The common signalling subunit, hβc in the myeloproliferative disorder, Polycythemia Vera

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Abstract

PV is an acquired, late onset myeloproliferative disorder characterised by the clonal hyperproliferation of cells of the erythroid, myeloid and megakaryocytic haemopoietic lineages. Committed progenitor populations isolated from PV patients show hypersensitive responses to multiple haemopoietic growth factors (HGF). Consistent with the dysregulation of a molecule or molecules commonly utilised by the signalling pathways of multiple HGF receptors, PV is thought to be due to an accumulation of genetic events in an early, multipotent haemopoietic stem cell.

Several studies are consistent with the common β subunit of the HGF receptors for IL3, GM-CSF and IL5 (hβc) playing a role in PV. PV progenitors are hypersensitive to IL3 and GMCSF (approximately 50 x and 100 x, respectively). hβc and activating mutant forms have been shown to interact with other receptor subunits including those for HGFs involved in PV. Transgenic mice expressing an activating form of hβc develop myeloproliferative disease with similar characteristics to the PV phenotype. In particular, these mice display expansion of multiple lineages and committed progenitors are hypersensitive to several HGFs. Thus hβc alterations can lead to effects on multiple HGF receptor components *in vitro* and to profound multilineage effects *in vivo*.

The aim of this study therefore was to determine whether mutations of the gene encoding hβc (*CSF2RB*) are present in PV patients. I have screened a defined target region in peripheral blood mononuclear cells and used a novel approach to examine this region in BFU-E isolated from PV patients. Two possible PV specific single nucleotide substitutions were detected that result in non-conservative amino acid substitutions, F451S and R472I, within the transmembrane and the intracytoplasmic box 1 motifs of hβc. The potential significance of these alterations with regard to receptor signalling and PV is discussed.

In addition, a novel, alternatively spliced variant of hβc (Δ1041-1045) was identified in both normal and PV patient samples. This mRNA species encodes a

truncated, soluble form of the receptor. Further analysis determined that the Δ1041-1045 splice variant is expressed at a low level in PBMNC.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published by any other person, except where due reference has been made in the text. I give consent for this thesis to be made available for loan and photocopying.

Signed,

Carolyn M. Butcher

1st April, 2003

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Publication

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Abbreviations

A	adenine
AML	acute myeloid leukemia
(α-^{32}P)dATP	α-labelled 2'-deoxyadenosine-5'-triphosphate
bp	base pair
BFU-E	erythroid blast forming unit
BM	bone marrow
BMMNC	bone marrow mononuclear cells
BSA	bovine serum albumin
C	cytosine
cDNA	deoxyribonucleic acid complementary to ribonucleic acid
CFU-E	erythroid colony forming unit
CML	chronic myeloid leukemia
CR	cytokine receptor
CRD	cytokine receptor domain
CRM	cytokine receptor module
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTPs	dATP, dCTP, dGTP, dTTP
E.coli	*Escherischia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EPO	erythropoietin

FCS	foetal calf serum
G	guanine
GCSF	granulocyte colony stimulating factor
GMCSF	granulocyte-macrophage colony stimulating factor
GMR	GMCSF receptor
h	human
HEPES	N'-[2-hydroxyethyl]piperazine-N'-2-ethanesulphonic acid
HGF	haemopoietic growth factor
IGF-1	insulin-like growth factor-1
IL-	interleukin
IMDM	Iscove's modified Dulbecco's medium
JAK	Janus kinase
JMML	juvenile myelomonocytic leukaemia
LB	Luria broth
M	moles per litre
Meg	megakaryocyte
mg	milligram
min	minute
ml	millilitre
MPLV	myeloproliferative leukemia virus
NF1	nuclear factor 1
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBMNC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTK	protein tyrosine kinase

PTP	protein tyrosine phosphatase
PV	polycythemia vera
R	receptor
rbc	red blood cell
RNA	ribonucleic acid
RT	reverse transcriptase
RTK	receptor tyrosine kinase
RTPCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SH2	*src* homology 2
SNuPE	single nucleotide primer extension
SOCS	suppressor of cytokine signalling
STAT	signal transducers and activators of transcription
T	thymidine
Taq	*Thermus aquaticus*
TGFβ	transforming growth factor β
Tpo	thrombopoietin
Tris	tris[hydroxymethyl] amino methane
WT	wild type

Chapter 1 Introduction

Part 1 Polycythemia Vera

1.1 The haemopoietic system

In the adult human approximately 10^{10} red blood cells and 10^8-10^9 leukocytes are produced every hour in order to maintain the populations of the eight cellular lineages that comprise the haemopoietic system. These mature cells perform such diverse functions as oxygen transport, the immune response and the clotting cascade and are derived by a series of maturational and divisional steps from quiescent, undifferentiated stem cells that reside in the bone marrow.

The haemopoietic system can be viewed as a series of overlapping, functional compartments (Figure 1.1). The ***stem cell compartment*** consists of rare, primitive, multipotent cells from which the cells of all 8 haemopoietic lineages are derived. They can be induced to undergo differentiation and proliferation to produce the diverse range of functional mature haemopoietic cells. In order to maintain the stem cell pool these cells have high self-renewal capacity and the majority are quiescent or slowly cycling (Graham, 1997; Morrison et al., 1995; Zipori, 1992). Due to difficulties in obtaining sufficient number and purity of these cells, the regulation of this compartment has not been thoroughly investigated. Similarly, the process termed commitment, in which stem cells transit to the ***progenitor cell compartment*** is also incompletely understood. Committed progenitors are characterised by a more restricted capacity to differentiate and proliferate but have the ability to form colonies *in vitro*, and have therefore been studied more extensively. The progenitor pool consists mainly of cells that are unipotential together with a low frequency of bi- or multi-potential cells. Finally, the ***mature cell compartment*** comprises highly specialised and terminally differentiated cells with readily recognisable and lineage-specific morphologies.

The entire process of haemopoiesis, including stem cell self-renewal, progenitor cell survival, proliferation, terminal differentiation and mature cell activation, is tightly regulated by a complex network of haemopoietic growth factors (HGFs). These soluble factors are released by a range of cell types and have important regulatory effects on the cellular viability, differentiation, proliferation and function of all cell compartments of haemopoiesis. Cells of different lineages and stages of differentiation respond differentially to HGFs depending on their acquisition or loss of receptors to which the HGFs bind and of downstream signalling molecules that mediate receptor responses. Contributing to further complexity, HGFs often act synergistically with each other, and have overlapping functions forming a highly complex network. Abnormal expression or function of HGFs, their receptors or the receptor associated signalling molecules can lead to dysregulated haemopoiesis resulting in myeloproliferative disorders and leukaemia (Gonda and D'Andrea, 1997; Ward et al., 2000; Goyal and Longmore, 1999; Lang et al., 1987).

The focus of this work is the myeloproliferative disease Polycythemia Vera that arises due to the abnormal growth of a multipotent stem cell and is characterised by the expansion of multiple haemopoietic lineages and the abnormal response of committed progenitors to several HGFs.

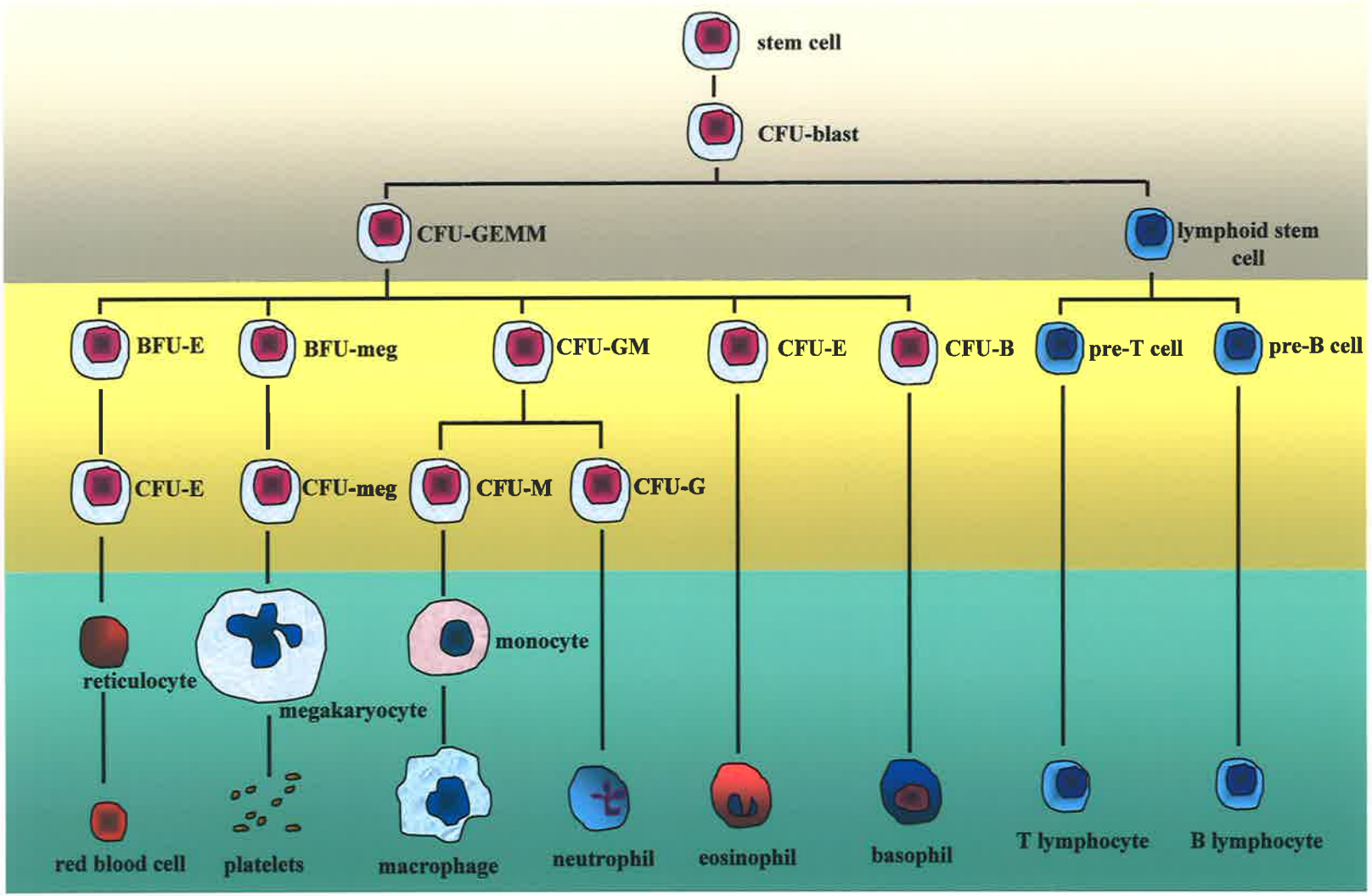
1.2 Polycythemia Vera

1.2.1 Introduction

Polycythemia Vera (PV) is one of several human haemopoietic diseases grouped together as the myeloproliferative disorders (MPD) by William Dameshek in 1951 (Dameshek, 1951). These disorders are characterised by hyperproliferation of one or more haemopoietic lineages, originating from the clonal expansion of a multipotent stem cell and with myelofibrosis in the terminal phase. MPD also include the disorders

Figure 1.1 The haemopoietic system

The stem (■), progenitor (■) and mature cell (■) compartments of haemopoiesis are shown. The primitive stem cell gives rise to multipotential and committed progenitors, which are classified on the basis of colony types (CFU and BFU) derived *in vitro*. Committed progenitors then terminally differentiate to produce the mature blood cell types of the eight lineages of haemopoiesis.


stem cell
CFU-blast
CFU-GEMM
lymphoid stem cell
BFU-E
BFU-meg
CFU-GM
CFU-E
CFU-B
pre-T cell
pre-B cell
CFU-E
CFU-meg
CFU-M
CFU-G
reticulocyte
megakaryocyte
monocyte
red blood cell
platelets
macrophage
neutrophil
eosinophil
basophil
T lymphocyte
B lymphocyte

chronic myeloid leukaemia (CML), idiopathic myelofibrosis and essential thrombocytosis.

PV is a late onset, acquired, progressive disease of typically 3 phases extending over 10-20 years with diagnosis usually made after the age of 60. Approximately 1-2 cases of PV are diagnosed per 100,000 people per year in western countries (Murphy, 1999). This disease is characterised primarily by an accumulation of morphologically normal red cells, granulocytes and platelets in the peripheral blood and increased granulocytes and megakaryocytes in the bone marrow, and is thought to originate from the expansion of an early haemopoietic stem cell capable of differentiation to the erythroid, myeloid and megakaryocytic lineages. In addition to increased numbers of mature cells, primitive progenitors expressing the surface antigen, CD34 are elevated in the peripheral blood, indicating mobilisation of the progenitor cell compartment from the bone marrow (de Wolf et al., 1994; Ash et al., 1982). Although B and T lymphocytes are not expanded in PV, there are conflicting reports with regard to the involvement of the lymphoid lineage (Raskind et al., 1985; Prchal and Prchal, 1999).

1.2.2 Diagnosis

The diagnosis of PV can be problematic since a robust diagnostic tool to identify the disease has not been identified. Further, diagnosis is complicated by the evolution of this disease and by the existence of other pathologies in elderly patients. The Polycythemia Vera Study Group (PVSG) was founded in 1967 to develop criteria for the diagnosis of PV and also to evaluate the efficacy of various clinical treatments for the disease (Murphy, 1999; Michiels and Juvonen, 1997; Michiels and Thiele, 2002).

PV is the most common of two diseases where erythrocytosis occurs in the absence of elevated growth factors or other external factors (Adamson, 1968). These diseases, termed the primary polycythemias, are caused by an intrinsic defect in the haemopoietic progenitors involved in the disease. PV is an acquired primary polycythemia whilst the other primary polycythemia is a rare congenital disease caused

by truncation of the EPO receptor, leading to its hyperactivation (Gregg and Prchal, 1997; de la et al., 1993b). Erythrocytosis in other disorders, termed the secondary polycythemias, is due to extrinsic factors that lead to decreased blood oxygen, resulting in increased serum levels of the erythroid specific growth factor, erythropoietin (EPO) and subsequent red cell mass increase (Pearson et al., 2000).

In the early erythrocytic phase of PV, an elevated red blood cell (rbc) mass may be the only clinical manifestation of the disease. Thus, patients usually present with erythrocytosis in the presence of normal arterial oxygen levels and normal or low serum EPO levels (Adamson, 1968). This may be accompanied by other symptoms, such as leukocytosis, thrombocytosis and enlargement of the spleen, however these may only become evident as the disease progresses. Involvement of other lineages may otherwise be revealed by bone marrow biopsy where hypercellularity of the erythroid, myeloid and megakaryocytic lineages (trilineage hyperplasia) occurs in this disease (Cashman et al., 1988). At presentation approximately 20% of PV patients have an abnormal acquired karyotype, which confirms the presence of a clonal disorder (see Chapter 1, section 1.2.5). A key observation relates to the ability of bone marrow and peripheral blood cells from PV patients to give rise to colonies of erythroid origin in colony assays containing serum, but without added EPO, which is normally essential for their growth. These colonies are termed 'endogenous' colony forming units-erythroid (CFU-E) and blast forming units-erythroid (BFU-E), and are a hallmark feature of PV (Prchal and Axelrad, 1974). Although the growth of endogenous BFU-E and CFU-E are highly indicative of PV (Zwicky et al., 2002), its use as a major diagnostic marker has not been advised due to limited availability and difficulty with standardisation of the assay. The diagnostic tests outlined above have been recommended by the PSVG as major and minor criteria for the formation of a diagnosis of PV (Pearson et al., 2000).

1.2.3 Disease progression

PV is a progressive disorder with patients typically moving through identifiable stages for up to 20 years. The ***erythrocytic phase*** is characterised by erythrocytosis, granulocytosis, thrombocytosis and splenomegaly. The spleen during this phase is congested with mature erythrocytes but there is no significant extramedullary haemopoiesis (Wolf et al., 1988). Symptoms may include headache, weakness, weight loss, pruritus, sweating, visual disturbances and joint symptoms due to excessive blood cell proliferation and peripheral vascular disease. However, the major cause of morbidity and mortality in the erythrocytic phase is vascular thrombosis and haemorrhage, causing 40% and 20% of the deaths, respectively that occur during this phase. Neurologic abnormalities also occur in 60-80% of patients due to increased blood viscosity and reduced cerebral blood flow.

The ***post polycythemic myeloid metaplasia*** (PPMM) or spent phase occurs in 10-15% of PV patients, with onset an average of 10 years post diagnosis. During this phase the rbc mass may be normal however bone marrow fibrosis can develop and splenomegaly becomes increased causing excessive discomfort. Extramedullary haemopoiesis involving the erythroid, myeloid and megakaryocytic lineages is seen in the spleen and anaemia often occurs as the result of splenic pooling, short rbc life span and bone marrow failure. Haemorrhage due to thrombocytopenia or platelet functional abnormalities may also occur during this phase. 25-50% of PPMM patients progress to the development of acute leukaemia within a few years (Hoffman, 1995).

Leukaemic transformation due to PV occurs at a frequency of 1-2% in patients receiving phlebotomy alone but rarely develops before 8 years post diagnosis. The phenotype for PV patients is overwhelmingly myeloid ie. acute myeloid leukaemia (AML). The evolution of PV to leukaemia has been shown to be due to the clonal malignancy, however there is clear evidence that some therapeutic interventions,

particularly the myelosuppressive agent chlorambucil, increase the frequency of leukaemia in PV to 10-15% (Landaw, 1986).

1.2.4 Therapy and Prognosis

Survival of PV patients is dependent on the duration of the erythrocytic phase, appropriate therapies, the nature and severity of symptoms, and the time for transition to the PPMM and leukaemic phases. Uncontrolled erythrocytosis results in a high risk for life threatening thromboses within 1 or 2 years. The PSVG has carried out a series of trials over a period of 20 years to study the benefits and adverse effects of several PV therapies and has formulated therapy guidelines. Thus the survival time for most patients extends for over 10 years, however the choice of therapy remains an area of active debate with advantages and disadvantages for each therapeutic option. Individualised therapy based on age and presence of other disease, for example, cardiovascular disease, is recommended (Berk et al., 1981).

Phlebotomy is the major treatment of choice in the erythrocytic phase of PV to maintain normal blood volume and hematocrit levels. This is generally used alone or in conjunction with myelosuppressive treatments such as radiotherapy with Phosphorous^{32} (P^{32}) (Najean and Rain, 1997), or chemotherapeutic drugs such as hydroxyurea, chlorambucil, or busulfan where leukocytosis requires treatment. However, several studies have shown an increased incidence of leukaemic transformation with some chemotherapeutic agents or P^{32} treatment. For example, it has been shown that phlebotomy alone or phlebotomy combined with P^{32} therapy achieve similar survival times in PV cases but result in different disease complications (high thrombosis risk and high leukaemic risk, respectively) (Berk, 1997; Tefferi et al., 1997; Brandt and Anderson, 1995; Tefferi et al., 1997; Brandt and Anderson, 1995).

Therapy during the PPMM phase is also problematic. Anaemia may require transfusion therapy since massive erythrocytosis is induced by iron supplementation during this phase. Pressure symptoms associated with marked splenomegaly may be

treated with radiotherapy or low dose chemotherapy, however splenectomy is a last resort since 25% of PV patients die with this surgery due to a high risk of haemorrhage. Chemotherapy for AML is usually unsuccessful and is not recommended at all for elderly patients. 70% of patients that progress to the PPMM or leukemic stages of PV have a survival time of less than 3 years (Landaw, 1986).

1.2.5 Clonality and cytogenetics

PV is a late-onset, clonal malignancy, suggesting that it is caused by the acquisition of one or more genetic alterations that change the growth properties of multipotent and committed progenitors. Clonality has been demonstrated in PV using X chromosome gene inactivation studies in heterozygous females where only one allele from an X-linked gene is expressed. This indicates that all the cells within the tissue are derived from a single clone. For example, erythrocytes, platelets, granulocytes and monocytes expressed only one allele of an X-linked gene (eg. glucose-6-phosphate dehydrogenase) in PV patients. T lymphocytes always expressed both alleles whilst in some patients the B-lymphocytes were also clonal (Raskind et al., 1985; Adamson et al., 1976; Prchal and Prchal, 1994). This type of analysis has more recently been performed using polymerase chain reaction (PCR) techniques, requiring only nanogram amounts of DNA and enabling clonality to be demonstrated in haemopoietic colonies grown in culture (Fialkow, 1974; Gilliland et al., 1991).

Chromosomal abnormalities with low penetrance have been found in PV. Approximately 20% of untreated patients and 60% of patients treated with cytotoxic agents have karyotype abnormalities, reflecting the leukaemogenic effects of myelosuppressive treatments. The duration and stage of the disease is also associated with the acquisition of abnormalities and 71-80% of patients who develop myelofibrosis or leukaemia have an abnormal karyotype. However, it is important to note that cytogenetic abnormalities occur in the absence of cytotoxic treatment and that PV can progress to acute leukaemia where a normal karyotype exists (Hoffman, 1995).

In 2 studies of 104 and 285 PV patients the more common abnormalities found include deletion of 20q (with a frequency of approximately 10%), deletion of 13q (5%), trisomy 8 (9%) and trisomy 9 (9%) (Diez-Martin et al., 1991; Hinshelwood et al., 1997). Such a range of chromosomal aberrations may indicate the location of several genes involved in the pathogenesis of the disease. However, none of these alterations are fully penetrant suggesting that the PV phenotype results from a collection of heterogeneous genetic changes that lead to the dysregulated growth of an early stem or progenitor cell.

Deletion of the long arm of chromosome 20 (del 20q) is the second most common primary structural abnormality seen in haematological disorders (after the Philadelphia chromosome) and is associated with several other myeloproliferative disorders and in myeloid leukaemias (Bench et al., 2000). A common deleted region (CDR) on 20q has been identified between D20S174 and D20S17 (Asimakopoulos et al., 1994). One approach to identifying a genetic lesion for PV and other disorders with this CDR has been to look for mutations of candidate genes in this region, which might be expected if this region is important for disease pathogenesis, however no mutations have been detected to date (Bench et al., 2000; Bench et al., 1998b; Bench et al., 1998a; Bench et al., 1998a).

Other studies have focussed on genome wide searches for alterations that may contribute to PV pathogenesis. cDNA subtraction studies using mRNA isolated from PV and normal early progenitor cells identified reduced expression of the tumour suppressor H19 (Nunez et al., 2000), and the consistent over-expression of neutrophil alloantigen NB1 mRNA (also termed PRV-1, CD177), related to the uPAR/Ly6/CD59/ snake toxin family of proteins, in granulocytes from PV patients. However, the function of this GPI-linked protein has yet to be determined, its surface expression is not upregulated in PV and recent Southern and fluorescence in situ hybridisation (FISH) analyses of the PRV-1 gene in PV did not detect any gross rearrangements. Therefore

the functional of this finding remains unclear (Temerinac et al., 2000; Bettinotti et al., 2002; Klippel et al., 2002; Najfeld et al., 2003).

A search for loss of heterozygosity (LOH) in a rare case of familial PV identified a region of LOH on chromosome 11 however this finding is yet to be confirmed in other family members with PV or in non-familial PV patients (Kralovics, 1999). More recently, a genome wide screen of 6 PV patients identified 3 LOH regions on chromosomes 9p, 10q and 11q. Interestingly, 9p LOH was found in 6/20 patients and 19 candidate genes from this region were further investigated. Although no mutations were detected, mRNA and protein expression of the transcription factor NF1-B was increased in progenitor and mature cell populations from PV patients with 9p LOH. NF1-B is a member of the nuclear factor 1 (NF1) family of transcription factors implicated in the control of cell proliferation. Specifically, the overexpression of this gene and another NF1 family member (NF-X) results in cellular resistance to transforming growth factor β1 (TGF-β1), an important negative regulator of early haemopoiesis Thus, the overexpression of NF1-B in PV may contribute to hyperproliferative effects via altered sensitivity to TGF-β (Kralovics et al., 2002; Gronostajski, 2000; Ruscetti and Bartelmez, 2001; Hu and Zuckerman, 2001; Fortunel et al., 2000). Other studies have shown decreased levels of the TGF-β1 receptor (TGF-βR11A) mRNA expression in PV however mutational analysis revealed no abnormalities (Li et al., 2001; Rooke et al., 1999). The role of TGF-β1 in PV therefore remains unclear and further study of this important pleiotropic regulator of stem cell quiescence and its receptor signalling pathways is required to rule out its involvement in disease pathogenesis.

1.2.6 HGF requirements of PV progenitors *in vitro*

HGFs have a major regulatory role in the growth and differentiation of early stem cells and committed progenitors and therefore have been a major focus in the field of PV research for more than a decade. The ability of haemopoietic progenitors to grow

in vitro in semi solid media in the presence of specific HGFs has enabled the determination of HGF requirements of progenitors isolated from the bone marrow and peripheral blood of PV patients. Since the most prominent clinical manifestation of PV is marked erythrocytosis, early investigations focused on HGFs regulating the *in vitro* differentiation and growth of committed erythroid progenitors. However more recently, altered responses to several other HGFs by myeloid and megakaryocytic PV progenitors have also been described.

1.2.6.1 Erythropoietin

Erythropoietin (EPO) is the principle growth factor that promotes the survival, proliferation and differentiation of late erythroid progenitor cells. In response to low oxygen levels the serum EPO concentration is elevated, resulting in the increased production of rbc. Abnormally high levels of EPO can cause erythrocytosis however in PV patients the serum EPO concentration is normal or reduced (Messinezy et al., 2002).

Early studies with PV progenitors showed the growth of blast forming units-erythroid (BFU-E) and colony forming units-erythroid (CFU-E) without exogenously added EPO in colony assays containing serum (Weinberg et al., 1989; Biljanovic-Paunovic et al., 1990; Dudley et al., 1990). EPO dose response experiments demonstrated that both normal and defective progenitors were present in PV bone marrow. One population, termed endogenous BFU-E displayed a hypersensitive response to EPO (< 0.001Units/ml), whilst a second population responded normally to EPO at 1U/ml. Further, antibodies to EPO caused a dose dependent inhibition of normal BFU-E growth, but did not affect endogenous BFU-E growth and differentiation further indicating their reduced requirement for EPO (Eaves and Eaves, 1978; Fisher et al., 1994). However, a later study in serum free assays showed that the reduced requirement for EPO by these progenitors was in fact due to marked hypersensitivity to insulin-like growth factor-1 (IGF-1) found at low levels in serum and that the response of PV cells to EPO is normal (Correa et al., 1994) (see Chapter 1, section 1.2.6.3).

1.2.6.2 Interleukin-3 (IL3) and Granulocyte-Macrophage Colony Stimulating Factor (GMCSF)

The cytokines IL3 and GMCSF have distinct but overlapping roles in the regulation of the cellular proliferation, differentiation and survival of early haemopoietic stem cells and committed progenitors of the myeloid and erythroid lineages and modulation of the mature cell responses of the monocytic and granulocytic lineages. IL3 exerts its effects on multilineage and committed progenitors in synergy with other HGFs, however as these cells differentiate further they lose IL3 responsiveness (Nicola, 1994; Sawada et al., 1991). The activity of GM-CSF is more restricted but overlaps extensively with that of IL-3. GMCSF acts mainly on the differentiation and proliferation of more committed myeloid progenitors and is a potent activator of the mature cell activities of the monocytic and granulocytic lineages (Nicola, 1994; Sawada et al., 1991; Gasson, 1991; Gillessen et al., 2001).

Despite these effects on early and committed progenitors, studies in mice deficient for responses to GMCSF and IL3 suggest their roles in embryogenesis and steady state haemopoiesis can be compensated for by other HGFs (Wrana et al., 1992; Nishinakamura et al., 1996; Metcalf, 1997). Nevertheless, HGFs such as IL3 and GMCSF that exert influences on early stem cells and multipotent progenitor populations may play a role in the multilineage nature of PV pathogenesis. In serum free colony assays BFU-E from PV patients show a reduced dependence on IL3 for survival *in vitro* compared with BFU-E from normal subjects and display marked hypersensitivity to both IL3 and GMCSF (38x and 48x, respectively) (Dai et al., 1991a). In addition, CFU-GM and CFU-Meg are hypersensitive to both cytokines (Dai et al., 1992). Therefore, progenitors from the 3 lineages that are affected in PV display a hypersensitive response to IL3 and GMCSF thus implicating these HGFs and their receptors in the pathogenesis of this disease.

1.2.6.3 Insulin-like growth factor-1 (IGF-1)

IGFs are a family of ubiquitously produced proteins that stimulate a variety of cellular responses including effects on cell survival, proliferation and differentiation (Zumkeller and Burdach, 1999). In the haemopoietic system, IGF-1 promotes cell division and prevents programmed cell death (Sell et al., 1995; Muta and Krantz, 1993). Specifically, IGF-1 rescues promyeloid cells from apoptosis induced by serum starvation, and permits their maturation to granulocytes (Liu et al., 1997). High levels of IGF-1 have been shown to substitute for EPO in the growth of BFU-E from normal peripheral blood mononuclear cells (PBMNC) however, the IGF-1 concentration required is 100 fold higher than that required for EPO, and addition of both IGF-1 and EPO is not synergistic, suggesting that the cellular response to these two cytokines occurs via different stimulatory pathways (Correa and Axelrad, 1991).

In serum-containing colony assays the growth of EPO independent BFU-E from PV patients was found to be due to low levels of IGF-1 present in the serum and was abolished by neutralising antibody to IGF-1. In assays without serum, these progenitors were 100 fold more sensitive to IGF-1 compared with normal BFU-E (Correa et al., 1994). IGF-1 is implicated in a PV case in which the patient had acromegaly due to elevated IGF-1 and growth hormone levels caused by a pituitary gland tumour. After removal of the tumour, hormone levels normalised and the haematological abnormalities resolved (Weinberg, 1997). However, elevated levels of IGF-1 or growth hormone have not been reported in further cases of PV, therefore the significance of this case is unclear.

Most of the circulating IGF-1 is bound to specific high affinity binding proteins, termed IGF binding proteins (IGFBP), which function as carriers of circulating IGF and modulate IGF activity. Altered levels of several IGFBP have been reported in PV and this may be a mechanism by which IGF-1 responses are altered in the disease (Michl et al., 2001)

1.2.6.4 Stem cell factor

Stem cell factor (SCF) is a growth factor with important roles in embryogenesis and the proliferation of many cell types in human and mouse. In addition to effects on the nervous system and the gut, SCF has a potent, synergistic action with other HGFs, such as IL-3, GMCSF, EPO and IGF-1, in the enhancement of the proliferation of multi-potential stem cells and early erythroid, myeloid and lymphoid progenitors (Lyman and Jacobsen, 1998; Zsebo et al., 1990) and reviewed in (Broudy, 1997; Ashman, 1999; Zsebo et al., 1990). Mice deficient for functional SCF receptors (W and *Steel* mice) are severely anaemic due to a deficiency in haemopoietic stem cells and bone marrow stromal cells and also have profound defects in pigmentation and gametogenesis (Chabot et al., 1988; Broxmeyer et al., 1991; Bernstein et al., 1990).

Whilst EPO mediates BFU-E and CFU-E survival and differentiation to late erythroblasts, SCF is the principle factor required for expansion of these progenitors and has been shown to act directly in serum free conditions to increase the growth of BFU-E and CFU-E *in vitro* (Dai et al., 1991b). The multilineage effects of this HGF suggest that abnormal SCF responses may be involved in PV. In support of this, colony assays with purified PV BFU-E progenitors have shown marked hypersensitivity to SCF compared with normal BFU-E, however this was not the consequence of alteration to the SCF receptor profile (Dai et al., 1994a).

1.2.6.5 Thrombopoietin (TPO)

TPO is the major regulator of the proliferation and differentiation of megakaryocytic precursors and also plays a role in the production of early multipotent progenitors, (reviewed in Kaushansky 1998). It has been shown to synergise with other early acting HGFs to induce colony formation from primitive murine progenitors (Ku et al., 1996b; Ku et al., 1996c). Mice deficient for TPO expression are severely thrombocytopenic due a deficiency in megakaryocytes, and also display a reduction in the number of multilineage and committed progenitor cells (Alexander et al., 1996b;

Gurney et al., 1995; Carver-Moore et al., 1996). Megakaryocyte numbers in bone marrow and peripheral blood are increased in PV patients. Thus, due to its profound effects on this lineage and on multipotent progenitors, TPO has been the subject of further investigation in PV pathogenesis. Megakaryocyte colonies (CFU-Meg) from bone marrow and peripheral blood cells of PV patients were found to be independent of exogenously added TPO, indicating that these progenitors are hypersensitive to or independent of this HGF in addition to IL3 and GMCSF (Li et al., 1994; Kobayashi et al., 1993; Kimura et al., 1988).

Part 2 HGF receptor studies in PV

2.1 HGF receptor families

The specificity of the HGFs is mediated by the lineage and stage-restricted expression of HGF receptors on the cell surface of target cells. HGF receptors are membrane-anchored glycoproteins to which HGFs bind, resulting in the transduction of intracellular signals and regulation of transcriptional events. Receptors for HGFs are grouped into two major classes based on their associated protein tyrosine kinase (PTK) activity, the receptor tyrosine kinase (RTK) family, which contains intrinsic PTK activity, and the cytokine receptor (CR) supergene family, which associates with members of the Janus kinase (JAK) family of cytoplasmic kinases. Members within these receptor superfamilies share common structural features and activate many common signal transduction pathways.

2.1.1 Receptor tyrosine kinases (RTK)

The RTK family is characterised by intrinsic protein tyrosine kinase (PTK) activity within the cytoplasmic domain, enabling direct tyrosine phosphorylation of the receptor itself and the subsequent phosphorylation of a number of cytoplasmic signalling molecules. RTK family members, which include the receptors for IGF-1, SCF, epidermal growth factor (EGF) and macrophage-colony stimulating factor (M-

CSF) have conserved consensus motifs within their cytoplasmic domains (Schlessinger and Ullrich, 1992; Nicola, 1994).

Several receptor tyrosine kinase oncogenes including FLT3, c-Fms (M-CSF receptor) and c-kit (SCF receptor) are involved in the normal regulation of haemopoiesis and typically function to prolong cell viability and enhance proliferation, reviewed in (Scheijen and Griffin, 2002). However, altered forms of RTKs have been shown to be involved the dysregulation of haemopoiesis. For example, two separate translocations of the c-Abl gene result in the BCR-ABL and TEL-ABL proteins. BCR-ABL translocations occur in virtually all cases of CML and in a proportion of acute lymphocytic and chronic neutrophilic leukaemias. All variants result in increased tyrosine kinase activity, inhibition of apoptosis and a high transforming ability in haemopoietic cells (Scheijen and Griffin, 2002; Pierce et al., 2002). Other, less profound examples include mutations of FLT3 involving cytoplasmic tandem duplications, point mutations and activating loops found in a significant proportion of AML cases where they are associated with a poor prognosis. When expressed in mice, a FLT3 activating mutation results in myeloproliferative disease (Kelly et al., 2002; Zwicky et al., 2002; Gilliland and Griffin, 2002; Spiekermann et al., 2002; Quentmeier et al., 2003). Point mutations of the c-kit receptor have been identified in human mast cell lines and in mastocytosis and myelodysplasia (Broudy, 1997) and mutated forms of c-fms are implicated in a small number of AML cases (Scheijen and Griffin, 2002). Thus altered forms of RTKs appear to be directly oncogenic.

2.1.2 Cytokine receptors (CR)

Members of the CR supergene family are defined by conserved structural characteristics. The extracellular domains of all CR contain either one or two copies of a conserved motif of approximately 200 amino acids, termed the cytokine receptor module (CRM). Each CRM is composed of two sub domains, referred to as the cytokine receptor domains (CRD), each forming a fibronectin-like barrel of 7 β-strands. The

membrane proximal CRM contains a WSXWS motif which is a unique and highly conserved region of the CR superfamily.

CR family members are classified into subfamilies based on receptor subunit composition. ***Subfamily I*** members bind ligand as homodimers and include the receptors for EPO, TPO, granulocyte-colony stimulating factor (G-CSF), growth hormone (GH) and prolactin (Prl). ***Subfamily IIa*** receptors consist of 2 subunits, an α-subunit that binds ligand and a common β-subunit (hβc) that increases ligand affinity and transduces receptor signalling. This subfamily includes the receptors for IL3, GMCSF and interleukin 5 (IL5). ***Subfamily IIb*** receptors are characterised by the shared common signalling unit gp130, and include the interleukin 6 (IL6), interleukin 11 (IL11) ciliary neurotrophic factor (CTNF), leukaemia inhibitory factor (LIF) and oncostatin M (OSM) receptors. ***Subfamily III*** receptors are composed of 2 or 3 subunits and share the interleukin-2 δ-subunit. This group comprises the receptors for the interleukins 2,4,7,9,13 and 15 (Watowich et al., 1996).

Members of the CR family, in contrast to the RTK family, lack intrinsic kinase activity. Rather, signalling by these receptors is mediated by association with members of the Janus kinase (JAK) family of protein tyrosine kinases via conserved motifs in the cytoplasmic domain. In general, cytokine binding initiates receptor aggregation, which mediates JAK transphosphorylation and activation. JAKs then phosphorylate multiple tyrosine residues on the receptor which form docking sites for further signalling molecules containing *Src* homology 2 (SH2) domains. These molecules then activate signalling pathways such as those involving signal transducers and activators of transcription (STATs), PI-3 kinase and Ras/MAPK leading to regulation of the transcription of specific target genes in the cell nucleus (Ihle et al., 1995).

Like members of the RTK family, altered expression of CRs have been implicated in dysregulated haemopoiesis. Murine bone marrow cells, enriched for primitive progenitors and infected with MPLV *in vitro,* yield growth factor independent

cell lines of multiple lineages, indicating the potential multilineage effects of *Mpl* activating mutations (Souyri et al., 1990). Further, a truncated constitutively active form of *Mpl* (*v-Mpl*), introduced into mice using a retrovirus (MPLV), induces a myeloproliferative disorder characterised by erythrocytosis, thrombogranulosis and splenomegaly (Wendling et al., 1989). Activated mutants of hβc have also been described that confer factor independent growth on murine haemopoietic cell lines and induce leukaemia or MPD when expressed in mice (Chapter 1, section 3.1).

