The Isolation and Characterization of Revertants of Mammalian Cell Transformation.

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Abstract

In an effort to identify the components of the signal transduction cascades involved in tumorigenesis, a genetic approach was undertaken. Revertants of v-*abl* and v-Ha-*ras* mediated transformation were isolated by a novel method utilizing fluorescence activated cell sorting (FACS). The analysis of the revertants of v-*abl* transformation led to the isolation of a dominant negative v-*abl* gene. The dominant negative v-*abl* blocks Rat-1 fibroblast cellular transformation by the polyoma Middle T, v-*abl*, v-*fms*, v-*erbB* and *neu* oncogenes while having no effect on cellular transformation mediated by v-Ha-*ras*, v-Ki-*ras*, v-*mos*, v-*gag-fos-fox*, v-*src* and *tpr-met* oncogenes. The mutant was also assessed for its ability to affect cellular signalling in other cellular contexts. PC12 neuronal differentiation is potentiated in cells overexpressing the mutant v-*abl*. No detectable effect on B-cell and T-cell receptor signalling was detected. Cloning and sequence analysis of the mutant v-*abl* revealed that the protein product encoded a largely truncated form of the wild type $p160^{v\text{-}abl}$. The mutant protein has retained the *gag* moiety and SH2 domain of the wild type protein but lacks the entire tyrosine kinase domain and C-terminal portion of the protein. The structure of the mutant coupled with preliminary attempts at determining the mode of action of the mutant have suggested that the mutant may interfere with the proper modification of a critical signal transducer. The analysis of the revertant line obtained from Mink cells transformed with v-Ha-*ras* suggested a potential role for the *mdr* gene product in transformation. Somatic cell hybrid experiments suggest that the revertant line obtained has sustained at least two different genetic events. In order to assess the contribution of the *mdr* gene product to v-Ha-*ras* mediated transformation, two separate approaches were used. Firstly, a construct designed for overexpression of the mouse *mdr1b* gene was tested for its ability to induce transformation in Mink cells. Secondly, a construct designed to block expression of *mdr* genes, an antisense construct, was assayed for its ability to block v-Ha-*ras* mediated

transformation in Mink cells. The results obtained suggest that *mdr* gene expression does not play a substantial role in v-Ha-*ras*-mediated transformation.

Résumé

Afin d'identifier les composantes des voies métaboliques impliquées dans la transformation cellulaire, une approche génétique a été utilisée. Des révertants furent isolés à partir de lignées cellulaires transformées par les oncogènes v-*abl* et v-Ha-*ras* à l'aide d'une technique basée sur le triage de cellules teintes par des fluorochromes (FACS). Au cours de l'analyse de révertants v-*abl*, un rétrovirus v-*abl* mutant fut isolé. L'expression du mutant bloque la transformation cellulaire de fibroblastes Rat-1 induite par les oncogènes v-*abl*, Middle T (du virus polyoma), v-*erbB*, v-*fms* et *neu*. Cependant, l'expression du mutant n'a aucun effet sur la transformation médiée par les oncogenes v-Ha-*ras*, v-Ki-*ras,* v-*mos*, v-*gag-fos-fox*, v-*src* ou *tpr-met*. De plus, le mutant facilite la différenciation cellulaire des cellules PC12 en neurones. L'analyse de l'expression du mutant dans des cellules B et T n'a pas révélé d'effet sur la signalisation des récepteurs B et T. Nous avons cloné et séquencé le rétrovirus mutant. Le gène mutant code pour une protéine qui ne contient que les portions *gag* et SH2 du virus souche. Les résultats de certaines expériences préliminaires et la structure de la protéine suggère que le mutant affecte la modification d'une composante, qui demeure inconnue, des voies de signalisation cellulaire. Le révertant v-Ha-*ras* semblait issu de deux évènements génétiques. Néanmoins, au cours de l'analyse de cette lignée cellulaire, nous avons fait certaines observations suggèrant un rôle pour les gènes *mdr* dans le processus de transformation. Nous avons employé deux approches complémentaires pour examiner le rôle de ces gènes: nous avons surexprimé le gène murin *mdr1b* dans la lignée cellulaire Mink afin de déterminer s'il pouvait agir d'oncogène dans ce contexte cellulaire. Deuxièment, une construction antisense a été utilisée dans le but de bloquer l'expression de ces gènes dans les cellules Mink transformées avec v-Ha-*ras*. Les résultats obtenus suggèrent que l'expression des gènes *mdr* n'est pas essentiel pour la transformation cellulaire médiée par l'oncogène v-Ha-*ras*.

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Preface

The following statements have been included in accordance with thesis specifications outlined in in "Guidelines to Thesis Preparation":

> Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.
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>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include**: A Table of Contents, an abstract in English and french, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
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Chapters 2 and 4 will be submitted as manuscripts to peer reviewed scientific journals.

Contributions to Original Knowledge

1. The truncated mutant *abl* protein described in the first part of this thesis represents a unique example of a dominant negative cytoplasmic tyrosine kinase which inhibits transformation by different classes of oncogenes.

2. Our findings also suggest that v-*abl* mediated transformation shares a critical step with transformation by other oncogenes of a different class.

3. The dominant negative mutant potentiates PC12 neuronal differentiation even though it blocks transformation (by a selective set of oncogenes) in fibroblasts. Potentiation and stimulation of PC12 differentiation is more often associated with oncogenic or mitogenic signalling molecules.

4. Our studies into the role of MDR in transformation were fueled by several observations which suggested a potential involvement in malignant transformation. Our results suggest that the *mdr* gene does not play a substantial role in v-*Ha-ras* mediated transformation in epithelial cells.

5. The transformed cell line 1532N harbors an amplification of the *mdr* locus even though this cell line was not subjected to selection with drugs from the MDR spectrum. This constitutes a unique example of *mdr* gene amplification in the absence of selection.

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List of Abbreviations

ABC transporters	ATP-binding cassette transporters
ALL	Acute lymphoblastic leukemias
AML	Acute myelogenous leukemias
A-MuLV	Abelson murine leukemia virus
bcr (gene)	Break cluster region
BCR	B cell receptor complex
CA (p30)	Major capsid protein
CAT	Chloramphenicol acetyl transferase
C. elegans	*Caenhorhabditis elegans*
CFTR	Cystic Fibrosis transporter protein
CML	Chronic myelogenous leukemias
CNS	Central nervous system
CSF-1	Colony stimulating factor 1
C-terminus	Carboxyl terminus
CTD	C-Terminal domain of RNA polymerase II
DM	Double minutes
D. melanogaster	*Drosophila melanogaster*
DMSO	Dimethylsulfoxide
E. coli	*Escherichia coli*
EGF	Epidernal growth factor
EGFR	Epidernal growth factor receptor
EMS	Ethyl methyl sulfonate
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
FBSI	Fetal bovine serum heat-inactivated
FGF	Fibroblast growth factor
FTase	Farnesyl transferase
gag	Group antigen gene
GAP	GTPase activating activity
GDI	Guanine-nucleotide-dissociation inhibitor
GDS	Guanine-nucleotide-dissociation stimulator
GEF	Guanine nucleotide exchange factor
GNRP	Guanine-nucleotide-releasing protein
GST	Glutathione-S-transferase
GVBD	Germinal Vesicle Breakdown
HBD	Hormone binding domain
HBV	Hepatitis B virus
HDA	Haploinsufficiency dependent on an *abl* mutant
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HSR	Homogeneous staining regions
IPTG	Isopropyl-β-D-thiogalactopyranoside.

