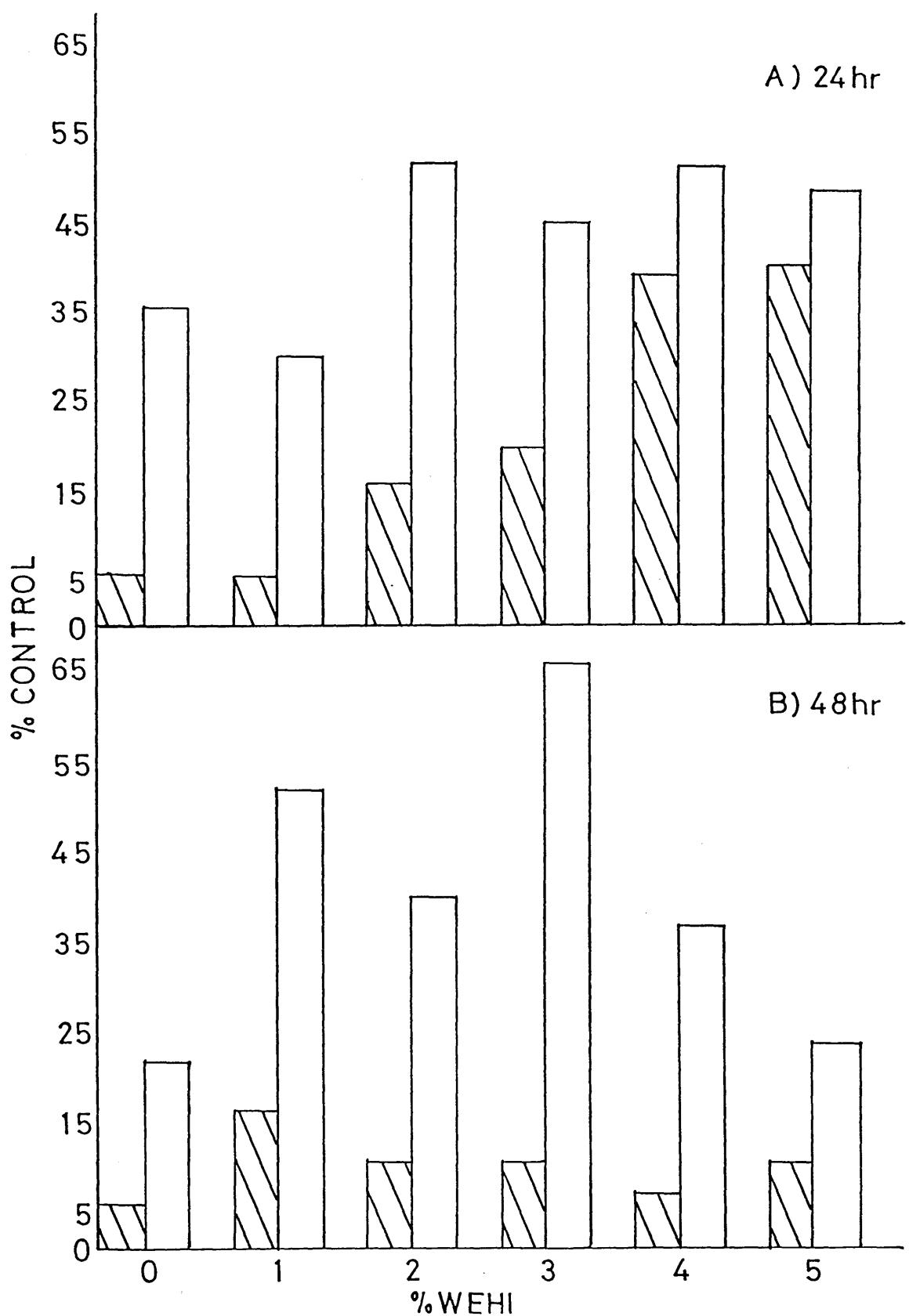
it is possible that much of the loss of stem cells observed when only the non-adherent cells are assayed, as described previously, (Fig. 6.1) is due to stem cells adhering to the bottom of the tissue culture flask.