Despite such profound effects *in vitro* and in murine models, few mutations of CRs have been described in human disease. EPOR is over-expressed or rearranged in several independent human erythroleukemia cell lines and truncating mutations of EPOR cause a rare form of primary familial polycythemia in humans, characterised by the excessive production of red blood cells. These mutations result in hypersensitivity of the receptor in response to EPO due to the loss of negative regulatory regions within the cytoplasmic tail (Gregg and Prchal, 1997; Kralovics et al., 1997; de la Chapelle et al., 1993a; Watowich et al., 1999; de la et al., 1993b). Recently, an activating mutant in the transmembrane domain of the granulocyte colony stimulating factor (G-CSF) receptor that confers factor independent growth in murine cell lines has been detected at low frequency in AML patients (Forbes et al., 2002).

2.2 HGF receptor defects in PV

As discussed in Chapter 1, section 1.2.6, PV progenitors from the erythroid, myeloid and megakaryocytic lineages display hypersensitive responses to several HGFs *in vitro*. Therefore, many of the receptor and signalling molecules that elicit these responses have been targeted for further investigation in studies of PV pathogenesis, and are summarised in the following sections. Investigations involving the GMCSF and IL3 receptors are discussed in further detail (see Chapter 1, section 3.5) as these receptors are the focus of this study.

2.2.1 The EPO receptor, EPOR

Due to the profound erythrocytosis found in PV and studies demonstrating the hypersensitivity of PV progenitors to EPO, many investigations have focussed on searching for alterations to the EPOR. In addition to roles in the erythroid lineage, effects of EPO and EPOR on cells of the megakaryocytic lineage have also been reported. For example, megakaryopoiesis and erythropoiesis are increased in mice expressing the oncogenic murine EPOR mutant, R129C (Longmore et al., 1993). Therefore mutated forms of EPOR may explain the erythrocytosis and contribute to the megakaryopoiesis in PV but it is more difficult to explain the effects on myelopoiesis by a defect in the EPOR. Despite this, several groups have proceeded to study EPOR gene structure in PV. PV EPOR gene defects have not been detected by southern analysis and no structural changes were detected in the entire coding region of the gene by sequencing, PCR and SSCP analysis (Hess et al., 1994; Mittelman et al., 1996; Emanuel et al., 1992). The EPOR undergoes rapid tyrosine phosphorylation in response to EPO and this is comparable between normal and PV erythroid progenitor cells (Damen and Krystal, 1996; Dai et al., 1997).

Another study has shown the marked decrease of mRNA encoding a truncated form of EPOR (EPOR-T) in 8/8 PV patients but not in other myeloproliferative disorders (Chiba et al., 1997). This variant binds EPO with similar affinity to full length EPOR and acts as a dominant negative regulator EPOR-T is normally expressed in immature progenitors and is downregulated in maturing erythroid cells, and it has been postulated that under normal steady state conditions when EPO concentration is low, early progenitors may not undergo erythropoiesis due to co-expression of EPOR-T. However during stress mediated erythropoiesis when EPO levels are increased, the inhibitory effect of EPOR-T may be overcome. Therefore, EPOR-T may be a key regulator of erythrocyte production and its reduced expression in early progenitors could explain the deregulation of erythrocyte production that occurs in PV. However, a

decrease in EPOR-T does not readily explain the presence of EPO independent colonies or multiple HGF hypersensitivity and its role in expansion of the myeloid and megakaryocytic lineages remains unclear (Nakamura et al., 1992; Andersson et al., 1997).

Due to these studies and further important work in serum-free assays demonstrating that endogenous erythroid colony formation is due to low levels of IGF-1 found in serum (Correa et al., 1994), it is now generally accepted that the EPOR is unlikely to play a role in PV.

2.2.2 The IGF-1 receptor, IGF-1R

The mature form of the IGF-1 receptor (IGF-1R) is comprised of 2 ligand binding extracellular α-subunits, disulphide linked to 2 membrane spanning β-subunits. Upon IGF-1 binding to the receptor, the β-subunits are transphosphorylated thereby increasing kinase activity and resulting in the phosphorylation of intracellular substrates (Zumkeller and Burdach, 1999). Since PV cells display an increased sensitivity to IGF-1 *in vitro*, the kinase activity of IGF-1R was investigated. Basal and ligand-induced tyrosine phosphorylation of the IGF-1R is increased in PV (Mirza et al., 1995), however the mechanism by which this occurs is unclear, as mutational analysis of the receptor by single strand conformation polymorphism analysis (SSCP) failed to detect any receptor mutations (Michl et al., 2001). Thus, in the absence of any alteration to the receptor itself, there may be alteration of a signalling component or accessory receptor component that directly increases or desensitises its kinase activity.

2.2.3 The thrombopoietin receptor, *Mpl*

Expression of the receptor for TPO (*Mpl*) is restricted to primitive haemopoietic stem cells, megakaryocytes and platelets (Debili et al., 1995). In *Mpl* deficient mice platelet levels are reduced by 90% consistent with TPO being the major growth factor regulating megakaryopoiesis. Multilineage and committed progenitor numbers are also reduced in these mice due to defects in the stem cell compartment, consistent with

studies indicating a role for *Mpl* in early haemopoiesis (Carver-Moore et al., 1996; Solar et al., 1998; Alexander et al., 1996a). These effects indicate that TPO and *Mpl* have the potential to contribute to the dysregulated proliferation of multiple lineages as seen in PV.

To date, gross abnormalities of *Mpl* have not been detected in PV patients from studies using Southern and Northern analysis. However, TPO induced tyrosine phosphorylation of *Mpl* is impaired in PV platelets and a novel isoform of *Mpl* has been detected using an antibody to the intracellular domain. This PV specific isoform (Mpl B) has a lower molecular weight (80-82kD cf. 85-92kD) and higher isoelectric point (6.5 cf. 5.5) than normal *Mpl* and has increased sensitivity to endoglycosidase H digestion, suggesting that the 2 isoforms differ in their post-translational processing. The significance of this finding is unclear, as the functional properties of Mpl B have not been reported to date and Mpl B expression was not detected in newly diagnosed patients but rather increased in expression in only a proportion of patients with disease duration (Moliterno and Spivak, 1999; Le Blanc et al., 2000). It is possible that the altered glycosylation of *Mpl* may result from a secondary defect affecting post-translational modification and therefore may not represent a primary lesion in PV.

2.2.4 The SCF receptor, *c-kit*

The receptor *c-kit* is broadly distributed within the haemopoietic lineages including primitive stem cells, and erythroid, myeloid and lymphoid progenitors. High affinity *c-kit* receptors are present on normal human CFU-GM, BFU-E and CFU-E, however there is a loss of receptor expression with further differentiation (Broudy, 1997). As shown in colony assays, activation of this receptor enhances the size and number of colonies derived from committed progenitors (Dai et al., 1991b; Broudy et al., 1996).

Since PV progenitors display hypersensitivity to SCF *in vitro*, *c-kit* has been investigated in the disease, although mutational analysis has not been reported.

Expression of *c-kit* did not differ from normal levels at any stage of differentiation and affinity for SCF was comparable to normal cells. Receptor phosphorylation and internalisation also appeared unaffected in PV cells (Dai et al., 1994b). Again, the unaltered receptor responses suggest that the increased sensitivity of progenitors to SCF may be a secondary event in PV.

2.3 Signalling defects in PV

2.3.1 Introduction

Signalling pathways that transmit survival, proliferation and differentiation signals from the receptor upon HGF binding are mediated via SH2 containing molecules that bind to phosphorylated tyrosine residues of the receptor intracytoplasmic tail. Whilst the pathways elicited by these molecules are complex and incompletely understood, it is known that RTKs and CRs utilise several common regulatory molecules and pathways in order to transmit their signals. Since a receptor abnormality has not been detected that could account for the hypersensitivity of PV progenitors to multiple HGFs, a number of recent studies have focussed on several common signalling molecules. The dysregulation of such a component may explain the hypersensitivity of progenitors to multiple HGFs in the absence of any profound HGF alterations. Although some studies have focussed on regulators of apoptosis (Silva et al., 1998) it is unlikely that a primary defect involves an apoptotic regulatory molecule as this would not account for HGF hypersensitivity.

2.3.2 Intracellular signalling molecules

The activation of members of the JAK family of non-receptor PTKs is the first detectable event in CR signalling (reviewed in Ihle et al., 1994; Parganas et al., 1998; Ward et al., 2000) and JAKs are also activated in response to stimulation of some of the RTKs. Specifically, the family member, JAK2 has been shown in numerous studies to play a critical role in the signalling pathways of receptors involved in PV pathogenesis

(EPOR, GMR, *Mpl,* IGF-1R and *c-kit*) however this molecule has not been investigated in PV (Li et al., 1994; Gual et al., 1998; Weiler et al., 1996; Witthuhn et al., 1993; Quelle et al., 1994; Mizuguchi and Hatakeyama, 1998).

One function of JAKs is to mediate the phosphorylation of signal transducer and activator of transcription (STAT) molecules thereby mediating their nuclear translocation and the activation of cytokine response genes, reviewed in (Rane and Reddy, 2002). STAT proteins are a family of transcription factors that mediate a broad range of processes including proliferation, differentiation, apoptosis, transformation, inflammation and the immune response. Aberrant signalling via JAK and STAT molecules contributes to malignant transformation in murine models and in human leukaemias and myeloproliferative disease. For example, in CML, a chromosomal translocation characterised by the Philadelphia chromosome, generates the BCR-ABL gene resulting in BCR-ABL fusion proteins. These are constitutively activated tyrosine kinases that result in hyperactivation of the JAK/STAT pathway and HGF independent proliferation and cell transformation. The TEL-JAK2 and TEL-PDGFβR fusion proteins, generated by translocation of the translocated ets leukemia (TEL) gene fused to the catalytic domain of the JAK2 and platelet derived growth factor (PDGF)β receptor genes, respectively, are found in human AML, ALL and CML. These oncogenes are also constitutively activated and result in constitutive tyrosine phosphorylation of several of the STAT proteins and HGF independent proliferative responses (Ho et al., 1999; Lacronique et al., 2000; Ilaria, Jr. and Van Etten, 1996; Benekli et al., 2002; Coffer et al., 2000).

The STAT family member, STAT3, is utilised by numerous receptors implicated in PV including EPOR, IL3R, GMR, GCSFR and TPO and its hyperactivation results in the enhanced transcription of anti-apoptotic genes (Biethahn et al., 1999; Rane and Reddy, 2002). Thus this transcription factor could be postulated to contribute to the multiple growth factor hypersensitivities of PV cells. In support of this, constitutive

phosphorylation and activation of STAT3 has been shown in granulocytes isolated from 4/14 PV patients consistent with a potential role in some cases (Roder et al., 2001).

STAT target genes such as the recently described suppressors of cytokine signalling (SOCS) proteins have been shown to negatively regulate CR signalling following induction via the JAK/STAT pathway (Dey et al., 1998). Although there have been no reports of investigation of SOCS proteins in PV, altered expression of such a molecule may be implicated in the disease.

2.3.3 Phosphatases

The phosphorylation status of tyrosine residues essential for receptor signalling is determined by a balance of PTK and the protein tyrosine phosphatases (PTP) activities that regulate phosphorylation. Thus, an impaired balance of these molecules has a clear potential to lead to disorders of cell growth. For example, the IGF-1R is hyper-phosphorylated in PV (see Chapter 1, section 2.2.2) and in the absence of any intrinsic receptor alteration, a defect in a negative regulatory molecule or phosphatase could be implicated in this altered receptor phosphorylation and activation. Further, such a defect would be consistent with the marked hypersensitivity of PV progenitors to multiple HGFs.

Three major haemopoietic PTPs (SHP-1, SHP-2 and CD45) have been identified and extensively studied. The intracellular phosphatase, SHP-1 (*src* homology phosphatase-1), negatively regulates signalling from the receptors for EPO, SCF, and IL3. For example, SHP-1 associates via its SH2 domains with phosphorylated tyrosine residues in the cytoplasmic domain of EPOR and terminates proliferative signals (Klingmuller et al., 1995). Mice expressing either no SHP1 or a defective SHP-1 protein due to splice site mutations (*motheaten*), display severely disrupted haemopoiesis characterised by marked expansion of monocytic cells causing patchy dermatitis, extramedullary haemopoiesis and splenomegaly. Several HGF receptors are involved in the *motheaten* phenotype. For example c-kit, GMR, IL3R and macrophage-CSFR

(MCSFR) all transduce enhanced mitogenic signals. Thus, analyses of these mice have shown a critical role for SHP-1 in the negative regulation of a broad range of haemopoietic cells and receptors (Tsui et al., 1993; Zhang et al., 2000; Lyons et al., 2003).

Little is known about the role of PTPs in the proliferation and differentiation of haemopoietic stem and progenitor cells and to date only SHP-1 has been studied in PV cells. PV progenitors and mature cells show normal mRNA expression and protein levels of SHP-1 however the PTP inhibitor orthovanadate had a reduced effect on PV cells compared with normal erythroid progenitors, suggesting a possible defect in phosphatase activity (Asimakopoulos et al., 1997; Dai et al., 1997; Andersson et al., 1997). Subsequently, a novel membrane associated, erythroid specific PTP (E-PTP 170) was identified in PV cells also in highly purified normal erythroid progenitors where expression declined rapidly with cell maturation. PV progenitors however had increased E-PTP 170 activity and showed a slower loss of E-PTP 170 expression with differentiation (Sui et al., 1997). However, the mechanism by which E-PTP 170 expression is altered in PV has not been determined and further functional analysis will be required to elucidate its importance for signalling in this disease.

Part 3 IL3, GMCSF and IL5 receptors

3.1 Receptor components

The IL3, IL5 and GMCSF receptors (IL3R, IL5R and GMR respectively) are each composed of a unique α subunit, and the common β subunit, hβc (see Figure 1.3). The association of an α subunit and hβc is absolutely required for activation of the signalling events elicited by the cytokines (Guthridge et al., 1998). The α-subunits can bind the cognate ligand alone, but cannot confer signalling in the absence of hβc, however, conserved regions of α-subunit cytoplasmic domains have been shown to be

important for signal transduction and specificity by the IL3R and GMR complexes (Evans et al., 2002; Lilly et al., 2001). hβc is comprised of 2 CRM and therefore has 4 CRD in the extracellular domain. These have been designated CRD1-4 from the n-terminus. The transmembrane domain is followed by a long cytoplasmic tail of 423 amino acids, which contains two highly conserved membrane proximal motifs (box 1 and box 2) and 8 tyrosine residues important for the recruitment of downstream signalling molecules (see Chapter 1, section 3.2 and Figure 1.4).

The mechanism of formation and stoichiometry of the high affinity complexes for the IL3, IL5 and GMCSF receptors has been an area of active research and several modelling and mutagenesis studies have led to some understanding of the interactions involved. It is currently thought that each β-subunit in the β:β dimer is activated by association with a ligand: α subunit complex mediated via a combination of weak interactions. This results in a receptor complex with a 2:2:2 stoichiometry that is stabilised by a disulphide bond formed between the α and β subunits, possibly via cysteine residues in CRD1 of hβc and the α subunit extracellular domain. The high affinity complex appears to be further stabilised via interactions involving residues in the membrane proximal CRD4 of hβc which interact with ligand. Specifically, ligand has been shown to be associated with the β subunit via key residues in the B-C and F-G loops of CRD4 (Stomski et al., 1998; Carr et al., 2001; Bagley et al., 1997; Rossjohn et al., 2000).

3.2 Signalling mechanisms

Ligand binding and oligomerisation of the receptor subunits results in homodimerisation and rapid trans-phosphorylation of JAK2 molecules that are constitutively associated with the membrane proximal box 1 motif of the hβc cytoplasmic domain. This region is highly conserved across species and among several cytokine receptors and is critical for JAK2 binding to the receptor. JAK2 activation

results in the phosphorylation of tyrosine residues in the cytoplasmic tail of hβc, which are target binding sites for the SH2 domains of downstream signalling molecules. JAK2 has been shown to be crucial in the early hβc signalling response in several studies. Deletion of box 1, but not deletion of box 2, expression of a dominant negative JAK2 in a murine cell line and progenitor responses in JAK2 -/- mice have demonstrated the essential role of JAK2 for ligand mediated hβc tyrosine residue phosphorylation and signalling (Parganas et al., 1998; Itoh et al., 1996; Liu et al., 2002). In addition to these early events, a number of other tyrosine kinases are implicated in hβc signalling including Lyn, Tec, Syk, Btk, Hck, Fyn, Lnk, Rac, JAK1, Tyk2, Fps/Fes and several members of the STAT proteins, although their roles in signalling are not fully elucidated (Geijsen et al., 2001; Velazquez et al., 2002; Arai et al., 2002; Dahl et al., 2000; Yousefi et al., 1996; de Groot et al., 1998).

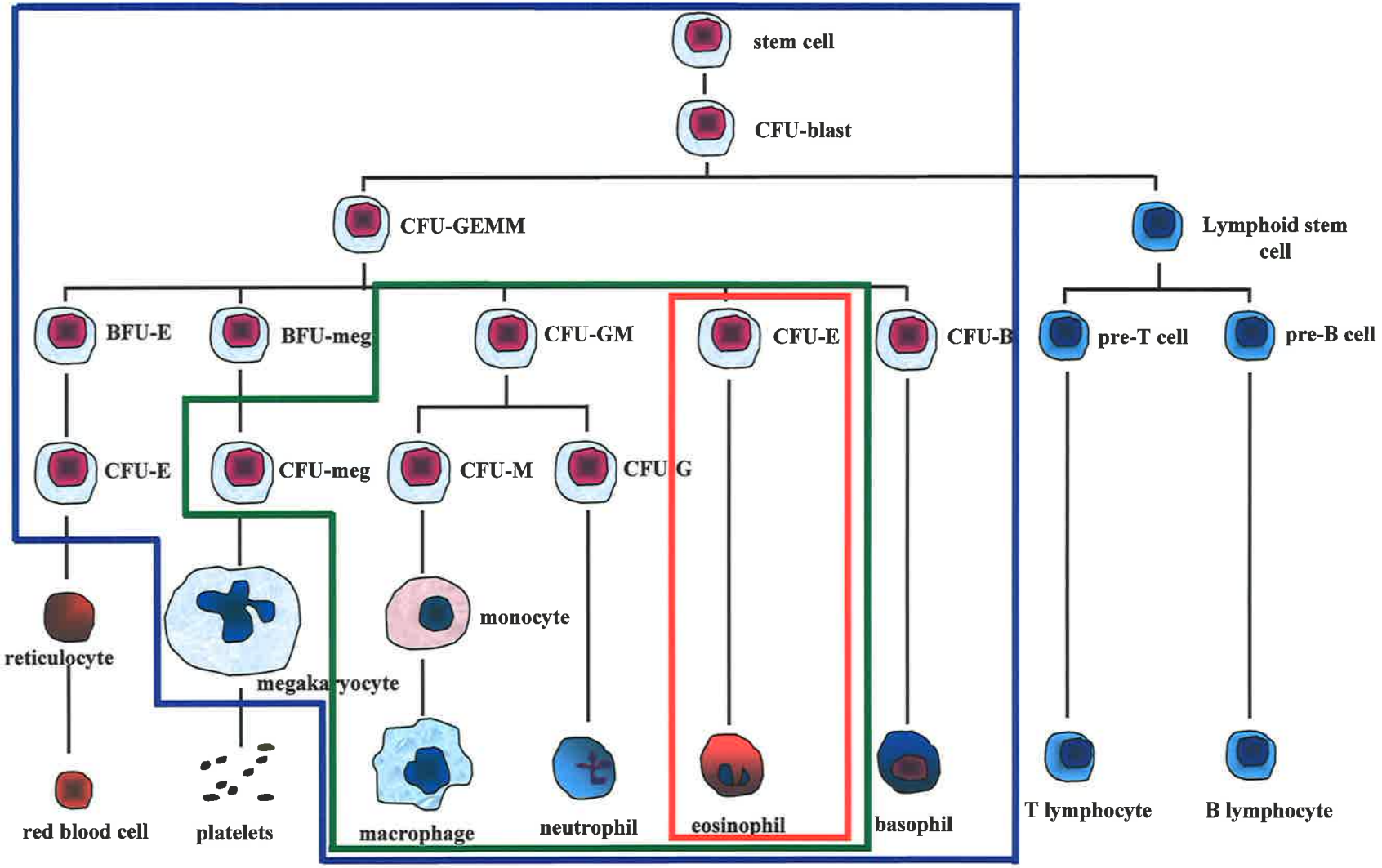
3.3 Role in haemopoiesis and leukaemogenesis

3.3.1 Role and expression in normal haemopoiesis

The precise distribution and timing of HGF receptor expression has been difficult to resolve however several studies have now shown IL3R and GMR expression patterns utilising several approaches in several species (summarised in Figure 1.2). Importantly, these collective results are in overall agreement with the known biological responses of cells to these cytokines. The IL3R is expressed on multipotent progenitor cells and all committed progenitors of the erythroid and myeloid lineages, however it is not detectable on the earliest recognisable stem cell. The GMR is restricted to multipotent progenitors and cells of the macrophage and neutrophil lineages but is not present on committed erythroid progenitors whilst the IL5R is expressed only on committed progenitors and mature cells of the eosinophilic lineage (Wognum et al., 1994; Billia et al., 2001; Lund-Johansen et al., 1999b; McKinstry et al., 1997; Berardi et al., 1995).

Figure 1.2 The biological activities of IL3, IL5 and GMCSF

The distribution of receptors for IL3 (—), GMCSF (—) and IL5 (—) with regard to haemopoietic cell lineage and stage of differentiation are shown.


stem cell
CFU-blast
CFU-GEMM
Lymphoid stem cell
BFU-E
BFU-meg
CFU-GM
CFU-E
CFU-B
pre-T cell
pre-B cell
CFU-E
CFU-meg
CFU-M
CFU-G
reticulocyte
megakaryocyte
monocyte
red blood cell
platelets
macrophage
neutrophil
eosinophil
basophil
T lymphocyte
B lymphocyte

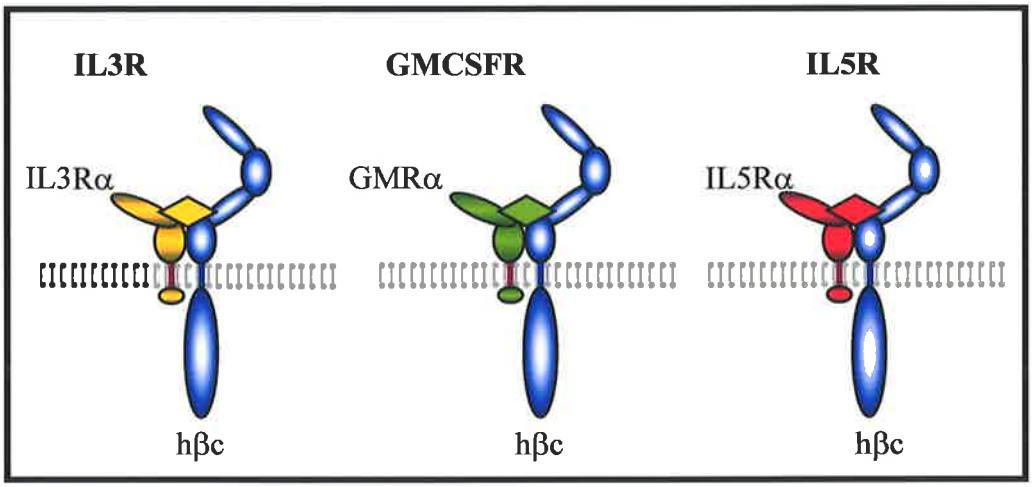


Figure 1.3 IL3, IL5 and GMCSF receptor complexes
Schematic representation of the α:β:ligand complexes formed for IL3R, GMR and IL5R. The cognate ligands are represented by diamond shapes.

Signal peptide sequence	**1- 16**
Extracellular domain	**17- 439**
Transmembrane domain	**440- 460**
Intracytoplasmic domain	**461- 881**
Box 1 & 2 motifs	**470-481 (box1)**
	534-546 (box 2)

Figure 1.4 The structure of hβc

Schematic representation and the amino acid positions of the conserved domain structure of hβc. The amino acid locations of conserved domains and motifs are shown and these regions are schematically represented as follows; (▨) signal sequence; (▢) extracellular domain consisting of CRD 1-4; (■) transmembrane domain; (▬) intracytoplasmic domain; (▬) box 1 and 2 motifs.

An alternatively spliced variant form of hβc encoding a truncated receptor (termed β-intracytoplasmic truncation, β_{IT}) has been identified at a frequency of 20% in normal myeloid cells. This form has a truncated cytoplasmic tail, C-terminal to box 1. Further characterisation of β_{IT} has shown that JAK2 associates with this isoform and is tyrosine-phosphorylated in response to ligand stimulation, however it was unable to transduce a mitogenic signal when expressed in CTLL-2 cells, or when co-expressed with hβc in Baf-3 cells, despite forming a high affinity complex with the GM-CSFα or IL-3α subunits (Gale et al., 1998; Wagner et al., 2001). Thus, β_{IT} is severely deficient in normal hβc signalling capacity and appears to act as a dominant negative regulator of receptor function.

In murine haemopoiesis, a second β subunit exists (βIL3) which in contrast to the hβc homologue, is specific for signalling via IL3. Mice lacking functional mβc or βIL3, or both, develop abnormal lungs characterised by infiltrations of macrophages, alveolar proteinosis, accumulation of surfactant and susceptibility to parasitic infections via disrupted IL5 signalling. However these mice otherwise develop normally and steady state haemopoiesis remains intact despite the lack of responsiveness of progenitors to IL3, IL5 and GMCSF. Thus, signalling via mβc can be compensated for by other mechanisms and appears, with the exception of IL5 mediated eosinophil responses, to be redundant with regard to haemopoiesis (Stanley et al., 1994; Reed et al., 2000).

3.3.2 Human leukaemogenesis

The role of hβc in leukaemia has been investigated in several studies however no mutations have been reported in human disease to date. The extracellular and transmembrane domains, and the cytoplasmic domain of hβc have been analysed for mutations in AML patients in 2 separate studies. A number of point mutations were detected, however none of these were thought to be of pathological significance

(Freeburn et al., 1998; Freeburn et al., 1996). A similar study in patients with the myeloproliferative disorder, juvenile myelomonocytic leukaemia (JMML) also failed to detect significant hβc mutations (Freeburn et al., 1997). Interestingly, the truncated form of hβc, β_{IT}, is found at markedly increased levels in blast cells from 75% of AML patients. Given the significant mRNA levels detected for this isoform in normal individuals and its elevation in AML patients, β_{IT} may play a significant role in hβc signalling responses in dysregulated haemopoiesis (Gale et al., 1998).

3.3.3 Activating mutants of hβc

3.3.3.1 in vitro studies

A number of hβc mutations have been identified that confer factor independent growth on factor dependent murine cell lines. Many of these mutations involve residues in CRD4 of the extracellular region. Indeed, a comprehensive saturation mutagenesis study of the entire molecule identified 10 constitutive extracellular point mutants, all clustered within this region of the receptor (Jenkins et al., 1998). Additionally, mutants encoding either a 37 amino acid duplication within CRD4 (FIΔ), or N-terminal truncation of the receptor at CRD4 (ΔQP) each confer constitutive activation (D'Andrea et al., 1994; Jones et al., 2001; D'Andrea and Gonda, 2000). This is consistent with the importance of residues in CRD4 for receptor activation, in agreement with modelling and mutagenesis studies of normal receptor structure and function, (see Chapter 1, section 3.1). The mechanism by which these mutants induce activation is unclear, however a proposed model suggests that normal receptor activation involves a conformational change of the extracellular domain upon association with the ligand:α-subunit complex. Thus, alteration of critical residues in CRD4 in these mutants may mimic the activated conformation in the absence of ligand (Gonda and D'Andrea, 1997; Jones et al., 2001). Although these studies suggest the importance of CRD4 for hβc activation, spontaneously occurring mutations in two murine cell lines that have HGF independent growth responses and constitutive activation of JAK2, involve n-terminal

truncation of murine βc at the transmembrane domain and lack CRD4. Interestingly, this βc mutant also required the complete loss of both murine mβc and the juxtapositioned second murine receptor for IL3 (IL3R) on the second allele for its factor independent activity in both cell lines (Hannemann et al., 1995).

Random mutagenesis studies have also identified activating point mutations located within the transmembrane and cytoplasmic domains of hβc (Jenkins et al., 1995; Jenkins et al., 1998; Jenkins et al., 1999). The transmembrane mutant V449E may act by inducing the close association of hβc molecules required for functional activation of JAK2 molecules. The cytoplasmic mutants R461C/H and H544R are located proximal to the transmembrane domain and within the box 2 motif, respectively. Proximity of the R461 residue to the transmembrane region may indicate a role in facilitation of homodimerisation of hβc as in the other CR transmembrane mutants. The role of the box 2 motif in hβc is unclear since its deletion (in contrast to the deletion of box 1) did not affect ligand mediated proliferation however, it has been postulated that the H544R mutant either alters the conformation of this region to enhance JAK activation, or facilitates dimerisation of the cytoplasmic region of hβc (Jenkins et al., 1998; Itoh et al., 1996).

All activating hβc mutants were isolated on the basis of the ability to confer factor independent growth on the murine GMCSF/IL3 dependent cell line, FDCP-1. However, differential activity of the mutant forms of hβc is evident when they are expressed in the murine pro-B cell line (BaF-B03). Whilst the transmembrane mutant V449E can confer factor independent growth on the BaF-B03 cell line, the extracellular mutants require the presence of a second human or murine CR subunit such as EPOR, *Mpl* or mGMR, for constitutive signalling in this cell line (Jenkins et al., 1999; Blake et al., 2002). This data, together with differences observed in tyrosine phosphorylation of the receptor mutants is consistent with the extracellular mutants conferring constitutive

signalling via heterodimeric receptor complex formation as distinct from the hβc homodimers predicted to form with the V449E mutant (Gonda and D'Andrea, 1997).

The ability of CR mutants to confer factor independence on haemopoietic cell lines is generally indicative of tumorigenic potential. In support of this, when FDCP-1 cells expressing the V449E, FIΔ, ΔQP and I374N mutants were injected into syngeneic mice, solid tumours developed within 3 weeks while cells expressing hβc were not tumorigenic (Jenkins et al., 1995; D'Andrea et al., 1994). Further, when expressed in primary murine haemopoietic cells by retroviral transduction, the extracellular mutants confer factor independence on cells of the neutrophil and monocytic lineages only while the V449E mutant confers factor independent growth on all cells of the erythroid and myeloid lineages (McCormack and Gonda, 1999). These *in vitro* effects indicate potent constitutive signalling responses by the activating mutants in multiple haemopoietic cell lineages.

3.3.3.2 in vivo studies

The altered *in vitro* growth properties and tumorigenicity of the activated hβc mutants has led to *in vivo* studies investigating the leukaemogenic potential of these forms of the receptor. The extracellular duplication mutant, FIΔ, was expressed in transgenic mice using a constitutive promoter derived from the phosphoglycerate kinase gene, *PGK-1*. These mice (termed FIΔ mice) displayed perturbations in haemopoiesis, characterised by splenic enlargement and increased numbers of mature erythroid, myeloid and megakaryocytic cells in the peripheral blood. Increased numbers of early haemopoietic progenitors were found also in the peripheral blood, and CFU-E and CFU-GM isolated from spleen and bone marrow formed colonies *in vitro* independently of exogenously added growth factors (IL3, GMCSF or EPO). Thus, the FIΔ mutant expressed ubiquitously in mice induces a myeloproliferative disorder involving alterations to the HGF responsiveness of progenitors and expansion of several haemopoietic lineages (D'Andrea et al., 1998).

In a separate study, the effects of several of the hβc mutants were examined using murine bone marrow reconstitution experiments. Donor cells were transduced with retroviral constructs containing hβc mutants prior to bone marrow engraftment. Reconstitution of lethally irradiated mice with cells expressing the extracellular mutants FIΔ or I374N resulted in an early onset chronic myeloproliferative disorder resembling that of the FIΔ mice and the transmembrane mutant V449E led to an acute leukaemic phenotype with anaemia, thrombocytopenia and blast cell expansion (McCormack and Gonda, 1999). Together, these studies highlight the potential of activating hβc mutations to induce human myeloproliferative and leukemic disorders.

3.4 Receptor crosstalk

A number of recent studies have demonstrated further complexity in receptor and signalling pathways in several systems, including haemopoiesis. These studies have shown interactions between heterologous receptors leading to altered ligand responsiveness. This phenomenon has been termed receptor crosstalk and although its biological significance is an area of active debate (Scott et al., 2000) it is postulated that this is a mechanism by which alteration to one receptor could affect the responses of others.

In the haemopoietic system physical association and transphosphorylation of the EPOR and c-kit has been shown (Dumont et al., 2001; Wu et al., 1995; Wu et al., 1997) and several further examples of receptor crosstalk implicate hβc in PV. EPOR has been shown to associate with hβc and *c-kit* to induce their tyrosine phosphorylation in murine cell lines and TPO has been shown to induce the tyrosine phosphorylation of hβc in an erythroleukaemic cell line (Jubinsky et al., 1997; Chin et al., 1997; Hanazono et al., 1995; Wu et al., 1995; Ooi et al., 1998). In support of this, a recent study shows direct association *in vitro* of FIΔ with EPOR or *Mpl* to induce JAK2 activation and factor independent growth in BAF-B03 (Blake et al., 2002). Although the functional

consequences of these interactions *in vivo* are unclear, it remains feasible that an alteration to normal hβc activity could also affect signalling via other HGF receptors. In PV, receptor crosstalk could be postulated to lead to the multiple HGF sensitivities of PV progenitors and may explain some of the receptor and signalling defects shown in previous studies.

3.5 Studies implicating hβc in PV pathogenesis

One study investigating the IL3R α subunit did not detect any alteration to ligand affinity or receptor number in PV (Dai et al., 1992). However, a number of factors suggest that hβc may play an important role in PV pathogenesis. Firstly, PV erythroid and myeloid progenitors are markedly hypersensitive to IL3 and GMCSF (see Chapter 1, section 1.2.6.2). Secondly, the expression pattern of IL3R and GMR in normal haemopoiesis is identical to the cell lineages that are clonally expanded in PV, that is, hβc is expressed in mutipotent and committed progenitors of the erythroid and myeloid lineages (see Chapter 1, section 3.3.1). Thirdly, as discussed above (see Chapter 1, section 3.4), hβc has been shown to interact *in vitro* with further HGF receptors implicated in PV eg. *Mpl* and *c-kit*. Activation or sensitisation of such receptors in the presence of hβc mutants may be a mechanism by which hypersensitivity to multiple HGFs occurs. For example, transgenic mice expressing the FIΔ mutant display trilineage hyperplasia, splenomegaly and HGF independent growth of progenitor populations similar to the PV phenotype in humans (D'Andrea et al., 1998). These abnormalities may be explained by the effect of FIΔ signalling on the sensitivity of erythroid and megakaryocyte progenitors to EPO and TPO, via its direct interaction with EPOR or *Mpl*.

These findings together led us to propose that a mutant form of hβc could represent a clonal stem cell defect that contributes to multiple HGF hypersensitivity and clonal expansion of the erythroid, myeloid and megakaryocytic lineages in PV.

3.6 Project aims and approach

The aim of this study is to determine whether mutations of hβc are present in PV. Identification of such a mutation would significantly enhance the understanding of the molecular mechanisms underlying this disease, and also contribute to the understanding of normal stem cell regulation. Further, a comprehensive analysis of hβc would be a valuable addition to studies by others that have investigated several other HGF receptors implicated in PV. The lack of mutations detected in these studies supports the hypothesis that this disease results not from the direct alteration of HGF receptors but from a defect in a shared signalling molecule that leads to altered HGF sensitivities. If this study fails to identify a PV specific mutation this would suggest that hβc is also not directly involved and would therefore be an important validation of such a hypothesis.

Cells isolated from the peripheral blood of PV patients will be screened for the presence of mutations in a target region of the gene encoding hβc, *CSF2RB*. Previously identified activating mutations of hβc are clustered within this region. In order to determine the PV specificity of any mutations found, screening of the same region will be performed in cells from haematologically normal individuals. The screen will initially involve the isolation of cDNA from the peripheral blood mononuclear cell (PBMNC) populations of a number of PV patients and normal individuals from which the hβc target region will be amplified, cloned and sequenced. This part of the study may be limited by the low contribution of cells derived from the PV clone, to the PBMNC population. Therefore, if PV specific mutations are not detected, or to confirm the specificity of any mutations found, the same region will be screened in endogenous

BFU-E obtained from PV patients compared with EPO dependent BFU-E obtained from normal individuals. This approach will increase the likelihood detecting alterations specific to PV since endogenous BFU-E have been shown to be derived from the PV clone (Gilliland et al., 1991).

Thus, this study represents a comprehensive mutational analysis of hβc in PV patients that will further our understanding of the molecules and mechanisms involved in the clonal expansion of a multipotent haemopoietic stem cell in PV and since a high number of these patients develop AML, with the potential to give insight into the early events of leukaemogenesis.