List of Abbreviations (continued)

IRS-1	Insuliin receptor substrate 1
JNK	c-Jun specific kinase
LTR	Long terminal repeat
MA (p15)	Matrix associated protein
MAIDS	Murine acquired immunodeficiency syndrome
MAPK	Mitogen activated protein kinase
MEK or MAPKK	MAPK kinase
MEKK or MAPKKK	MEK kinase
MDR	Multidrug Resistance
MMTV	Mouse mammary tumor virus
MoMuLV	Moloney murine leukemia
MuLV	Murine leukemia viruses
neo	Neomycin
NF1	Neurofibromatosis type 1
NGF	Nerve growth factor
NK	Natural Killer
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
Pgp	P-glycoprotein
Ph^1	Philadelphia chromosome
PH	Pleckstrin homology domain
PLCγ	Phospholipase C gamma
PKC	Protein kinase C
pTyr	Phosphotyrosine
puro	Puromycin
PVDF	Polyvinylidine fluoride
SDS	Sodium dodecyl sulfate
SH	SRC homology domain
TCR	T cell receptor complex
Tris	Tris(hydroxymethyl)aminomethane
TLCK	L-1-Chloro-3-[4-tosylamido]-7-amino-2heptanone
X. Laevis	*Xenopus laevis*

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 The *Abl* Oncogene

1.1.1 Introduction

The *v-abl* oncogene was first identified as the transforming gene product of the Abelson Murine Leukemia virus (A-MuLV), a retrovirus which causes pre-B lymphomas in mice (1-3). The v-*abl* oncogene is the result of the transduction of the murine c-*abl* into a Moloney Murine Leukemia (MoMuLV) virus genome (4). Homologs of the murine c-*abl* have been isolated from human, *Drosophila melanogaster*, *Caenorhabditis elegans*, as well as from a Feline retrovirus, the Hardy-Zuckerman-2 Feline Sarcoma Virus (5-7). The human c-*abl* locus is rearranged in 90-95% of Chronic Myelogenous Leukemias (CML) as well as in a lesser proportion of Acute Lymphoblastic Leukemias (ALL) and Acute Myelogenous Leukemias (AML) (8). The characteristic or classical cytogenetic abnormality, termed the Philadelphia chromosome (Ph^1), results from a translocation between chromosomes 9 (*c-abl*) and 22, more specifically t(9;22)(q34;q11) (8). As a result of this translocation a hybrid mRNA is formed between the *bcr* gene, for break cluster region (9), and the c-*abl* gene. The gene product encoded by this hybrid mRNA is called $p210^{bcr/abl}$. It should be noted that in some forms of ALL a slightly shorter region of *bcr* is fused to *abl* thus giving rise to a $p185^{bcr/abl}$. The human *bcr/abl* proteins as well as the murine $p160^{v\text{-}abl}$ represent activated forms of the $p150^{c\text{-}abl}$. It is believed that increased gene expression, as well as relocalization and increased tyrosine kinase activity of the *abl* gene product, enable these activated forms to escape normal cellular controls (10,11). An increasing body of evidence suggests that c-*abl* is a negative regulator of growth thus, unlike other protooncogenes, overexpression is not sufficient to render it fully oncogenic (12). Although v-*abl* and *bcr/abl* can give rise to a myeloproliferative disease in mice reminiscent of CML (13), there exist some differences in their ability to transform cells. For instance, $p210^{bcr/abl}$ can not transform NIH 3T3 cells while $p160^{v\text{-}abl}$ can effectively transform these cells (14). These phenotypic differences may reflect structural differences between these activated forms which in turn

could translate into unique interactions with specific signal transducers. Section 1.1.2 examines the molecular characteristics of the *abl* genes and gene products while section 1.1.3 focuses on the substrates, effectors and regulators of the Abl proteins .

1.1.2 The Molecular Structure of *abl* Genes

The murine and human *c-abl* genes are surprisingly similar in their genomic organization (figure 1). Both have 10 or 11 common exons which are separated from alternative 5' exons (4,15). In the mouse genome there exist four alternative 5' exons while in the human genome there exist only two. Murine type 1b (also known as type IV) and 1a (also known as type 1) exons are used predominantly in mouse somatic tissues. The level of type 1b transcript varies very little from tissue to tissue, conversely, the level of type 1a can fluctuate up to an order of magnitude (21). The levels of type II and III murine c-*abl* mRNAs are very low. In humans, the two alternate exons also designated 1a and 1b give rise to two different c-*abl* transcripts. The human and murine type 1b transcripts give rise to protein products with potential myristylation signals, encoded by the unique 5' exons. In comparing the activated forms of *abl* with c-*abl*, we can identify important deletions and substitutions in the Abl proteins which will likely affect its physiological activity (figure 2).

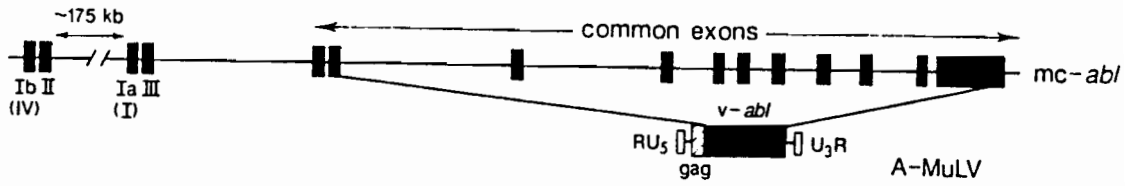
Traditionally, activated forms have been assayed for their ability to transform fibroblasts, transform lymphoid cells as well as their ability to render cell lines (myeloid or lymphoid) growth factor independent (11). The contribution of each of the domains of these proteins to these physiological activities will be discussed in the following sections.