Fig. 6.3 documents the results of an experiment designed to investigate the presence of CFU-S within developing _in vitro_ CFU-A colonies. Although bone marrow incubated for 24h does contain CFU-S (Figs. 6.1 and 6.3) it was thought of some importance to demonstrate that the _in vitro_ colonies which developed in the _in vitro_ CFU-A assay using 24h incubated bone marrow, do contain CFU-S. CFU-S can be detected in 24h incubated bone marrow but the cells which form CFU-A colonies may be a distinct population in the manipulated bone marrow, however the ability to detect low numbers of CFU-S within developing CFU-A colonies as shown in Fig.6.3, suggests that the developing colonies do still originate from a primitive cell population with stem cell characteristics.

6.2   Retroviral infection of bone marrow progenitor and stem cells.

Fig 6.4 shows the frequency with which bone marrow progenitors (CFU-C) and stem cells (CFU-A), present within normal and 5-fluorouracil (5FU) treated bone marrow, are infected with pVneo virus from the $\Psi 2$ producing clone 38 (Fig.4.3). Bone marrow from NIH mice (obtained from Olac) were co-cultivated for 24h with clone 38 as described in Materials and Methods, after which non-adherent cells were removed and assayed _in vitro_ for progenitor cells (CFU-C) or stem cells (CFU-A). Bone marrow from mice treated 10 or 14 days previously with 5FU was used since bone marrow stem cells (CFU-A and CFU-S) show an increase in frequency around this time after 5FU treatment (Pragnell et al., 1988). The frequency of infection was assessed by adding G418 at 1mg/ml to the semi-solid medium. Thus colonies which develop in the presence of 1mg/ml G418 express the retrovirally encoded neo (Fig. 6.8). Fig 6.4 shows that CFU-C progenitor cells can be infected with a high efficiency irrespective of the marrow used. However the frequency of G418 resistant CFU-A stem cells colonies was variable. It might be expected from the data of Pragnell et al. that bone marrow 14 days after 5FU treatment would contain an increased frequency of CFU-A stem cells and therefore represent an increased cell population for viral infection in comparison to normal bone

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Figure 6.3

Presence of CFU-S in manipulated bone marrow.

Bone marrow which had been incubated for 24h on 25cm^2 flasks (5 flasks) at a density of 1-2 x10^7 nucleated cells per flask (10ml) in the presence of WEHI was assayed for the presence of CFU-S stem cells immediately after the incubation period and after a period of CFU-A colony formation in methocellulose. Incubated bone marrow was assayed in vitro for CFU-A and developing colonies picked using a dissection microscope after 5, 6, 7 or 8 days after commencement of growth in semi-solid medium. Developing colonies from 5×6cm plates (2 x 10^4 cells per ml, 4ml per plate) were picked and pooled for each time point in 10ml ice cold SLM growth medium. The cells were then spun at 1.5K for 5min and resuspended in SLM. The numbers of cells injected per mouse is shown in the figure.

PRESENCE OF CFU-S IN MANIPULATED BONE MARROW

EXPERIMENTAL CONDITION	NO. OF CELLS INJECTED/MOUSE	COLONIES/SPLEEN	MEAN COLONIES/ SPLEEN	FOCI/ 10^5 CELLS
Non Transplanted	0	0,0,0,0,0,1	0.17	–
Normal marrow	5×10^4	19,12,19,19	17	34
24hr Liquid Culture	1×10^5	5,10,13,10,15,8,13	10.5	10.5
5 Days in Methocel	2.8×10^4	1,0,4,6	2.7	9.8
6 Days	4×10^4	0,0,4,3,1	1.6	4
7 Days	5×10^4	3,0,0,0	0.7	1.4
8 Days	1.2×10^4	8,0,2,3,1	2.8	23

Figure 6.4

Virus infection of bone marrow progenitor cells and stem cells by pVneo.

$1-2$ x 10^7 nucleated bone marrow cells were incubated for 24h with the virus producing cell line cl-38 which releases pVneo virus. After 24h the non-adherant cells were harvested and assayed for CFU-A stem cell colony and CFU-C progenitor cell colonies resistant to 1mg/ml G418.

The source of bone marrow was from normal mice (0 5FU treated) or bone marrow treated 10 or 14 days previously with a single dose of 5 flurouracil (5FU).