Chapter 2 Materials and Methods

2.1 Reagents and chemicals

2.1.1 Chemicals

Standard chemicals were generally of analytical grade and were purchased from Ajax chemicals (NSW, Australia) or Sigma Chemical Company (MO, USA). The sources of other chemicals and reagents were as follows,

Phenol: chloroform: isoamyl alcohol 25:24:1 (v:v:v): Life Technologies, MD, USA

Glycogen: Roche, Mannheim, Germany

Agarose: Scientifix, Victoria, Australia

Bacto-agar, Bacto-tryptone and Bacto-yeast extract: Difco Laboratories, MA, USA

dNTPs: Roche, Mannheim, Germany

Lymphoprep : Nycomed, Uppsala, Sweden

Acrylamide, bis-acrylamide: Biorad Laboratories, Hercules, CA, USA

TEMED: Promega, Madison, WI, USA

Taq polymerase: Roche, Mannheim, Germany

Acrylamide: bis-acrylamide solution (37:5:1) as Protogel^{TM}: National Diagnostics, Hessel Hill, England

TRIZOL: Life Technologies, Maryland, USA

2.1.2 Solutions and buffers

6 x loading buffer Type III (6xLB)	0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, filter sterilised, aliquots stored at –20°C

Formamide loading dye	95% formamide, 20mM EDTA pH 8.0, 0.05% bromophenol blue (w/v), 0.05% xylene cyanol (w/v), stored at 22°C
TAE buffer	40mM Tris pH 8.2, 20mM sodium acetate, 1mM EDTA
TBE buffer	900mM Tris borate pH 8.2, 2mM EDTA
10% ammonium persulphate (APS)	10% ammonium persulphate, filter sterilised, aliquots stored at –20°C
TELT buffer	50mM Tris pH 7.5, 62.5mM EDTA, 5.5mM LiCl, 0.4% Triton X100
Denaturing PAGE gel (20%)	7M Urea, 20% acrylamide, 1% bis-acrylamide (w/v) in TBE buffer, polymerised by addition of 0.1% TEMED (v/v) and 1% APS solution (v/v)
Non-denaturing PAGE gel (20%)	ProtogelTM stock solution in TBE buffer, polymerised by addition of 0.1% TEMED and 1% APS solution (v/v)
Buffered glycerol	10% glycerol in 1mM HEPES pH 7.0, sterilized by autoclaving

10% glycerol solution	10% glycerol in Milli Q water, sterilized by autoclaving
Bicarbonate coating buffer	8.6g Na2CO3, 17.2g NaHCO3 in 1 litre, pH adjusted to 9.5 using 10M HCl
Phosphate buffered saline (PBS)	130mM sodium chloride, 10mM di-sodium orthophosphate, 10mM sodium di-hydrogen orthophosphate, pH 7.2

2.1.3 Restriction endonucleases

The restriction endonucleases and appropriate dilution buffers used for this study were purchased from New England Biolabs (Beverly, MA, USA).

2.1.4 Haemopoietic Growth Factors

Recombinant human IL-3 (hIL3) was a kind gift from Dr. Ian Lewis, Hanson Centre for Cancer Research, Adelaide South Australia.

Recombinant human GMCSF (hGMCSF), produced in *Eschericia coli* and purified by anion exchange and reverse phase high-performance liquid chromatography was a gift from Professor Angel Lopez, Hanson Centre for Cancer Research, Adelaide, South Australia.

Recombinant human EPO (hEPO) as Eprex 4000^{TM} was purchased from Jansen-Cilag, NSW, Australia

Recombinant human SCF (hSCF) was kindly provided from Assoc. Prof. Leonie Ashman, Hanson Centre for Cancer Research, Adelaide, South Australia.

2.1.5 Monoclonal antibody

The affinity purified monoclonal antibody 8E4, raised in mouse, was used in an ELISA for the detection of soluble hβc. This antibody has been epitope mapped to CRD1 to 3

of the hβc protein and was a generous gift of Professor Angel Lopez, Hanson Centre for Cancer Research, Adelaide, South Australia (Sun et al., 1996).

2.1.6 Radiochemicals

(α-^{32}P)dATP and (α-^{32}P)dTTP were purchased from Bresatec, Adelaide, South Australia

2.1.7 Bacterial cell culture media

Luria Broth (LB)	1% (w/v) bacto-tryptone, 1% (w/v) bacto-yeast extract in Milli-Q water, pH adjusted to 7.5 with sodium hydrochloride, sterilised by autoclaving
L-agar	LB containing 1% (w/v) bacto-agar, sterilised by autoclaving
L-Amp plates	L-agar, cooled to 55°C, containing 100ug/ml ampicillin, dispensed into 10cm petri dishes, allowed to solidify at 22°C and stored at 4°C until required.
SOC medium	2% Bacto-tryptone (w/v), 0.5% Bacto-yeast extract (w/v), 10mM NaCl, 2.5mM KCl, 20mM $MgCl_2$, 100mM D-glucose, sterilized by autoclaving.
ψ broth	2% Bacto tryptone (w/v), 0.5% Bacto-yeast extract (w/v), 20mM $MgSO_4.7H_2O$, 10mM NaCl, pH adjusted to 7.6 with 0.1M KOH, sterilised by autoclaving.
Transformation buffer 1 (Tfb 1)	

30mM KAc, 100mM RbCl, 10mM $CaCl_2$ ($2H_2O$), 50mM $MnCl_2$ ($4H_2O$), 15% glycerol (v:v), pH adjusted to 5.8 with 0.2M acetic acid, filter sterilised.

Transformation buffer 2 (Tfb 2)

10mM MOPS (acid), 10mM RbCl, 75mM $CaCl_2$ ($2H_2O$), 15% glycerol (v:v), pH adjusted to 6.5 with 0.1M KOH, filter sterilised.

2.1.8 Mammalian cell culture media

Dulbecco's modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) was supplemented with HEPES, L-glutamine and antibiotics

Iscove's modified Dulbecco's Medium (IMDM) (Gibco, Grand Island, NY, USA) was supplemented with L-glutamine and antibiotics

Foetal Calf Serum (FCS) (CSL Laboratories, Victoria, Australia) was heat inactivated at 56°C for 1 hour

Methylcellulose medium as Methocult™ (Stem Cell Technologies, Vancouver, Canada), consisted of 0.9% methylcellulose in α-modified Eagle's medium containing 30% FCS, 1% bovine serum albumin, 10^{-4}M 2-mercaptoethanol and 2mM L-glutamine.

2.1.9 Molecular weight standards

DNA Plus™ DNA markers (Gibco, Grand Island, NY, USA) were used at a final concentration of 0.9ug in 1µl, with each fragment consisting of approximately 50ng DNA. Fragment sizes were 100, 200, 300, 400, 500, 650, 850, 1000, 1650, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000 and 12000 bp. Alternatively, 1Kb DNA ladder (Gibco, Grand Island, NY, USA) was used at a concentration of 0.9ug in

1µl, with each fragment consisting of approximately 40 ng DNA. Fragment sizes for this marker were 75, 134, 154, 201, 220, 298, 344, 396, 510, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198 and 12216 bp.

2.1.10 Preparation of competent *Escherichia coli (E.coli)*

2.1.10.1 electrocompetent cells

A single colony of JM109 strain *E.coli* was used to inoculate a 10ml culture, which was incubated overnight at 37°C with shaking. The 10ml culture was transferred into 400mls of LB and grown for approximately 2 hours at 37°C with shaking until the OD_{600} determined using a DU-64 spectrophotometer (Beckman Coulter, Bucks, UK), reached between 0.4 and 0.8. The culture was chilled on ice for 30 mins and centrifuged at 4500x g at 4°C for 10 mins in a Beckman J6 centrifuge. The cell pellet was resuspended in 20mls of buffered glycerol and centrifuged as above. Cells were similarly washed with a further 2x buffered glycerol washes followed by 2x 10% glycerol washes. The cell pellet was finally resuspended in 400µl of 10% glycerol and 50µl aliquots frozen in liquid nitrogen. The aliquots were maintained in –80°C storage until required.

2.1.10.2 chemically competent cells

A single colony of JM109 strain E.coli was used to inoculate 2mls of ψ broth for incubation with shaking at 37°C for 16 hours. 330µl of this culture was subcultured into 10mls ψ broth and incubated for approximately 90 mins at 37°C until the OD_{600} reached approximately 0.6. Cells were then chilled on ice for 5 mins, centrifuged at 4000x g, 4°C for 5mins and resuspended in 40mls Tfb 1 buffer. Cells were again chilled on ice, 5 mins, then resuspended in 4mls Tfb 2 buffer and incubated on ice for a further 15 mins. Cells were frozen in liquid nitrogen in 125µl aliquots and stored at –80°C until required.

2.2 Mammalian cell techniques

2.2.1 Peripheral blood collection

Peripheral blood samples from PV patients undergoing phlebotomy therapy were obtained by venesection by the Haematology Day Clinic, Royal Adelaide Hospital, South Australia. Blood samples of two patients were obtained by Clinpath Laboratories, Adelaide, South Australia. All patients provided written consent for involvement in the study. Normal control samples were obtained from consenting staff members of the Hanson Centre for Cancer Research, Adelaide, South Australia.

2.2.2 Mononuclear cell (MNC) fractionation

Peripheral blood or bone marrow obtained from consenting patients and healthy donors was anticoagulated with EDTA, layered carefully over Lymphoprep^{TM} (Nycomed, Norway) and centrifuged at 400x g for 30 minutes at 22°C without braking. The MNC fraction was collected from the gradient interface by pipetting into 20mls DMEM/10% FCS. Cells were then washed twice in the same medium by centrifugation at 450x g for 5 mins.

2.2.3 Freezing and thawing of MNC preparations

MNC preparations were pelleted by centrifugation, chilled on ice and resuspended at a concentration of 10^7-10^8 cells/ml in cold FCS containing 10% DMSO. Aliquots were frozen in nitrogen vapour phase for a minimum of 3 hours, and stored in liquid nitrogen until required. Frozen MNC aliquots were thawed quickly by immersion in a 37°C water bath followed by drop wise addition of 10mls DMEM/20% FCS. Cells were washed twice in the same medium by centrifugation at 450x g for 5 mins and removal of the supernatant. Cell viability was assessed by 0.2% trypan blue exclusion, and cell number determined by standard haemocytometer counting procedure.

2.2.4 Blast Forming Units -Erythroid (BFU-E) Assay

MNC previously prepared from peripheral blood or bone marrow were thawed from liquid nitrogen storage, washed twice in IMDM containing 20% FCS, and resuspended to a concentration of 10^7 cells/ml. Cells were mixed vigorously with Methocult^{TM} methylcellulose medium containing haemopoietic growth factors. 300µl cell suspension

per well was dispensed into 24 well tissue culture trays (Nalge Nunc, IL, USA) using a 1ml syringe. Wells surrounding the methylcellulose cultures were filled with sterile Milli-Q water to maintain high humidity and the trays were incubated for 12-14 days at 37°C with 5% CO_2. BFU-E were identified by their size, morphology and red colour and were manually scored using light microscopy.

2.3 Immunochemistry

2.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

96 well Maxisorp Immunoplates™ (Nalge Nunc,IL, USA) were incubated at 37°C for 2 hours with the purified capture monoclonal antibody 8E4 diluted to 10μg/ml in bicarbonate coating buffer. The wells were blocked by incubation at 37°C for 90 mins with bicarbonate coating buffer containing 1% BSA and stored at 4°C until required. Wells were washed with PBS/Tween20 using an automated plate washer LP35 (4 x 200μl washes). 100μl of 1% BSA/PBS and 100μl of serum samples or standard appropriately diluted in 1% BSA/PBS were added to the wells and incubated at 22°C for 90 mins. Wells were washed as above three times then incubated with 100μl of biotinylated 8E4 monoclonal antibody diluted to 1mg/ml in PBS/ 0.05% Tween20 for 90 mins at 22°C , followed by washing as above. The biotinylated 8E4 antibody was detected and the signal amplified using a Streptavidin:biotinylated horseradish peroxidase (HRP) complex. 100μl of Streptavidin:HRP (Amersham Pharmacia, NJ, USA) diluted to 1:3000 in PBS/0.05% Tween20 was added to the wells and incubated for 90 mins at 22°C . Wells were washed as previously. The amount of HRP bound was measured by addition of 200μl of o-phenylenediamine substrate as Sigma *FAST* ™ OPD reagent and incubation for 30 mins at 22°C in the dark. Colour development was terminated with the addition of 50μl 3M H_2SO_4. Absorbance at 492nm was determined using an automated plate reader.

2.4 Molecular biology techniques

2.4.1 RNA Extraction

2.4.1.1 MNC

RNA was prepared from freshly isolated BMMNC or PBMNC pelleted at 450x g for 5 mins then lysed in TRIZOL™ reagent at a concentration of 10^7 cells/ml. Samples were incubated at 22°C for 5 mins. RNA was extracted by incubation at 22°C for 3 mins with 200µl chloroform per ml sample. Samples were centrifuged at 12000x g for 15 mins at 4°C and the aqueous phase removed to a fresh tube. RNA was precipitated by incubation with 500 µl isopropyl alcohol at 22°C for 10 mins and pelleted by centrifugation at 12000x g at 4°C for 10 mins. The RNA pellet was washed with 1ml 75% ethanol, dried at 37°C and resuspended in Milli Q water.

2.4.1.2 BFU-E

Single BFU-E were aspirated using a pipette tip using light microscopy into 10 µl PBS. The colonies were lysed in 100µl TRIZOL™ reagent in the presence of 1 µl glycogen (20mg/ml stock). Samples were extracted with 10 µl chloroform by incubation on ice for 5 mins followed by centrifugation at 12000rpm for 15 mins. The aqueous phase containing the RNA was removed to a fresh tube and precipitated by addition of 50 µl isopropanol and incubation overnight at –20°C. Samples were then centrifuged at 12000x g for 15 mins to pellet the RNA, followed by brief washing with 75% ethanol, vortexing and centrifugation at 12000x g for 10mins. The RNA pellet was then dried briefly, dissolved in 25 µl MilliQ and stored at –70°C.

2.4.2 Genomic DNA Extraction

After complete removal of the aqueous phase as described for RNA preparation (section 2.5.1) genomic DNA was precipitated from the remaining interphase and organic phase with 300µl ethanol per 1ml TRIZOL™ lysate. Samples were mixed by inversion, incubated for 3 mins at 22°C and centrifuged at 2000x g, 4°C for 5 mins. The DNA pellet was washed twice in 0.1M sodium citrate/10% ethanol then once in 75% ethanol

with 30 mins incubation at 22°C followed by centrifugation at 2000x g, 4°C for 5 mins, for each wash. The pellet was dried and dissolved in 8mM NaOH.

2.4.3 Reverse Transcriptase (RT) Reaction

RNA samples obtained from PBMNC or BFU-E were reverse transcribed using Omniscript™ or Sensiscript™ Reverse Transcriptase Kits (QIAGEN, CA, USA) respectively, according to the manufacturer's instructions. Briefly, mixed together on ice in a 20 µl reaction volume were 2 µl Buffer RT, 2 µl dNTP mix (consisting of 5mM of each dNTP), 2 µl 10µM Oligo-dT primer, 1 µl Omniscript or Sensiscript reverse transcriptase (RT) enzyme and template RNA, 100-500 ng (for Omniscript procedure) or <50 ng (for Sensiscript procedure). Additional control reactions for each RNA sample were performed in the absence of RT enzyme. Reactions were incubated for 60 mins at 37°C then stored at –20°C. 5 µl aliquots were used for PCR amplification (see section 2.4.4).

2.4.4 Polymerase Chain Reaction (PCR)

Reactions were performed in a total volume of 50µl, in the presence of 2.5 units Amplitaq or Amplitaq Gold DNA polymerase enzyme, 1x *Taq* DNA polymerase buffer (Perkin Elmer, CT, USA), 0.2mM dNTPs, 100ng of each primer, 1.5mM $MgCl_2$ and 1-5 µl cDNA or genomic DNA. Thermal cycling conditions were as described in Figure legends and were performed using a DNA Thermal Cycler (Perkin Elmer, CT, USA). PCR products were analysed by agarose gel electrophoresis as described in section 2.4.5.

2.4.5 Agarose gel electrophoresis

Analytical and preparative DNA electrophoresis was performed in agarose gels consisting of 1 or 2% agarose (w/v) in TAE buffer. Gels were submerged in TAE buffer, 2mm or 20mm wells for analytical or preparative samples, respectively, were loaded with DNA samples (previously mixed with 6xLB) and electrophoresed at 100 volts for approximately 20 mins. DNA was visualised by staining in ethidium bromide

solution (10ug/ml) for 5 mins, destaining in tap water for 15 mins and photographed using either Polaroid positive land film, type 667 (Polaroid, Hertfordshire, England) or an Imagemaster™ Gel Documentation System (Amersham, Pharmacia Biotech, NJ, USA).

2.4.6 Cloning

2.4.6.1 Gel Purification of insert and vector DNA

Insert and vector DNA, appropriately digested where required, were purified prior to ligation using preparative gel electrophoresis and visualised as described above. The required fragments were excised from the gel using a scalpel blade. DNA was recovered using the BRESACLEAN™ DNA extraction kit (Bresagen, Adelaide, South Australia). Briefly, the gels slices were incubated at 55°C for 7 mins with 800µl Bresa Salt™. 5µl glass milk suspension was added to the melted gel solution and incubated at 22°C for 5 mins. The DNA bound to the glass milk was centrifuged at 12000x g for 20 secs, washed thrice with 800µl New Wash™ and eluted with 11µl Milli Q water. The yield of DNA was approximated by agarose gel electrophoresis of 1µl and comparison to molecular weight standards.

2.4.6.2 Ligation Reactions

Ligation reactions consisted of 50ng pGEMT or pGEM4Z vector DNA (Promega, WI, USA), 4-fold molar excess of insert DNA, 1x T4 DNA ligase buffer (Promega, WI, USA) and 0.1 units DNA ligase (New England Biolabs, MA, USA). Reactions were incubated overnight at 14°C and stored at –20°C.

2.4.6.3 Bacterial transformation

L-Amp plates were spread prior to plating of the transformation with indicator medium containing 50mM IPTG and 7.5 mg/ml X-gal (Promega, WI, USA) to enable blue/ white selection of colonies containing recombinant vector.

i) *electroporation method*

A 50µl aliquot of JM109 electrocompetent cells was thawed on ice, transferred to a 0.2mM electroporation cuvette (Biorad, CA, USA) and held on ice for a further 10 mins with 2-5µl DNA ligation mix. The cells were electroporated using a Gene-Pulser (Biorad, CA, USA) at 2kV and 25mF. 150µl of SOC buffer was added immediately and cells were incubated at 22°C for 1 hour prior to spreading on L-Amp plates. Cultures were incubated overnight at 37°C then sealed and stored at 4°C.

ii) heat shock method

A 125µl aliquot of JM109 chemically competent cells was thawed slowly on ice and incubated for 25 mins with 5µl DNA ligation mix. Cells were heat shocked for 2 mins at 42°C, cooled on ice for 2 mins and incubated for 1 hour at 37°C with SOC buffer. Cells were centrifuged at 4500x g for 1 min, the pellet resuspended in 100µl SOC buffer and spread onto L-Amp plates for overnight incubation at 37°C.

2.4.6.4 Minipreparation of plasmid DNA

A sterile toothpick was used to inoculate 2mls LB + Amp media from a single fresh colony of *E.coli*. The culture was incubated for 16 hours at 37°C with shaking. 1.5mls of culture was chilled on ice and centrifuged at 12000x g for 1 minute. The supernatant was removed and the pellet resuspended in 250µl TELT buffer. Cells were lysed with the addition of 20µl lysozyme (20mg/ml) and incubation on ice for 10 mins. Samples were heated at 95°C for 2 mins, cooled on ice for 10 mins and centrifuged at 13,000rpm for 20 mins. The pellet was removed with a toothpick and DNA precipitated with the addition of 500 µl ethanol. The precipitate was pelleted by centrifugation at 12000x g for 10mins at 4°C. Pellets were washed with 1ml 70% ethanol and air dried at 55°C for 10 mins. DNA was dissolved in 32 µl of Milli-Q water.

2.4.6.5 Restriction endonuclease digestion

2µl of plasmid DNA minipreparation was digested using a minimum of 10 units of restriction enzyme (New England Biolabs, MA, USA) in a total volume of 10µl

containing the appropriate digestion buffer. Digestions were performed at 37°C for a minimum of 1 hour and DNA fragments resolved by agarose gel electrophoresis.

2.4.6.6 Phenol/ chloroform extraction of plasmid DNA

Plasmid DNA was prepared for sequence analysis by dilution to 100µl with Milli Q water in the presence of 0.2M NaCl. DNA was extracted by addition of 100µl phenol: chloroform: isoamyl alcohol solution. The mixture was centrifuged at 12000x g for 2 mins and 100µl of the aqueous phase containing DNA was removed to a fresh tube. DNA was precipitated by addition of 500 µl ethanol and pelleted by centrifugation at 13,000 rpm for 10 mins at 4°C. The DNA was then washed 3 times with 800 µl 70% ethanol, air dried at 55°C for 10 mins, and resuspended in 25µl Milli-Q water.

2.4.6.7 DNA Sequencing

DNA sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase, FS (Applied Biosystems, CA, USA) 100-500ng of chloroform/ phenol extracted plasmid DNA was sequenced with 100ng SP6 or T7 primer consisting of vector sequences and 4 µl Big Dye Terminator version 2 or 3 (Applied Biosystems, CA, USA) in a 20 µl reaction volume. Cycle sequencing was performed using a DNA Thermal Cycler (Perkin Elmer, CT, USA) for 25 cycles of 96°C, 30 secs, 50°C, 15 secs, 60°C, 4 mins. The DNA was then precipitated in the presence of 1 µl glycogen (20mg/ml) with 80 µl 75% isopropanol and incubation for 30 mins. The precipitate was centrifuged at 4°C for 30 mins washed in 200 µl 70% ethanol and air dried at 55°C. Sequencing was performed by the Institute of Medical and Veterinary Science Sequencing Facility (Adelaide, South Australia) using an ABI Prism 377 DNA Sequencer (Applied Biosystems, CA, USA).

2.4.7 Single Nucleotide Primer Extension (SNuPE) and semi-quantitative (Q) SNuPE

Added together in a 10µl reaction mix were 2-5µl gel purified DNA, 1x PCR buffer, 0.6 µl Mg^{2+} (2mM final), 1 unit *Taq* polymerase, 100ng SNuPE primer (see Appendix A) and 1 µl ^{32}PdATP (SNuPE), ^{32}PdATP or ^{32}P-dTTP (QSNuPE) with a total activity of 2µCi. A single cycle of 94°C, 3 mins, 48°C, 1 min, 72°C, 1 min was applied using a DNA thermal cycler (Perkin Elmer, CT, USA) and samples cooled on ice. Formamide loading dye (10µl) was added and a 10µl aliquot from each sample was electrophoresed on a 20% denaturing polyacrylamide gel at 400-500 volts for 1 hour. The wet gel was sealed in plastic and exposed to autoradiographic film (Amersham Pharmacia Biotech, NJ, USA) or phosphorimage analysis. For size estimations the migration of radiolabelled bands were compared to the migration pattern of the dyes bromophenol blue (9bp) and xylene cyanol (29bp) in 20% denaturing PAGE analysis.

Chapter 3 Enrolment and diagnosis of PV patients

3.1 PV diagnostic criteria

The accurate diagnosis of PV can be challenging in the absence of a specific diagnostic marker for the disease. The Polycythemia Vera Study Group (PVSG) was established in 1968 with the initial aim of forming a set of clinical criteria for diagnosis. This group has published guidelines for PV diagnosis over several decades and the most recent criteria, published in 2002 (Michiels and Thiele, 2002) are summarised in Table 3.1. Patients with PV usually present with marked erythrocytosis, defined as a peripheral blood red cell mass of 25% or more above the normal range (Fruchtman S.M. et al., 1998). Erythrocytosis can be further subdivided into primary and secondary erythrocytoses. Secondary erythrocytosis is due to the response of normal bone marrow to an extrinsic pathological process resulting in increased levels of EPO, eg. hypoxemia caused by heart disease, lung dysfunction or high altitude. Therefore, these patients can be distinguished from those with primary erythrocytosis by an elevated serum EPO level. Primary erythrocytosis however, occurs as the result of an intrinsic defect of the haemopoietic progenitor cell compartment and is the common term for 2 diseases, a rare, congenital primary erythrocytosis involving only the erythroid lineage caused by truncation and subsequent hypersensitivity of the EPO receptor, and acquired primary erythrocytosis of which Polycythemia Vera is the only recognised form (see Chapter 1).

Therefore, elevated red cell mass in the absence of a cause of secondary erythrocytosis is the major diagnostic criterion for PV. Other recommended criteria that further distinguish PV from secondary erythrocytosis include splenomegaly detectable by palpitation or ultrasound scanning, acquired abnormal bone marrow karyotype and normal or reduced serum EPO. Diagnostic criteria that distinguish PV from congenital primary erythrocytosis are thrombocytosis or leukocytosis, which indicate involvement

Table 3.1 Recommended criteria for the diagnosis of Polycythemia Vera

(referenced from Michiels et al., 2002)

A1 Elevated red cell mass (>25% above mean normal predicted value)

A2 Absence of cause of secondary erythrocytosis

A3 Palpable splenomegaly

A4 Acquired abnormal bone marrow karyotype

B1 Thrombocytosis

B2 Leukocytosis

B3 Splenomegaly demonstrated on ultrasound scanning

B4 Endogenous BFU-E

B5 Reduced serum Epo level

A1 + **A2** + **A3** or **A4** establishes a diagnosis of PV, or

A1 + **A2** + two of **B** establishes a diagnosis of PV

of the megakaryocytic and myeloid lineages. Lastly, the growth of endogenous BFU-E is highly specific to PV patients and has been previously recommended as a major diagnostic tool, however it is currently recommended as only a minor criterion due to difficulties associated with test availability and standardisation (Michiels and Thiele, 2002).

In South Australia, a diagnosis of PV is generally formed with presentation of erythrocytosis in the absence of extraneous factors that may lead to the elevation of EPO and secondary erythrocytosis. That is, elevated peripheral red blood cell mass in the presence of normal serum EPO levels and blood oxygen levels indicates primary polycythemia. Splenomegaly and involvement of the myeloid and megakaryocytic lineages aids PV diagnosis however these symptoms often occur only as the disease progresses. Karyotype analysis and endogenous BFU-E assays are not usually performed.

3.2 Human ethics approval

The Royal Adelaide Hospital Research Ethics Committee under the Declaration of Helsinki and National Health and Medical Research Council Guidelines on Human Experimentation granted human ethics approval for the current study (Appendix B). Patients with a diagnosis of probable PV were provided with a patient information sheet (Appendix C), and asked to complete a consent form (Appendix D). A number of haematologically normal individuals also consented to donation of bone marrow and/or peripheral blood, and were used as controls for the study. The Haematology Outpatient Clinic, Royal Adelaide Hospital and the Division of Haematology, Institute of Medical and Veterinary Science collected the samples.

3.3 Clinical history of enrolled patients

Eleven patients consented to donation of peripheral blood samples, which were obtained at the time of phlebotomy treatment. A summary of patient details is presented below (Table 3.2). Patients were of mature age (between 53 and 75 years) at diagnosis. Disease duration was variable, ranging from recently diagnosed to 9 years post diagnosis. Six patients were female and 5 male. All patients had primary erythrocytosis for which they were undergoing phlebotomy therapy at the time of peripheral blood collection for this study. Six patients had involvement of the megakaryocytic and/or myeloid lineages and several of these patients were receiving hydroxyurea treatment to suppress myeloid cell numbers. One patient was receiving, in addition to phlebotomy and hydroxyurea, γ-interferon treatment to manage elevated platelet numbers. Enlargement of the spleen was documented for 3 patients.

Bone marrow biopsy had previously been performed on only 1 patient with no karyotype abnormalities detected in this sample. In several cases a diagnosis of probable PV was made on the basis of the presence of erythrocytosis with normal oxygen and EPO levels but without other detectable PV symptoms. Endogenous BFU-E growth was not assessed for the diagnosis of any of the patients. Thus, to confirm the diagnosis of PV in all patients enrolled in the study, replicate BFU-E assays were performed with PBMNC samples for assessment of endogenous BFU-E growth (see section 3.4).

3.4 BFU-E formation

The growth of EPO independent BFU-E from PV BMMNC has previously been regarded as an important diagnostic criterion, however the most recent guidelines from the PVSG recommend the presence of these colonies as a minor criterion only. Cost of the procedure and difficulty with standardisation of this test between institutions has been cited as a problem (Fruchtman S.M. et al., 1998). However, given that 'endogenous' BFU-E have been described as the 'hallmark' of PV diagnosis and that

Table 3.2 Summary of PV patients clinical history

Patient ID	Sex	Age (yrs)	Disease duration (yrs)	Lineage involvement*	Enlarged spleen	Treatment
AK	F	64	9	E+M+P	yes	phlebotomy
JD	M	54	5	E	yes	phlebotomy hydroxyurea
AP	M	64	n/a	E+M	no	phlebotomy hydroxyurea
JL	F	75	9	E+M+P	no	phlebotomy
MB	F	72	1	E	yes	phlebotomy
MD	M	n/a	n/a	E	n/a	phlebotomy hydroxyurea
AG	M	72	6	E+G+P	n/a	phlebotomy hydroxyurea
YD	F	73	n/a	E+M+P	n/a	phlebotomy
MG	M	55	1	E	no	phlebotomy
PD	F	53	2	E+M+P	no	phlebotomy hydroxyurea γ-interferon
MW	F	n/a	n/a	E	n/a	phlebotomy

* Involvement of the erythroid, myeloid and megakaryocytic lineages is indicated by increased numbers of erythrocytes (E), myeloid cells (M) and platelets (P) respectively, in the peripheral blood.

n/a indicates where information was not available.

greater than 95% of PV patients give rise to these colonies from their bone marrow (Zwicky et al., 2002), this assay was undertaken to confirm the diagnosis of PV in the patients used for this study. The detection of endogenous BFU-E from PV peripheral blood samples is reported to be more difficult (Westwood and Pearson, 1996) however we established the assay using peripheral blood mononuclear cells (PBMNC) as bone marrow (BM) samples were not available from PV patients.

MNC were incubated in methylcellulose cultures at various frequencies (5.10^4-5.10^5 PBMNC or BMMNC per well) in order to obtain between 20 and 100 BFU-E per well in the presence of EPO. This enabled accurate scoring and also optimisation of the assay for endogenous colony detection since these are formed at lower frequency. BFU-E were detected in both PV and control samples in the presence of hEPO however in the absence of EPO, BFU-E were detected only in PV samples (Figure 3.1 panel A). BFU-E morphology was heterogeneous with regard to depth of colour and shape. Some samples produced colonies of a compact, spherical appearance whilst others gave rise to clusters of irregularly shaped colonies. However, in general the endogenous BFU-E obtained from PV samples were smaller and of paler appearance than the BFU-E from PV and control samples that grew in the presence of EPO (Figure 3.1 panel B).

A single assay representative of 7 assays is shown (Figure 3.2). Endogenous BFU-E were detected in all PV PBMNC samples but not in control samples of BMMNC. For some samples, plating at high cell number resulted in greater than 200 BFU-E per well with EPO which, given the dispersion of BFU-E within methylcellulose were difficult to score with accuracy (see section 2.2.5). These samples were scored as >200 colonies per well and were included in the data for normal individuals as they were useful in determining the number of EPO independent BFU-E obtained at this frequency of cells plated. For example, endogenous BFU-E were not detected in normal samples even when >200 EPO dependent BFU-E grew per well (see Figure 3.2, sample CH).

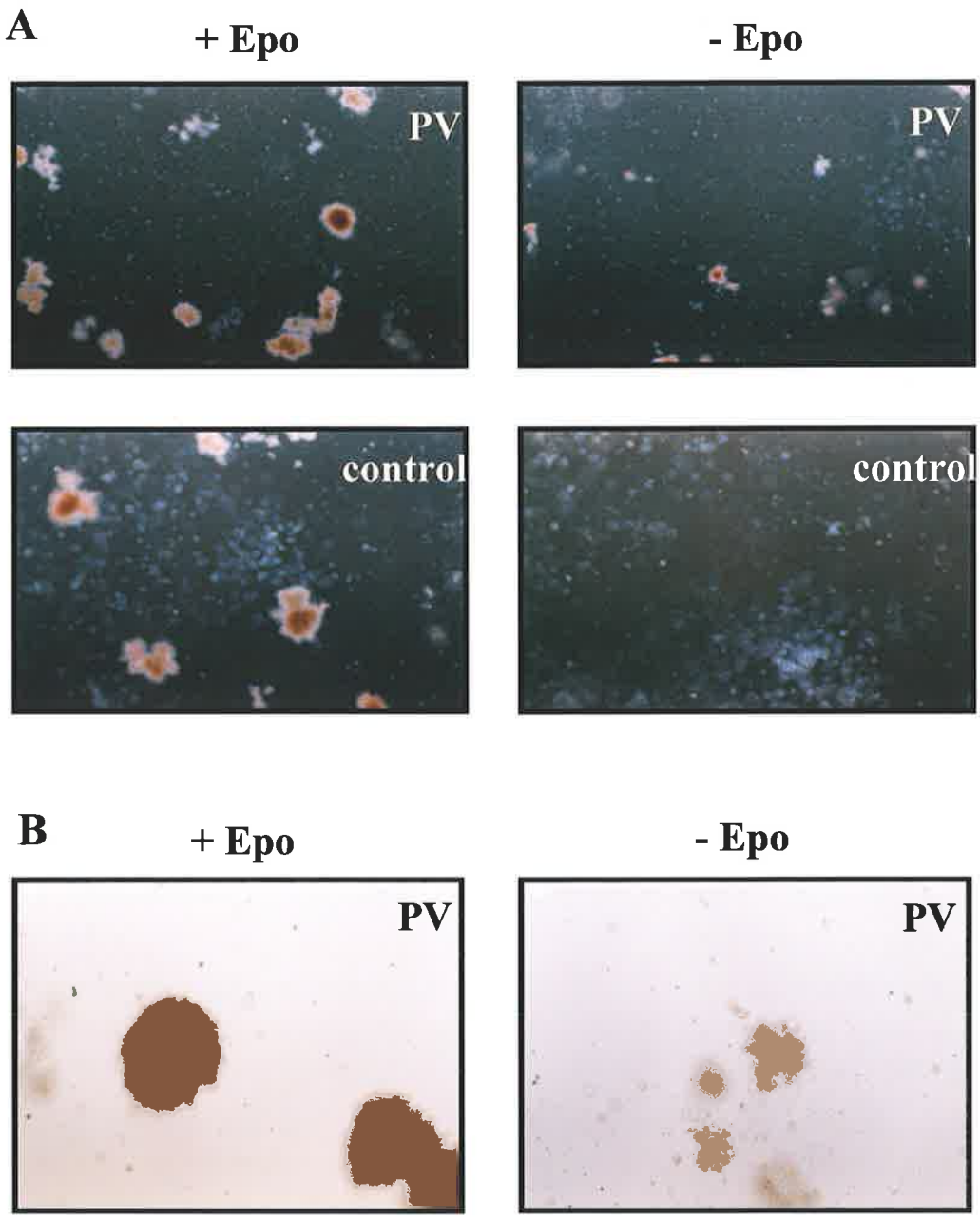


Figure 3.1 Morphology of PBMNC derived BFU-E

PBMNC samples were incubated for 14 days in methylcellulose cultures in the presence of hIL-3, hSCF and in the presence or absence of hEPO. (A) Colony formation from normal or PV patient PBMNC samples. Endogenous BFU-E were detected in PV samples but not in control samples. Photographed at 40x magnification. (B) Representative photographs at 100x magnification of BFU-E grown in the presence or absence of hEPO from a PV patient PBMNC sample.

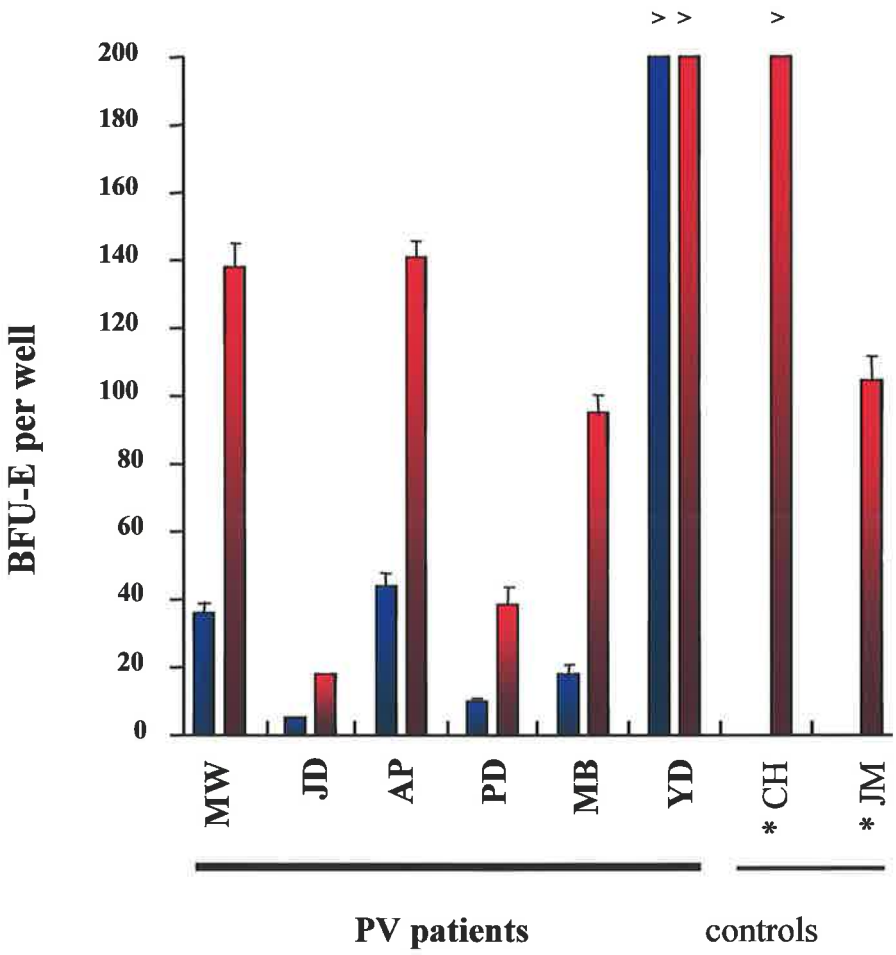


Figure 3.2 Endogenous BFU-E formation by PV PBMNC samples

A single BFU-E assay is shown as a representation of 7 experiments performed. PBMNC or BMMNC (**) samples were plated in methylcellulose cultures in the presence of hIL-3, hSCF and in the absence (■) or presence (■) of hEPO. BFU-E numbers were determined after 12-14 days of culture. Endogenous BFU-E were obtained from PV samples but not from control samples. Bars represent the mean of quadruplicate cultures and error bars indicate the standard error of the mean. > indicates samples where >200 colonies were obtained per well and were not able to be accurately counted.