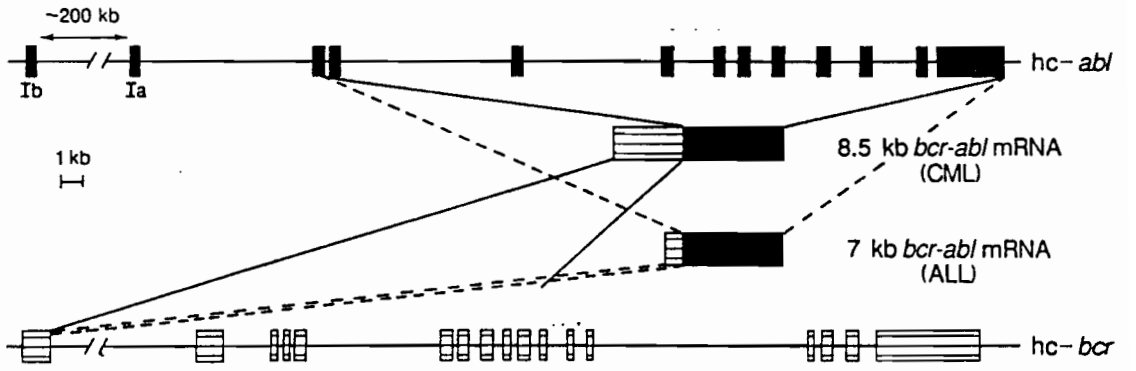
1.2.1 The N-Terminus

As mentioned previously the two predominant forms of *c-abl* differ in their amino(N)-termini, type 1b has a 46 amino acid residue N-terminus which encodes a myristylation signal while type 1a has 26 unique amino acids lacking this same signal

Figure 1. Schematic Representation of the Genomic Organization of the Murine and Human c-*abl* Locus.

The murine c-*abl* (mc-abl) and v-*abl* (A-MuLV) are illustrated in the top portion of this figure while the human c-*abl* (hc-abl) and *bcr* fusions are illustrated in the bottom portion. The small black boxes represent *abl* exon sequences while the striped boxes are *bcr* exon sequences. Cross-hatched boxes represent *gag* encoding sequences derived from Moloney Murine Leukemia virus (MoMuLV). The relative position of these exons within the murine and human activated *abl* genes are indicated. The mRNA species derived from these recombination events are also depicted. Adapted from (11).


~175 kb
common exons
Ib II
(IV)
Ia III
(I)
mc-abl
v-abl
RU5
U3R
gag
A-MuLV


~200 kb
Ib
Ia
hc-abl
1 kb
8.5 kb bcr-abl mRNA
(CML)
7 kb bcr-abl mRNA
(ALL)
hc-bcr

(22). Although most *in vitro* generated activated forms maintain the c-*abl* N-terminus, it is often replaced in naturally occurring variants (figure 2).

A) A-MuLV

In the A-MuLV, the N-terminus of p150$^{c\text{-}abl}$ has been substituted with 236 MoMuLV *gag* (group antigen) amino acids which include the entire p15 (matrix associated protein, MA), p12 (a protein of unknown function) and 20-21 amino acids of the p30 (major capsid protein, CA) protein (figure 2) (23). It should be noted that in the context of p160$^{v\text{-}abl}$ these Gag proteins are not processed into their subunits.Work by Goff and his colleagues has suggested that MoMuLV Gag proteins can oligomerize, thus it also plausible that the activation of the Abl protein can be accounted for by oligomerization of the Abl protein kinase to allow transphosphorylation as was suggested previously by other groups (24,25). The substitution of Gag amino acids has also resulted in the loss of the SH3 domain, a negative regulator of the tyrosine kinase activity of the Abl protein (described in detail in section 1.1.2.2.B). The p15 moiety of p160$^{v\text{-}abl}$ harbours a myristylation signal which confers localization to the plasma membrane (26). The myristylation signal appears critical for fibroblast transformation while the rest of the Gag amino acids appear largely unnecessary for fibroblast transformation since v-*abl* variants with large deletions encompassing most of the *gag* except the N-terminal myristylation signal retain fibroblast transformation activity (27,28). In contrast, these same large deletions affect lymphoid transformation by rendering the p160$^{v\text{-}abl}$ unstable in this cellular context (29). Furthermore, N-terminal myristylation is not required for transformation of Ba/F3 to growth factor independence and tumorigenecity (30). Thus the relative importance of each subdomain is strongly dictated by the cellular context in which it is expressed.

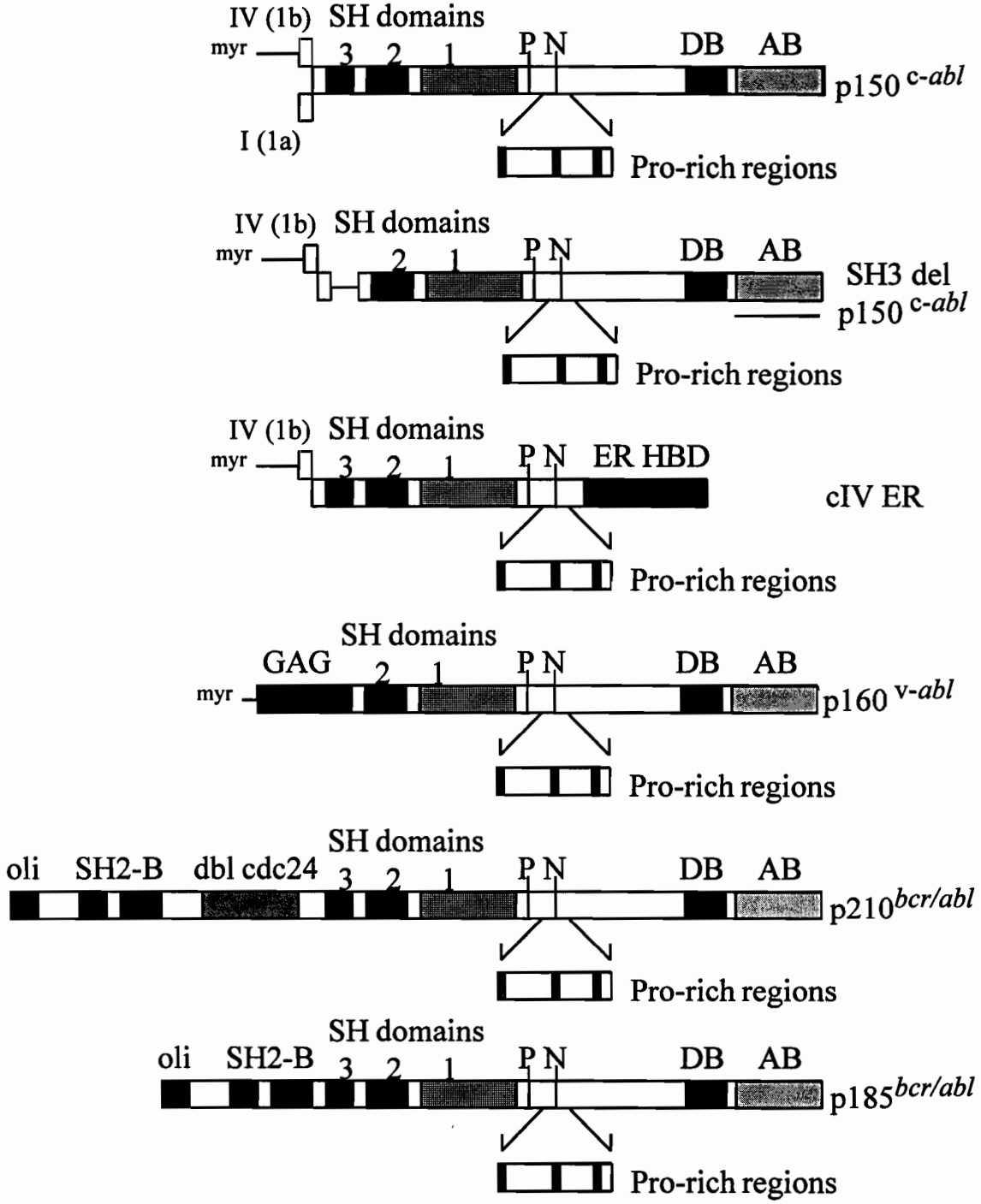
Figure 2. Schematic Representation of Abl Protein Structure.