CFU-A assay 10 x10 cm petri dishes per point were used for selection for G418 resistance. 5 x10 cm petri dishes per point were used for unselective conditions. All plates were overlayed with 10ml of 0.3% agar conytaining 8 x 10^3 nucleated cells per ml.

CFU-C assay 5 x 3 cm petri dishes were used per point containing 7.5 x 10^4 cells per ml (1ml per plate). Control unincubated bone marrow was plated out in the CFU-A assay at 5 x 10^3 cells per ml in triplicate 6cm plates (4ml per plate) +/- 1mg/ml G418 and at 5 x 10^3 cells per ml for the CFU-C assay in triplicate 3cm plates (1ml per plate).

VIRUS INFECTION OF OLAC NIH MICE BY

cl-38 (pVneo)

5-FU BONE MARROW	G418	ASSAY			
		CFU-C COLONIES/ 10^5 CELLS	% INFECTION	CFU-A COLONIES/ 10^5 CELLS	% INFECTION
0	−	61		34	
	+	45	74	11	33
10	−	140		55	
	+	89	64	11	21
14	−	149		56	
	+	145	97	4	8
CONTROL	−	249		215	
	+	0	−	0	−

marrow. The low frequency of infection of 14 day post 5FU bone marrow suggests that perhaps the timing of bone marrow recovery after 5FU may be critical. It is also possible that insufficient cells were plated to obtain accurate estimations on infection frequency. Alternatively, it may be that any advantage theoretically present in using 5FU, treated marrow is lost upon co-cultivation with the virus producing cells.

One problem encountered during the scoring of the G418 resistant CFU-A colonies in this experiment was their apparent reduction in size. In the original protocol described by Pragnell et al. (1988) CFU-A colonies are those which attain a diameter of greater than 2mm after 11 days in culture. Although there were many small colonies ranging from the microscopic upwards on plates containing 1mg/ml G418, very few were greater than 2mm. Thus there was some difficulty in scoring CFU-A colonies as the smaller colonies (less than 2mm), may have been G418 resistant CFU-A colonies whose development had been impaired due perhaps, to poor expression of viral neo. Alternatively, the small colonies may represent G418 resistant progenitor colonies. Returning these plates to the incubator for a further 7 days did not improve resolution.

Fig. 6.5 shows the use of a liquid culture pre-selection step in 2mg/ml G418 after a 24h co-cultivation period prior to plating the non adherent cells in the CFU-C and CFU-A assays. The virus producing cell line used in this experiment was clone 41, which releases $mos^{-3}neo$ virus. Pre-selection in 2mg/ml G418 reduces the number of CFU-A colonies per 10^5 cells from 80 to 55 in the bone marrow used in the co-cultivation with Cl-41 (Fig. 6.5) and from 80 to 30 CFU-A per 10^5 cells in the uninfected control bone marrow. On selection with 1.5mg/ml G418 during colony developnent, although the actual number of G418 resistant colonies did not differ greatly between pre-selected and non-selected $mos^{-3}neo$ infected marrow, the reduction in total CFU-A colonies observed with pre-selection, effectively increased the frequency of infected CFU-A colonies. Therefore although this experiment was only carried out once, there may be some value in including a pre-selection step for the infection of CFU-A stem cells. A similar effect of preselection was not obvious using the CFU-C progenitor assay, although infection of progenitor type cells was achieved.

The infection shown in Fig 6.5 differs from that in Fig 6.4 since bone marrow used for the pre-selection experiments (Fig. 6.5) was from NIH mice bred at the Beatson Institute, whereas the previous experiment (Fig. 6.4) used marrow from NIH mice obtained directly from the suppliers Olac. Also in the pre-selection experiment the marrow was infected with mos^{-3}neo virus in comparison to pVneo. Fig 6.6 shows infected or uninfected pre-selected bone marrow which has been assayed for CFU-A in the presence of G418. It can be seen that there are no obvious size differences between the G418 resistant colonies and the control colonies. This is a repeatable observation using bone marrow infected with mos^{-3}neo and thus the difficulties encountered with scoring CFU-A colonies in the experiment described in Fig. 6.4 and discussed previously are not encountered.