All 11 PV samples gave rise to some level of endogenous BFU-E formation in replicate assays (Table 3.3). The percentage of endogenous BFU-E (grown in the absence of EPO) compared with those that grew in the presence of EPO varied considerably between PV patients (within the range of 0.6 to 66%), which may reflect variation in the degree of progenitor cell mobilisation and therefore BFU-E numbers in the peripheral blood. There was also considerable variation between replicate assays for individual samples with regard to the percentage of endogenous BFU-E obtained. This may be due to differences in viability of progenitors in the samples after thawing or to variation in methylcellulose batches. Nevertheless, PBMNC samples from all 11 PV patients consistently gave rise to endogenous BFU-E. In contrast, PBMNC and BMMNC samples from 6 control individuals did not produce a single endogenous BFU-E under these assay conditions. This was important in confirming the diagnosis of PV for all patients participating in this study.

3.5 Discussion

The formation of a diagnosis of PV can be problematic. Therefore, the aim of this part of the study was to determine whether the 11 patients recruited fulfilled the recommended criteria for PV diagnosis as outlined recently by the PVSG.

All patients fulfilled the 2 major diagnostic criteria of elevated peripheral red blood cell mass that occurred in the absence of a cause of secondary erythrocytosis. In addition, 6 of the patients had involvement of the myeloid, megakaryocytic, or both lineages and 3 had splenomegaly. All 11 patients had a diagnosis of probable PV and this was confirmed in this study by the detection of endogenous BFU-E from PBMNC samples from all PV patients. In our hands this assay proved to be robust with no endogenous BFU-E detected over 7 assays from either PBMNC or BMMNC samples from six haematologically normal individuals.

Table 3.3 Summary of endogenous BFU-E assays

Sample	Phenotype	Number of experiments	* Mean % endogenous BFU-E ± SD
AK	PV	3	16.4±16.2
MD	PV	3	28.7±25.4
JL	PV	3	0.8±1.4
AG	PV	3	9.2±12.1
MG	PV	2	0.6±0.8
JD	PV	3	14.3±13.9
AP	PV	3	51.4±17.6
MW	PV	3	27.6±6.5
PD	PV	3	18.3±13.3
MB	PV	3	9.5±9.5
YD	PV	2	66.6±24.6
CH**	N	1	0
HE**	N	2	0
FS	N	1	0
CY	N	3	0
HO	N	3	0
JM**	N	1	0

* The mean number of endogenous BFU-E scored from quadruplicate wells for each sample in 1-3 experiments, relative to the mean number of Epo dependent BFU-E was determined for each sample in each assay. Mean % endogenous BFU-E refers to the average of this number in replicate experiments ± standard deviation.

** samples were BMMNC, all other samples were PBMNC

The BFU-E assay results together with clinical details obtained from the physicians of the PV patients enrolled for this study indicate that all 11 patients fulfil the recommended criteria for PV diagnosis.

Chapter 4 Mutation screening of hβc in PV patients

4.1 Introduction

The primary lesion in PV leads to the dysregulated growth of haemopoietic progenitor cells, therefore a number of studies have focussed on the HGF requirements, receptors and signalling pathways of progenitors from PV patients. Extensive research over several decades however, has failed to identify a causative lesion. Identification of a genetic abnormality that contributes significantly the pathogenesis of PV would have major implications in the field and could provide a genetic test for PV that would aid diagnosis of the disease. Furthermore, it is likely that such a finding will have important implications with regard to our understanding of the processes of *self-renewal* whereby the stem cell pool is maintained, and *commitment* whereby stem cells undergo the gradual process of differentiation to cells of the progenitor compartment. In addition, the identification of a lesion that leads to altered HGF responses of stem cells and committed progenitors would make a major contribution to our understanding of HGF receptor signalling mechanisms.

A role for hβc is implicated in PV pathogenesis from several studies. Firstly, PV progenitor cells from the erythroid, myeloid and megakaryocytic lineages display marked hypersensitivity to IL3 and GMCSF in colony assays (Dai et al., 1992). hβc expression correlates with the lineages affected in PV. For example, hβc expression has been shown on committed progenitors from the erythroid, megakaryocytic and myeloid lineages and functional GMR has been demonstrated on more primitive haemopoietic progenitors. In the murine system, mRNA transcripts for GMRα and IL3Rβ are detectable in early and committed progenitors of the erythroid, megakaryocytic, macrophage and granulocyte lineages (Lund-Johansen et al., 1999a; Wognum et al.,

1994; Lund-Johansen et al., 1999b; McKinstry et al., 1997; Berardi et al., 1995; Billia et al., 2001).

Most importantly, murine models expressing two constitutively active extracellular mutants of hβc (FIΔ and I374N) display a PV-like myeloproliferative disease with massive increases in rbc counts and concomitant expansion of the myeloid and megakaryocytic lineages. CFU-GM and CFU-E isolated from these mice grow *in vitro* in the absence of exogenously added GMCSF and EPO, respectively (D'Andrea et al., 1998; McCormack and Gonda, 1999). Thus, activating mutations of hβc can lead to expansion of multiple haemopoietic lineages *in vivo* and result in hypersensitive responses to HGFs *in vitro* as seen in PV.

Finally, there is recent evidence that hβc has the capacity to influence signalling by other receptors *in vitro*, providing a mechanism by which these *in vivo* multilineage effects may be mediated. For example, phosphorylation of hβc occurs following EPO or TPO stimulation of cells (Jubinsky et al., 1997; Ooi et al., 1998; Hanazono et al., 1995) and the hβc mutant FIΔ, has been shown to co-operate with EPOR and *Mpl* to induce factor independent growth in the murine cell line BaF-B03 (Blake et al., 2002).

Together these findings suggest that intrinsic alteration to hβc can lead to reduced factor requirements by progenitors *in vitro* and have profound multilineage effects *in vivo*, possibly via interaction with other receptors. Therefore, the aim of this study was to determine whether hβc mutations are present in PBMNC isolated from patients diagnosed with PV.

4.2 The target region of *CSF2RB*

A 619 bp region of *CSF2RB* was targeted as the area most likely to contain activating mutations (Figure 4.1). Selection of this region, between nucleotides 1002 and 1621 of the cDNA sequence, according to the original published sequence (Hayashida et al., 1990) was based on saturation mutagenesis studies of the entire

CSF2RB coding region in which numerous activating point mutations were detected (Gonda and D'Andrea, 1997). Additionally, the FIΔ mutation (consisting of duplication of amino acid residues 396-431), which gives rise to a myeloproliferative disorder in transgenic mice, lies within this region (Jones et al., 2001; D'Andrea et al., 1998). Primers *hbc3* and *hbc12* (see Chapter 2) were designed for PCR amplification of the region between amino acid residues 326 and 531 of hβc. This region spans the C-terminal region of CRD3 and entire CRD4 of the extracellular domain, the transmembrane domain and the box 1 motif of the intracellular domain (see Figure 4.1).

4.3 Screening PBMNC by RT-PCR

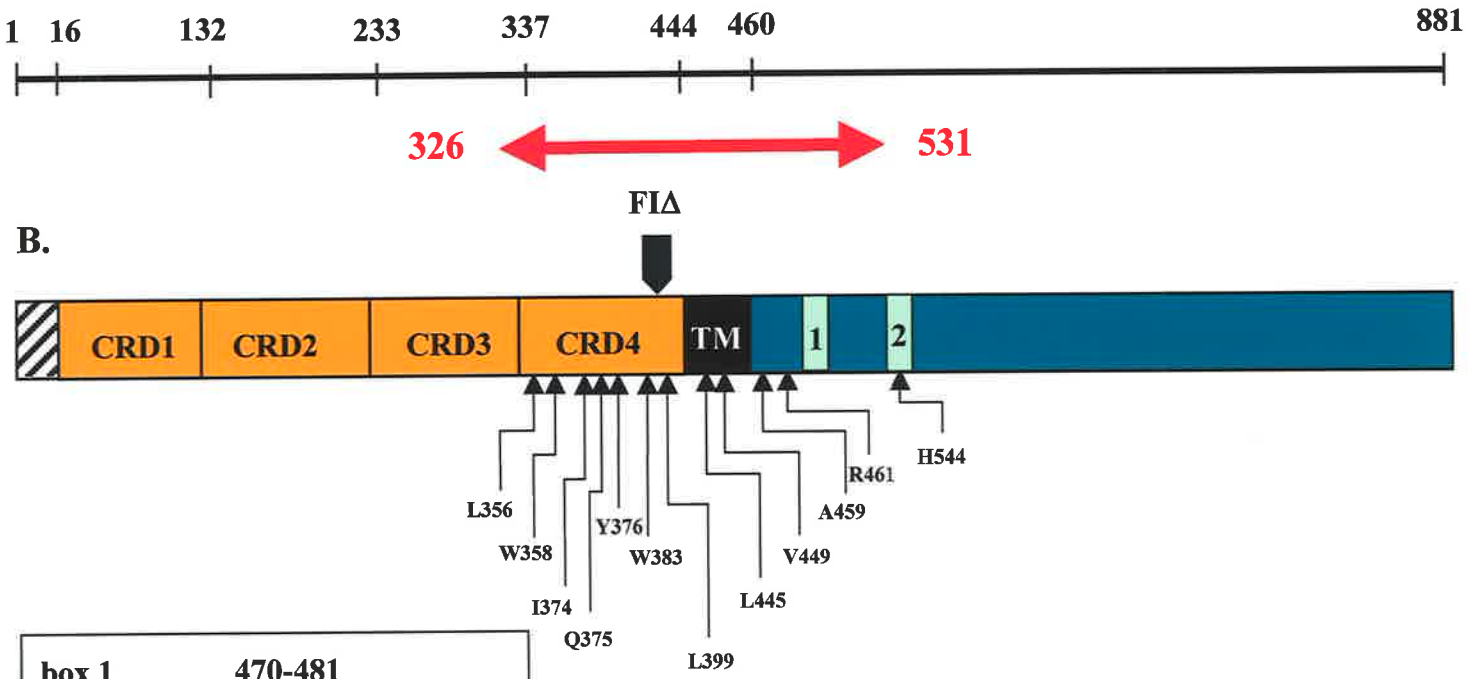
Bone marrow samples from PV patients were not readily available for this study, therefore peripheral blood samples were obtained from normal individuals and from PV patients undergoing phlebotomy therapy. RNA was extracted from purified PBMNC and the 619bp target region was amplified by RT-PCR. The amplicons obtained were cloned into the pGEM T vector (Promega, WI, USA), and a number of independent clones derived from 4 PV patient samples and 5 normal samples were sequenced using primers directed to vector sequences (Table 4.1). The sequences obtained were aligned with *CSF2RB* sequence (accession number NM000395) using the DNA analysis programs DNasis™ and DNAstar™.

From a total of 113 clones isolated from 4 PV patients and 163 clones isolated from normal individuals, a number of clones were detected that contained sequence differing from published *CSF2RB* sequence (Table 4.2). These sequences were confirmed by reverse sequencing of the complementary cDNA strand. 26 clones contained single base alterations that occurred in single clones only, and thus the possibility that they are attributable to *Taq* polymerase errors during PCR amplification cannot be excluded. Only base alterations that occurred in multiple clones or those that had been previously reported were considered further.

Figure 4.1 The *CSF2RB* target region

A. The amino acid positions of the protein domains are indicated by numbers in black type. B. Conserved motifs are schematically represented as follows; (▨) signal sequence; (■) extracellular domain consisting of CRD 1-4; (■) transmembrane domain; (■) intracytoplasmic domain; (■) box 1 and 2 regions. The single amino acid mutants resulting in constitutive activation of the receptor are indicated by arrows with wild type amino acid code followed by the amino acid number of each position in black type. The position of the FIΔ duplication mutant is shown with a solid black arrow and the region of hβc targeted in this study is indicated by the red arrow and numbering of amino acid positions. The amino acid positions of the FIΔ mutant and box 1 and 2 motifs are also indicated.

A. amino acid residues



box 1	470-481
box 2	534-546
FIΔ mutant	396-431 duplication

Table 4.1 Summary of the number of clones sequenced in the RTPCR screen of *CSF2RB* in PBMNC samples.

PBMNC sample	Phenotype	Number of clones sequenced	Total clones sequenced
MJ	PV	26	
JL	PV	20	
AK	PV	13	113
MD	PV	15	
MD(2)*	PV	39	
HR	N	15	
FS	N	38	
PM	N	14	163
KM	N	59	
SP	N	26	
SP(2)*	N	11	

* indicates duplicate PCR samples from the same PBMNC cDNA sample.

Table 4.2 ***CSF2RB*** **target region sequence alterations detected in cDNA clones isolated from PV patients and control individuals PBMNC.**

BASE CHANGE	AA CHANGE	PV CLONE ID.	CONTROL CLONE ID.
A1038G	N337S	**JL8**	KM2
		MD144	
T1041 insertion (15bp)	5 aa insertion		KM7
Δ1041-1045 del;etion	N337 frameshift	**JL4**	HR15, HR22
		MJ10, MJ11, MJ23, MJ24	KM2, KM19, KM26,
		MD3, MD5, MD6, MD9,	KM42
		MD(2)126, MD12	FS12,FS38
C1059A	S344Y	**JL2**	
A1106G	T360A		KM50
T1110C	M361T	**MD(2)146**	FS31
T1116C	M363T		SP5
A1126G	E366D	**AK17**	
A1148C	I374L		KM4
TT1142CG	F374R		HR1
G1158 insertion (1bp)	Y376 frameshift		SP9
A1179G	K384R	**MJ1**	
G1180A	K384L		KM53
A1188G	K387R		KM59
A1212G	H395R	**JL13**	
C1221T	A398V	**JL1, MJ2**	FS23, FS24, FS10
A1247G	R407G	**MD154**	
G1256A	A410T		KM56
A1271G	R415G		SP22
A1274G	T416A		KM34
T1277C	S418P		KM35
G1286A	G420S		SP25
			KM52
G1329A	W434 stop	**MD(2)106**	

G1343 insertion (36bp)	12 aa insertion	**MD(2)171**	PM11,PM29 KM39 SP77
G1356A	W443stop		HR2
T1380C	F451S	**MD4, MD1**	
T1422C	I464T		SP10
T1430C	Y468H		HR2
G1443T	R472I	**MJ4, MJ7, MJ12, MJ15**	HR1, HR2, HR9
		MD1, MD10 MD(2)166	FS52
A1466G	I465V	**AK19**	
A1476G	K483R		HR9
A1491G	Q488R		KM38
Δ1492-1596 deletion	Q488 frameshift	**MJ3 AK11, AK18**	HR5, PM12 SP9, SP15
		MD(2)166, MD(2)108,	SP(2)40, SP(2)81
		MD(2)128,MD(2)116,	KM2, KM12,KM5,KM27
		MD(2)120,MD(2)128	KM30,KM31,
		MD(2)122, MD(2)117	KM32, KM54 KM56, KM21
			KM39, KM43 KM44,KM51
C1577T	nil		SP59
C1063T	nil	**JL4**	
A1081G	nil		FS45
A1120G	nil		KM6, KM43
C1306T	nil	**AK1, AK6, AK7, AK11**	HR2, HR4, HR5, HR7, HR10
		AK17 AK19	PM 21, PM24, PM13
			KM5, KM6, KM8, KM15,
			KM21, KM22, KM29, KM34,
			KM53, KM55, KM56, KM24,
			KM40 KM42, KM54, KM57
			KM35, KM36, KM18
T1417C	nil	**JL8**	

Ten sequence changes that occurred in both normal and PV samples were detected (summarised in Table 4.3). Several of these alterations correspond to previously reported *CSF2RB* variants. A 36bp insertion at position 1343 was found in 1 PV and 3 normal samples and a 15bp insertion at position 1041 was detected in a single clone from a normal individual. These insertions consist of intron sequence and occur at intron/ exon junctions indicating that they are derived from alternative splicing events and were previously identified in the original cloning of hβc from the human erythroleukemia cell line TF-1 (Hayashida et al., 1990). A 104bp deletion at position 1492 that was detected in a total of 32 clones from 3 PV and 4 normal individuals has also been previously described. This form results from the removal of an entire exon by alternative splicing and encodes a truncated form of hβc, termed β intracytoplasmic truncation (β_{IT}) and has been the subject of further studies (Gale et al., 1998; Wagner et al., 2001). A single base substitution (C1306T), which does not confer an amino acid change, was detected in clones from 3 normal samples and a single PV patient sample at relatively high frequency (between 21 and 46%). This polymorphism has been described previously with an allele frequency of 0.13 in the general population (Freeburn et al., 1998; Freeburn et al., 1997; Freeburn et al., 1996).

In addition to known variants of *CSF2RB*, a 5bp deletion, found at nucleotide position 1041, termed Δ1041-1045, was detected in 19 clones from 3 PV and 3 normal individuals. Although this alteration does not appear to be involved specifically in PV, it had not been previously described and was the subject of further investigation (see Chapter 5).

Five single base substitutions were detected that also have not been previously reported. An A1120G base substitution occurred in 2 normal samples and does not confer an amino acid alteration. Two further base substitutions, A1038G and T1110C, occurred at low frequency in both normal and PV samples. An additional single base substitution, T1380C, occurred in 2 clones from a single PV patient sample. This

Table 4.3 Summary of *CSF2RB* sequence alterations detected in cDNA from normal and PV patient PBMNC.

Nucleotide change	Amino acid change	# clones in PV patient samples (n= 4)	# clones in normal samples (n= 5)	Previously described
at T1041	15bp insertion	0	1 (1)*	yes
at G1343	36bp insertion	1 (1)	4 (3)	yes
Δ1492-1596	104bp deletion	11 (3)	21 (4)	yes
Δ1041-1045	5bp deletion	11(3)	8 (3)	no
A1120G	nil	0	2 (2)	no
A1038G	N337S	2 (2)	1 (1)	no
T1110C	M361T	1 (1)	1 (1)	no
T1380C	F451S	2 (1)	0	no
G1443T	R472I	7 (2)	4 (2)	no
C1306T	nil	6 (1)	27 (3)	yes

* numbers in brackets refer to the number of individuals detected with the sequence change.

change confers a non-conservative amino acid substitution (phenylalanine to serine) at position 451. Whilst his alteration did not occur in further PV patient samples, screening for its presence in further PV samples may be warranted as this alteration lies within the transmembrane domain of the receptor and in close proximity to the activating mutant V449E previously described (McCormack and Gonda, 1999; Jenkins et al., 1995). The G1443T substitution confers the non-conservative amino acid substitution (arginine to isoleucine) at amino acid residue 472. This is a highly conserved residue that lies within the box 1 consensus region of the cytoplasmic domain of a number of cytokine receptors (Bagley et al., 1997). This sequence alteration was found in PBMNC samples from 2 PV patients and 2 normal individuals and will be discussed further in section 4.5 and in Chapter 6.

4.4 Screening endogenous BFU-E by RT-PCR

The detection of PV specific *CSF2RB* mutations in PBMNC RT-PCR screen may be difficult frequency of cells derived from the PV clone within the samples may be low. To overcome this limitation, a novel approach was undertaken to screen for hβc mutations in cells known to be derived from the PV clone, that is, in endogenous BFU-E. Clonality studies have shown that endogenous BFU-E are derived the PV clone and their presence was confirmed in all PV PBMNC samples analysed (see section 3.4) (Gilliland et al., 1991). Therefore endogenous BFU-E derived from a PV patient PBMNC sample, and EPO dependent BFU-E isolated from a normal individual, were screened by RT-PCR for changes within the *CSF2RB* target region.

Two individual endogenous BFU-E derived from a PV patient (MD) and three EPO dependent BFU-E from a normal individual (FS), were removed from day 14 methylcellulose cultures, RNA was extracted and the target *CSF2RB* region amplified by RT-PCR as for the PBMNC screen. The success of this procedure was variable given the reportedly low expression of hβc in these progenitors and the small number of cells

in the colonies, however PCR products were obtained from 5 individual BFU-E (see fig 4.2). These were cloned into pGEM-T vector and the sequences of numerous clones determined, summarised in Table 4.4.

A number of single base substitutions were detected in single clones that were not detected in the PBMNC screen. These again were discounted, as they may be attributable to Taq polymerase errors during the PCR reaction. Two further sequence changes were detected that occurred with some frequency in the PBMNC screen in both these individuals. The Δ1041-1045 variant was detected in a single clone of 13 from the MD derived endogenous BFU-E #1, a frequency of 7.7%. This may reflect the normal mRNA expression level of this splice variant in PBMNC from this patient since Δ1041-1045 was detected in 6 from a total of 54 clones from this patient in PBMNC cDNA, (11.1%).

A single clone of 4 sequenced from a normal BFU-E (FS BFU-E #2) contained the G1443T base substitution also found at a frequency of 5.6% in the PBMNC cDNA screen from this individual. Interestingly, G1443T was the only sequence obtained in all 13 clones from one of the endogenous BFU-E (MD BFU-E #2). All 13 clones from a second endogenous BFU-E from the same patient (MD BFU-E #1) were wild type at this position. G1443T was detected at a frequency of between 2.7 and 20 % in the PBMNC cDNA of 4 (from a total of 9) individuals, including MD and FS (Table 4.5). These results are consistent with G1443T being a polymorphism that is expressed at a frequency below that of the normal allele. The presence of G1443T as possibly the only allele expressed in an endogenous BFU-E isolated from a PV patient, together with the potential importance of the resultant amino acid substitution suggested a potential significance of this variant for the disease. Therefore we undertook further investigation of its prevalence in PV patients and normal individuals.

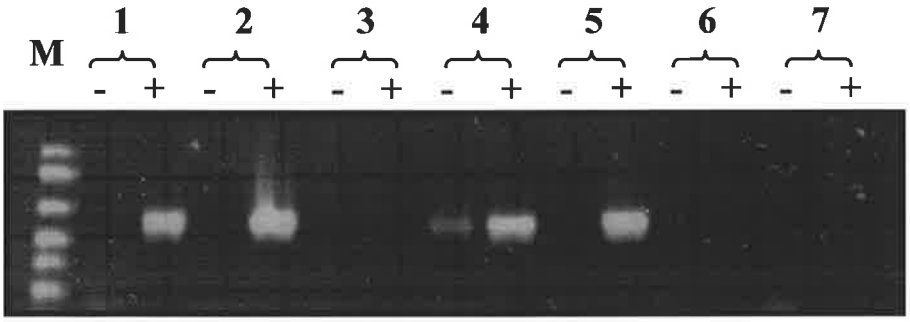


Figure 4.2 RTPCR of the *CSF2RB* target region from individual BFU-E. A one step RTPCR technique was used to amplify the target region of *CSF2RB* from individual BFU-E using primers *hbc3* and *hbc12*. Specificity was determined by the presence (+) or absence (-) of the reverse transcriptase enzyme in the reaction and products were cloned for sequencing only where a product was not obtained in the corresponding RT- reaction. Thermal cycling conditions used were 94°C, 30 secs followed by 35 cycles of 94°C, 45 secs; 58°C, 45 secs; 72°C, 40 secs. Lane numbers correspond to endogenous BFU-E samples from the PV patient MD (1-3) and EPO dependent colonies from the normal individual FS (4-7). The lane marked M contains DNA PLUS™ molecular weight marker.

Table 4.4 Sequence changes detected in the screen for *CSF2RB* alterations in BFU-E by RT-PCR

Sample	Phenotype	BFU-E #	Sequence changes	# clones	Total # clones sequenced
MD	PV	1	nil	7	
			Δ1041-1045	1	
			G1364A	1	
			A1256G	1	13
			G1111A	1	
			T1224C	1	
			T1383A	1	
		2	G1443T	13	13
FS	N	1	nil	9	
			T1536G	1	11
			A1132G/A1184G/C1484T	1	
		2	nil	5	5
		3	nil	3	4
			G1443T	1	

Table 4.5 Frequency of the G1443T alteration in cDNA clones isolated from PBMNC samples

PBMNC samples	Phenotype	Total number of clones sequenced	Number of G1443T clones	Frequency of G1443T (%)
MJ	PV	26	4	15.4
MD	PV	54*	3	5.6
JL	PV	20	0	0
AK	PV	13	0	0
HR	N	15	3	20.0
SP	N	37*	1	2.7
FS	N	38	0	0
PM	N	14	0	0
KM	N	37	0	0

* clones were derived from two separate PCR amplifications

Table 4.6 Frequency of G1443T in *CSF2RB* clones isolated from PBMNC genomic DNA

PBMNC samples	Phenotype	Total number of clones sequenced	Clones with G1443T	Frequency of G1443T (%)
MJ	PV	17	0	0
MD	PV	22*	0	0
HR	N	8	3	37.5

* clones were derived from two separate PCR amplifications

4.5 The G1443T base substitution

The low frequency of clones detected with the G1443T substitution in PBMNC and BFU-E of normal individuals suggests a low level of expression relative to the normal allele in individuals carrying this polymorphism. However, in the screen of BFU-E cDNA, G1443T was the *only* sequence detected in 13 clones derived from a single endogenous BFU-E. This PV patient also expressed the variant in PBMNC. To test whether G1443T is a polymorphism, the genomic DNA sequence at this position was determined in PV patients and normal individuals from PBMNC samples.

Genomic DNA was isolated from PBMNC samples that had previously shown the G1443T substitution in cDNA and the *CSF2RB* region surrounding G1443 was amplified by PCR using primers *hbc30* and *hbc31* (see Appendix A). PCR products were cloned into the pGEM 4Z vector and the sequence of a number of clones determined to identify the base at position 1443 (Table 4.6). It was predicted that the altered residue would be detected in approximately 50% of genomic DNA clones in individuals heterozygous for the G1443T polymorphism.

The frequency of PCR amplified genomic DNA clones containing the G1443T alteration in the normal sample (HR) was 37.5% thereby confirming the presence of a G1443T polymorphism in this individual. Surprisingly however, the G1443T substitution did not occur in numerous clones isolated from the PV patient samples MD and MJ (22 and 17 clones respectively). This was notable given that these samples gave rise to G1443T clones in the PBMNC cDNA screen and in the case of MD, also in BFU-E. This raises the possibility that in these 2 individuals, the G1443T alteration is not a polymorphism but is an acquired somatic mutation.

4.6 Discussion

The PBMNC screen for *CSF2RB* mutations by RT-PCR identified a number of sequence alterations that were present in multiple samples and independent PCR. These

included known splice variants and polymorphisms and additional novel variants, including a splice variant encoding a soluble form of hβc. However, apart from a single base substitution detected only in product from one PV patient (T1380C) none of these alterations appear to be specific to patients with PV. The low frequency of a PV-specific change may be explained by a low contribution of PV cells to the PBMNC samples, which could not be determined due to the absence of a PV specific marker.

In a direct approach to identifying a lesion in PV, endogenous BFU-E were screened for *CSF2RB* mutations by RT-PCR analysis. This analysis identified the G1443T substitution as the only sequence obtained in product from a single endogenous BFU-E isolated from a PV patient. This was in striking contrast with all other PCR data from PBMNC (normal and PV samples) and BFUE (FS #1) where this variant was identified at low frequency. Given that this result is obtained from a single endogenous BFU-E the finding requires confirmation in a separate PCR and in other BFU-E, however, it is consistent with either the presence of G1443T on both alleles (which is unlikely and inconsistent with the PBMNC data) or by a loss of heterozygosity (LOH) in this region in these cells.

The G1443T substitution was detected in PBMNC cDNA at a frequency of 5.6% in the same PV patient. It was also detected in 2 normal individuals and a second PV patient sample, consistent with its presence as a polymorphism. Genomic DNA analysis of PBMNC confirmed the polymorphism in a normal individual however interestingly, only wild type sequence was detected in the 2 PV patient samples that expressed G1443T. This raises the possibility that G1443T is an acquired lesion in these individuals. The G1443T base substitution results in a potentially important amino acid substitution at amino acid position 472 of the hβc protein. A number of approaches to resolving the nature of this substitution in PV and the impact of its expression on hβc function are discussed in Chapter 6.

The T1380C substitution identified in the PBMNC screen from the patient, MD, was not detected in product derived from the cDNA of 2 endogenous BFU-E from this individual. This alteration results an interesting amino acid substitution and is therefore discussed further in Chapter 6, however since any PV specific alterations would be expected to be present in endogenous BFU-E, the significance of T1380C in the disease remains unclear.

In summary, the screen for PV associated alterations of *CSF2RB* in PV patients PBMNC and endogenous BFU-E samples has identified 2 single base substitutions that result in non-conservative amino acid residue substitutions in critical regions of the hβc protein. Further studies will be required to determine their importance for the disease.

Chapter 5 Identification of a novel hβc splice variant, Δ1041-1045.

5.1 Introduction

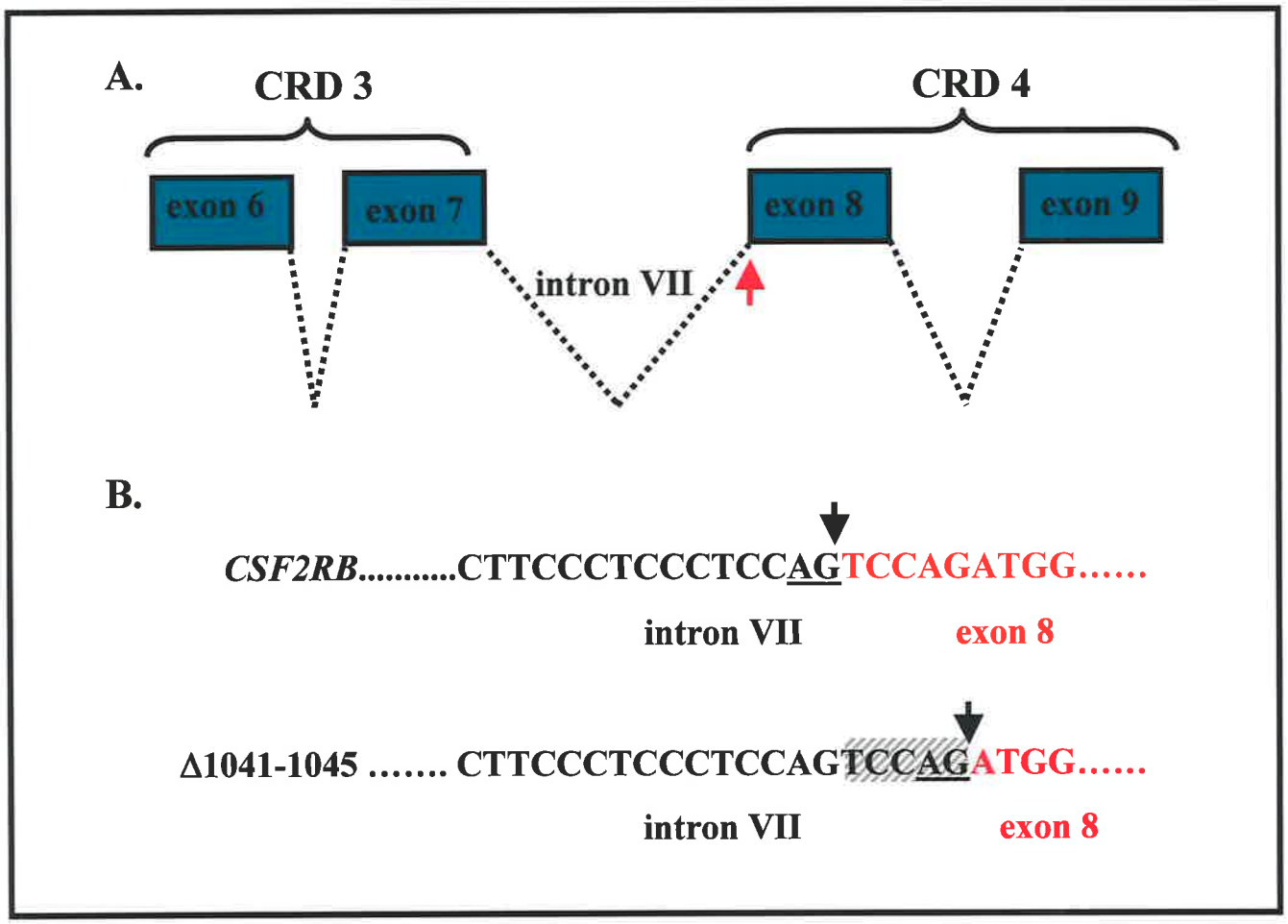
A 5bp deletion at position 1041 of the cDNA (Δ1041-1045) was detected in the screen for mutations within a target region of *CSF2RB*, occurring at an average frequency of 11.67 ± 4.33% (SEM) in PBMNC samples from 6 out of 9 individuals (see Chapter 4). Analysis of the structure and sequence of *CSF2RB* in this region suggests that the deletion arises as the result of an alternative splicing event at the junction of intron VII and exon 8 (Figure 5.1 A). It is likely that the 3' intron splice sequence (*AG*) that produces the major form of hβc has not been recognised, and a secondary *AG* sequence 5 bp downstream has been utilized, thereby generating a 5 bp deletion of the 5' end of exon 8, corresponding to nucleotides 1041-1045 of the major cDNA form (5.1 B).

Three consensus sequence elements within pre-mRNA are critical for cleavage and ligation reactions involved in the splicing process. These are consensus sequences at the 5' and 3' intron splice recognition sites and branch point sites within the intron. Alteration to these sequences may lead to alternative splice site recognition or selection. The splice recognition sequence at the intron VII/ exon 8 junction of the *CSF2RB* gene, AG/TCCAGA, is a poor match to the consensus splice recognition sequence AG/GUPuAGU which may explain mis-splicing at this site (Goldstrohm et al., 2001).

It was noted that one PBMNC sample derived from a PV patient (MD) initially gave rise to a high number of clones containing the Δ1041-1045 deletion. A frequency of 33.3% Δ1041-1045 clones (5 clones from a total of 15) were detected compared to a frequency of between 0 and 15.4% seen with all other PV and normal samples (Table

Figure 5.1 Genomic structure and sequence of the *CSF2RB* intron VII / exon 8 splice junction.

A. Exons encoding CRD 3 and 4 are shown as green boxes and adjoining dashed lines indicate the position of intron sequences. **B.** The genomic sequence of the intron VII/exon 8 splice junction is indicated by intron VII sequence (black) and exon 8 sequence (red). The splice recognition sequence (AG) utilised by the full length and Δ1041-1045 forms of hβc is underlined. The red arrow indicates the position of the intron VII and exon 8 splice junction and black arrows indicate putative splice sites. The sequence deleted from the cDNA of Δ1041-1045 is shaded grey.


A.
CRD 3
CRD 4
exon 6
exon 7
exon 8
exon 9
intron VII
B.
CSF2RB.....CTTCCCTCCCTCCAGTCCAGATGG.....
intron VII
exon 8
Δ1041-1045 ..... CTTCCCTCCCTCCAGTCCAGATGG.....
intron VII
exon 8

5.1). It was hypothesized that an increased frequency of Δ1041-1045 mRNA might be present in cells derived from the PV clone in this patient. To investigate this further, genomic DNA isolated from PBMNC from this patient was used to amplify the region of the *CSF2RB* intron VII / exon 8 splice junction to determine whether alteration to sequence at this site was contributing to the increased frequency of Δ1041-1045 mRNA in this patient.

The alternative splicing that gives rise to Δ1041-1045 mRNA results in a frameshift that generates 34 residues of novel peptide sequence. This sequence is unremarkable with regard to residue content and hydrophobicity and literature and database searches reveal that it bears no significant homology to any known protein. The Δ1041-1045 splice variant of *CSF2RB* has not been described previously, however given that Δ1041-1045 was detected in this study in RT-PCR products derived from several individuals it is likely that it is expressed at low level in normal PBMNC mRNA. Therefore, to confirm its expression and establish the potential biological significance of Δ1041-1045, further studies were conducted. A number of approaches were employed with the aim of more rapidly screening samples from a number of cell types and cell lines for the presence of the Δ1041-1045 splice form and to determine the level of Δ1041-1045 compared with the major mRNA form of *CSF2RB*. Demonstration of a significant level or of differential regulation would provide supportive evidence of a biological role for this alternatively spliced form of hβc.

5.2 Results

5.2.1 Screening for genomic mutations in the region of the *CSF2RB* intron VII/exon 8 splice junction

To determine whether a mutation in the 3' splice recognition sequence of intron VII may be affecting the frequency of alternative splicing in PV, this region was amplified from genomic DNA from PBMNC isolated from the patient MD and the PCR

Table 5.1 Frequency of Δ1041-1045 clones isolated by RTPCR amplification of the *CSF2RB* target region from PBMNC samples.

PBMNC sample	Phenotype	# total clones	# Δ1041-1045 clones	% Δ1041-1045
MD	PV	15	5	33.3
MD(2)	PV	39	1	2.6
AK	PV	13	0	0
JL	PV	20	1	5.0
MJ	PV	26	4	15.4
HR	N	15	2	13.3
SP	N	26	0	0
SP(2)	N	11	0	0
FS	N	38	2	5.3
PM	N	14	0	0
KM	N	59	4	6.8

Sequence data was obtained from the *CSF2RB* target region screen of PBMNC cDNA (see section 4.3).
(2) indicates that these clones were derived from a duplicate PCR reaction.

product cloned into the vector pGEM-4Z. To ensure that clones representing normal and PV-derived haemopoietic cells were sequenced, bi-directional sequence was determined for 33 clones (Table 5.2). 30 clones consisted of wild type *CSF2RB* sequence. No clones were detected that contained altered sequence at the 3' splice recognition site however, single base changes were identified in 3 individual clones, all within exon sequence. One of the mutations, A1081G, was also detected in the PBMNC RT-PCR screen in a single clone from a PBMNC sample isolated from a normal individual (see Figure 4.2) and most likely represents a polymorphism. The remaining two mutations (A1134G and A1148G) were not found in the PBMNC RT-PCR screen and may be attributable to Taq polymerase errors during PCR amplification.