The structures are shown approximately to scale. In this figure the prototypical p150$^{c\text{-}abl}$ is compared to:1) two activated forms of p150$^{c\text{-}abl}$(IV), the SH3 deleted (SH3 del) and the hormone-conditional transforming variant (cIV ER) (22,54); 2) the murine p160$^{v\text{-}abl}$ (23) 3) the two forms of the *bcr/abl* fusion protein, p185 and p210 (8). Some of the common features are represented: Myr, myristic acid group added to N-terminal glycine; IV(1b) and I (1a) alternative 5' exons, murine IV and I, human 1a and 1b; the SH domains, 3 for SH3, 2 for SH2, 1 for SH1 also tyrosine kinase domain (26); P, CDC2 phosphorylation site (115); N, nuclear translocation signal (81); Pro-rich regions, proline rich regions magnified to show the three distinct SH3 binding sites (56); DB, DNA binding domain (82,83); AB, F and G actin binding domains (69,93).

For p160$^{v\text{-}abl}$, GAG represents MoMuLV *gag* amino acids (23).

HBD is a small portion of the estrogen receptor hormone binding domain sufficient to confer estrogen conditional activation of this protein.

Within the *bcr/abl* proteins, we find : oli, the oligomerization domain; SH2-B, the bipartite SH2 binding domain, Bcr domain 2; dbl cdc24, racGEF domain with homology to the dbl and cdc24 proteins (148).


IV (1b)
SH domains
myr
3
2
1
P
N
DB
AB
p150 c-abl
I (1a)
Pro-rich regions
IV (1b)
SH domains
myr
2
1
P
N
DB
AB
SH3 del
p150 c-abl
Pro-rich regions
IV (1b)
SH domains
myr
3
2
1
P
N
ER HBD
cIV ER
Pro-rich regions
SH domains
GAG
2
1
P
N
DB
AB
myr
p160 v-abl
Pro-rich regions
SH domains
oli
SH2-B
dbl cdc24
3
2
1
P
N
DB
AB
p210bcr/abl
Pro-rich regions
SH domains
oli
SH2-B
3
2
1
P
N
DB
AB
p185bcr/abl
Pro-rich regions

B) BCR/ABL

The p185*bcr/abl* and p210*bcr/abl* proteins contain an N-terminal region which is encoded by the *bcr* gene and lacks the unique 5' exon of c-*abl* (8). The *bcr* gene encodes a 160kDa protein with many functional domains including serine/threonine kinase and *rac* GTPase activating activities (*rac*GAP) (31). Experiments with *bcr* (-/-) mice have revealed a role for Bcr in regulating oxidative bursts in neutrophils (32). It appears that its ability to regulate Rac2 activity may be responsible for the phenotype observed. These mice will likely prove to be an invaluable tool in determining the role of Bcr in the progression of leukemia. In CML, the p210*bcr/abl* protein is the result of the fusion of the first 902 or 927 amino acids of the Bcr to the c-*abl* common exons encoded amino acids (33,34). The p185*bcr/abl*, which is more often associated with ALL, has the first 406 amino acids of Bcr (33). The fused Bcr sequences activate the tyrosine kinase, actin-binding (domain found in C-terminus) and transforming functions of the Abl protein (35).

A large body of work has shed some light on the mechanism of action of this portion of Bcr. Firstly, through the analysis of deletion variants, it was determined that two domains contained in exon 1 of Bcr were responsible for its "activating" potential (35). Domain number 1 stretching from amino acids 1-63 was shown to be essential for the activation of actin binding and deletion of amino acids 3-37 (contained within this domain) rendered the protein unable to transform fibroblasts and lymphoid cells. One model suggests that domain 1 contains a coiled-coil oligomerization domain which induces tetramerization of *bcr/abl* proteins. This, in turn, would favour intermolecular cross-phosphorylation of the Abl tyrosine kinase domain which would play a role in its activation (36,37).

The second domain critical for transforming activity is located between amino acids 176-242: on its own, this domain only weakly activates tyrosine kinase activity and microfilament binding activity but interacts with the Abl SH2 domain in a phosphotyrosine independent manner (35,38). It is believed that this interaction may

disrupt the negative regulatory function of the Abl SH3 (38) or SH2 domains (37,39). Further analysis of this SH2 binding capacity of Bcr has revealed that a limited number of SH2 domains can interact with this domain and that the interaction is of relatively lower affinity than phosphotyrosine dependent interactions. This interaction may also depend on other domains of the protein (40). In their initial studies Pendergast and colleagues had demonstrated that two serine threonine-rich regions of Bcr, between amino acids 192-242 and 298-413, could bind to the Abl SH2 domain (38). Loss of both of these regions resulted in a transformation-defective p185*bcr/abl* although the presence of one was sufficient for transforming activity. Even though these interactions are potentially weaker and more selective, some suggest that they may be involved in the initial recruitment of substrates to SH2 harbouring proteins while others believe that they enhance SH2 function (37,40).

Domain 2 also contains a tyrosine residue, Y177, which when phosphorylated binds the adaptor molecule GRB2 thus potentially coupling BCR/ABL to the Ras signaling pathway (41,42). Mutation of this residue to phenylalanine prevents autophosphorylation which eliminates Grb2 SH2 binding and impairs the transforming activity of p185*bcr/abl* in primary bone marrow cells as well as in Rat-1 fibroblasts (41). In Rat-1 cells expressing p185*bcr/abl*, p185*bcr/abl*-Grb2-Sos 1 complexes are detected as well as Grb2-Shc complexes, providing further evidence that p185*bcr/abl* oncogenesis is mediated partly by the Ras cascade (42).