Fig 6.7 documents an attempt to infect bone marrow stem and progenitor cells with a GM-CSF virus, pVneoGM. Previous results on the viral expression of GM-CSF in the FDCP1 cell line (chapter 5) suggested that both pVneoGM and mos^{-3}neoGM could generate what appeared to be factor independent FD colonies after infection, but on further analysis, cell lines expressed sub-optimal levels of GM-CSF for maximal proliferation . It was therefore decided that any effects of virally expressed GM-CSF on colony formation, in terms of factor independent proliferation, may only be observed if low levels of growth factor were supplied at a suboptimal level for stimulation of normal progenitor, and stem cells. Bone marrow cells were co-cultivated for 24h with either clone 41 (mos^{-3}neo) or clone 15 (pVneoGM) followed by 48h pre-selection in 2mg/ml G418. The non-adherent cells were then added to semi-solid medium and assayed for either CFU-C or CFU-A. The source of stimulation in the CFU-C assay was recombinant GM-CSF (rGM-CSF), which was added at 10% (875pg/ml), 0.25%. 0.1% and 0%. Using bone marrow co-cultivated with Cl-41 (mos^{-3}neo) G418 resistant colonies were obtained as before in the presence of 10% rGM-CSF. Small clusters of cells also proliferated on plates supplemented with 0.25% rGM-CSF but no colonies formed in the absence of rGM-CSF or in 0.1% rGM-CSF. No G418 resistant colonies were formed in co-cultivation with Cl-15 (pVneoGM) and thererfore it was impossible to prove that viral infection had occurred. Also there were no factor independent colonies, and so even if viral infection had occurred the viral genes are not expressed at

Figure 6.5

Pre-selection in liquid culture during virus infection.

$1-2 \times 10^7$ nucleated bone marrow cells were incubated for 24h either with or without virus producing cells, Cl-41 in 25cm^2 flasks. Non-adherent cells were then removed, split into two new 25cm^2 flasks +/- 2mg/ml G418, +WEHI CM and incubated for a further 48h. The non-adherent cells were again removed and assayed for CFU-C progenitor and CFU-A stem cells resistant to 1.5mg/ml G418 in methocellulose. Cells to be assayed for CFU-A content were plated out in 5 x 6cm petri dishes per point at 5×10^3 cells/ml. Cells to be assayed for CFU-C content were plated out in 5 x 3cm petri dishes per point at 7.5×10^4 cells /ml (1ml per plate).

INFECTION OF (BEATSON NIH MICE) BONE MARROW CELLS BY mos^{-3}neo VIRUS.   USE OF PRESELECTION STEP

| CLONE	VIRUS	PRESELECTION 2mg/ml G418	SELECTION 1.5mg/ml G418	ASSAY CFU-C COLONIES/ 10^5 CELLS	% INF.	CFU-A COLONIES/ 10^5 CELLS	% INF.
cl-41	mos^{-3}neo	−	−	26		80	
		−	+	12	46	10	12
		+	−	20		55	
		+	+	8	40	15	27
CONTROL	UNINFECTED	−	−	74		85	
		−	+	0	−	0	−
		+	−	48		30	
		+	+	0	−	0	−

Figure 6.5

G418 resistant CFU-As.

1-2 x 10^7 nucleated bone marrow cells were incubated for 24h either with or without virus producing cells, cl-41 in $25cm^2$ flasks. Non-adherent cells were then removed, transfered to a $25cm^2$ flasks +2mg/ml G418, +2% WEHI CM and incubated for a further 48h. The non-adherent cells were again removed and assayed for CFU-A stem cells resistant to 1.5mg/ml G418 in methocellulose. Cells to be assayed for CFU-A content were plated out in 5 x 6cm petri dishes per point at 5 x 10^3 cells/ml.

A)   No virus, -G418

B)   No virus, +G418

C)   Plus virus, -G418

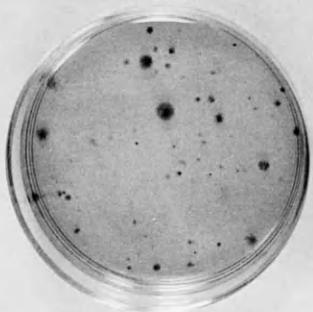D)   Plus virus, +G418
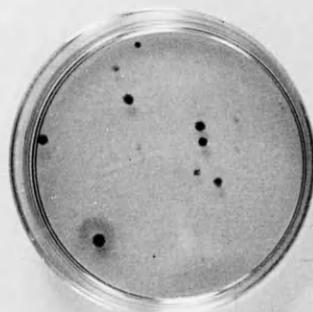
<u>Figure 6.7</u>

Co-cultivation of bone marrow with a GM-CSF virus producer cell line.