Subsequent to this work, a second PCR amplification of the target region of *CSF2RB* was performed on the same PBMNC cDNA sample to confirm the increased frequency of Δ1041-1045 in the patient MD. The clones isolated from this PCR (MD(2)) did not reveal the same frequency of Δ1041-1045 (see Table 5.1) suggesting that the increased frequency of clones containing the Δ1041-1045 mutation in the initial PCR may have been due to preferential amplification in this reaction. In support of this, statistical analysis was performed on the final Δ1041-1045 sequence data derived from the entire PBMNC screen but with the exception of the initial MD results. The number of Δ1041-1045 clones obtained from 4 PV patients compared with 5 normal individuals was not significantly different ($p > 0.680$ by Pearson Chi-Square analysis and $p > 0.4$ by Fisher's Exact Test) and the number of Δ1041-1045 clones obtained was not significantly different between PV patient samples ($p > 0.12$ by Pearson Chi-Square analysis). Therefore, the Δ1041-1045 form of hβc appears to be expressed in both normal and PV PBMNC samples, is not increased in PV patients and is unlikely to be the result of an acquired lesion in genomic DNA in patients with PV.

5.2.2 Detection and quantitation of Δ1041-1045 mRNA

5.2.2.1 Δ1041-1045 amplification by RT-PCR

Table 5.2 Genomic DNA sequencing of the *CSF2RB* intron V11/ exon 8 splice site

Genomic DNA sequence change	Number of clones detected	cDNA sequence change	Amino acid sequence change
nil	30	nil	nil
A10728G	1	A1134G	D369G
A10675G	1	A1081G	G351G
A10742G	1	A1148G	I374V

Nucleotides 10506 to 10769 (a total of 263 bp) were amplified from a PBMNC genomic DNA sample from a PV patient (MD) using the primers *hbc22* and *hbc23* (see Appendix A) and the thermal cycling conditions 94°C, 30"; 54°C, 45"; 72°C, 30" for 40 cycles. The amplified product was cloned into pGEM4Z with a total of 33 clones isolated and sequenced.

A PCR strategy was designed to specifically amplify cDNA containing the 5 bp 1041-1045 deletion. A 5' primer *(hbc24)* spanning the 5bp deletion site with 4bp 3' of the deletion, and a reverse primer *(hbc12)* were designed to enable amplification of a 595 bp fragment from Δ1041-1045 DNA (Figure 5.2). As positive controls, highly diluted plasmid preparations of full length hβc and Δ1041-1045 clones were used (lanes 2 and 3, respectively) and additional reactions using the same plasmid DNA samples were performed with PCR primers (*hbc3* and *hbc12*) that together amplify the entire 619bp hβc target region. After 30 cycles of PCR, Δ1041-1045 plasmid DNA was specifically amplified using the *hbc24* and *hbc12* primer pair, however Δ1041-1045 was not detectable in PBMNC cDNA samples (lanes 4 and 5). When the number of cycles was increased to 35 or 40 to increase the sensitivity of the reaction, full length hβc plasmid DNA also became amplified (lane 2). Thus, the deleted form was not specifically detectable in PBMNC cDNA using a primer spanning the deletion.

5.2.2.2 Resolution of Δ1041-1045 PCR amplicons by PAGE

An alternative PCR strategy was utilised to produce a small amplicon enabling differentiation of Δ1041-1045 from the full length form based on differential mobility in 20% non-denaturing polyacrylamide gel electrophoresis. Two sets of primers were designed to produce amplicons of 111 bp (*hbc3* and *hbc21*) and 64 bp (*hbc3* and *hbc25*) from full length hβc cDNA encompassing the region of Δ1041-1045. These primers generated PCR products from plasmid DNA containing Δ1041-1045 of 106 bp and 59 bp respectively. The products generated using *hbc3* and *hbc21* primers from full length (111bp) and Δ1401-1045 (106bp) plasmid DNA were not easily distinguishable in gel electrophoresis (fig 5.3 A, lanes 2 and 3, respectively). However, the 59 bp fragment amplified from Δ1041-1045 plasmid DNA could be clearly differentiated from the 64 bp fragment amplified from full length plasmid using the *hbc3* and *hbc25* primer pairs (5.3 B, lanes 2 and 3). The primer dimer formed in the latter reaction in the absence of DNA (5.3 B, lane 1) migrates slightly faster than the 59bp amplicon and was found to

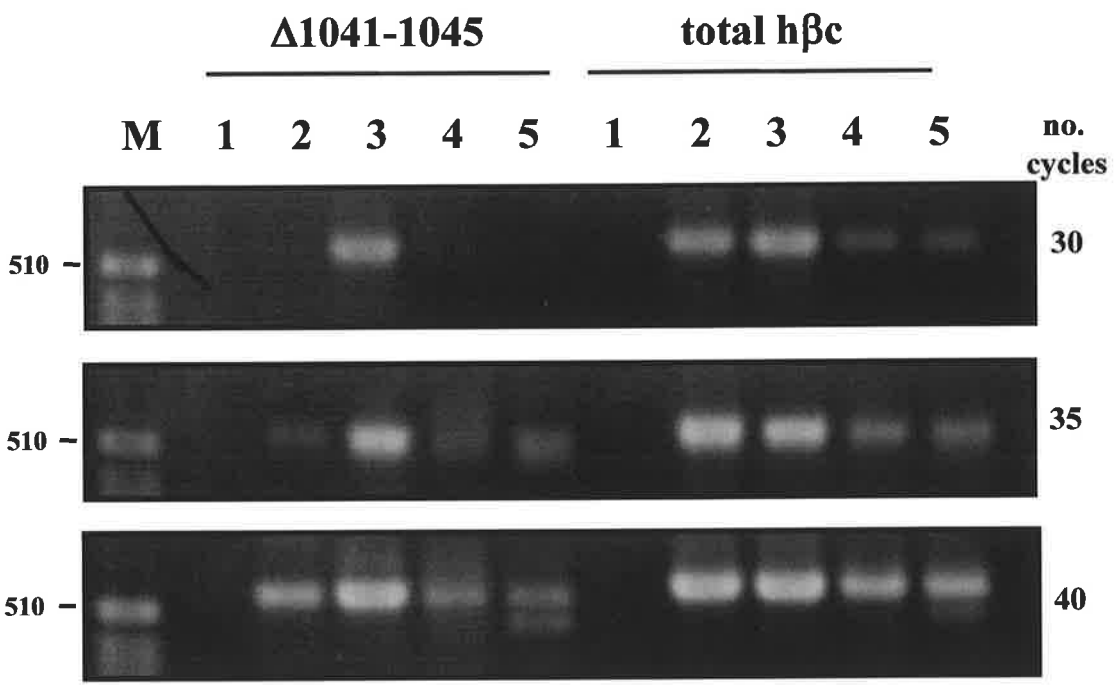


Figure 5.2 Detection of Δ1041-1045 by deletion specific RT-PCR.

cDNA was derived from PBMNC samples by RT reaction and the Δ1041-1045 form amplified by PCR using primer *hbc12* and the deletion specific primer *hbc24*. Total hβc was amplified from identical samples using primers *hbc3* and *hbc12*. Thermal cycling conditions used were 94°C, 30"; 55°C, 45"; 72°C, 45" with samples removed from the thermal cycler for gel analysis at 30, 35 and 40 cycles. Purified plasmid preparations of full length and Δ1041-1045 clones, diluted at 1:10000 with Milli-Q water were used as positive controls and a negative control reaction was performed containing no DNA. Lane numbers are (1) no DNA; (2) full length hβc plasmid DNA; (3) Δ1041-1045 plasmid DNA; (4) normal PBMNC cDNA; (5) PV patient PBMNC cDNA. The lane marked M contains 1Kb DNA molecular weight marker.

be detectable only in samples containing no DNA. Using both primer sets the Δ1041-1045 form of hβc was not detectable in total cDNA derived from PBMNC samples indicating that the level of Δ1041-1045 mRNA is significantly lower than full length hβc (5.3 A, lanes 4-12 and B, lanes 4-8).

In an attempt to determine the sensitivity of Δ1041-1045 detection by this method, highly diluted (1:10000) Δ1041-1045 and full length hβc plasmid DNA were mixed at various ratios prior to amplification using the primer pair *hbc3* and *hbc25*. Gel analysis indicates that at a ratio of 1:1 (full length hβc: Δ1041-1045) approximately equivalent amounts of product are obtained (5.3C, lane 4) but at ratios of 10:1 to 60:1 (full length hβc: Δ1041-1045) the deleted form was undetectable (5.3C, lanes 5-7). This result is consistent with Δ1041-1045 mRNA representing less than 50% of total hβc mRNA in PBMNC however the alternative form may exist at a much lower level.

Although this technique was not sensitive enough to detect Δ1041-1045 in total cDNA samples its presence was confirmed since 2 Δ1041-1045 clones from a total of 12 clones derived from RT-PCR amplification of the target hβc region from PBMNC samples were detected using primers *hbc3* and *hbc25* (fig 5.4, clones 15 and 22). The sequence of these clones was confirmed by bi-directional sequencing.

5.2.2.3 Detection of Δ1041-1045 by RT-PCR single nucleotide primer extension (SNuPE) assay

Although the previous technique confirmed that Δ1041-1045 cDNA could be specifically detected, screening numerous samples by cloning was not feasible. Therefore a more sensitive assay was developed to enable detection in total cDNA samples. The SNuPE reaction enzymatically extends by a single radiolabelled nucleotide, a primer that terminates at the 3' nucleotide adjacent to the site of interest (Singer-Sam and Riggs, 1993). Extension of the primer sequence can be demonstrated by resolution of products using polyacrylamide gel electrophoresis, followed by autoradiography or phosphoimage analysis and quantitation.

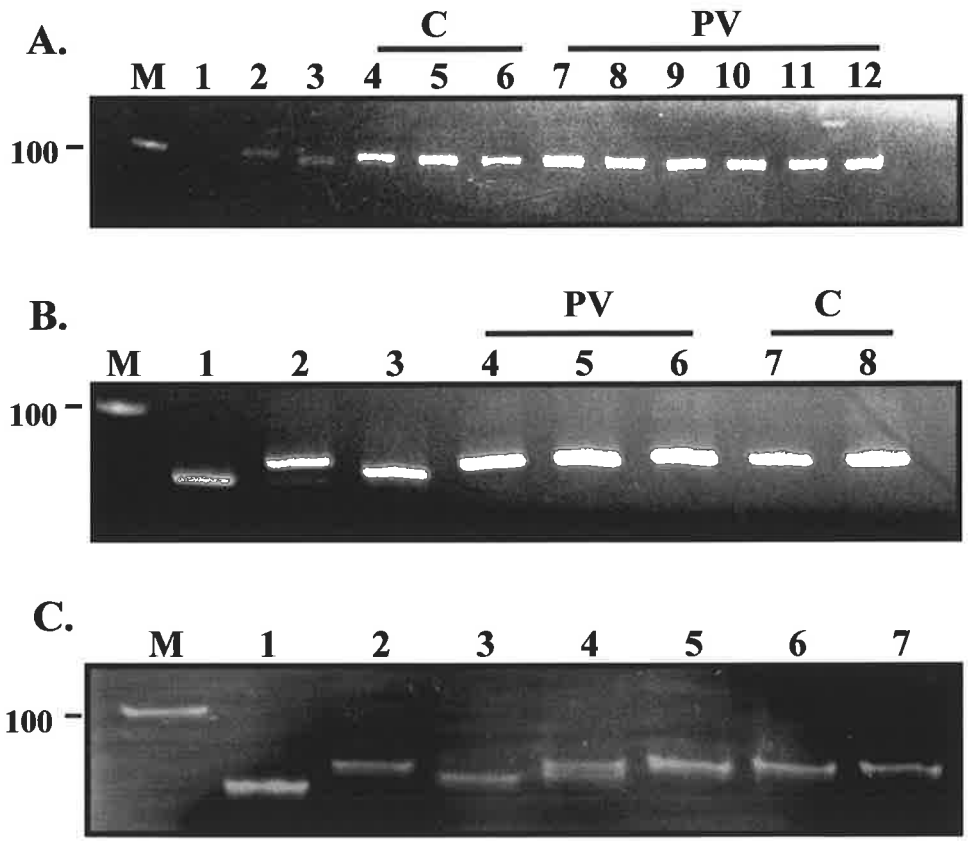


Figure 5.3 Detection of Δ1041-1045 by RTPCR and 20% PAGE analysis. cDNA was prepared by RT reaction from PBMNC samples from PV patients and control subjects. The Δ1041-1045 form of *CSF2RB* was distinguished from full length by amplification of a small region of cDNA encompassing the deletion using primer pairs *hbc3* and *21* (panel **A**) and *hbc3* and *25* (panels **B** and **C**). Thermal cycling conditions used were 94°C, 30"; 55°C, 45"; 72°C, 15" for 35 cycles. Products were resolved using 20% non-denaturing PAGE analysis and compared to samples containing no DNA (lane 1), full length *CSF2RB* plasmid DNA diluted to 1:10000 (lane 2) and Δ1041-1045 plasmid DNA diluted to 1:10000 (lane 3). The Δ1041-1045 form of plasmid DNA migrates slightly slower than the full length form. Δ1041-1045 was not detectable in samples containing total cDNA from either PV patient or control subject PBMNC cDNA samples using either *hbc3* and *hbc21* (111bp amplicon) or *hbc3* and *hbc25* (64 bp amplicon) primer pairs (**A**, lanes 4-12 and **B**, lanes 4-8, respectively). The sensitivity of Δ1041-1045 detection was determined by mixing Δ1041-1045 plasmid DNA with full length hβc plasmid DNA at various ratios (**C**). Lanes containing products derived from various ratios of full length: Δ1041-1045 plasmid DNA are (4) 1:1; (5) 10:1; (6) 20:1; (7) 40:1. Lanes marked M contain 1Kb PLUS DNA molecular weight markers.

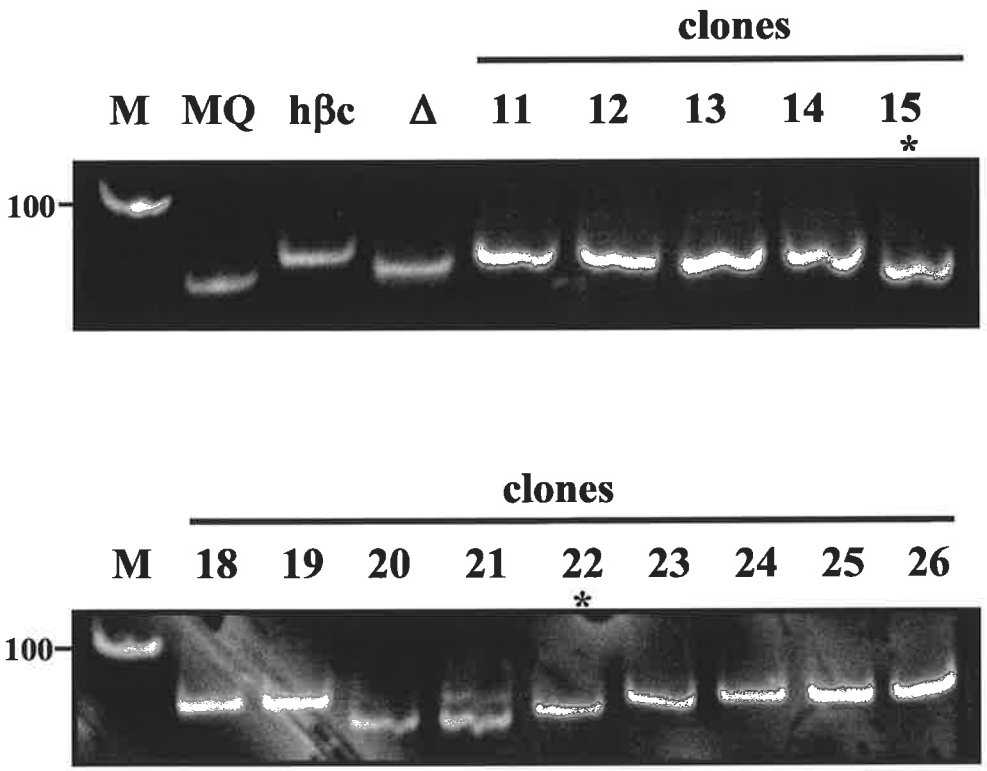


Figure 5.4 Detection of PBMNC cDNA clones containing Δ1041-1045 by RTPCR and 20% PAGE analysis

Clones obtained by RTPCR of the *CSF2RB* target region from PBMNC of a control individual were subjected to further PCR analysis using primers *hbc3* and *hbc25* and the thermal cycling conditions 94°C, 30"; 55°C 45"; 72°C, 15"for 35 cycles. 64 bp and 59 bp amplicons were obtained from clones containing full length *CSF2RB* and Δ1041-1045 sequences, respectively and were resolved using 20% non-denaturing PAGE analysis. These samples were compared to samples containing no DNA (MQ), full length *CSF2RB* plasmid DNA diluted to 1:10000 (hβc) and Δ1041-1045 plasmid DNA diluted to 1:10000 (Δ). Lanes labelled with an asterisk indicate clones containing Δ1041-1045 products as determined by differential migration in 20% PAGE analysis and confirmation by bi-directional sequencing. Lanes marked M contain 1Kb PLUS DNA molecular weight markers.

SNuPE reactions were performed using products obtained by RT-PCR amplification of the 619bp *CSF2RB* target region, or from a smaller amplicon of 111 bp (see section 5.2.2.2). For verification, initial assays were conducted using primers *hbc26* and *hbc27* that were extended following annealing to full length or Δ1041-1045 plasmid DNA, respectively (Figure 5.5, lanes 1 and 2). A band present in the full length *CSF2RB* plasmid sample (lane 2) of the *hbc27* primer extension appears to migrate slightly faster than bands obtained with other samples. Therefore extension of this primer appears to be specific as in the case of *hbc26* primer extension where a band was obtained from full length but not Δ1041-1045 plasmid DNA.

Using this approach both the full length and Δ1041-1045 forms of *CSF2RB* were detected in all RT-PCR samples derived from control and PV patient PBMNC samples (lanes 4-8). To semi-quantitate the amount of Δ1041-1045 cDNA in the PBMNC samples, a single primer (*hbc26*), was used in subsequent assays. The experiment shown in Figure 5.6 demonstrated that extensions using either 32P-dATP or 32P-dTTP were specific for full length and Δ1041-1045 cDNA template, respectively. This assay was termed quantitative-SNuPE (Q-SNuPE) and the qualitative and quantitative determination of Δ1041-1045 compared with full length *CSF2RB* mRNA was assessed using phosphoimage analysis. The accuracy of the quantitation was determined by mixing full length and Δ1041-1045 control plasmid DNA preparations at various ratios, followed by PCR amplification and Q-SNuPE assay. Δ1041-1045 plasmid DNA was detectable at $1/10^{th}$ of full length hβc input plasmid DNA (Figure 5.6, A and B) and calculation of the signal output ratios compared to actual DNA input ratios demonstrated that this assay is at least semi-quantitative at this level (Figure 5.6, C).

Subsequent experiments detected Δ1041-1045 mRNA in PBMNC samples from all 16 individuals tested at levels only slightly above the limit of detection for the Q-SNuPE assay (fig 5.7 A). Calculation of the ratio of full length hβc: Δ1041-1045 signal indicates a range of between 10:1 and 60:1, with an average of 33 ± 3.79: 1 (mean ±

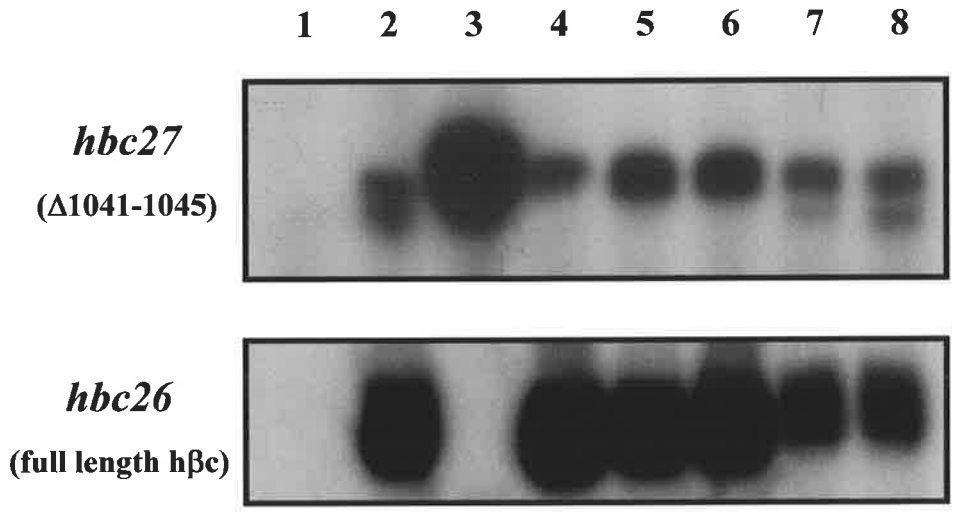
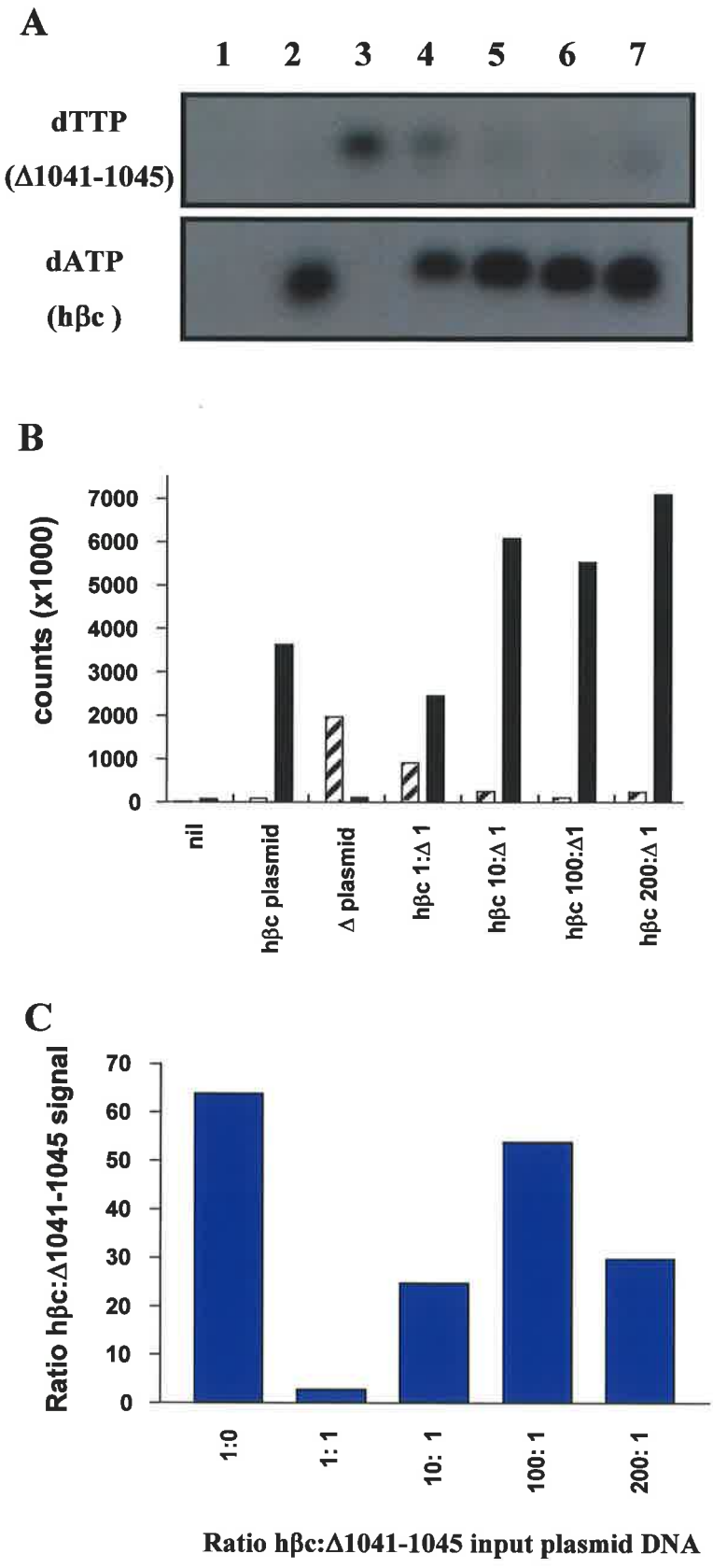


Figure 5.5 SNuPE analysis specifically detects Δ1041-1045 cDNA

PCR products were amplified from cDNA derived from PBMNC samples or from full length and Δ1041-1045 plasmid DNA using the primer pairs *hbc3* and *hbc12,* or *hbc3* and *hbc21* to yield 620 or 111 bp amplicons, respectively. These amplicons were used as template for extension of the primers *hbc26* (full length *CSF2RB*) or *hbc27* (Δ1041-1045) using ^{32}P-dATP and a single denaturation, annealing, and extension cycle of 94°C, 3'; 48°C, 1'; 72°C, 1'. The extended primers were resolved by 20% PAGE and exposed to X-ray film for 3 hours (*hbc26*) or 6 hours (*hbc27*). Lane numbers are (1) no DNA; (2) full length *CSF2RB* plasmid (620bp amplicon); (3) Δ1041-1045 plasmid (620bp amplicon); (4-6) PBMNC samples from control individuals (111bp amplicon) (7,8) PBMNC samples from PV patients (620bp amplicon).

Figure 5.6 Q-SNuPE analysis of Δ1041-1045 DNA is semi-quantitative

619bp PCR products were amplified from full length hβc and Δ1041-1045 plasmid DNA either alone or mixed together at various ratios using primers *hbc3* and *hbc12.* These were used as template for extension of the primer *hbc26* by a single nucleotide with either ^{32}P-dATP in the presence of full length hβc DNA, or with ^{32}P-dTTP in the presence of Δ1041-1045 DNA. **A.** Lanes are (1) no DNA; (2) full length hβc plasmid DNA; (3) Δ1041-1045 plasmid DNA and full length: Δ1041-1045 plasmid DNA mixed at (4) *1:1* (5) *10:1* (6) *100:1* (7) *200:1*. **B**. The intensity of each band (counts) corresponding to Δ1041-1045 (◪) and full length hβc (■) was determined by phosphoimage analysis using the Imagequant™ data analysis program. **C.** The ratio of full length *CSF2RB* signal compared to Δ1041-1045 signal was calculated from the counts obtained.


A
1 2 3 4 5 6 7
dTTP
(Δ1041-1045)
dATP
(hβc)
B
counts (x1000)
7000
6000
5000
4000
3000
2000
1000
0
nil
hβc plasmid
Δ plasmid
hβc 1:Δ 1
hβc 10:Δ 1
hβc 100:Δ1
hβc 200:Δ 1
C
Ratio hβc:Δ1041-1045 signal
70
60
50
40
30
20
10
0
1:0
1: 1
10: 1
100: 1
200: 1
Ratio hβc:Δ1041-1045 input plasmid DNA

SEM) for these samples. Therefore, these results confirm the presence of Δ1041-1045 mRNA at levels between 1.66 and 10% of full length *CSF2RB* mRNA (Figure 5.7, C).

Further Q-SNuPE assays were performed with the aim of detection and quantitation of Δ1041-1045 mRNA in other cell types. However in normal neutrophils, bone marrow, placenta and in the cell lines TF-1 (human erythroleukemia), Mo7e (human megakaryoblasic leukaemia)) and HEL900 (human erythroleukemia) the signal for the alternative splice form was below the background signal obtained from WT plasmid DNA only (fig 5.8). Therefore the presence of Δ1041-1045 mRNA in these cell types was not confirmed.

5.2.3 Δ1041-1045 encodes a soluble cytokine receptor

5.2.3.1 Rationale

The Δ1041-1045 deletion results in a frame shift which generates 34 amino acids of carboxyl-terminal sequence and truncates the receptor prior to the transmembrane domain (fig 5.9). The derived protein therefore lacks the transmembrane and cytoplasmic domains and constitutes a soluble form of hβc, consisting of CRD1, 2 and 3 of the full length receptor (fig 5.10). Given that Δ1041-1045 mRNA was detected at between 1 and 10% in PBMNC, it was postulated that this cell population may be generating a soluble form of hβc encoded by Δ1041-1045 and that this may be detectable in serum samples.

5.2.3.2 Soluble hβc protein detection by ELISA

An immunological assay was utilised in an attempt to detect the putative soluble form of hβc encoded by Δ1041-1045 mRNA in human serum samples.

Antibodies specific for hβc were used in a sandwich type ELISA technique previously developed to detect recombinant soluble hβc (McClure et al., 2003). This assay was undertaken in the current study for detection of soluble hβc protein in human serum samples that may be present due to the Δ1041-1045 mRNA identified in PBMNC samples. A total of 36 serum samples from normal and diseased individuals, including

Figure 5.7 Q-SNuPE detection of Δ1041-1045 cDNA from normal and PV PBMNC samples

The 619bp target region of *CSF2RB* was amplified from PBMNC samples from several PV patients and normal controls. Q-SNuPE was performed using primer *hbc26* and extension with either ^{32}P-dTTP to detect Δ1041-1045 cDNA (panel A) or with ^{32}P-dATP to detect full length *CSF2RB* cDNA (panel B). Controls included in the analysis were; no DNA (nil) and amplified products from full length *CSF2RB* (hβc plasmid) and Δ1041-1045 (Δ plasmid) plasmid DNA. The counts obtained for each sample were determined by phosphoimage analysis using Imagequant™ data analysis. The ratio of full length: Δ1041-1045 signal was determined for each sample (panel C).

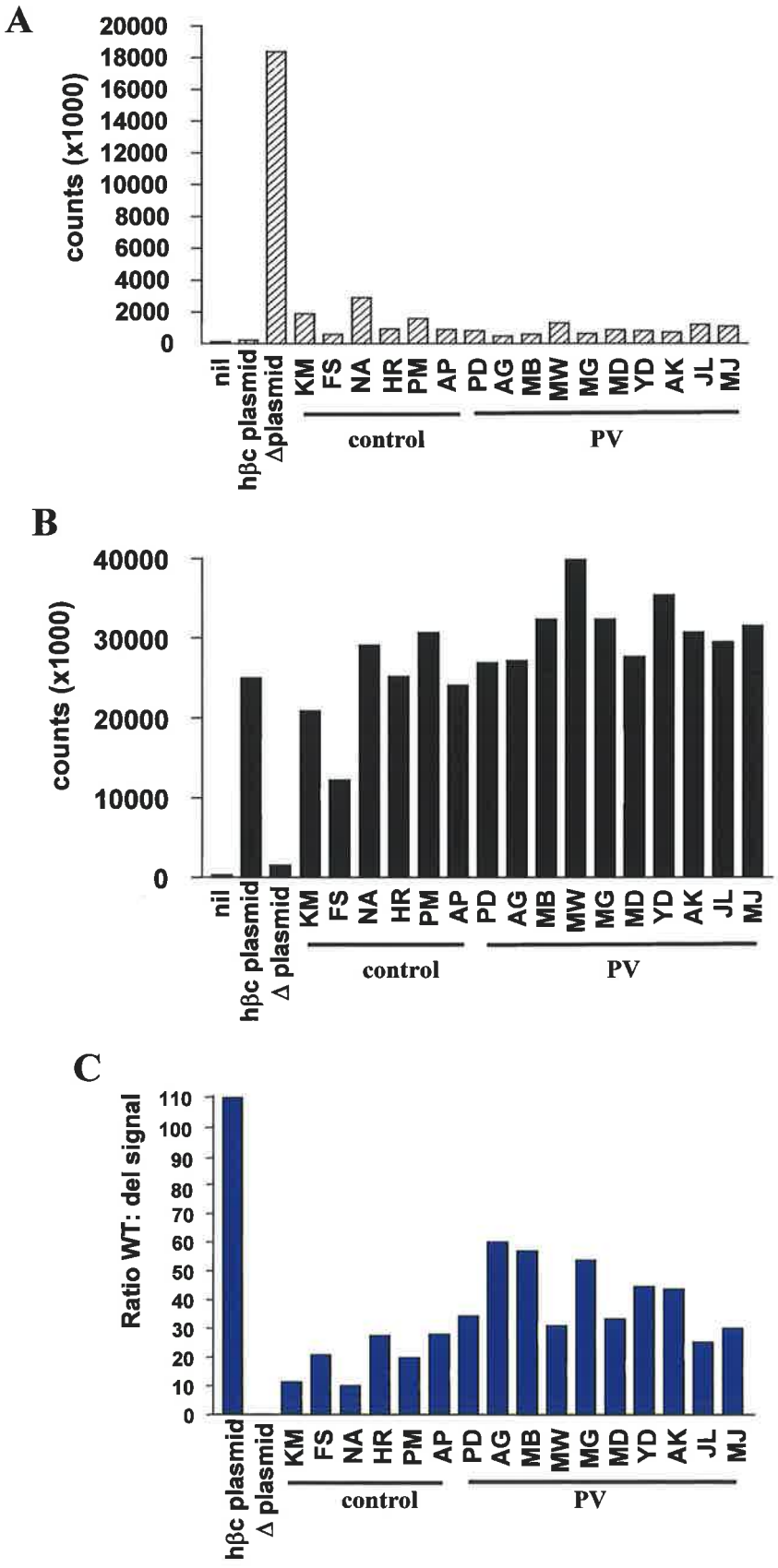
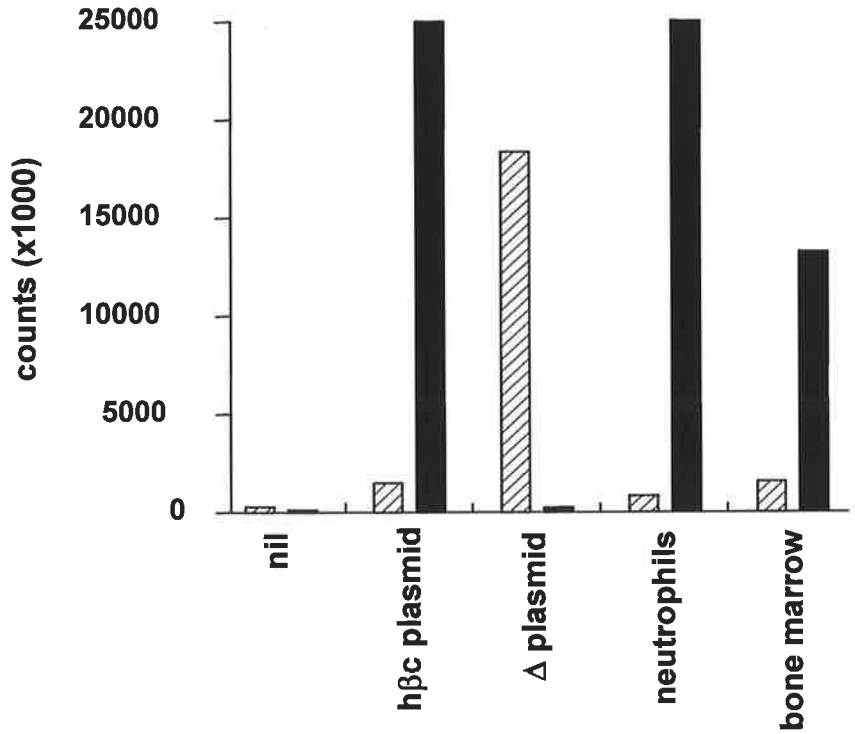

A
counts (x1000)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
nil
hβc plasmid
Δplasmid
KM
FS
NA
HR
PM
AP
PD
AG
MB
MW
MG
MD
YD
AK
JL
MJ
control
PV
B
counts (x1000)
40000
30000
20000
10000
0
nil
hβc plasmid
Δ plasmid
KM
FS
NA
HR
PM
AP
PD
AG
MB
MW
MG
MD
YD
AK
JL
MJ
control
PV
C
Ratio WT: del signal
110
100
90
80
70
60
50
40
30
20
10
0
hβc plasmid
Δ plasmid
KM
FS
NA
HR
PM
AP
PD
AG
MB
MW
MG
MD
YD
AK
JL
MJ
control
PV

Figure 5.8 Q-SNuPE detection of Δ1041-1045 cDNA from various human tissues and cell lines.

The 619 bp target region of *CSF2RB* was amplified from the cDNA of normal human neutrophils, bone marrow and placenta and also from the cell lines TF-1, Mo7e and HEL900 and used to quantitate the level of Δ1041-1045 mRNA compared with full length *CSF2RB* mRNA in 2 separate assays (panels A and B). The primer *hbc26* was extended by either ^{32}P-dTTP to detect Δ1041-1045 cDNA (◪) or ^{32}P-dATP to detect full length *CSF2RB* cDNA (■). Controls included in the analysis were; no DNA (nil) and amplified products from full length *CSF2RB* (hβc plasmid) and Δ1041-1045 (Δ plasmid) plasmid DNA. The counts obtained for each sample were determined by phosphoimage analysis using the Imagequant™ data analysis program.