Finally, the portion of Bcr contained in *bcr/abl* proteins has also been shown to interact with the Bap-1 protein a member of the 14-3-3 proteins which are believed to be involved in mitogenic and cell cycle controls (43). The physiological relevance of this interaction awaits a better understanding of the precise role of the 14-3-3 proteins in cellular processes.

1.1.2.2 The SRC Homology Domains (SH)

The SRC homology domain 2 (SH2) was first identified as a region of sequence identity N-terminal to the tyrosine kinase domain, also termed SH1, of cytoplasmic tyrosine kinases (44). Additionally, the presence of another domain of high sequence identity among these cytoplasmic tyrosine kinases was identified and termed the SH3 domain (45). A large body of work has recently shed some light on the role of these domains in mediating protein-protein interactions involved in signal transduction associated with a large range of cellular phenomena including cellular transformation (26,46-51).

A) The SH3 domain

The SH3 domain is a modular protein binding domain of approximately 50 amino acids long (figure 2). The Abl SH3 domain has long been considered an inhibitory domain since deletions and point mutations in this domain appear to activate the tyrosine kinase activity and transforming potential of Abl proteins (22,30,52,53,17). Interestingly, this inhibitory action is overriden when the hormone binding domain (HBD) of the estrogen receptor (ER) is fused to the C-terminus of a truncated c-*abl* type IV protein (54). It is suggested that overexpression or oligomerization of this variant may override the SH3 inhibitory function. A mutation in a conserved phenylalanine (F420 in the SH1) activates the fibroblast transforming potential of c-*abl* type IV even if the SH3 is present (53). The position of the SH3 domain with respect to the tyrosine kinase domain also appears critical (55). Although the mutational and positional studies may hint at an intramolecular interaction, the candidate SH3 binding motifs (see figure 2 and section 1.1.2.3B) within the Abl proline-rich domains bind relatively weakly *in vitro* to the Abl SH3 domain (56). Furthermore, work by Pendergast and colleagues suggests that the regulation of the p150$^{c\text{-}abl}$ tyrosine kinase activity occurs through another cellular component (36). Their studies had revealed that overexpression of the c-*abl* kinases increased *in vivo* kinase activity through a mechanism other than transphosphorylation

since coexpression of kinase-inactive variants with kinase-active forms does not lead to phosphorylation of the kinase inactive p150$^{c\text{-}abl}$. Furthermore, the level of kinase activation appeared to be related to the level of overexpression. This suggested that overexpression had effectively titered out a molecular repressor. Finally, it was determined that the p210$^{bcr/abl}$ and p150$^{c\text{-}abl}$ kinases are dephosphorylated at similar rates. Thus, the lower level of phosphotyrosine present on the c-*abl* gene product is due to a lower intrinsic tyrosine kinase activity. This also suggests that the negative regulator does not affect the rate of dephosphorylation thus it is unlikely that it is a phosphotyrosine phosphatase (30).

The apparent involvement of the SH3 domain in the regulation of p150$^{c\text{-}abl}$ kinase activity suggested that it may interact with the cellular component responsible for this process. A high affinity "ligand" for the Abl SH3 domain has been identified but its molecular characteristics are more consistent with a function in linking the Abl protein to another signaling pathway, than in the negative regulation of the tyrosine kinase activity of Abl (57). Alternatively, the Abl SH3 could exert its function through localization effects as has been suggested for the SH3 domains of phospholipase C gamma (PLCγ) and Grb2 (58). However, this is inconsistent with the positional studies where even subtle changes in the position of the SH3 within p150$^{c\text{-}abl}$ affected its inhibitory activity (55).

To summarize, the Abl SH3 domain may negatively regulate the tyrosine kinase activity of Abl through an direct or indirect (via the high affinity ligand) interaction with a yet unidentified cellular inhibitor.

B) The SH2 domain

SH2 domains were first identified as regions of high sequence identity in the amino terminus of cytoplasmic tyrosine kinases (44). It was later found in a large variety of proteins with a range of enzymatic ativities as well as in proteins with no obvious catalytic domain (26,46-51). It has been demonstrated extensively that the SH2 domain mediates protein-protein interactions important in all aspects of cellular life .

It is widely recognized that SH2 domains bind phosphotyrosine residues in a specific amino acid context (59). They may also bind phosphoserine or threonine but with relatively weaker affinity (40,60). Work by Songyang and colleagues (61,62) with phosphopeptide libraries has revealed that SH2 domains recognize specific phosphotyrosine containing motifs. It appears that SH2 specificity is dictated by the next four residues (for some it extends to six residues (63)) C-terminal to the phosphotyrosine residue. These amino acids residue are designated the +1,+2,+3,+4 residues. Nuclear magnetic resonance (NMR) solution structure and X-ray crystallographic studies have helped determine the critical residues in the SH2 domain which affect phosphotyrosyl binding as well as substrate specificity (64-66). The structural studies supported biological data which suggested that the arginine (R) residue of the highly conserved FLVRESES motif of SH2 domains is critical in phosphotyrosine binding (67). Even a conservative substitution of arginine to lysine is not tolerated in the Abl SH2 of an SH3 deleted $p150^{c\text{-}abl}$IV, the resulting protein is impaired in phosphotyrosine binding as well as transformation activity in NIH 3T3 fibroblasts (67). The structural studies determined that the two terminal nitrogens of arginine would form hydrogen bonds with two of the phosphate oxygens (66). Thus, no other residue can substitute for this highly conserved arginine. The precise position of the residues involved in the recognition of the +1,2,3,4 residues of the substrate have also been determined (64-66). It was demonstrated that a single residue change in a predicted +3 binding residue in the SH2 domain of Src can modify its selectivity to that of Grb2 *in vivo* (68). A Grb2 protein containing the mutant Src SH2 domain with Grb2 selectivity can substitute for wild type Grb2 in vulval induction in *C. elegans*. Therefore the biological activity of an SH2 domain and its binding specificity are tightly linked.

This model has shown great predictive value. For instance, in identifying the region involved in the binding of Grb2 to Bcr sequences, the Bcr sequences were searched for the consensus Grb2 SH2 binding site as determined by the phosphopeptide

studies. It was shown that a region of Bcr which contained this potential Grb2 binding site was also implicated in the binding (41). The Abl SH2 selects motifs with the following general properties: phosphotyrosine (pTyr)-hydrophilic-hydrophilic-Ile/Pro with a strong preference for asparagine (N) and proline (P) at the +3 and +4 positions respectively (61). Surprisingly, even though many potential substrates for the SH2 domain of Abl have been identified none have been substantiated physiologically.