$1-2$ x 10^7 nucleated bone marrow cells were co-cultivated with either Cl-41 or Cl-15 virus producer cell lines ot in the absence of a virus producer line (control). After 24h co-cultivation the non-adherent cells were removed and cultured for a further 48h in 25cm^2 flasks (10ml) in the presence of 2% WEHI CM and 2mg/ml G418. Non-adherent cells were again removed and CFU-A and CFU-C present in this population was assayed for resistance to 1.5mg/ml G418 and response to sub-optimal growth factor stimulation. Cells assayed for CFU-A frequency were plated at 1.5 x 10^4 cells/ml in 3 x 6 cm plates per point. Cells assayed for CFU content were plated at 10^5 cells/ml in 5 x 3cm plates (1ml per plate). Dilutions of recombinant GM-CSF (rGM-CSF) were added to some plates in the CFU-C assay. Conditioned media from the AFI and the L929 cell lines were used as a source of stimulation in the CFU-A assay (Pragnell <u>et</u> <u>al</u>., 1988.

*[1,2,3,4] = microscopic clusters or colonies of cells .

A. INFECTION OF CFU-C

CLONE	VIRUS	G418	% rGM-CSF	COLONIES/ 10^5 CELLS	% INFECTION
CL-41	mos^{-3}neo	–	10	82	
		+	10	23	28
		–	0.25	*[1]	
		–	0.1	0	
		–	0	0	
cl-15	pVneoGM	–	10	74	
		+	10	0	0
		–	0.25	*[2]	
		–	0.1	0	
		–	0	0	
CONTROL		–	10	96	
		+	10	0	

B. INFECTION OF CFU-A

CLONE	VIRUS	G418	L929	AF1	COLONIES/ 10^5 CELLS	% INFECTION
cl-41	mos^{-3}neo	–	+	+	32	
		+	+	+	18	56
		–	+	–	*[3]	
		–	–	–	0	
cl-15	pVneoGM	–	+	+	45	
		+	+	+	0	0
		–	+	–	*[4]	
		–	–	–	0	
CONTROL		–	+	+	53	
		+	+	+	0	

Figure 6.8

Titration of G418 on bone marrow stem cells.