A


25000
20000
15000
10000
5000
0
counts (x1000)
nil
hβc plasmid
Δ plasmid
neutrophils
bone marrow

B

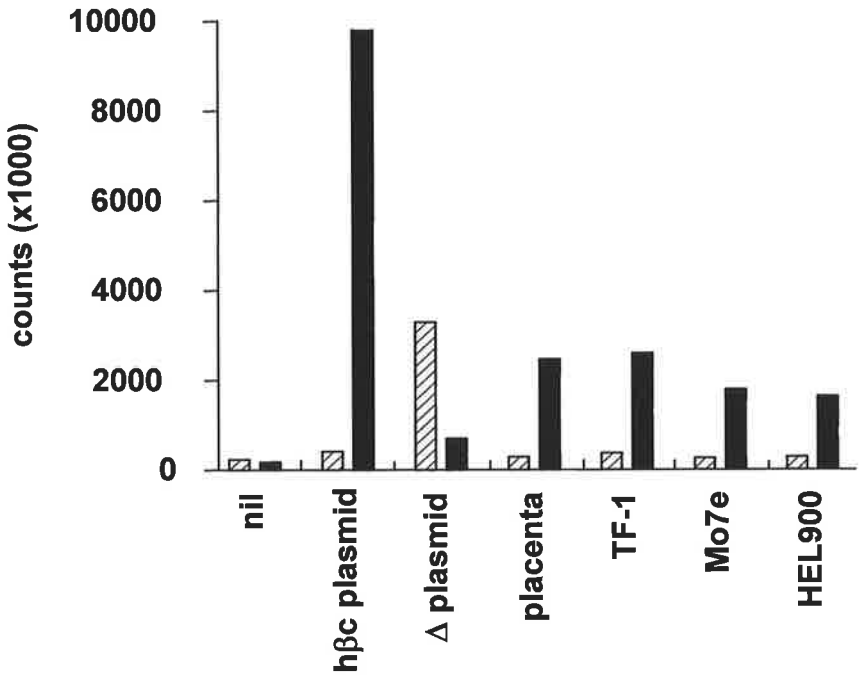
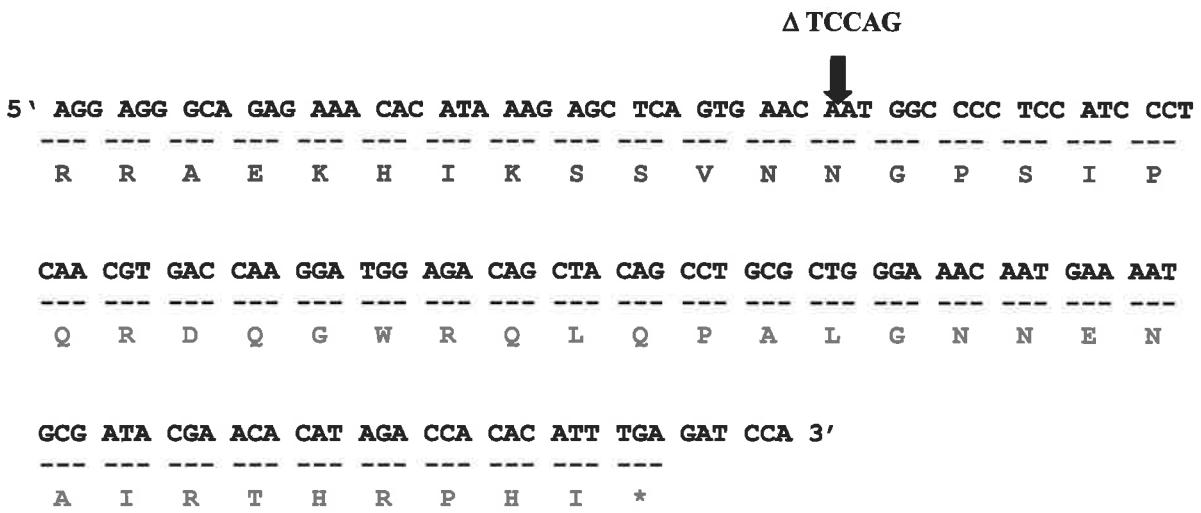

10000
8000
6000
4000
2000
0
counts (x1000)
nil
hβc plasmid
Δ plasmid
placenta
TF-1
Mo7e
HEL900

Figure 5.9 cDNA and amino acid sequence of Δ1041-1045.

The 5' to 3' cDNA sequence of Δ1041-1045 extending from nucleotides 1005 to 1149 is shown in black lettering. Amino acid residues are shown blue where the sequence is full length hβc and red to indicate the deduced Δ1041-1045 amino acid sequence. A red asterisk denotes a stop codon. The 5 bp deletion occurs at position 1041 as indicated by the red arrow.


Δ TCCAG
5' AGG AGG GCA GAG AAA CAC ATA AAG AGC TCA GTG AAC AAT GGC CCC TCC ATC CCT
R R A E K H I K S S V N N G P S I P
CAA CGT GAC CAA GGA TGG AGA CAG CTA CAG CCT GCG CTG GGA AAC AAT GAA AAT
Q R D Q G W R Q L Q P A L G N N E N
GCG ATA CGA ACA CAT AGA CCA CAC ATT TGA GAT CCA 3'
A I R T H R P H I *

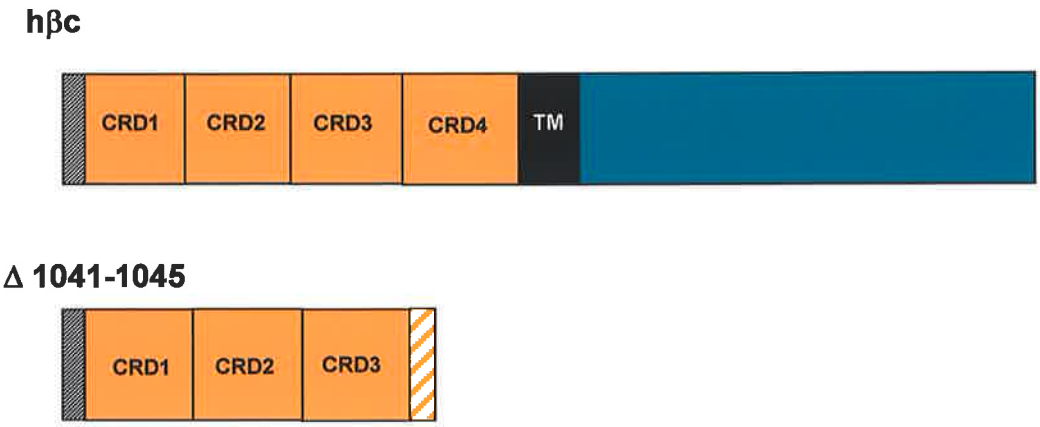


Figure 5.10 The predicted protein structure of Δ1041-1045 compared to full-length hβc.

Schematic representation of the predicted structure of Δ1041-1045 compared to full length hβc. Motifs are indicated as follows; (▨) signal sequence; (■) extracellular domain; (■) transmembrane domain; (■) cytoplasmic domain; (▨) novel 34 amino acid tail.

PV patients, were tested for the presence of soluble hβc. Recombinant shβc was detectable at a concentration of 155 pg/ml as shown by standard curve analysis however, soluble hβc was not detectable in any serum samples (fig 5.11). Therefore the secretion of protein encoded by Δ1041-1045 mRNA into human serum was not confirmed.

5.3 Discussion

The generation of a novel splice variant of *CSF2RB*, Δ1041-1045, was confirmed at low frequency in PBMNC cDNA samples from several individuals. This form arises due to an alternative splicing event at the intron VII/ exon 8 junction of the gene, resulting in a 5 bp deletion at position 1041-1045 of the cDNA sequence that is predicted to encode a soluble form of the hβc protein. A soluble form of hβc has not been previously described, however soluble isoforms exist for a number of other haemopoietic growth factor receptors. The aim of this part of the study was to gain supportive evidence of the biological significance of Δ1041-1045 by determining the abundance of Δ1041-1045 mRNA relative to total hβc mRNA, investigating the differential expression of this variant in various haemopoietic cell types and attempting to detect the putative protein in human serum samples. The potential mechanisms by which such a protein could contribute to receptor activation pathways will be discussed in Chapter 6.

RT-PCR of the hβc target region followed by cloning and sequencing generated a number of Δ1041-1045 clones derived from several PBMNC samples. This procedure confirmed the presence of the splice variant in several PV and normal individuals however, a simpler and more rapid detection system was sought. Several PCR-based assays were employed to specifically detect and quantitate the Δ1041-1045 form in cDNA samples. Plasmid DNA containing the Δ1041-1045 form was detectable by PCR using a specific primer, however an increased cycle number was required to detect this

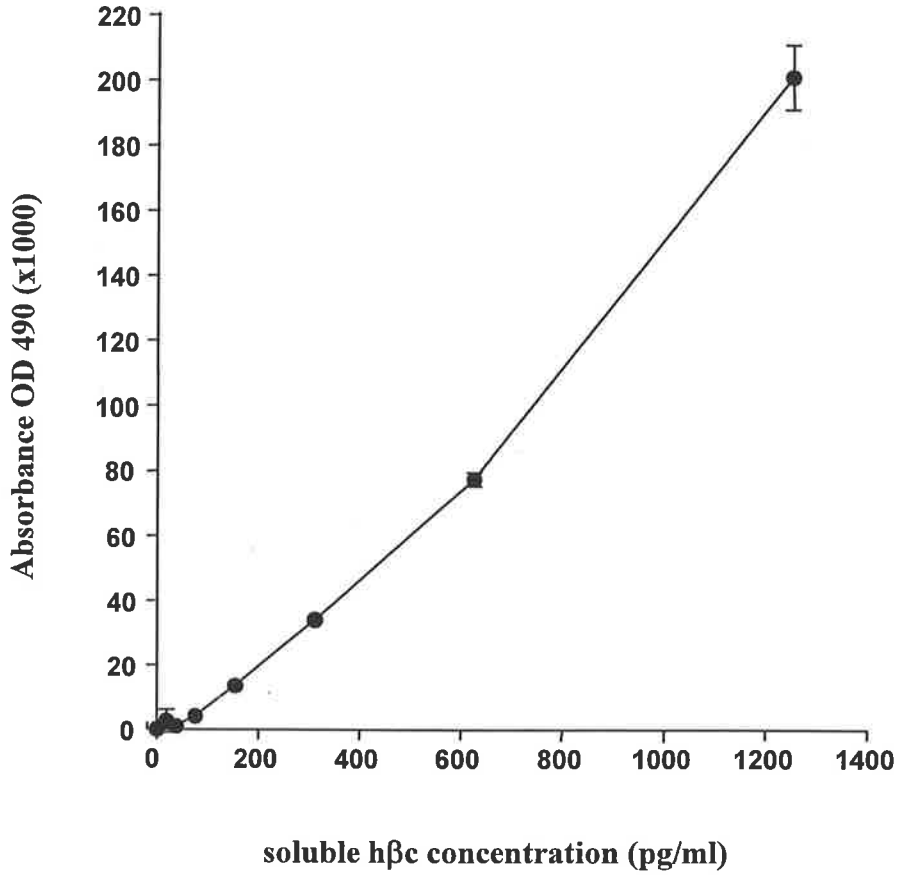


Figure 5.11 ELISA detection of soluble hβc.

The sensitivity of the ELISA detection of soluble hβc was determined using standard curve analysis of recombinant soluble hβc at concentrations within the range of 19 to 1250 pg/ml. Points represent the mean of duplicate absorbance determinations at 490 nm ± SD. The graph is representative of 2 separate ELISA assays.

form in total cDNA samples and under these conditions, specificity was lost as full length hβc plasmid DNA also became amplified.

In a second approach, PCR primer pairs were designed to generate small products that could be differentially resolved by gel electrophoresis. Full length hβc clones isolated by RT-PCR and T vector cloning, were distinguishable from those containing the Δ1041-1045 variation, however the deletion form was again not detectable in samples of total PBMNC cDNA.

SNuPE analysis was more sensitive than the previous methods employed and confirmed the presence of Δ1041-1045 in total PBMNC cDNA samples, although at low level. This assay was shown to be semi-quantitative and although the Δ1041-1045 signals obtained were only slightly above background, it is estimated from this and the PCR data that Δ1041-1045 mRNA represents less than 10% of total hβc mRNA in these cells.

Differential expression of Δ1041-1045 compared with full length hβc mRNA in other cell types would give further insight into a possible role for the alternative form. Other cell types, included in the SNuPE analysis were neutrophils, bone marrow, placenta and the cell lines TF-1, Mo7e and HEL900, all of which express the full length form of hβc (Hayashida et al., 1990; Li et al., 1995; Diamond et al., 2001). However, since Δ1041-1045 mRNA was not detectable in these cell types in these experiments, its presence was not confirmed.

Whilst several of these approaches were successful in detection of the Δ1041-1045 form in PBMNC cDNA samples, all relied upon an initial PCR amplification of the target region of *CSF2RB*. Thus, the potential for preferential amplification by PCR based methods is of concern although the Δ1041-1045 variant has been detected in several independent samples and experiments. Alternative approaches such the RNase protection assay or Real Time PCR technology may be useful to gain improved

accuracy and sensitivity in the quantitation of low level mRNA species such as Δ1041-1045.

The putative protein encoded by Δ1041-1045 is predicted to be a soluble form of hβc and therefore may be secreted by cells. Soluble forms of several HGF proteins have been previously described. For example, soluble GM-CSFRα is present in serum at a concentration of 36 ± 17 pmol (Sayani et al., 2000) and soluble IL-6 receptor is found at 50-80 ng/ml in peripheral blood (Peters et al., 1998). An ELISA specific for soluble hβc was performed to detect Δ1041-1045 derived protein in human serum samples. A standard curve using recombinant shβc demonstrated a detection limit of 155pg/ml however, serum samples had no detectable protein. This result may not be surprising given that Δ1041-1045 mRNA levels appear to be between 1 and 10% of total hβc mRNA, however the presence of such a protein in serum below a concentration of approximately 150pg/ml remains possible.

The Δ1041-1045 splice variant of hβc was shown to be a novel, low level mRNA species in samples obtained from all 16 haematologically normal and PV individuals, analysed by a variety of techniques. Its expression does not appear to be altered in PV. Semi-quantitative analysis indicates that this variant is present at levels between 1 and 10% of total hβc mRNA in PBMNC, however its presence in other cell types was not confirmed.

Chapter 6 Concluding Discussion

6.1 Outline

The aim of this study was to determine whether mutations in the gene encoding hβc (*CSF2RB*) contribute to the disease Polycythemia Vera. There is convincing but indirect evidence that hβc may be involved in PV pathogenesis. The pronounced hypersensitivity of PV progenitors to IL3 and GMCSF and the emerging evidence of hβc involvement in crosstalk with other receptors, a mechanism that could explain how signalling responses of multiple receptors are affected in the disease, have been discussed in Chapter 1, sections 3.4 and 3.5. Further evidence that hβc mutations may be involved in PV has been obtained from experiments with mice expressing mutant forms of hβc. These mice develop a PV-like myeloproliferative disease, characterised by expansion of multiple haemopoietic lineages and the hypersensitivity of progenitors to multiple HGFs (Chapter 1, sections 3.3.and 3.5).

An extensive screen of a 620bp target region of *CSF2RB* by RT-PCR, using PBMNC RNA identified only one putative PV-specific lesion, which was a single base substitution (T1380C). This alteration occurred in 2 clones of 15 isolated from a single PV patient RT-PCR product. PV PBMNC samples may contain a low frequency of cells derived from the PV clone, therefore despite the large number of clones sequenced in the study, detection of PV specific alterations is expected at low frequency and may be difficult. Thus, to confirm the presence of the T1380C change and to increase the possibility of detecting any further PV specific lesions in the disease, the *CSF2RB* target region was screened in cDNA derived from endogenous BFU-E. This approach was important as it allowed cells derived from the PV clone to be screened in isolation, thereby overcoming the putative low frequency of such cells in the PBMNC population. However, this proved to be a difficult undertaking technically, since BFU-E have been

shown to express only small amounts of hβc (Billia et al., 2001) and consist of only between 50-100 cells. Sequence was analysed from several clones derived from RT-PCR products from 5 individual (2 PV and 3 normal) BFU-E.

The T1380C alteration that was detected in the PBMNC screen as possibly PV specific, was not detected in endogenous BFU-E from the same patient and therefore the significance of this alteration for PV remains unclear. Further BFU-E RT-PCR and genomic DNA analysis will be required to confirm the importance of this change. A single base substitution, G1443T, that was detected at low frequency in clones derived from several normal and PV PBMNC samples was represented at high frequency in clones from RT-PCR product derived from an endogenous BFU-E. This raises the possibility that it may represent an important lesion in PV. To pursue this further, the frequency of the G1443T allele in genomic DNA and cDNA samples from normal and PV PBMNC was determined. These results and the potential effects of this substitution on hβc function are discussed in sections 6.6 and 6.7.

In addition to the above changes, possibly associated with PV, a novel alternatively spliced variant (Δ1041-1045) encoding a soluble form of hβc was detected at low level in several normal individuals and PV patients. Further analysis using a more sensitive and semi-quantitative detection method confirmed the presence of this variant in all normal and PV PBMNC samples tested although it was not detected in other cell populations. The putative protein encoded by this form was undetectable in serum samples using a sensitive ELISA method. In the sections below (6.2 and 6.4) the potential importance of this variant is discussed.

6.2 Splice forms of hβc

Alternative splicing of pre-mRNA is one of several mechanisms whereby protein diversity is achieved in eukaryotic cells. It has been predicted that at least 20,000 human genes undergo alternative splicing which results in the production of

several mRNA species from each gene. These gene products may be differentially expressed in various cell types and can yield proteins of diverse functions (reviewed in Modrek et al., 2001). Thus, the process of alternative splicing provides an important mode for tissue specific regulation of gene expression and contributes significantly to genetic diversity.

The cDNA for hβc was originally cloned in 1990 and a number of splice variants of the receptor were reported (Hayashida et al., 1990). The 620 bp target region examined in this study spans the location of two of these variants, a 36bp insertion in the extracellular domain, and a 104bp deletion occurring in the cytoplasmic domain of the receptor. In this study I detected these two variants and the frequent polymorphism, C1306T, previously identified in SSCP studies with AML and JMML samples (Freeburn et al., 1998; Freeburn et al., 1997; Freeburn et al., 1996), thereby confirming their presence in both normal individuals and PV patients.

6.3 A novel splice form of hβc, Δ1041-1045

In addition to known splice variants, a novel splice form (Δ1041-1045) was detected at low frequency in RT-PCR products derived from PBMNC of 19 individuals (normal and PV patients). The putative structure of this variant and other previously reported splice variants of hβc are summarised in Figure 6.1.

The Δ1041-1045 5bp deletion in hβc cDNA is the result of an alternative splicing event at the junction of intronVII and exon 8 of the *CSF2RB* gene, causing a frameshift and generating novel C-terminal amino acid sequence. The resultant protein consists of CRD1, 2 and 3 of the extracellular domain of hβc, followed by 33 amino acids of novel, unremarkable sequence. Thus, the protein encoded by Δ1041-1045 mRNA is predicted to represent a soluble form of the receptor. Although soluble forms have been described for many other cytokine and HGF receptors, a soluble form of hβc

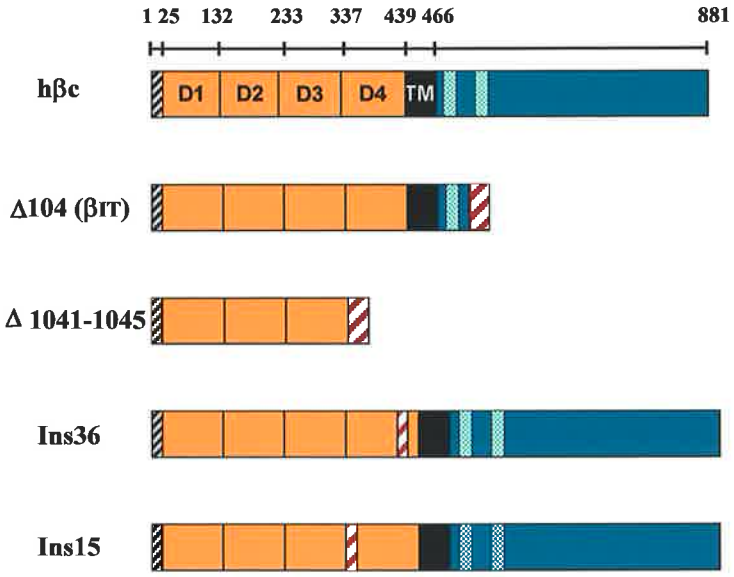


Figure 6.1 The structure of hβc splice variants

The domain structure of hβc and splice variant forms identified in the current study are shown. Amino acid positions of conserved domains are indicated at the top of the figure. Conserved domains and motifs are schematically represented as follows; (▨) signal sequence; (■) extracellular domain consisting of CRD 1-4; (■) transmembrane domain; (■) cytoplasmic domain; (■) box 1 and 2 motifs. Novel sequences generated by alternative splicing are indicated by (▨).

has not been previously identified. The potential role of this variant of hβc is discussed in the following section.

6.4 Soluble HGF receptors

Soluble HGF receptor isoforms are generally highly conserved among species suggesting they have biological significance. They are, almost without exception, created either by the proteolytic cleavage of an expressed membrane-bound receptor, or by alternative mRNA splicing resulting in a specific transcript encoding a soluble receptor (reviewed in (Heaney and Golde, 1996)). For example, the soluble receptors for TNF, IL-1, IL-2, and PDGF (Smith et al., 2003; Loppnow et al., 2002; Tiesman and Hart, 1993; Kim et al., 2001; Breunis et al., 2003) are derived by proteolytic cleavage, whereas the soluble receptors for GM-CSF (Ashworth and Kraft, 1990; Hu and Zuckerman, 1998; Raines et al., 1991), G-CSF, IL-4, IL-5, IL-7, IL-9, LIF, EGF, IFNα, TPO and EPO (Korte et al., 2000; Gessner and Rollinghoff, 2000; Renauld et al., 1992; Pitard et al., 1998; Ku et al., 1996a; Fukunaga et al., 1990; Liu et al., 2002; Baynes et al., 1993) arise by alternative splicing. Most of these alternatively spliced soluble receptors arise by exclusion of the exon encoding the transmembrane domain (for example GMRα soluble receptors). However, other soluble receptor isoforms such as those for IL-4R, IL-5R and LIF arise similarly to Δ1041-1045 where alternative splicing results in protein termination before the transmembrane exon.

The potential importance of soluble cytokine receptors in disease pathology is suggested by an increase in serum concentration in some disease states. For example, soluble IL-2Rα is elevated in human serum in a number of disorders including asthma, rheumatoid arthritis, Hodgkin's lymphoma, CLL, and numerous solid tumours (Chrobak, 1996; Chilosi and Pizzolo, 1995; Rose et al., 1994). Plasma concentrations of soluble gp130 are elevated in patients with systemic sclerosis (Hasegawa et al., 1998; Hasegawa et al., 1999), multiple sclerosis (Padberg et al., 1999) and chronic renal

failure (Frieling et al., 1999). Soluble IL-6R is similarly increased in multiple myeloma, where it has been shown to increase the sensitivity of myeloma cells to IL-6 by 10 fold, indicating a role for this receptor in disease pathogenesis (Barille et al., 2000). It has also been shown that the expression of soluble forms may be differentially regulated from the membrane bound form of the receptor. For example, membrane associated and soluble GMRα are independently regulated in HL-60 cells, possibly as a result of preferential mRNA splicing (Heaney et al., 1995).

Soluble receptors have been shown to alter HGF receptor responses by several mechanisms including facilitating receptor activation, playing an antagonistic role or acting as soluble binding proteins that protect the cognate ligand from degradation or clearance in the extracellular space. For example, the soluble form of growth hormone receptor (GHR), GH binding protein (GHBP), prolongs the half-life of its ligand and is thought to stabilize GH levels during fluctuations of hormone secretion (Baumann, 2002). Soluble receptors can also facilitate ligand mediated signalling as seen in the IL-6 receptor family where soluble IL-6 receptor confers increased sensitivity to IL-6 in cells expressing gp130 (Jones and Rose-John, 2002). Further, a sIL-6R/IL-6 complex has been shown to trigger transmembrane gp130 dimerisation and signalling in the absence of membrane anchored IL6R (Jostock et al., 2001). Alternatively, a number of soluble cytokine receptors have been shown to mediate antagonistic responses by sequestering ligand from the membrane bound form of the receptor, as in the case of soluble GMRα (Brown et al., 1995).

In multi-subunit receptor families such as the IL-3, IL-5 and GM-CSF receptor family where signalling occurs following formation of complexes containing ligand and α and β subunits, the action of soluble receptor components may be complex. With regard to hβc, it has been demonstrated that an engineered soluble form of hβc (sβc) comprising the entire extracellular region (CRD1-4) but lacking the transmembrane and cytoplasmic domains formed di-sulphide linked homodimers and associated with

membrane anchored GMRα in transfected cells. However, this complex could not bind GM-CSF with high affinity as seen with the full length hβc:GMRα complex. Therefore, this soluble form produced from cells by transfection, may have the capacity to sequester GMRα from the full hβc form. sβc was also shown to associate with soluble GMRα and this complex was able bind GMCSF, an interaction that could be inhibited by a blocking monoclonal antibody to CRD4 of the receptor. Thus, association of this soluble form of hβc with soluble GMRα can occur and may sequester GMCSF and reduce its availability to membrane anchored GMCSF receptor complexes. (Woodcock et al., 1997; McClure et al., 2003).

The soluble form of hβc encoded by Δ1041-1045, however, lacks CRD4. Since residues in this domain are critical for ligand binding and association with GMRα (see Chapter 1, section 3.1) the interaction of Δ1041-1045 with other receptor components or ligand seems unlikely. Thus, with our current understanding of receptor activation mechanisms, it is difficult to predict how the soluble Δ1041-1045 form might affect hβc signalling. Further study of this isoform may not be justified unless detection of the novel mRNA splice form or the derived soluble protein is observed at significant levels in specific cell types or in disease states.

To address this point some preliminary investigations were carried out. Quantitation of the Δ1041-1045 mRNA levels in a number of other cell types was attempted and serum samples were assayed for the presence of the putative soluble protein. Although Δ1041-1045 mRNA was detected in PBMNC samples from all 19 individuals tested (normal and PV), the level of this form was between 1 and 10% of the total hβc mRNA. The methods employed for this were at the limit of detection and the Δ1041-1045 splice variant was not detected in several other haemopoietic cell types. Additionally, an ELISA technique that detected recombinant soluble hβc at 150pg/ml did not detect soluble hβc in human serum samples. Clearly, proteins can have biological activity at concentrations below this level (eg. sgp130 serum concentration is

in the range 100-300ng/ml), therefore further attempts to detect protein expression may be warranted (Hasegawa et al., 1998).

6.5 Possible PV associated forms of hβc

Two interesting changes in *CSF2RB* sequence that are potentially important for PV were detected in the RT-PCR PBMNC screen of 4 PV patients and 5 control individuals. A G1443T substitution was found in several clones derived from 2 normal and 2 PV patient products. This substitution was also present at increased frequency in RT-PCR product derived from an endogenous BFU-E (see section 6.6). Only one nucleotide alteration (T1380C) was detected in the PBMNC RT-PCR screen that was specific for PV. Since this change was detected in only one PCR product, its significance must be clarified by screening of independent RT-PCR samples from this patient. The presence of these changes at such low frequency in this screening approach may not be surprising given that the frequency of cells derived from the PV clone in PBMNC populations could be quite low. All patients contain endogenous BFU-E in peripheral blood but at variable frequency (see Table 3.3). Although numerous clones were screened for each PBMNC RT-PCR product, there may be only a small possibility of detecting an alteration specific to the PV clone. To overcome this limitation, a screen of the same target region in individual endogenous BFU-E was undertaken.

6.6 Screening BFU-E for *CSF2RB* alterations

The amplification of cDNA derived from individual colonies grown in methylcellulose, and indeed from single cells, has been reported previously (Chiang and Melton, 2003; Kishino et al., 2002). It has also been previously shown that endogenous BFU-E are clonal and therefore represent part of the PV clone (Gilliland et al., 1991). Furthermore, we have shown in this study that endogenous BFU-E can be derived consistently from all 11 PV patients using PBMNC but not from normal

individuals PBMNC or BMMNC samples (see section 3.4). Therefore in utilising endogenous BFU-E derived *in vitro*, isolated PV cells could be screened for hβc mutations. This is a novel and direct approach for identification of gene abnormalities in PV that may be useful for further gene analysis studies.

PCR products were successfully obtained from 5 individual BFU-E. Control PCRs using methylcellulose medium alone or lacking RT enzyme did not generate products indicating that the products were derived from BFU-E RNA. To identify the sequences present, products were cloned and several independent clones were sequenced. Interestingly, the G1443T substitution, detected in cDNA from normal and PV PBMNC samples was also identified in the BFU-E RT-PCR screen. This sequence was obtained in all clones derived from a single endogenous colony isolated from a PV patient. Genomic DNA sequence analysis of PBMNC DNA from 2 patients and a single normal individual indicated that the normal individual was polymorphic for this change. However surprisingly, two PV patients did not appear to carry this change in genomic DNA indicating that the presence of G1443T in the PBMNC mRNA of these 2 individuals must be the result of an acquired somatic mutation. The presence of G1443T as the only sequence in product from an endogenous BFU-E from one of these patients raises the possibility that the mutation has been acquired on one allele and that the remaining *CSF2RB* allele is lost (LOH) in the BFU-E. Clearly, given that this result is from a single BFU-E, further work will be required to establish the sequence of this region from several endogenous BFU-E from this patient, other patients and normal individuals.

6.7 Potential significance of the G1443T and T1380C substitutions

G1443T confers the non-conservative amino acid change arginine (positively charged, hydrophilic) to isoleucine (hydrophobic) at position 472 in the membrane proximal region of the cytoplasmic domain of hβc (R472I). R472 is a highly conserved

residue within box 1, a proline rich motif that is conserved amongst many cytokine receptors (see fig 6.2). This region has been shown to be critical for JAK association and phosphorylation and for mitogenic signalling responses by numerous receptors including hβc, EPOR, IL4R, prolactin receptor, and gp130 (Joneja and Wojchowski, 1997; Harada et al., 1998; Pezet et al., 1997; Greiser et al., 2002). For example, JAK2 has been shown to associate constitutively with hβc and become activated specifically via the box 1 motif (Sakamaki et al., 1992; Quelle et al., 1994). The substitution of a hydrophobic amino acid residue for a hydrophilic residue would be predicted to alter the properties of the box 1 motif potentially altering JAK2 activation and subsequent signalling events. Although this residue in particular has not previously been targeted for study, the orientation of the box 1 motif has been shown using insertional mutagenesis studies to be critical for JAK mediated receptor responses. In addition, other mutations in this membrane proximal region can affect receptor responses (Gurney et al., 1995; Greiser et al., 2002; Lodish et al., 1995) (discussed further in section 6.8).

The single base substitution T1380C was detected in a PBMNC RT-PCR product from a PV patient but not in products from PBMNC of normal individuals. However, this alteration was not detected in RT-PCR product derived from endogenous BFU-E from the same patient or in other PV PBMNC samples, therefore the significance of this finding is unclear. If its presence is confirmed in further independent RT-PCR products from the same patient or in other PV samples, T1380C may be of importance for the disease. This alteration results in a non conservative phenylalanine to serine amino acid substitution at position 451 (F451S) in the transmembrane domain of hβc. Previous activating mutations have been described in this region of hβc that result in factor independent proliferation when expressed in murine cell lines *in vitro* and confer a leukemic phenotype *in vivo* (Jenkins et al., 1995; McCormack and Gonda, 1999).

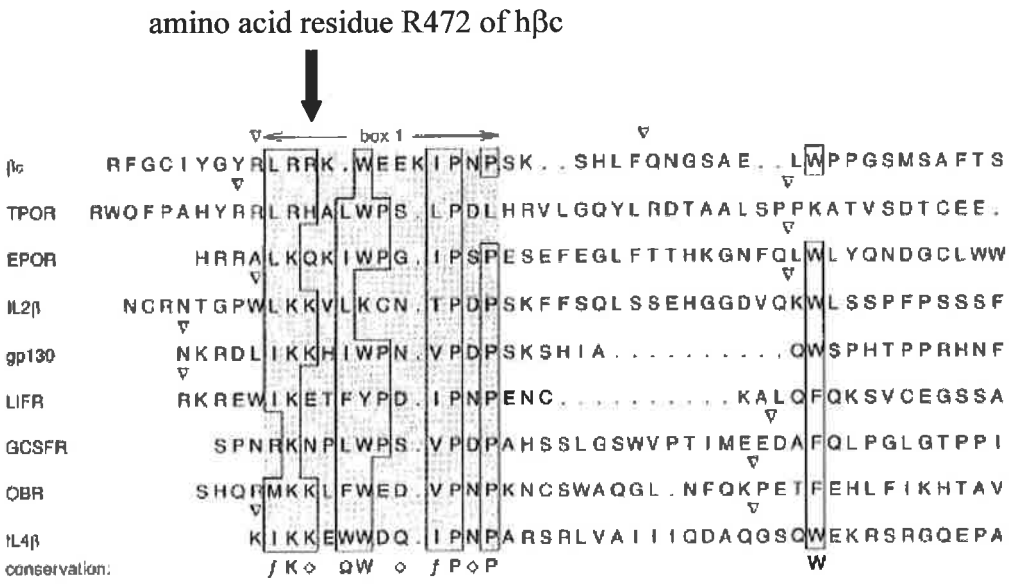


Figure 6.2 Conservation of the box 1 motif between human cytokine receptor subunits

The box 1 motif is highly conserved amongst cytokine receptor. The hβc amino acid residue R472 altered as a result of the G1443T nucleotide change in *CSF2RB* is indicated by the red arrow.

This figure is taken from Bagley et al., 1997

6.8 Implications of the R472I and F451S substitutions for PV

Whilst the specificity of both the R472I and F451S substitutions for PV have not been confirmed, it is possible to speculate regarding mechanisms by which they could contribute to the disease. Since the amino acid substitutions involved are non-conservative, they are likely to result in conformational changes that could cause hypersensitivity or hyperactivation of the receptor. Conformational alteration of the box 1 motif by R472I may result in ligand independent activation of JAK2. Conformational alteration to the transmembrane domain by F451S may result in ligand independent homodimerisation of the receptor and associated JAK2 molecules, leading to their phosphorylation and activation. Similar activating mutations within box 1 have not been previously described, however cytoplasmic activating mutations have been reported. Transmembrane substitutions in close proximity to residue F451 have also been described which result in constitutive activation of hβc and JAK2 (Jenkins et al., 1995; Jenkins et al., 1999). Since the phosphorylation and activation of JAK2 is also involved in signalling of a number of the receptors implicated in PV (IGF-1R, c-kit, EPOR and *Mpl*)(Weiler et al., 1996; Gual et al., 1998; Deberry et al., 1997; Witthuhn et al., 1993), it is conceivable that increased JAK2 activation via hβc alteration may also result in hyperactive responses by other receptors. In support of this, an activated mutant form of hβc has been shown to associate directly with EPOR and *Mpl* and lead to ligand independent phosphorylation of JAK2 associated with these receptors (Blake et al., 2002).

It is interesting that the R472I and F451S changes were not detected in the saturation mutagenesis study carried out by Jenkins and Gonda (Jenkins et al., 1998). It is possible that certain activating mutations may not have been detectable in the system given the requirement of mutants to induce complete factor independence in mouse cells. For example, a truncated form of murine βc (mβc) encoded by one allele in a

factor independent variant of the D35 promyelocytic cell line was associated with deletion or functional inactivation of the second normal mβc and βIL3 alleles. In fact, when normal mβc was re-introduced in these cells, factor independent growth was markedly attenuated (Alexander and Nicola, 1998; Hannemann et al., 1995). Since FDCP-1 cells express mβc and βIL3, the ability of some hβc mutations to cause altered growth of these cells may be masked. With this in mind, it is interesting that the G1443T change was the only sequence derived from an endogenous BFU-E RT-PCR product. This raises the possibility that in these cells the second *CSF2RB* allele is absent. This may also provide a mechanism whereby the G1443T change can be tolerated in normal individuals. Further work will be required to investigate this hypothesis (discussed further in section 6.9).

Whilst the identification of hβc alterations in PV is important for our understanding of mechanisms underlying the disease it seems likely that the PV phenotype results from a heterogeneous collection of genetic changes. Thus, putative hβc alterations described in this study may represent just some of these changes and may not be fully penetrant in the disease. Nevertheless, the identification of a genetic lesion in a significant number of cases that has the potential to affect multiple receptors would contribute significantly to the understanding of the underlying mechanisms of PV.

6.9 Further studies

Although the T1380C and G1443T changes in the *CSF2RB* cDNA that lead to the R472I and F451S alterations were detected in PBMNC and endogenous BFUE their association with the disease requires further clarification.

6.9.1 T1380C

The T1380C substitution was detected in 2 clones from a total of 54 isolated from a single PCR product derived from the PV patient MD. Therefore the significance

of this variant will need to be confirmed in further independent PCR products from this individual. RT-PCR products obtained from 2 separate endogenous BFU-E derived from the same patient did not contain the T1380C change as would be expected if this alteration is present in the PV clone of this patient. Clearly, sequence from further BFU-E should be obtained before ruling out a role in PV. Alternatively, if the T1380C change can be confirmed in other BFU-E from this patient then further PV patients should be examined for the alteration. However, it is important to note that other specific lesions associated with haemopoietic disease states can be present at very low frequency in patient cohorts. For example a transmembrane point mutation in the GCSF receptor transmembrane domain was recently described in only 2 of 555 AML patients (Forbes et al., 2002).

6.9.2 G1443T

Genomic DNA analysis demonstrated that a normal individual (HR) was heterozygous for the G1443T alteration and expressed this form at low level in PBMNC. However 2 PV patients (MD and MJ) appear to have acquired this mutation as they express this form in PBMNC while it was not found in clones derived from genomic DNA. To determine whether the acquisition of G1443T is a significant event in PV a more extensive screen for this alteration in genomic DNA from PV and normal cell populations and in endogenous BFU-E is warranted. The screening of a large number of samples to establish whether there is a higher frequency of this change in a cohort of PV patients relative to normal controls could be facilitated by the use of SSCP analysis. It is also important to determine whether endogenous BFU-E display loss of the normal *CSF2RB* allele. To examine this further, we aim to perform FISH analysis of endogenous BFU-E chromosomes with probes covering the *CSF2RB* gene.