The contribution of the SH2 domain to the biological activities of Abl genes varies. It appears that for promoting factor-independent growth the Abl SH2 domain is not required (69) while it is essential for its transforming activity (55,67,70). Interestingly, other SH2 domains can substitute for the Abl SH2 domain in this capacity (55). The substitution of the Src SH2 domain for the Abl SH2 domain in p160$^{v\text{-}abl}$ does not affect its ability to transform fibroblasts as well as lymphoid cells. From these studies, it was concluded that unlike the Src SH2 domain, the Abl SH2 domain does not dictate cell type specificity (71). Cell-type specificity appears to be dictated by a domain located C-terminal to the Abl tyrosine kinase domain since replacement of this domain with pp60$^{v\text{-}src}$ sequences results in a chimeric protein that has lost its ability to transform preB cells while retaining its transforming activity in fibroblasts. In the activated forms of Abl proteins the SH2 domain plays a largely positive role in mediating transformation. In contrast, the substitution of the RasGAP N-terminal SH2 domain for the Abl SH2 into the type 1b c-*abl* activates its transforming potential (39), suggesting that the Abl SH2 contributes to the negative regulation of p150$^{c\text{-}abl}$. It has been suggested that the SH2 domains serves either a transforming or negative regulatory function. Hence, activated forms of Abl including the c-*abl*-RasGAP SH2 substituted form have uncoupled the negative regulatory capacity of the SH2 domain from its ability to elicit transforming signals (39). Since the substitution of the Abl SH2 domain for the RasGAP SH2 leads to a predominantly cytoplasmic membrane-bound form akin to the activated Abl proteins,

modifying the subcellular localization of these proteins may effectively uncouple the negative regulatory function of the Abl SH2 (39).

At the molecular level, the Abl SH2 domain does not appear to be involved in allosteric activation of the tyrosine kinase domain and does not affect protein localization (55). The model put forth by Mayer and Baltimore suggests that the SH2 domain does not dictate substrate specificity but essentially protects substrates which have been phosphorylated by the Abl kinase and now harbour the target phosphopeptide sequence for the Abl SH2 domain. In support of this model, more recent studies on tyrosine kinase specificity indicate that the tyrosine kinase domain of cytoplasmic tyrosine kinases like Abl preferentially phosphorylate peptides which are recognized by their SH2 domain (72,73). Thus the SH2 domain would protect the newly phosphorylated peptide sequence from the action of phosphatases (55,17). Secondly, this interaction would anchor the substrate to the Abl tyrosine kinase thus rendering it available to further modification by phosphorylation. Progressive phosphorylation could create novel binding sites for other signal transducing proteins thus creating a potential macromolecular signal transduction complex much like the one postulated for IRS-1, the so-called docking protein (74).

C) The SH1 domain

The tyrosine kinase domain of Abl is necessary for transformation of fibroblast, lymphoid cells and factor dependent cell lines (11). It shows substantial sequence identity with the tyrosine kinase domain of receptor tyrosine kinases and even greater homology to the catalytic domain of the cytoplasmic tyrosine kinases (10). As mentioned in the previous section, recent evidence (72,73) contradicts the long-held belief that the SH1 domain was largely non-specific (76). It is proposed that the SH1 and SH2 domains work together to bring about the progressive phosphorylation of signal transducers. The SH1 may also contribute to transformation mediated by the p185or p210$^{bcr/abl}$ through autophosphorylation at position Y1294. This site is analogous to tyrosine 416 of the Src kinases and is essential for transformation of lymphoid cells and fibroblasts but is

unnecessary for growth factor independence (77). Analysis of the pattern of phosphorylated proteins and *in vitro* tyrosine kinase assays from cell lines with the wild type and point mutant F1294 revealed no differences in the tyrosine kinase activity of the autophosphorylation mutant (77), unlike the equivalent c-*src* kinase autophosphorylation mutants (78-80). It is unlikely that this site plays a role in modulating tyrosine kinase activity. Consequently, the authors suggest that this phosphorylated tyrosine may recruit a critical signal transducer for transformation (77). This finding again establishes distinct requirements for transformation and growth factor independence.

1.1.2.3 The Carboxy (C)-Terminus

The C-terminal portion of Abl bears little or no homology to other cytoplasmic tyrosine kinases (10,11). It harbours many important domains believed to be required for the proper function of c-*abl* gene products as well for transformation by the *bcr/abl* proteins. The next section reviews the various domains which are contained within this unique C-terminal domain (see figure 2).

A) the Nuclear Localization Signal

The nuclear localization signal was found to be a penta-lysine motif contained in the C-terminal portion of the protein (81). This signal possibly ensures proper localization of the protein for it to effect its DNA binding function (82,83). The c-*abl* gene product must be localized to the nucleus to negatively regulate cell growth, its putative physiological role (12). Conversely, most of the activated forms of Abl appear to be excluded from the nucleus despite the presence of a nuclear localization signal. Thus the activation of the transforming potential seems to override the nuclear localization signal. Experiments with two *in vitro* generated variants of the c-*abl* protein appear to support this claim: 1) the hormone inducible variant translocates from the nucleus to the cytoplasm upon hormone treatment which activates its transforming potential (54), 2) *en bloc* substitution of the Abl SH2 domain for the RasGAP SH2 domain activates the

transforming potential of type1b c-*abl* as well as relocalizing the chimeric protein to the cytoplasm (39). Thus, the nuclear localization signal may be important for c-*abl* function but a number of activating mutations override the signal.

B) The SH3 binding region

It was originally discovered through the use of the two hybrid system (84) where the C-terminal portion of the p150$^{c\text{-}abl}$ protein (common to all Abl proteins save the HBD mutant) was used as bait (56). The SH3 binding region consists of three potential SH3 binding sites (56). *In vitro* studies revealed that these three potential sites were bound with different specificities by the Crk SH3 domains as well as the SH3 domains of other adaptor proteins namely Nck and Grb2 . *In vivo* it appears that the c-*abl* protein as well as the activated (SH3 deleted) variant of p150$^{c\text{-}abl}$ are capable of interacting with Crk when these proteins are overexpressed in 293 cells. Furthermore, deletion of the SH3 binding sites disrupts this *in vivo* association. A number of studies which support the existence of a physiolgical interaction between Abl and Crk proteins will be reviewed in the next section which deals with the potential substrates of the Abl kinases. It should be noted that p160$^{v\text{-}abl}$ mutants lacking this region retain full transforming activity for fibroblasts but decreased transforming potential for lymphoid cells (11).