The graph shows frequency of CFU-A colony formation with increasing concentrations of the antibiotic G418. Colony size became reduced at concentrations of 400µg/ml and over until at 1mg/ml only clusters of under 50 cells were observed with the aid of a dissection microscope. 2 x 6cm plates were used per point at a density of 4×10^3 cells/ml (4ml per plate). A similar sensitivity to G418 was observed for progenitor CFU-C cells and at 1mg/ml G418 no microscopic colonies of greater than 50 cells were observed (data not shown).
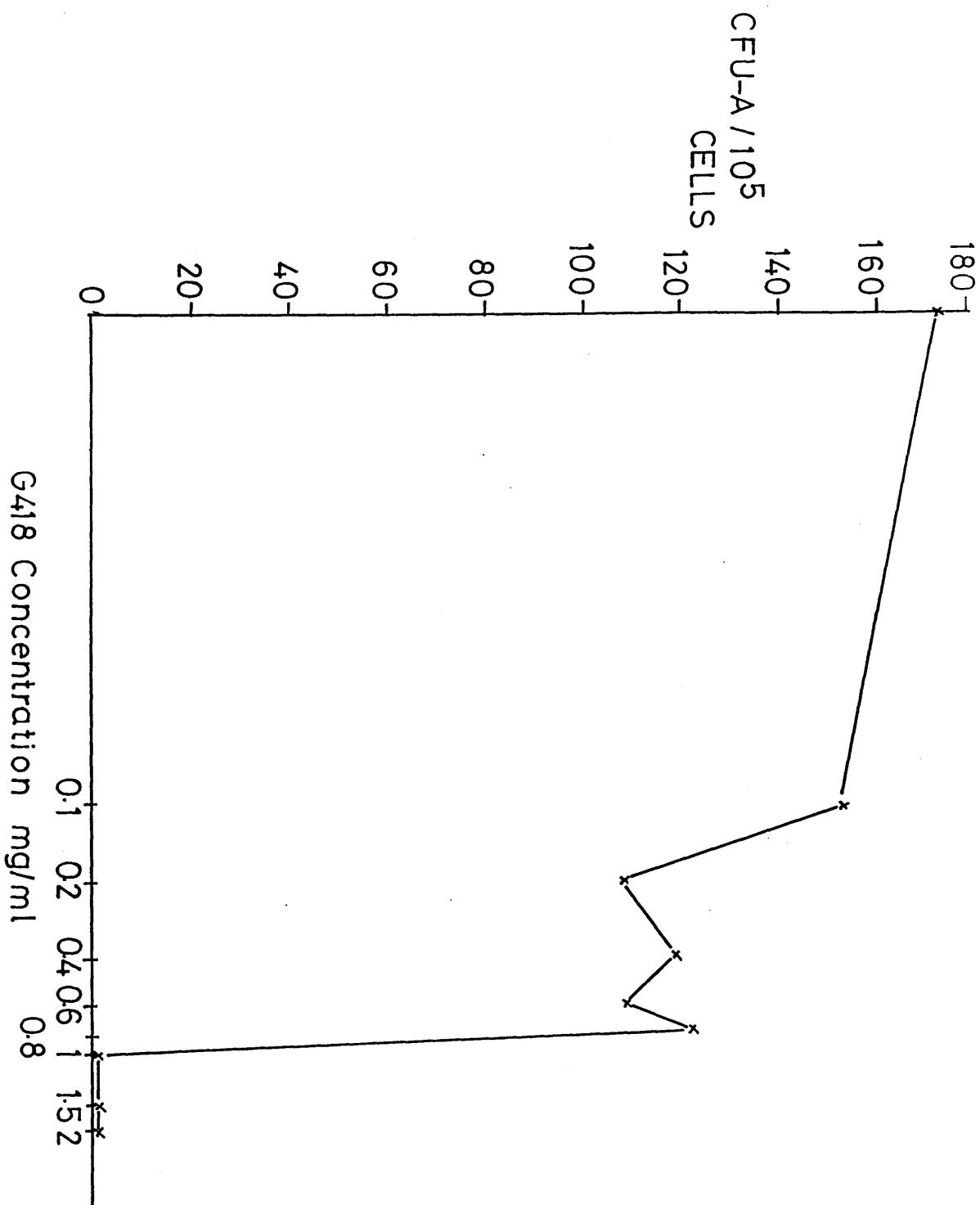
detectable levels.

A similar result was observed in the CFU-A assay described in Fig. 6.7. The CFU-A assay relies on stimulation by a cocktail of growth factors supplied by the conditioned medium from the L929 and AF1 cell lines. The conditioned medium from the AF1 cell line can be replaced by rGM-CSF (Pragnell et al., 1988) and so the effect of expressing a GM-CSF containing vector in CFU-A stem cells should theoretically be possible by adding L929 conditioned medium only. However as in the progenitor assay, viral infection with the GM-CSF virus could not be proven as no G418 resistant colonies were observed.

The bone marrow cells could be infected however by Cl-41 (Fig 6.7) and therefore the cells themselves are not refractory to infection by virus packaged by Ψ2 cells.

Discussion

The data shown in Figs. 6.4, 6.5 and 6.7 shows that normal bone marrow and 5FU treated marrow can be infected with retroviral vectors to yield G418 resistant colonies. No obvious advantage to using 5FU marrow can be concluded from Fig. 6.4 but these experiments would have to be repeated to confirm this. Infection of bone marrow with MoMLV based pVneo (clone 38) resulted in small colonies which were often difficult to distinguish from progenitor colonies on size basis. The above experiment was performed using NIH mice obtained through Olac.

The addition of a pre-selection step in liquid culture for 2 days in high G418 after co-cultivation, and before plating out in semi-solid medium, may have an effect on increasing the relative frequency of infection of CFU-A stem cells, but again this should be repeated. The preselection step did not appear to have any adverse affects on the assay other than lengthening it and so it is perhaps worth retaining.