Finally, functional analysis of these hβc variants is warranted if there is a clear association with PV. Specifically, the growth factor requirements of FDCP-1 or BAF-B03 cells expressing these forms will be assessed by analysis of cell proliferation,

survival and JAK2 association and activation. If loss of the normal *CSF2RB* allele is confirmed in association with the R472I mutation in endogenous BFU-E, expression of this form in cells that lack endogenous βc may be important. Cell lines such as the murine T cell line (CTLL) or primary cells from transgenic mice lacking both mβc and βIL3, would be suitable. In addition, it will be important to determine the ability of these mutants to affect signalling by other cytokine receptor subunits. Finally, bone marrow reconstitution or transgenic strategies could be utilised to investigate the effects of these mutant forms of hβc in vivo. Again, transgenic animals lacking mβc or m β_{IL3} can be generated to examine the effects of these variants in the absence of the normal βc allele.

Appendices

Appendix A	Primer sequences
Appendix B	Human Ethics Documentation
Appendix C	Patient Information Sheet
Appendix D	Patient consent form
Appendix E	*CSF2RB* genomic DNA sequence
Appendix F	C. M. Butcher and R. J. D'Andrea (2000) Molecular aspects of polycythemia vera (Review). International Journal of Molecular Medicine. *6*, 243-252.

Primer Sequences

Primer Name	Sequence	Application	Sequence position	Amplicon Size (bp)
hbc3 hbc12	5'caaggagggcagagaaacac3' 3'tccccgaatcctacagggaac5'	RT-PCR	*1002-1021 1620-1600	619
hbc3 hbc21	5'caaggagggcagagaaacac3' 3'cagcgcaggctgtagctgt5'	RT-PCR	*1002-1021 1101-1083	111
hbc3 hbc25	5'caaggagggcagagaaacac3' 3'ttgagggatggaggggcca5'	RT-PCR	*1002-1021 1065-1047	64
hbc24 hbc12	5'cataaagagctcagtgaacaΔatgg3' 3'tccccgaatcctacagggaac5'	RT-PCR	*1021-1049 1620-1600	594
hbc24a hbc12	5'acacataaagagctcagtgaacaΔa3' 3'tccccgaatcctacagggaac5'	RT-PCR	*1018-1046 1620-1600	597
hbc22EcoR1 hbc23BamH1	5'cgctagaattcgatccaaccatgggcagaac3' 3'cgctaggatccacgtggccgtgtctttcctgt5'	genomic PCR (intron VII/ exon8 splice site)	*f* 10506-10525 10769-10749	263
hbc30EcoR1 hbc31BamH1	5'cgctagaattcgaatgaatttccaagtgctggccc3' 3'cgctaggatccctgctgcaagtgctgcctatgt5'	genomic PCR (box1 motif)	*∂* 748-725 237-258	511
hbc26	5'cacataaagagctcagtgaaca3'	SNuPE	*1019-1040	
hbc27	5'cacataaagagctcagtgaacatcc3'	SNuPE	*1019-1043	
T7	5' taatacgactcactatagggcga3'	Sequencing		
SP6	5'attaggtgacactatagaatac3'	Sequencing		

Sequence positions refer to nucleotide numbering taken from Genbank entries: accession numbers are (*) NM000395 (*f*) see Appendix E and (*∂*) AL133392.

Appendix B

Royal Adelaide Hospital

Medical Administration
Level 3, Margaret Graham Building
Royal Adelaide Hospital
North Terrace, Adelaide 5000
South Australia
Telephone: (08) 8222 5345
Facsimile: (08) 8222 5936
Web: http://www.rah.sa.gov.au

9 November 1999

Dr R D'Andrea
Senior Research Fellow in Oncology
Head, Molecular & Development Haemopoiesis Laboratory
HANSON CENTRE FOR CANCER RESEARCH

Dear Dr D'Andrea,

Re: "The procurement of blood and bone marrow from polycythemia vera (PV) patients to provide a source of material for retroviral expression cloning of genes involved in the pathogenesis of the disorder." RAH Protocol No: 991104

I am writing to advise that ethical approval has been given to the above project. Please note that the approval is ethical only, and does not imply an approval for funding of the project.

Human Ethics Committee deliberations are guided by the Declaration of Helsinki and N.H. and M.R.C. Guidelines on Human Experimentation. Copies of these can be forwarded at your request.

Adequate record-keeping is important and you should retain at least the completed consent forms which relate to this project and a list of all those participating in the project, to enable contact with them if necessary, in the future. The Committee will seek a progress report on this project at regular intervals and would like a brief report upon its conclusion.

If the results of your project are to be published, an appropriate acknowledgment of the Hospital should be contained in the article.

Yours sincerely,

DR M JAMES
CHAIRMAN
RESEARCH ETHICS COMMITTEE

Appendix C

PATIENT INFORMATION SHEET
regarding a study entitled:

"The Procurement of Blood and Bone Marrow from Polycythemia Rubra Vera patients to provide a source of material for Retroviral cloning of genes involved in the pathogenesis of the disorder."

You have been diagnosed with a condition known as Polycythemia Rubra Vera. As would have been explained to you, this condition is due to your bone marrow producing excessive numbers of red blood cells. While we have a number of ways of controlling the disease, we still do not know exactly how this marrow abnormality develops. If we understood this better, we may be able to develop new treatments that more specifically target the cause of the disease. The study you have been asked to partake in, is attempting to improve our understanding of the abnormality in the bone marrow that causes the Polycythemia Rubra Vera. In particular, we are attempting to define the genes that may be activated abnormally in this disease. In order to define such genes, we need to obtain bone marrow and blood samples from patients with Polycythemia Rubra Vera. Having obtained these samples we would then use a number of special techniques to attempt to identify genes that are abnormally activated in disease marrow in comparison to marrow samples from normal individuals. Your involvement in this study would therefore be to provide a sample of bone marrow and blood taken at the time you are having a bone marrow biopsy performed as part of the management of your condition.

Specifically we will take between 5 and 10 mls of marrow and 50mls of blood. These samples will be frozen for later laboratory analysis. In addition, we will be asking your treating doctor for some details of the diagnosis of your condition.

The results from our laboratory tests will have no impact, either on the diagnosis of your disorder, or on subsequent clinical management but may provide information for the development of new approaches to this disorder in the future. You are not obliged to participate. If you do not wish to participate your medical treatment will not be affected in any way.

In consenting to involvement in this study, I understand that I give up all rights to any of the new findings that may result from the analysis of blood and bone marrow I have provided. I further understand that I can at any time discuss the details of my involvement in the study with the Chief Investigators, in particular, Dr Peter Bardy on and Dr Richard D'Andrea on You may also contact the Chairman, Research Ethics Committee on to discuss aspects of the study.

Appendix D

ROYAL ADELAIDE HOSPITAL
CONSENT FORM

PROTOCOL NAME: The procurement of blood and bone marrow from Polycythemia vera (PV) patients to provide a source of material for retroviral expression cloning of genes involved in the pathogenesis of the disorder.

INVESTIGATORS: Dr. Richard D'Andrea, Dr. Peter Bardy, A/Prof. Thomas Gonda, Ms. Carolyn Butcher

1. The nature and purpose of the research project has been explained to me. I understand it, and agree to take part.

2. I understand that I may not directly benefit from taking part in the trial.

3. I understand that, while information gained during the study may be published, I will not be identified and my personal results will remain confidential.

4. I understand that I can withdraw from the study at any stage and that this will not affect my medical care, now or in the future.

5. * I understand the statement concerning payment to me for taking part in this study, which is contained in the Information Sheet.

6. ** I have not been a volunteer in any other research projects which have involved radiation exposure in the last twelve months.

7. I have had the opportunity to discuss taking part in this investigation with a family member or friend.

Name of Subject: _____

Signed: _____

Dated: _____

I certify that I have explained the study to the patient/volunteer and consider that he/she understands what is involved.

Signed: _____
(Investigator)

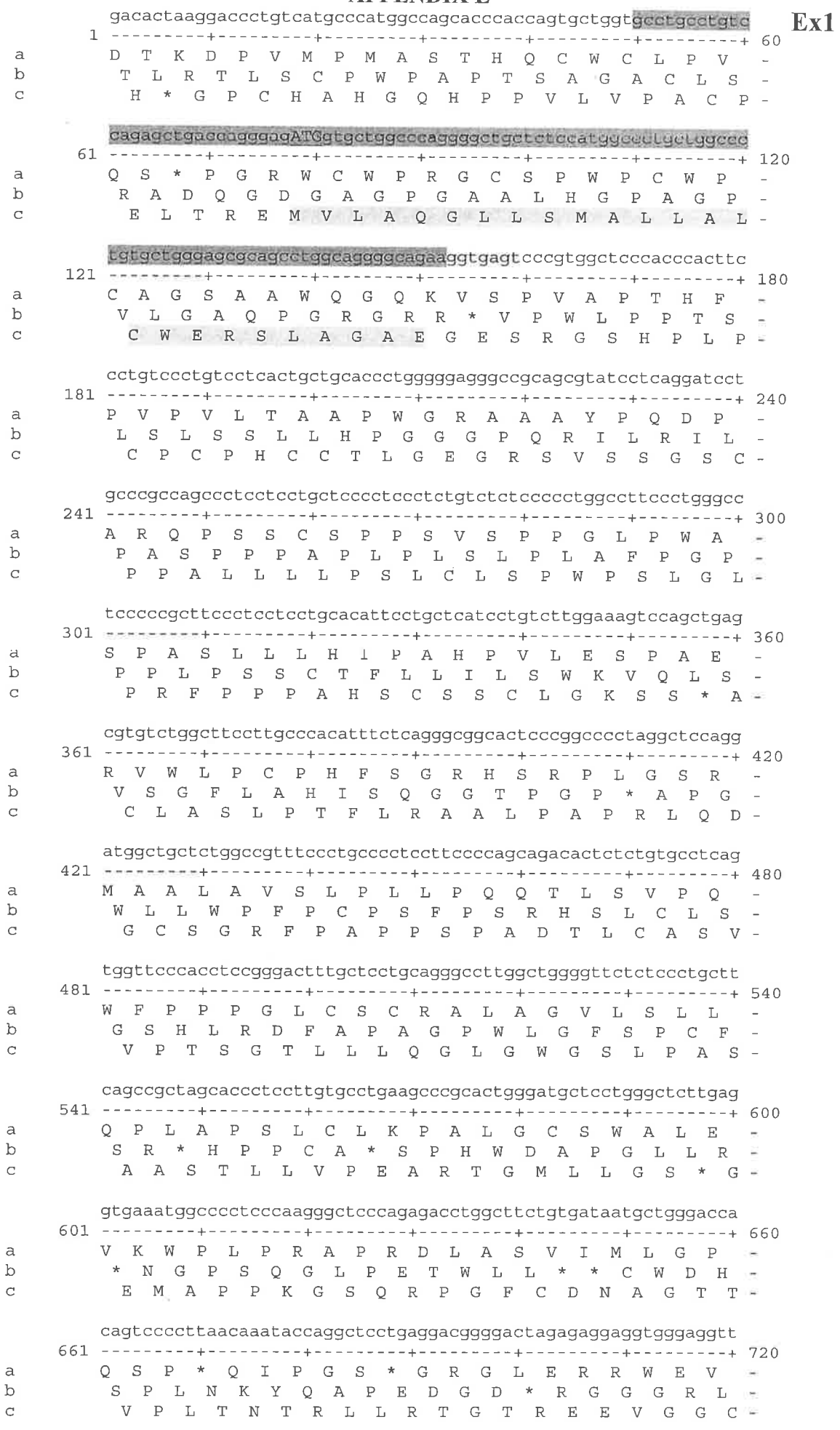
* Investigators are responsible for including an appropriate statement regarding payments to subjects on the information sheet.

** For protocols involving radiation exposure to volunteers.

APPENDIX E.

The sequence of *CSF2RB* is shown through to Exon 9. The initiation codon in Exon 1 is underlined. Exon sequences are highlighted in grey. The two primers *hbc22* and *hbc23* are boxed. In large introns some sequence has been deleted.

APPENDIX E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b T L R T L S C P W P A P T S A G A C L S -
c H * G P C H A H G Q H P P V L V P A C P -
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61 ---------+---------+---------+---------+---------+---------+ 120
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b R A D Q G D G A G P G A A L H G P A G P -
c E L T R E M V L A Q G L L S M A L L A L -
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121 ---------+---------+---------+---------+---------+---------+ 180
a C A G S A A W Q G Q K V S P V A P T H F -
b V L G A Q P G R G R R * V P W L P P T S -
c C W E R S L A G A E G E S R G S H P L P -
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181 ---------+---------+---------+---------+---------+---------+ 240
a P V P V L T A A P W G R A A A Y P Q D P -
b L S L S S L L H P G G G P Q R I L R I L -
c C P C P H C C T L G E G R S V S S G S C -
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241 ---------+---------+---------+---------+---------+---------+ 300
a A R Q P S S C S P P S V S P P G L P W A -
b P A S P P P A P L P L S L P L A F P G P -
c P P A L L L L P S L C L S P W P S L G L -
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301 ---------+---------+---------+---------+---------+---------+ 360
a S P A S L L L H I P A H P V L E S P A E -
b P P L P S S C T F L L I L S W K V Q L S -
c P R F P P P A H S C S S C L G K S S * A -
cgtgtctggcttccttgcccacatttctcagggcggcactcccggcccctaggctccagg
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a R V W L P C P H F S G R H S R P L G S R -
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c C L A S L P T F L R A A L P A P R L Q D -
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c G C S G R F P A P P S P A D T L C A S V -
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c V P T S G T L L L Q G L G W G S L P A S -
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c A A S T L L V P E A R T G M L L G S * G -
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b * N G P S Q G L P E T W L L * * C W D H -
c E M A P P K G S Q R P G F C D N A G T T -
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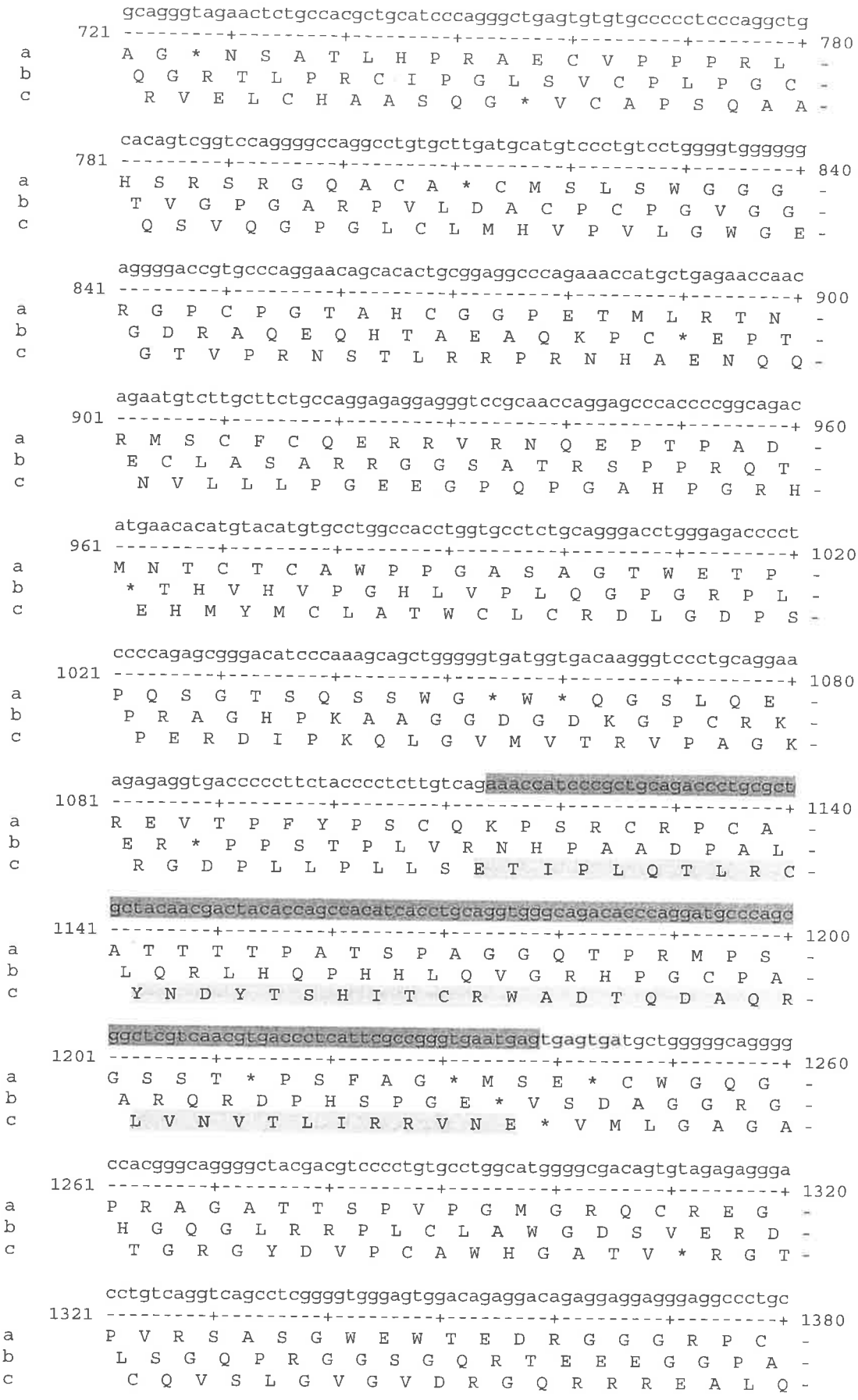
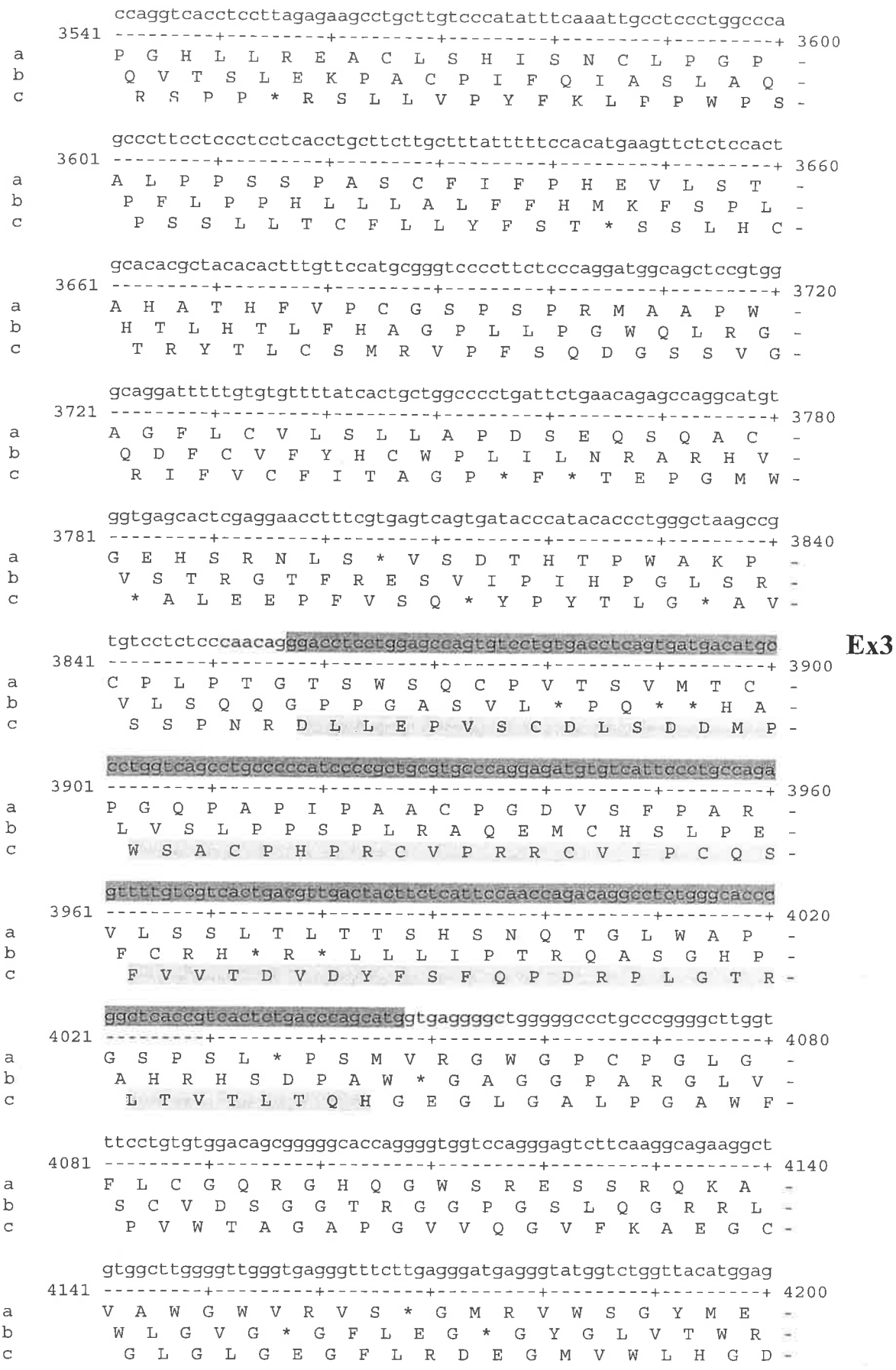
APPENDIX E

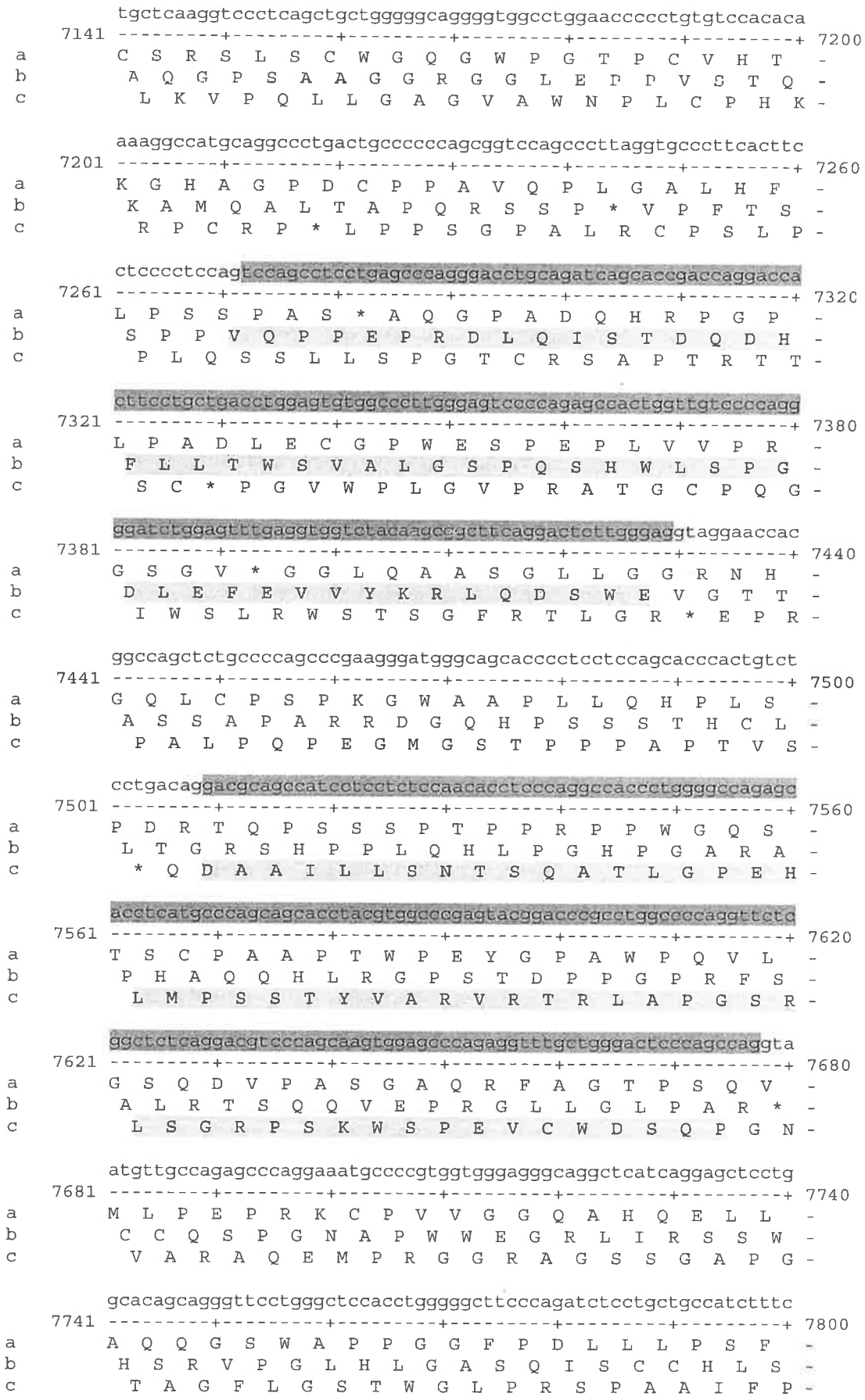


APPENDIX E



INTRON CONTINUES

APPENDIX E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c L K V P Q L L G A G V A W N P L C P H K -
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7201 ---------+---------+---------+---------+---------+---------+ 7260
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b K A M Q A L T A P Q R S S P * V P F T S -
c R P C R P * L P P S G P A L R C P S L P -
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Ex4
7261 ---------+---------+---------+---------+---------+---------+ 7320
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c P L Q S S L L S P G T C R S A P T R T T -
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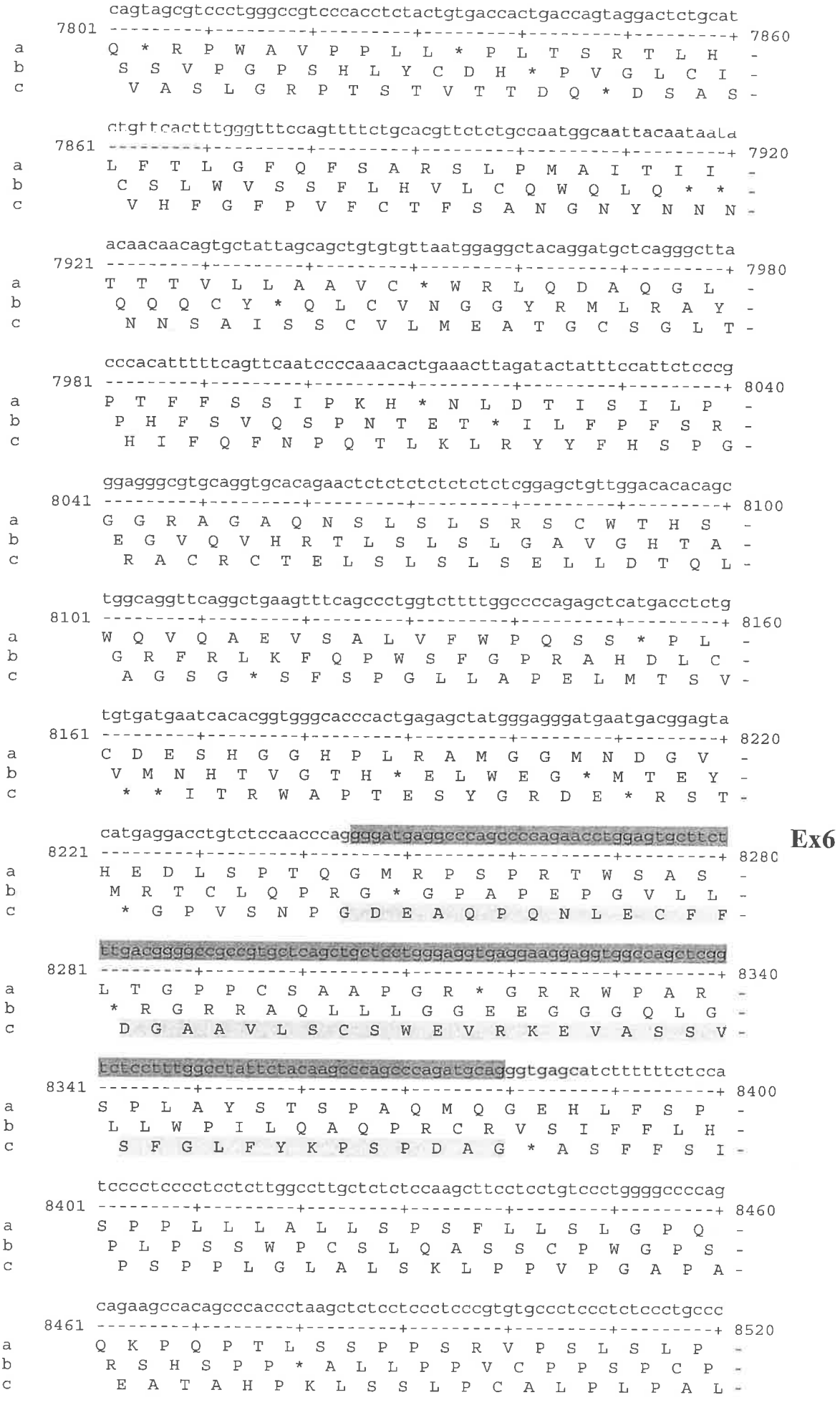
APPENDIX E



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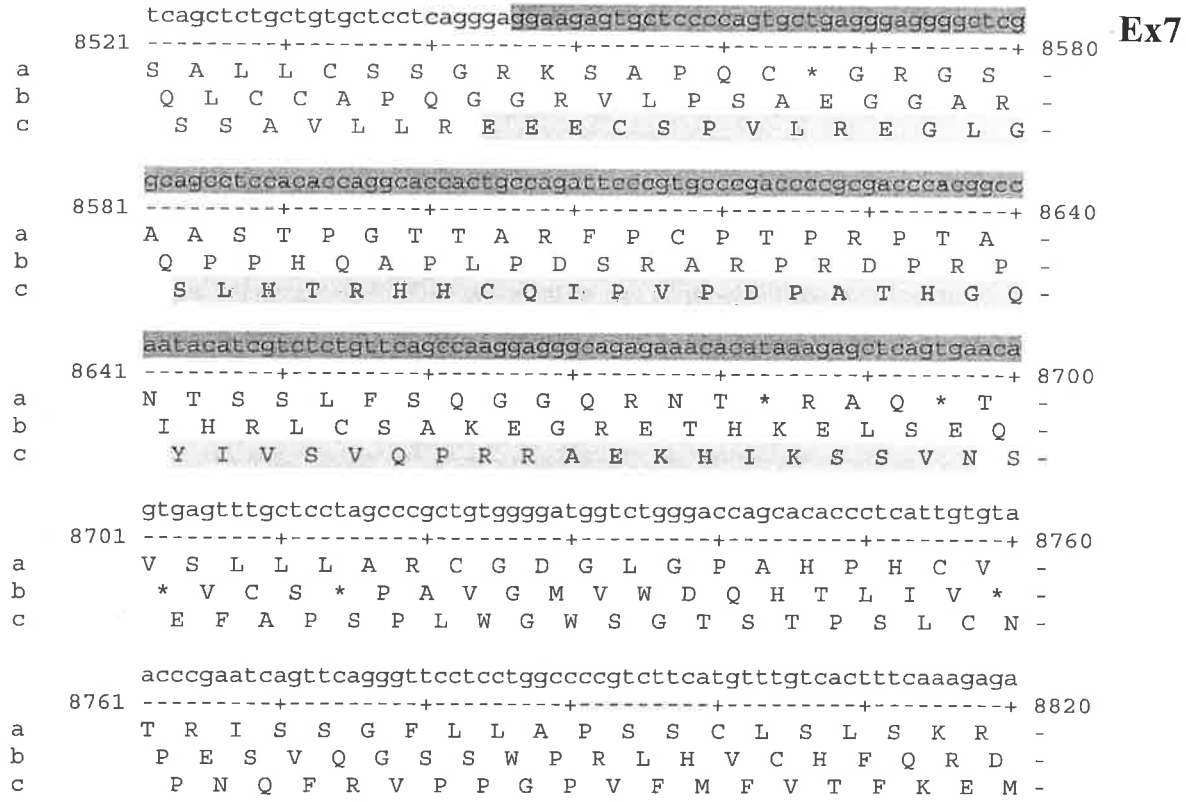

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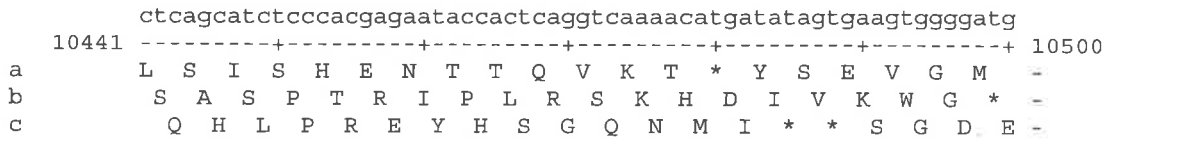

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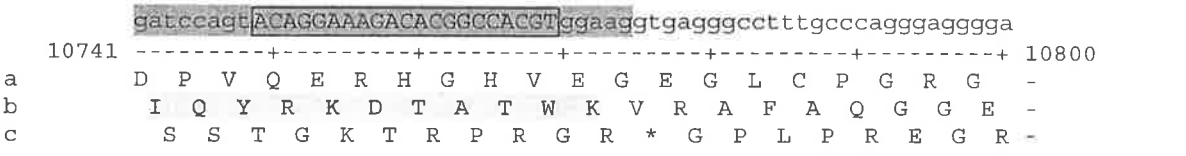
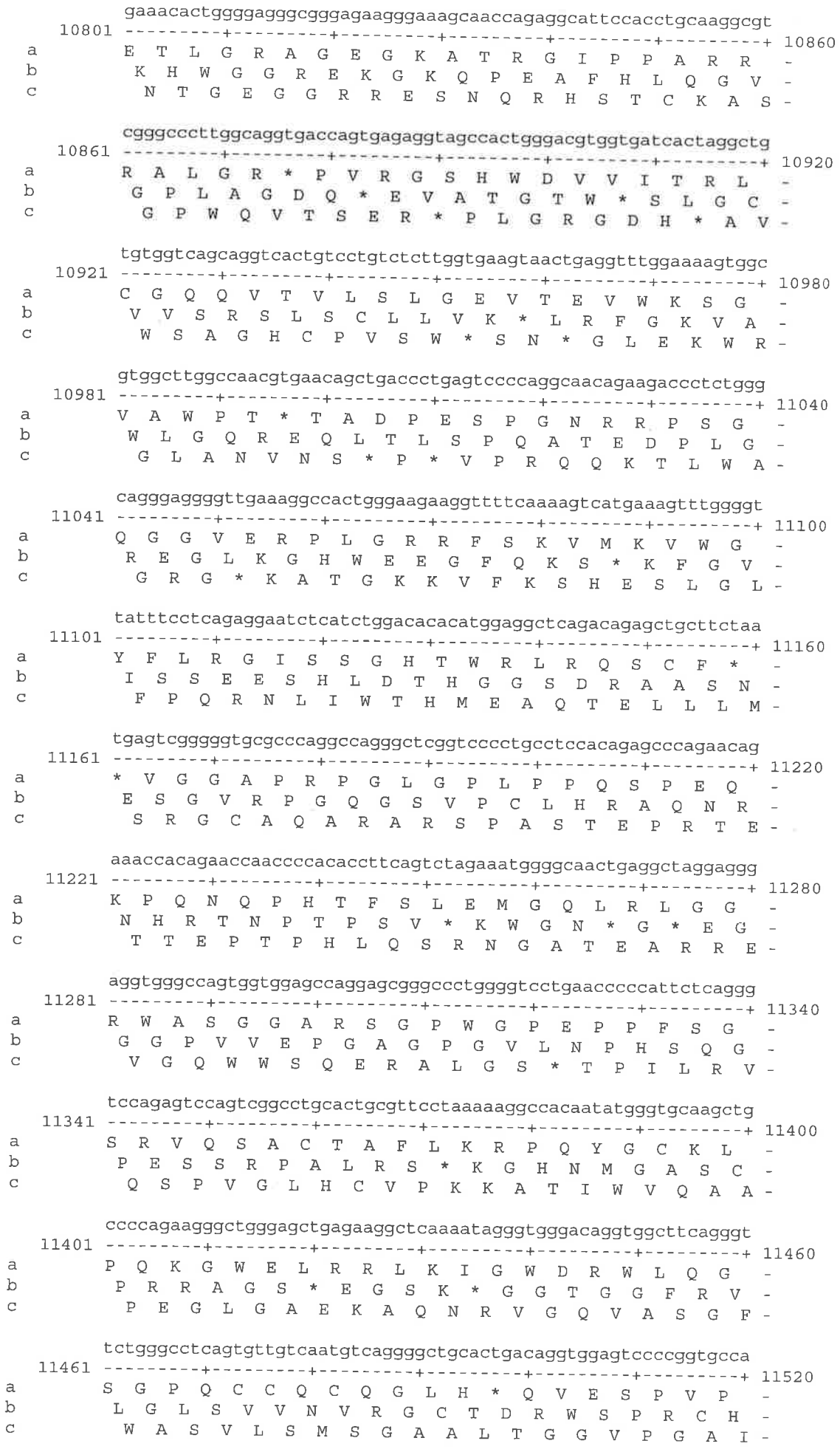


APPENDIX E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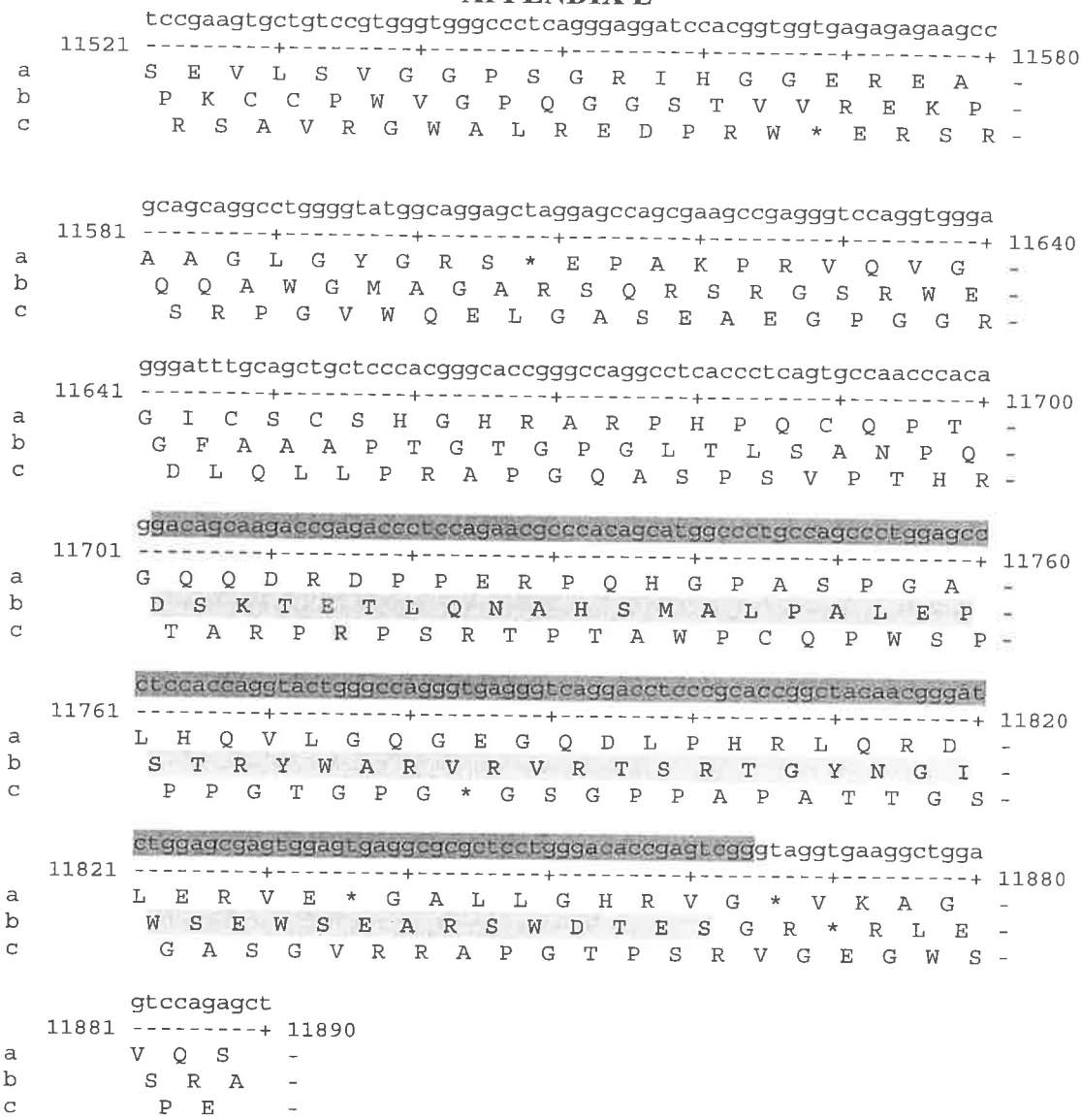
APPENDIX E



Ex9

Appendix F

Molecular aspects of polycythemia vera (Review)

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Abstract. Polycythemia vera (PV) is a rare, progressive myeloproliferative disorder thought to originate from the clonal expansion of a multipotent haemopoietic stem cell. This disease is characterised by hyperproliferation of the erythroid, myeloid and megakaryocyte lineages in the early phase, anaemia and fibrosis in the spent phase, and with a significant number of patients developing acute myeloid leukaemia (AML) in the final phase. Studies investigating the growth factor requirements of committed progenitors have shown hypersensitivity to a number of haemopoietic growth factors (HGF) *in vitro* and several HGF receptor and signalling molecule alterations have been reported. The findings to date, however, are unable to account for the transformation of a primitive stem cell and the many alterations to growth factor responses seen in PV progenitors. Identification of the primary lesion that leads to the pathogenesis of PV is of major importance given the profound effects on regulation of the haemopoietic stem cell compartment. In this article we focus on characteristics of the disease, research findings to date and possible mechanisms to explain altered growth factor responses, receptor alterations and signalling abnormalities in PV.