C) The C-terminal serine threonine phosphorylation sites

The C-terminal portion of p150$^{c\text{-}abl}$ is differentially phosphorylated during the cell cycle (85,86). The c-*abl* protein is phosphorylated on three ser/thr sites at interphase and then at a further seven sites during mitosis (85). *In vitro* kinase assays with purified preparations of Cdc2 kinase suggest that this important regulator of cell cycle progression is responsible for the mitotic phosphorylation events (85). It was demonstrated that these phosphorylation events diminish the DNA binding activity of p150$^{c\text{-}abl}$. Furthermore, dephosphorylation of p150$^{c\text{-}abl}$ *in vitro* restores DNA binding activity (82). Thus it appears that the ultimate role of Cdc2 mediated phosphorylation of p150$^{c\text{-}abl}$ is to modulate its DNA binding activity with no apparent affects on the kinase activity of the

Abl kinase. These sites could potentially be involved in modulating the activity of the SH2 domain since the Abl SH2 domain has been shown to bind phosphoserine (38,40).

D) The DNA Binding Domain

A C-terminal domain which bound the non-specific substrate of DNA-cellulose was defined as the DNA binding domain (82). A sequence specific enhancer binding activity was later described for p150$^{c\text{-}abl}$ (83). p150$^{c\text{-}abl}$ was identified as a component of the enhancer binding complex which binds an enhancer element found in the hepatitis B virus (HBV), termed the EP site. Interestingly, a similar element is found in the c-*myc* promoter. C-*myc* has been found to be activated transcriptionally in Abl transformed cells and it also appears to be involved in downstream events involved in Abl mediated oncogenesis (77,87,88). However, in gel shifts assays using the c-*myc* EP-like site and nuclear extracts from c-*abl* overexpressing cells, p150$^{c\text{-}abl}$ could not be detected in the EP-like binding complex (89). Furthermore, in transcriptional activation assays using the chloramphenicol acetyl transferase (CAT) reporter gene under the control of the c-*myc* EP sites, the DNA binding domain of p150$^{c\text{-}abl}$ was shown to be dispensable for transcriptional activation while the tyrosine kinase domain was required. In fact, the activated Abl proteins which have increased tyrosine kinase activity and are presumably localized in the cytoplasm, displayed increased c-*myc* transcriptional activation. These observations support the existence of an indirect mechanism for c-*myc* transcriptional activation (89). Finally, p150$^{c\text{-}abl}$ activation of *c-myc* transcription is not affected by mutations in the EP site. Thus, the DNA binding activity of p150$^{c\text{-}abl}$ does not appear to be required for transcriptional activation of specific genes. Alternatively, the DNA binding activity may simply target it to the nucleus for interactions with specific nuclear substrates or components of the transcriptional machinery (88). For instance, the C-Terminal Domain (CTD) of RNA polymerase II can be efficiently and apparently specifically tyrosine phosphorylated *in vitro* by p150$^{c\text{-}abl}$ (90).

Many lines of evidence suggest that DNA binding is not required for transformation: 1) activated forms of the Abl protein with deletions in this domain retain full transforming potential (11,69) 2) an activated variant of c-*abl* type 1b has sustained a deletion which removes 81 of 99 amino acids of the minimal DNA binding domain suggesting that the inability of this variant to bind DNA has resulted in the activation of its transforming ability in fibroblasts (91). This variant does not have elevated tyrosine kinase activity but has increased localization to the cytoplasm even though the nuclear localization signal is intact (91). Therefore, the DNA binding activity of Abl proteins is not required for transformation and may only serve in the proper localization of the c-*abl* gene products and in p150$^{c\text{-}abl}$ restricted functions.

E) The F and G Actin Binding Domains

The p210$^{bcr/abl}$ and p150$^{c\text{-}abl}$ IV protein had been shown to associate with filamentous structures in the cytoplasm which were similar to actin stress fibers (81,35,69). P210$^{bcr/abl}$ in K562 cells was later shown to colocalize with actin filaments (35,69). Deletion analysis revealed that the F actin binding domain was in the extreme C-terminal portion of these proteins (93). Although this domain is not necessary for fibroblast transformation by v-*abl* and the SH3 deleted activated c-*abl* proteins, deletions in this domain in p210$^{bcr/abl}$ reduce its ability to transform fibroblasts and a growth factor dependent cell line (28,69,94). The relatively high degree of homology in the last 130 amino acids of this protein in different species suggests a critical role in Abl mediated signal transduction (69). *Bcr/abl* proteins with deletions of this domain have a reduced ability to abrogate IL-3 dependence in Ba/F3 cells while the Bcr moiety appears to activate this actin binding activity. These observations have led to the speculation that this domain is responsible for a specific subcellular localization. Mutants lacking this domain are still cytoplasmic but do not display the charateristic punctate immunocytochemical reaction which correlates with F-actin colocalization. This refinement of its cytoplasmic localization would enable it to interact with critical

substrates on the actin cytoskeleton. This interaction could potentially interfere with cell maturation in myeloid cells mediated by cell adhesion molecules and their receptors which have an intimate connection with the cellular cytoskeleton (69).

A study with the isolated domains from p150$^{c\text{-}abl}$ helped define more precisely the domain responsible for F-actin binding to a 58 amino acid region of the C-terminus (93). The authors also demonstrated that these domains were sufficient to confer F-actin binding to an heterologous protein and that the binding was calcium independent. Furthermore, a proline rich region directs the interaction with G actin. Both domains collaborate to promote bundling of F-actin *in vitro* (93). It is unclear at this point if these bundling activities are functional *in vivo*. Although these data suggest that the Abl protein could affect the cytoskeleton through its bundling activities the authors concede that the low amount of p150$^{c\text{-}abl}$ in the cytoplasm (due to its predominantly nuclear localization) precludes a role for this protein as a major cellular actin sequestering or bundling protein. The authors speculate that p150$^{c\text{-}abl}$ may affect the cytoskeleton by a number of mechanisms. For instance, the Abl SH3 binding protein, 3BP-1(57), which has RhoGAP activity may be recruited to actin by p150$^{c\text{-}abl}$ in order to modulate Rho, a small G protein which has been demonstrated to have dramatic effects on the cytoskeleton (95). P150$^{c\text{-}abl}$ could also phosphorylate the cytoskeleton to bring about morphological changes. Finally, it could act as a signal transducer from the cytoplasm to the nucleus shuttling between these two subcellular compartments and interacting with specific targets (93). In short, although this domain has a very modest role in transformation it may be important in some yet unrecognized p150$^{c\text{-}abl}$ functions. A more rigourous assessment of p150$^{c\text{-}abl}$ trafficking and localization will be required to determine if these proposed functions are plausible.

The contribution of the various domains and structural determinants to the biological activities of the activated forms of ABL are summarized in table 1.