In an attempt to observe virally directed GM-CSF expression, bone marrow which had been exposed to virus (either mos^{-3}neo or pVneoGM) was plated out under conditions which should detect GM-CSF expression (Fig. 6.7). Thus a titration of rGM-CSF was used in the CFU-C progenitor assay and the conditioned medium from AF1 cells ommitted from the CFU-A assay. However, no viral infection was observed using clone 15 which releases pVneoGM virus particles. It is

possible that expression of a GM-CSF virus in primary haematopoietic cells is "toxic" to the cells and so colony development does not occur. In this situation one might expect a reduction in plating efficiency of bone marrow exposed to pVneoGM in comparison to $mos^{-3}neo$ virus. Fig 6.7 indicates that infection with a GM-CSF virus is probably not toxic to colony development. Unless clone 15 has stopped producing virus, (virus titre was not checked immediately prior to the bone marrow infection), the most likely explanation for the lack of infection with the MoMLV based pVneoGM is perhaps a poorer expression in haematopoietic cells, in comparison to the MPSV based $mos^{-3}neo$ virus. This may result in a low frequency of infection with pVneoGM, undetected under the assay conditions, which might be observed if a greater number of bone marrow cells were plated out after the co-cultivation with the virus producer clone. The results in Fig 6.4 demonstrate that MoMLV vector pVneo can infect and express in primary haematopoietic cells but the experiment in Fig 6.7 differed in using NIH mice bred at the Beatson Institute and a higher level of G418 (1.5mg/ml) to select for resistant colonies. Thus the initial success in infecting bone marrow cells with a MoMLV based vector may have been due to slightly different experimental conditions. The inability of MoMLV based vectors to infect bone marrow progenitor and stem cells was observed several times using NIH Beatson mice and 1.5mg/ml G418 selection conditions (data not shown), whereas infection of bone marrow cells by clone 41, which releases the MPSV derived $mos^{-3}neo$ was observed on both occassions it was used (Figs. 6.5 and 6.7). It is therefore possible that the slight advantage that MPSV vectors may show over MoMLV vectors in expressing neo in FDCP1 cells (chapter 5) may be magnified in primary haematopoietic cells. Theoretically, MPSV based vectors should be capable of expressing efficiently in haematopoietic cells (Ostertag et al 1980) and therefore it should not be surprising that at least $mos^{-3}neo$ infected stem and progenitor cells express G418 resistance as judged by frequency and size of resistant colonies. It must be remembered that all the viruses used in this study are packaged in the Ψ2 cell line and so have the same envelope proteins. Thus, frequency of infection is probably not a function of the efficiecy with which virus particles interact with target cell membrane, but is probably related to the efficiency with which the selected gene is expressed once the virus has entered the

cell, and integrated into the genome.

CHAPTER SEVEN

Conclusions

Autocrine stimulation of haematopoietic cells has been the subject of considerable research in recent years and a complex picture of regulation by both GM-CSF and other factors is beginning to emerge (Chapter 1.6, 5). This study has concentrated in particular on retroviral mediated gene expression in cell lines (Chapters 4 and 5) and primary bone marrow cells (Chapter 6).

Retroviral mediated expression of GM-CSF described in this thesis and elsewhere (Laker et al., 1987; Lang, 1985) demonstrates the possibility of altering the growth characteristics of a haematopoietic cell line. However the growth observed is dependent on the retroviral vector used, an imoprtant consideration for interpretation of any results concerning the interaction of autocrine produced GM-CSF with the cell line (Chapter 5). The final interpretation may also be dependent on the exact time point at which cells are taken for analysis (Laker et al., 1987). The importance of the choice of vector is demonstrated by the results described in Chapter 5 which reveal a higher conversion of FDCP1 cells to factor independence using the pVneoGM vector in comparison to the mos^{-3}neoGM vector.

The observed frequency with which the retroviral constructs used in this study convert FDCP1 cells to factor independence may be related to the observations in Chapter 4 that growth factor release from virus producing cells lines, is greater from cells harbouring pVneoGM than mos^{-3}neoGM, possibly via the efficiency of retroviral mediated expression of GM-CSF.