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2. Disease progression
3. Therapy and prognosis
4. Clonality and cytogenetics
5. Haemopoietic growth factors
6. Signalling pathways
7. Potential molecular basis for PV
8. Summary

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Key words: polycythemia vera, myeloproliferative disorder, haemopoietic growth factor, cytokine receptor, erythropoietin

1. Introduction

Polycythemia vera is one of several diseases grouped together as the myeloproliferative disorders (MPD) by William Dameshek in 1951 (1). These disorders are characterised by hyperproliferation of one or more haemopoietic lineage, originating from clonal expansion of a multipotent stem cell, and with myelofibrosis in the terminal phase. MPD also include the disorders chronic myeloid leukemia, essential thrombocytosis and idiopathic myelofibrosis.

PV is characterised primarily by elevated red blood cells and usually increased granulocytes and megakaryocytes in the bone marrow, and is thought to originate from an early haemopoietic stem cell capable of differentiation to the erythroid, myeloid and megakaryocytic lineages. Increased numbers of primitive progenitors expressing the surface antigen, CD34 are found in the peripheral blood compared with normal blood, indicating a shift in the stem cell compartment from bone marrow (2). Although B and T lymphocytes are not increased in PV, there are conflicting reports with regard to the involvement of the lymphoid lineage (3,4). PV is an acquired, progressive disease of typically 4 phases extending over 10-20 years. It is a late-onset, rare disorder with diagnosis usually made after the age of 60 and with approximately 1 case diagnosed per 100,000 people per year in western countries (5).

PV is the most common of a group of diseases where erythrocytosis occurs in the absence of elevated growth factors or other external factors. These diseases, termed the primary polycythemias, are caused by an intrinsic defect in the haemopoietic progenitors involved in the disease. Secondary polycythemias however, are diseases where stimulation of normal progenitors occurs as a result of increased levels of extrinsic factors e.g. increased production of the growth factor, erythropoietin (EPO) due to external factors such as high altitude, lung dysfunction or cardiac disorders. Characterisation of the intrinsic defect that leads to dysregulated growth of haemopoietic stem cells in primary polycythemias may provide insight into the intrinsic controls of stem cell growth.

The polycythemia vera study group (PVSG), established in 1968, have defined the clinical criteria required for the diagnosis of PV (5) and have used these criteria to obtain a uniform patient population with which to assess therapeutic outcomes, discussed below.

2. Disease progression

PV is a progressive disorder with patients typically moving through identifiable stages for up to 20 years. Patients initially present with erythrocytosis i.e. the haematocrit is increased, usually accompanied by leukocytosis in 66% of patients, thrombocytosis in 50% of patients and splenomegaly. Serum vitamin B12 and B12 binding protein levels and the leukocyte granule component alkaline phosphatase are increased in the majority of PV patients. Platelet aggregation and bleeding time are abnormal and neutrophil and monocyte oxidative responses are markedly reduced (6,7).

i) The erythrocytic phase is characterised by erythrocytosis, granulocytosis, thrombocytosis and splenomegaly. The spleen during this phase is congested with mature erythrocytes but there is no significant extramedullary haemopoiesis (8). Symptoms include headache, weakness, weight loss, pruritis, sweating, visual disturbances and joint symptoms due to excessive blood cell proliferation and peripheral vascular disease. However, the major cause of morbidity and mortality in the erythrocytic phase is vascular thrombosis due to the expanded rbc mass, and haemorrhage, causing 40% and 20% of the deaths, respectively, that occur during this phase. Neurologic abnormalities also occur in 60-80% of patients due to increased blood viscosity and reduced cerebral blood flow. Iron deficiency often causes secondary non-haematological symptoms. A serious complication in approximately 5% of patients with PV is development of Budd Chiari syndrome where liver malfunction occurs due to thrombotic obstruction of the hepatic vein (9).

Bone marrow biopsy reveals hypercellularity of the erythroid, myeloid and megakaryocytic lineages and bone marrow cells will proliferate continuously in long-term culture (10). Bone marrow cells and often peripheral blood cells from PV patients give rise to colonies of erythroid origin in colony assays containing serum, in the absence of added erythropoietin (EPO), the growth factor normally required for their growth (11). These colonies are termed 'endogenous' colony forming units-erythroid (CFU-E), and are the hallmark of PV diagnosis. Similarly, megakaryocyte colonies grow in the absence of exogenously added growth factors from bone marrow and peripheral blood of PV patients (12). With treatment, this phase is prolonged and may extend for 10-20 years.

ii) Post polycythemic myeloid metaplasia (PPMM), also termed the spent phase, occurs in 10-15% of PV patients, occurring an average of 10 years post diagnosis. During this phase the rbc mass may be normal however bone marrow fibrosis may develop and splenomegaly becomes increased causing excessive discomfort. Trilinear extramedullary haemopoiesis is now occurring in the spleen tissue and anaemia often occurs as the result of splenic pooling, short rbc life span and bone marrow failure. Haemorrhage due to thrombocytopenia or platelet functional abnormalities is a significant cause of morbidity during this phase. PPMM patients (25-50%) progress to development of acute leukaemia (9).

iii) Leukaemic transformation due to PV occurs at a frequency of 1-2% in patients receiving phlebotomy alone but rarely develops before 8 years post diagnosis. The phenotype is overwhelmingly myeloid i.e. acute myeloid leukemia (AML). The evolution of PV to leukaemia is a consequence of the clonal malignancy, however there is clear evidence that some therapeutic interventions, particularly the myelosuppressive agent chlorambucil, increase the frequency of leukemia in PV to 10-15% (13).

3. Therapy and prognosis

Survival of PV patients is dependent on duration of the erythrocytic phase, appropriate therapy during the erythrocytic phase, nature and severity of symptoms, and the time for transition to PPMM or AML. Uncontrolled erythrocytosis results in a high risk for life-threatening thromboses within 1 or 2 years. Several therapeutic strategies have prolonged the survival time to over 10 years for most patients, however the choice of therapy remains an area of active debate. The PVSG has carried out a series of trials over a period of 20 years to study the beneficial and adverse effects of several PV therapies and has formulated therapy guidelines (14). Individualised therapy based on age and presence of other disease, for example, cardiovascular disease, is recommended. Phlebotomy is used in the erythrocytic phase to maintain normal blood volume and haematocrit levels. This may be used alone or in conjunction with myelosuppressive therapy such as radiotherapy using ^{32}phosphorous (^{32}P) (15), or chemotherapeutic drugs such as chlorambucil, or busulfan. However, several studies have shown increased incidence of leukaemic transformation with chlorambucil or ^{32}P treatment. For example, it has been shown that phlebotomy alone or phlebotomy combined with ^{32}P therapy achieve similar survival times but different disease complications (high thrombosis risk and high leukaemic risk, respectively) (16-18).

More recent therapies (reviewed in ref. 17), which require long-term study include hydroxyurea (inhibitor of S phase specific ribonucleotide reductase) (19-21), anagrelide (inhibitor of megakaryocyte maturation) (22) and low dose aspirin to inhibit platelet aggregation (23). Interferon-α has been studied in clinical trials recently and shown decreased rbc and platelet levels in PV patients (24).

Patients developing Budd Chiari syndrome may require a liver transplant, however PV patients are at increased risk of life-threatening thrombosis and haemorrhage related to surgery. Therapy during the PPMM phase is problematic. Anaemia may require transfusion therapy since massive erythrocytosis is induced by iron supplementation during this phase. Pressure symptoms associated with marked splenomegaly may be treated with radiotherapy or low dose chemotherapy. Splenectomy is a last resort since 25% of PV patients die with this surgery due to excessive haemorrhage. Chemotherapy for AML is usually unsuccessful and is not recommended at all for elderly patients and 70% of patients that progress to the PPMM or leukaemic stages of PV have a survival time of less than 3 years (13).

4. Clonality and cytogenetics

PV is a late-onset, clonal malignancy, suggesting that it is caused by the acquisition of one or more genetic alterations

that change the growth properties of a multipotent progenitor. Clonality can be established using X chromosome inactivation studies in female patients (25). In clonal tissues only one allele from an X-linked gene will be present indicating that all cells within the tissue are derived from a single clone. This was shown to be the case in PV where erythrocytes, platelets, granulocytes and monocytes expressed only one allele of an X-linked gene, e.g. glucose-6-phosphate dehydrogenase in heterozygous females. T lymphocytes always expressed both alleles whilst in some patients the B lymphocytes were also clonal (3,26,27). This type of analysis has more recently been performed using polymerase chain reaction (PCR) techniques, requiring only ng amounts of DNA and enabling clonality to be established from haemopoietic colonies grown in culture (28).

Chromosome abnormalities with low penetrance have been found in PV. Approximately 20% of untreated patients and 60% of patients treated with cytotoxic agents have karyotype abnormalities, reflecting the leukaemogenic effects of myelo-suppressive treatments (discussed above). Duration of disease is also associated with the acquisition of abnormalities and 71-80% of patients who develop myelofibrosis or leukemia have an abnormal karyotype. However, it is important to note that cytogenetic abnormalities occur in the absence of cytotoxic treatment and that PV can progress to acute leukemia with a normal karyotype (9).

In two studies of 104 and 285 PV patients, respectively, the more common abnormalities found include deletion of 20q (with a frequency of approximately 10%), deletion of 13q (5%), trisomy 8 (9%) and trisomy 9 (9%). Deletions of chromosome 5 or 7 were commonly seen only after myelo-suppressive treatment and are unlikely to indicate genes underlying the PV phenotype (29,30). Deletions of the long arm of chromosome 20 (del 20q) are found in several other myeloproliferative disorders and in AML and a common deleted region (CDR) has been identified (31). These abnormalities may indicate the possible location of genes involved in the pathogenesis of PV. One approach to identifying a genetic lesion for PV or other disorders with the 20q deletion has been to reduce the size of the CDR and then look for mutations of candidate genes in this region, however no mutations have been detected to date (32,33). A genome-wide search for loss of heterozygosity (LOH) in one case of familial PV identified a region of LOH on chromosome 11 but no LOH on chromosome 20 (34), however this finding is yet to be confirmed in other family members with PV or in sporadic PV cases.

Most recently, a novel protein (PRV-1) with homology to the uPAR/Ly6/CD59/snake toxin-receptor superfamily has been identified by subtractive hybridisation comparing normal peripheral blood mononuclear cell cDNA with that from a PV patient (35). This protein was expressed in mature granulocytes from PV patients and also in normal granulocytes stimulated *in vivo* or *in vitro* with the cytokines G-CSF or GM-CSF, and is selectively expressed in normal bone marrow but not other haemopoietic tissues. The role of this protein in normal haemopoiesis and in the abnormal expansion of erythroid, myeloid and megakaryocytic lineages in PV remains to be elucidated.

5. Haemopoietic growth factors

Haemopoietic growth factors (HGF) have important regulatory effects on the survival, differentiation, proliferation and function of all haemopoietic cells. HGFs have therefore been a major focus in the field of haemopoiesis for more than a decade, and many groups have studied the HGF requirement of PV progenitors to identify differences that may relate to the pathogenesis of the disease. HGFs influence their target cells via interaction with ligand specific growth factor receptors. These interactions convey messages via signal transduction pathways to transcription factors in the cell nucleus (36). Cells respond differentially to HGF depending on the stage of differentiation and thus HGFs exert multiple biological functions at defined stages of haemopoiesis. In addition, HGFs often act synergistically with each other, forming a highly complex network and have overlapping functions with other HGFs, creating redundancy in the regulation of haemopoiesis by these factors. Abnormal expression or function of HGFs, their receptors or associated signalling molecules can lead to dysregulated haemopoiesis and disease states (37-40).

Receptors for HGFs are classified into two major classes based on their associated protein tyrosine kinase (PTK) activity. Many receptors are members of the receptor tyrosine kinase (RTK) family and have intrinsic PTK activity (41). Another important class of receptors that lack intrinsic PTK activity is the cytokine receptor (CR) superfamily (36). Members within these receptor superfamilies share common structural features and signal transduction pathways by which their effects are mediated.

The growth of haemopoietic progenitors in semi-solid media (i.e. colony assays) and liquid culture has enabled the investigation of HGF and receptor responses *in vitro*, however study of more primitive cells has been difficult. Whilst the defect in PV appears to involve an early multipotent progenitor cell, the difficulty in obtaining sufficient purity and number of these cells has limited their direct analysis. One early study assessing the function of clonal multipotential progenitor cells (CFU-GEMM) in colony assays containing serum, showed increased numbers in blood and bone marrow of PV patients compared with normal subjects (42). Most studies however, have investigated the growth properties of committed progenitors in PV. Since the principle clinical manifestation of PV is marked erythrocytosis, many investigations have focused on HGFs regulating the *in vitro* differentiation and growth of progenitors of the erythroid lineage, i.e. burst and colony forming units-erythroid (BFU-E and CFU-E). Interpretation of these results is complicated by the presence of low levels of cytokines found in serum and other components of the media used for these assays and differences in cell isolation and culture methods, contributing to conflicting reports in the literature. Key findings are summarised below.

i) Erythropoietin requirement of progenitors. Erythropoiesis is the process of production and renewal of the red blood cell mass in response to tissue oxygen levels and is regulated by numerous HGFs, receptors and transcription factors (43). EPO is the principle growth factor that promotes the survival, proliferation and differentiation of late erythroid progenitor cells. However, it does not appear to be required for generation

of early committed erythroid progenitors since null mutations to EPO or the EPO receptor (EPOR) genes in mice permit development of BFU-E and CFU-E but not erythroid precursor cells (44).

Serum EPO concentration is not elevated in PV, therefore PV erythrocytosis cannot be attributed simply to increased stimulation of EPOR by its ligand. Early work in colony assays containing serum, demonstrated the growth of 'endogenous BFU-E and CFU-E', that is, EPO-independent erythroid colonies, in contrast to normal CFU-E and BFU-E which are EPO-dependent (11). Subsequent EPO dose response experiments showed 2 populations of BFU-E and CFU-E from PV cells. One group were hypersensitive to EPO and were responsive at a concentration of <0.001 U/ml whilst the second population responded normally to EPO at 1 U/ml, supporting the observation that both normal and abnormal progenitors are present in the marrow of PV patients (45). Antibodies to either EPO or EPOR caused a dose dependent inhibition of PV BFU-E that are sensitive to EPO, however 'endogenous BFU-E' were able to proliferate and differentiate in their presence. This further supports evidence of the reduced EPO requirement exhibited by this progenitor population (46).

ii) EPO receptor. Mutations in EPO receptor (EPOR) have been identified in primary and familial polycythemia but comprehensive analysis has failed to identify mutations associated with PV. Mutant forms of EPOR showing hypersensitive responses to EPO both *in vivo* and *in vitro* have been described. Truncated forms of EPOR which lack a negative regulatory region in the cytoplasmic domain cause a hypersensitive response to EPO and have been identified in cases of primary and familial polycythemia (47,48).

In addition to profound effects on the erythroid lineage, effects of EPO and EPOR on cells of the megakaryocytic lineage have also been shown. Megakaryopoiesis and erythropoiesis are increased in mice expressing the oncogenic murine EPOR mutant, R129C (49). Therefore mutated forms of EPOR may explain the erythrocytosis and contribute to the megakaryopoiesis in PV. Several groups have studied EPOR gene structure in PV. Despite extensive investigation, PV EPOR gene defects have not been detected by Southern analysis (23,50) and no structural changes were detected in the entire coding region of the gene by sequencing, PCR and SSCP analysis (51). EPOR undergoes rapid tyrosine phosphorylation in response to EPO (52) and this is comparable between normal and PV erythroid progenitor cells (53). Consistent with these findings, important work in serum-free assays has subsequently shown that the hypersensitivity of PV erythroid progenitors to EPO was due, in fact, to hypersensitivity to low levels of insulin-like growth factor-1 (IGF-1) found in serum and that the response to EPO was normal (54) (see below).

A recent study has shown the absence, or marked decrease, in mRNA encoding a truncated form of the EPOR (EPOR-T) in 8/8 PV patients but not in other myeloproliferative disorders (55). EPOR-T is derived from an alternative splicing event and encodes a protein with a truncated cytoplasmic domain. This EPOR variant binds EPO with similar affinity to full length EPOR (EPOR-F), and acts as a dominant negative regulator (56,57). EPOR-T is predominantly expressed in immature progenitors and is downregulated in maturing erythroid cells, suggesting regulation of a splicing event may occur during erythroid development. It has been suggested that under normal conditions *in vivo*, when EPO concentration is low, early progenitors may not undergo erythropoiesis due to co-expression of EPOR-T. However during stress erythropoiesis when EPO levels are increased, the inhibitory effect of EPOR-T may be overcome (56,58). Therefore, EPOR-T may be a key regulator in the maintenance of erythrocyte homeostasis and a loss of expression in early progenitors may explain dysregulated erythrocyte production as seen in PV.

iii) The receptors for interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF). Pathways exerting influences on the myeloid and megakaryocytic lineages, in addition to the erythroid compartment may play a role in the pathogenesis of PV. *In vitro*, interleukin-3 (IL-3) exerts its effects mainly on multilineage and committed progenitor growth in synergy with other HGFs (59). As cells differentiate they lose the capacity to proliferate in response to IL-3 (60). Knockout mouse studies suggest IL-3 is not required for steady state haemopoiesis (61). GM-CSF also has synergistic effects on progenitor cell growth and in addition, has further stimulatory effects on mature cells of the myeloid lineage (59). It has been used clinically to stimulate neutrophil production in myelosuppressed patients, however, GM-CSF knockout mice undergo normal embryogenesis and development, and blood cell compartments are normal except for defective functioning of alveolar macrophages (reviewed in refs. 61,62). The high affinity receptors for IL-3 (IL3R) and GM-CSF (GMR) are comprised of specific ligand binding α subunits and a common ß-subunit (hßc), which is also shared with the receptor for the eosinophil specific cytokine, interleukin-5 (63). hßc is essential for activation of signalling pathways in response to ligand binding (64).

BFU-E, CFU-GM and CFU-Meg from bone marrow of PV patients displayed marked hypersensitivity to IL-3 and GM-CSF in serum-free cultures (IL-3 38x and GM-CSF 48x for BFU-E) (65). In addition, PV BFU-E from peripheral blood have a reduced dependence on IL-3 for survival *in vitro* compared with BFU-E from normal subjects (66). Despite altered sensitivity to IL-3, there is no detectable increase in IL-3 receptor number and IL-3 binding is comparable between PV cells and normal cells (65). Consistent with an involvement of the GM-CSF/IL-3 pathway in PV, transgenic mice expressing a constitutively active form of hßc (FIΔ, reviewed in ref. 67) displayed trilineage hyperplasia and splenomegaly similar to the phenotype in PV (68).

Autocrine GM-CSF induction is implicated in abnormal responses of PV erythroid progenitors to other HGFs. For example, growth of 'endogenous BFU-E' is enhanced by exogenous IL-1; this effect is mediated by autocrine release of GM-CSF since anti-GM-CSF antibody could abrogate the IL-1 effect (2). Additionally, IL-10 inhibited growth of 'endogenous BFU-E' to a greater degree than normal EPO-dependent BFU-E (81±12% cf. 45±12%). This may be partly due to suppression of spontaneous release of GM-CSF since GM-CSF mRNA levels were reduced in PV peripheral blood mononuclear cells (PBMNC) cultured for 48 h in the presence

of IL-10, and addition of exogenous GM-CSF could abrogate the IL-10 effect (69).

iv) Thrombopoietin and its receptor, Mpl. Thrombopoietin (TPO) is an important regulator of the proliferation and differentiation of megakaryocytic precursors and also the production of multipotent progenitors (reviewed in ref. 70). It has been shown to synergise with other early acting HGFs to induce colony formation from primitive murine progenitors (71,72). Expression of the receptor for TPO (Mpl) is restricted to primitive haemopoietic stem cells, megakaryocytes and platelets (73). In Mpl deficient mice platelet levels are reduced by 90% consistent with TPO being the major growth factor regulating megakaryopoiesis. Multilineage and committed progenitor numbers are also reduced in these mice due to effects on the haemopoietic stem cell compartment (74). Further, a constitutively active, truncated form of Mpl (v-Mpl), introduced into mice using a retrovirus (MPLV), induces erythropoiesis, thrombogranulosis and splenomegaly (75). Murine bone marrow cells, enriched for primitive progenitors and infected with MPLV *in vitro*, yield factor independent cell lines of several different lineages, again indicating the multilineage effects of Mpl activating mutations (76). These effects on the erythroid, megakaryocytic and myeloid lineages suggest that TPO and its receptor may have a role in the dysregulated proliferation of these lineages in PV.

There is marked heterogeneity in Mpl protein levels in PV, depending on treatment regime, however, it is unlikely that Mpl abnormalities are involved directly in pathogenesis of PV as Mpl levels are normal in approximately half of patients (77). Gross c-*mpl* gene abnormalities have not been detected in PV patients from studies using Southern and Northern analysis. However, TPO induced tyrosine phosphorylation of Mpl is impaired in PV platelets (78). In addition, 2 isoforms of Mpl have been observed in PV platelets using an antibody to the intracellular domain which recognises a PV specific isoform (Mpl B) (79). Mpl B has a lower molecular weight (80-82 kDa cf. 85-92 kDa) and higher isoelectric point (6.5 cf. 5.5) than normal Mpl and was shown to have increased sensitivity to endoglycosidase H digestion suggesting that the 2 isoforms differ in their post-translational processing. The significance of this is unclear since the functional properties of Mpl B have not been reported. The Mpl B glycosylation defect was not detected in newly diagnosed patients but emerged, and increased in parallel with disease duration and extent of extramedullary haemopoiesis in 24 PV patients. This could indicate either a progressive increase in severity of the defect generating this underglycosylated form of Mpl or a selective advantage for cells expressing the Mpl B isoform as the disease progresses. The fact that the defect is not present in patients at diagnosis raises the possibility that the altered processing is secondary to the primary lesion in PV.

v) IGF-1 and IGF-1 receptor. IGFs are a family of ubiquitously produced proteins that stimulate a variety of cellular responses including effects on cell proliferation and differentiation (80). IGF-1 promotes cell division and prevents programmed cell death in haemopoietic cells (81,82). Specifically, IGF-1 rescues promyeloid cells from apoptosis induced by serum starvation, and permits their differentiation to mature granulocytes (83). High levels of IGF-1 can substitute for EPO in the growth of BFU-E from normal PBMNC. The IGF-1 dose response was 100-fold higher than that for EPO, and addition of both IGF-1 and EPO was not synergistic, suggesting that the cellular response to these two cytokines occurs via different stimulatory pathways (84).

Although PV BFU-E and CFU-E grow in the absence of EPO in colony assays containing serum, subsequent work in serum-free assays has shown that the formation of 'endogenous' colonies is due to marked hypersensitivity (100-fold) of PV cells to low levels of IGF-1 present in serum. 'Endogenous' colony formation was abolished by neutralising antibody to the IGF-1 receptor. Further, PV cells responded normally to EPO in serum-free dose response experiments (54). Thus, PV cells are hypersensitive to IGF-1 and not EPO *in vitro*. A role for IGF-1 is further implicated in one PV case in which the patient had acromegaly (caused by a tumour of the pituitary gland) with elevated IGF-1 and growth hormone levels. After removal of the tumour, hormone levels normalised and the haematological abnormalities resolved (85). Elevated levels of IGF-1 or growth hormone have not been reported in other cases of PV, therefore the significance of this case is unclear.

The mature form of the IGF-1 receptor (IGF-1R), a member of the RTK family of receptors, consists of two ligand binding extracellular α-subunits, disulphide linked to two membrane spanning ß-subunits. Upon IGF-1 binding to its receptor the ß-subunits are autophosphorylated thereby increasing kinase activity and resulting in the subsequent phosphorylation of intracellular substrates (80). Since PV cells display increased sensitivity to IGF-1, the kinase activity of IGF-1R was investigated. Basal and ligand-induced tyrosine phosphorylation of the IGF-1R is increased in PV (86), however to date, no studies have investigated potential mutations in this receptor. In the absence of any receptor abnormalities, alteration of a signalling component that directly activates or desensitises the kinase activity of IGF-1R may be implicated in its hyperphosphorylation in PV.

vi) Stem cell factor and c-kit. Stem cell factor (SCF) is the ligand for the RTK *c-kit*. It is produced by stromal cells and has a potent, synergistic action with other HGFs, such as IL-3, GM-CSF, EPO and IGF-1, in enhancement of the proliferation of multi-potential stem cells and early erythroid, myeloid and lymphoid progenitors (87,88). Approximately 20,000 high affinity receptors for SCF are present on normal human BFU-E and CFU-E, however there is a loss of receptor expression with further erythroid differentiation (89). SCF is the principle factor required for increased DNA synthesis and expansion of erythroid cells, while EPO is the principle factor for progenitor viability and differentiation to late erythroblasts. SCF has been shown to act directly in serum-free conditions to increase the growth of BFU-E in colony assays. CFU-E proliferate in response to SCF also, however do not require SCF for survival in the presence of EPO (90).

Colony assays with purified PV BFU-E progenitors have shown hypersensitivity to SCF compared with normal BFU-E (91). Expression of the receptor did not differ from normal levels at any stage of differentiation, and receptor phosphorylation, internalisation and binding of SCF was

comparable with normal cells (89). Again, the lack of receptor changes suggest that increased sensitivity to SCF may be a secondary event in PV.

6. Signalling pathways

Signalling pathways which transmit survival, proliferation and differentiation signals from the receptor upon HGF binding, are complex and incompletely understood. However, it is known that upon binding of HGFs to their respective receptors, phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor occurs. This is thought to be initiated by receptor homodimerisation followed either by autophosphorylation, mediated by intrinsic RTK activity, or by recruitment and activation of non-receptor PTKs (e.g. Janus kinases; JAKs) by CRs (92). Whilst RTKs and CRs differ in early receptor activation events, some common regulatory molecules and pathways are utilised in downstream signalling.

Tyrosine phosphorylation is regulated by the balance of PTK and protein tyrosine phosphatase (PTP) activities, and an impaired balance of these molecules may lead to disorders of cell growth. As discussed above, the IGF-1 receptor which has intrinsic PTK activity is itself hyperphosphorylated in PV suggesting that a defect in a negative regulatory molecule could contribute to altered receptor phosphorylation and activation. This would also be consistent with the hypersensitivity of PV progenitors to several HGFs, and also with genetic studies suggesting a loss of function may be important in disease initiation (discussed above). Recent findings from studies investigating signalling properties in PV cells are summarised below.

i) Phosphatases. Three major haemopoietic PTPs (SHP-1, SHP-2 and CD45) have been identified and extensively studied in mature cells. The intracellular phosphatase, SHP-1 (*src* homology phosphatase-1), negatively regulates signalling from the receptors for EPO, SCF, and IL-3. For example, SHP-1 associates via its SH2 domains with phosphorylated tyrosine residues in the cytoplasmic domain of EPOR and terminates proliferative signals (93). However, little is known about the role of PTPs in the proliferation and differentiation of haemopoietic stem cells and progenitor cells and to date only SHP-1 has been studied in PV cells.

PV progenitors and mature cells show normal mRNA expression and protein levels of SHP-1 (94) however, the PTP inhibitor orthovanadate has a reduced effect on PV cells compared with normal erythroid progenitors suggesting a possible defect in phosphatase activity (53,57). Subsequently, a potentially novel, membrane associated PTP (E-PTP 170) has been identified and shown to be present in highly purified normal erythroid progenitors. E-PTP 170 did not cross-react with antibodies to SHP-1, SHP-2 or the haemopoietic specific PTP, CD45. In normal cells E-PTP 170 declined rapidly with cell maturation, whereas PV progenitors showed a slower loss of E-PTP 170 expression and had increased E-PTP 170 activity (95). The cause of altered E-PTP 170 expression in PV has not been determined and further functional analysis will be required to elucidate its importance for signalling in this disease.

ii) Apoptosis. In haemopoietic tissues, homeostasis of the stem cell and progenitor compartments, and turnover of mature cells is controlled by apoptosis, where cells are genetically programmed to die in response to a variety of stimuli e.g. lack of survival factors (reviewed in ref. 96). The signals involved are incompletely understood, however apoptosis is partially regulated by the Bcl-2 family of proteins, members of which possess positive or negative regulatory functions (97). Several HGFs implicated in PV pathogenesis have been shown to support the growth and differentiation of cells partly through suppression of apoptosis (reviewed in ref. 98).

Expression of Bcl-xl, a negative regulator of apoptosis from the Bcl-2 family, has been investigated in PV erythroid progenitors. Bcl-xl mRNA levels correlated with the ability of PV cells to survive *in vitro* without EPO. Bcl-xl protein expression, measured using an antibody recognising total Bcl-x (i.e. Bcl-xl and Bcl-xs), was significantly higher in bone marrow erythroid cells from untreated PV patients than from other myeloproliferative disorders. Normal mature erythroblasts express little or no Bcl-x while mature erythroblasts from PV patients express the highest level of Bcl-x. Additionally, Bcl-x levels were prolonged in PV cells through all stages of erythroid differentiation whereas in normal cells Bcl-x expression is restricted to the EPO dependent stages of erythroid development (99). The role of Bcl-x in myelopoiesis and megakaryopoiesis and in more primitive stem cells remains to be elucidated, however, the dysregulation of Bcl-x in erythroid progenitors is a possible mechanism for the accumulation of erythroid cells in PV patients. Alternatively, alterations in early receptor signalling events could lead to secondary effects on Bcl-x levels.

7. Potential molecular basis for PV

It is clear from the studies described above that PV progenitors are altered in their response to many HGFs and multiple signalling pathways are affected in these cells. Below we speculate on potential mechanisms that could lead to such effects on multiple receptor pathways.

i) Dysregulation of an intracellular signalling molecule. A component involved in intracellular signalling via multiple receptors could be implicated in PV. RTKs and CRs share some common signalling molecules. For example, members of the JAK family of non-receptor PTKs are universally activated in CR signalling (reviewed in refs. 100,101), and are also activated in response to IGF and SCF stimulation (102,103). One function of JAKs is to mediate phosphorylation of signal transducers and activators of transcription (STATs) to induce their nuclear translocation and activation of cytokine response genes. Various combinations of JAK and STAT molecules are thought to contribute to signalling specificity in haemopoiesis (reviewed in ref. 38). The JAK/STAT pathway has not been extensively investigated in PV. Particularly, JAK2 has not been studied despite its role in the signalling pathways of multiple CRs and RTKs including EPOR, GMR, Mpl and IGF-1R (12,102-106). Of particular relevance also, are the recently described suppressor of cytokine signalling (SOCS) proteins which have been shown to negatively regulate CR signalling following induction by the JAK/STAT pathway

(107). Altered expression of such a molecule has the potential to profoundly affect signalling in response to multiple HGFs.

ii) Dysregulation of a shared receptor component. The altered response of PV progenitors to multiple HGFs also raises the possibility that an alteration to one receptor may be affecting other receptor responses. This would be consistent with recent studies which have demonstrated interactions between heterologous receptors. For example, EPOR has been shown to associate with hβc and *c-kit* to induce their tyrosine phosphorylation in murine cell lines (108-111) and TPO has been shown to induce tyrosine phosphorylation of hβc in an erythroleukaemic cell line (112). Further, mice expressing a constitutively active form of hβc (FIΔ, reviewed in ref. 67) developed hyperplasia of the erythroid and megakaryocytic lineages in addition to the myeloid lineage. This may be explained by an effect of the hβc mutant receptor on the sensitivity of erythroid and megakaryocyte progenitors to EPO and TPO, via direct interaction with EPOR or TPOR (68). Given this ability of hβc to interact with other receptors it is worthy of further investigation in PV.

iii) Altered regulation of splicing. Dysregulation of a specific, developmentally regulated, splicing event provides a mechanism for the loss of the altered splice variant (EPOR-T) in PV progenitor cells. In view of recent findings regarding EPOR interactions with IL3R, Mpl and *c-kit*, (see above), it would be interesting to investigate the consequences of interaction of EPOR-T with these receptors.

iv) Altered receptor processing. The identification of a PV specific Mpl isoform exhibiting altered glycosylation raises the possibility that a defect in post-translational receptor processing may affect the function of several receptors in PV. Whilst a number of receptors have been investigated for genetic alterations and appear normal, their glycosylation has not been studied.

v) Autocrine production of HGF. PV progenitors have been shown to respond normally to the HGFs IL-4, IL-6, G-CSF, M-CSF and EPO *in vitro* (54,113). However, autocrine production of a novel, synergising HGF remains a possible mechanism to explain the reduced requirement of PV cells for certain HGFs. Stromal cells co-isolate with progenitors purified from bone marrow for *in vitro* studies, forming an adherent layer in liquid culture. Normal BFU-E from PBMNC preparations require the adherent cell population in addition to exogenously added HGFs for growth in serum-free conditions (84). However, PV BFU-E under the same conditions, were able to grow when adherent cells were depleted from the culture, requiring only hemin, IL-3 and IGF-1 to which they are hypersensitive (54). This suggests the lack of requirement for a soluble factor released by stromal cells and raises the possibility of an autocrine HGF affecting PV cell responses *in vitro*. The growth of normal BFU-E was comparable in the presence of conditioned medium from PV and normal cells (65). However, these experiments were not performed with limiting concentrations of HGFs and the possibility of autocrine production of a synergising HGF cannot be ruled out.

8. Summary

PV is a myeloproliferative disorder that arises as a consequence of clonal expansion of a multipotent stem cell. Despite numerous studies over several years, the underlying lesion that leads to PV pathogenesis remains unknown. Since our understanding of the molecular mechanisms involved in self-renewal and differentiation of the haemopoietic stem cell is limited, the elucidation of a lesion with such profound effects on this cell is of major importance. Furthermore, the identification of a clonal marker for PV would be of major clinical significance, facilitating rapid diagnosis.

Investigations so far in bi- or uni-potential progenitor populations from PV patient material show increased sensitivity to a number of HGFs. In addition, several receptor and signalling abnormalities have been described in PV cells. However, it is important to note the difficulty in interpretation of these results since these studies compare the characteristics of a clonal population of PV progenitors with a heterogeneous population of normal progenitors. It seems likely, nevertheless, that some of these effects are true abnormalities of the PV stem cell and that an alteration that has a global effect on HGF responses is responsible for the cellular expansion in PV.

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