Although as indicated above knowledge of the growth characteristics of haematopoietic cells immediately after retroviral infection does reveal information on the autocrine stimulation of FDCP1 cells, the analysis of cell lines derived from infection uncovers a complexity hitherto unobserved.

FDCP1 cell lines which contain the pVneoGM virus and which were initially selected for resistance to G418 demonstrate a variable relative replating efficiency under conditions which select for factor independent growth (Fig. 5.3) and maximal colony growth is not observed in comparison to stimulation with WEHI conditioned medium (Fig. 5.3). The low replating efficiency in factor independent conditions may be due to epigenetic suppression of growth factor

sequences in the vector (Emmerman and Temin, 1984) after selection for G418 resistance. However, FDCP1 cells lines containing pVneoGM which are selected for factor independence show an even poorer replating efficiency in factor independent conditions at low cell density (10^3/ml in Fig. 5.6) but it is unlikely that epigenetic suppression of growth factor sequences is the cause, as the cells were initially selected for expression of growth factor sequences. The low replating efficiency of pVneoGM containing cells is therefore unlikely to be caused by epigenetic suppression. The inability of the factor independent cell lines to replate into factor independent conditions at low densities can in part be attributed to their low replating efficiency even in the presence of WEHI CM (Fig. 5.6).

It was of interest that pVneoGM containing factor independent cell lines did form colonies at a high cell density and therefore efficient colony formation may require cross feeding between colonies. The hypothesis that the factor independent cell lines produced levels of growth factor sub-optimal for efficient colony stimulation at low cell density was tested by examining the cloning efficiency of these cell lines in response to exogenous growth factor (Figs. 5.7 and 5.8). From the experiments shown in these figures it can be concluded that factor independent clones respond to exogenous growth factor by increasing their cloning efficiency in semi-solid medium, thus giving them a proliferative advantage over the parental FDCP1 cell line in sub-optimal growth conditions. Therefore, although the virus pVneoGM is expressed in the FDCP1 cells it is presumably not at a high enough level to totally relieve the cells reliance on exogenous growth factor, a characteristic which is manifestd in a poor cloning efficiency at low cell density.

Initial results using FDCP1 clones derived by infection with mos^{-3}neoGM also suggest that expression of viral GM-CSF sequences is poor in these cell lines (Chapter 5). These results suggest that growth factor must be released from the growth factor independent cells and attain a critical concentration before efficient colony formation can occur, an event which can be substituted by the addition to the culture of low levels of exogenous growth factor. The results in this study therefore differ from those of Lang et al. (1985) on the expression of GM-CSF in FDCP1 cells but are similar to those of Laker et al. (1987) published during the course of this thesis.

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Returning to the central question of autocrine stimulation of primary bone marrow progenitor and stem cells as discussed in Chapters 1.6 and 1.7, the lessons learned on retroviral gene expression in cell lines (Chapter 5) were used to design experiments to address the above question.

Conditions were established in which retroviral gene transfer could be achieved by the manipulation of existing _in vitro_ colony assays (Chapter 6). However, despite success in expressing the neomycin gene in primary haematopoietic cells and considerable effort to optimise cell density and growth factor levels, which the results in Chapter 5 indicate may be critical in order to observe retroviral mediated GM-CSF expression, the autocrine stimulation of primary haematopoietic cells was not observed. However, failure to detect factor independent colonies in the experiments shown in Fig.6.7 is most likely due to poor viral infection and/or expression.

The retroviral expression of genes in short term _in vitro_ assays may give some indication as to the multiple steps involved in leukaemogenesis, which can then be examined in long term marrow cultures or _in vivo_. Therefore, the use of short term _in vitro_ assays to assess the efficiency of new vectors, in terms of gene expression in bone marrow cells is important. Over the past few years our knowledge of mechanisms by which retrovirus vectors are expressed in highly specialised cell types has increased considerably. By the application of fundamental observations in both retroviral expression and the manipulation of bone marrow _in vitro_, the optimal system could be generated to study autocrine stimulation in haematopoietic cells. This study and others (Laker _et al._, 1986; Lang _et al._, 1985) have gone some way to addressing whether autocrine stimulation results in a proliferative advantage over non autocrine stimulated cells and to the necessity or otherwise for growth factor release as part of autocrine stimulation.

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