Motif or Domain	Requirement for Biological Activity
N-terminal myristylation	Required for fibroblast transformation Not required for Ba/F3 transformation.
gag (v-*abl*)	Required for lymphoid transformation Unnecessary (largely) for fibroblast transformation
bcr (BCR/ABL)	Deletions of a.a 176-242 or 298-413: 1) impair fibroblast transformation 2) result in partial impairment of IL-3 independent growth and transformation of 32D cells 3) have no effect on BaF3 IL-3 independent growth and transformation
Y177 (BCR-ABL)	A Y177F mutant is impaired in 1) fibroblast and primary bone marrow cell transformation 2) but can still transform and confer factor independent growth to BaF3 and 32D cells.
SH2 (v-*abl* and BCR-ABl)	Required for fibroblast transformation. Not required for factor independent growth and transformation of BaF3 and 32D cells by Bcr/abl.
SH1	Tyrosine kinase activity required for all biological activities
Y1294 in p210$^{bcr\text{-}abl}$ (Y793 in p185$^{bcr\text{-}abl}$)	Required for pre B and fibroblast transformation Not required for factor independent growth and transformation of BaF3 and 32D cells

SH3 protein binding domain(v-*abl*)	Not required for fibroblast transformation. Required for lymphoid transformation*.
F and G actin binding domains for BCR/ABL	Required for transformation of fibroblast and factor dependent cell line by p185bcr-abl Ability to abrogate IL-3 dependence reduced

Table 1. Summary of Relevance of Structural Determinants to Biological Activity of Activated Forms of Abl.This table summarizes the contribution of each domain or important structural present in the activated forms of Abl to transformation and abrogation of factor dependent growth in various cellular contexts. The SH3 domain was omitted from this table since it is largely believed to play an inhibitory role in all these activities. The nuclear localization signal, CDC2 phosphorylation sites was not included since its contribution to these various activities have not been rigorously assessed.

* A single study reports the deletion of this domain but the deletion also encompasses other structural determinants.

1.1.3 The Substrates, Effectors and Regulators of Abl

The structural studies have hinted at the important domains for mediating the normal as well as the transforming functions of *abl* gene products. In the next sections, studies aimed at identifying the mediators and regulators of Abl functions will be reviewed.

1.1.3.1 Putative Substrates

A) The C-Terminal Domain of RNA polII

As mentioned previously $p150^{c\text{-}abl}$ has been shown to phosphorylate the C-terminal domain of RNA polymerase II (90). This activity appears to be specific since the $p60^{c\text{-}src}$ tyrosine kinase fails to phosphorylate the CTD using similar reaction conditions. However, in fibroblasts from c-*abl* knockout mice, the CTD appears to be phosphorylated to a similar extent as in wild type fibroblasts (12). Thus other CTD tyrosine kinase activities can make up for the loss of $p150^{c\text{-}abl}$. The precise role of these phosphorylation events in regulating CTD activity awaits the generation of point mutants of the precise tyrosines phosphorylated by $p150^{c\text{-}abl}$. The c-*abl* protein's predominantly nuclear localization as well as its affinity for DNA make it an attractive candidate for regulating gene expression but these claims remain to be substantiated.

B) Syp

The Syp phosphotyrosine phosphatase contains two SH2 domains (96,97). Its role in transducing signals from a receptor tyrosine kinase was recently ascertained (96,97). Unexpectantly, Syp acts predominantly as an adaptor protein to recruit critical substrates to the activated epidermal growth factor receptor (EGFR), while having no effect on the phosphorylation status of the activated receptor. Tauchi and colleagues have determined that Syp can interact *in vivo* with $p210^{bcr/abl}$ and Grb2 in transformed factor-dependent cell lines (98). *In vitro* studies with fusion proteins have demonstrated that the N-terminal SH2 domain of Syp can mediate binding to the $p210^{bcr/abl}$ while the C-terminal SH2 fusion protein can not bind $p210^{bcr/abl}$. Thus it appears that its association to

p210*bcr/abl* is direct and not due to Grb2 which has been shown to have affinity for both *bcr/abl* proteins and Syp (41,42). The physiological relevance of the Syp-p210*bcr/abl* complex remains unclear: the authors suggest that since Syp is capable of dephosphorylating p210*bcr/abl* *in vitro*, it could potentially modulate p210*bcr/abl* activity by dephosphorylation. Conversely, Syp activity could be affected by this association either through its phosphorylation or by binding of its SH2 domains by a specific phosphopeptide. The latter has been shown to be sufficient to modulate Syp activity (99,100). Finally, recruitment of Syp to a specific locale may bring it into proximity of specific substrates. Grb2 and Syp display similar preferences for phosphopeptide binding, thus these proteins may be competing for the same site (61). Interestingly, Syp also harbours an *in vivo* phosphorylation site which matches the consensus sequence for the SH2 domain of Grb2 (101). Thus at this point the exact role of this interaction, whether it be as a regulator, effector or recruiting protein for p210*bcr/abl* as well as the potential interactions with Grb2 are still a matter of speculation.

C) Elements of the Ras Cascade

Early studies with the microinjection of neutralizing antibodies to p21*c-ras* revealed the importance of Ras in mediating transformation by receptor tyrosine kinases as well as some cytoplasmic tyrosine kinases (102). A direct physical interaction between proteins involved in the Ras cascade and *bcr/abl* proteins also support a role for the Ras in mediating *bcr/abl* oncogenesis (41,42,103). As described in a previous section (section 1.2.1), the Bcr portion harbours a tyrosine phosphorylation site which binds the Grb2 adaptor protein (41,42). A large body of genetic (104-106) as well as biochemical evidence has established the Ras activation pathway (107-112). Grb 2 plays a critical role in recruiting Sos, a guanine nucleotide exchange factor, to the membrane where it can activate Ras (please refer to section 2.1). Hence, it couples receptor tyrosine kinases, and in this case an activated Abl kinase, to the Ras pathway. Furthermore, other studies have

demonstrated that a small percentage of another adaptor protein, Shc may be linked to *bcr/abl* proteins (42). Shc proteins are also involved in Ras signaling through their interaction with Grb2 (113). This interaction is modulated by phosphorylation of the Shc gene product, thus in the *bcr/abl* mediated oncogenesis, several modes of activation of the Ras signalling pathway could be involved. Several lines of evidence suggest that persistent activation of the MAPK/ERK pathway(s) may be responsible for eliciting a mitogenic response (114). In keeping with this hypothesis, it appears that the *bcr/abl* protein is capable of stimulating the Ras/MAPK signaling pathway through a number of inlets thus providing the persistent signal required for a mitogenic response (70).

p120RasGAP which may play a dual role as positive and negative effector of Ras signaling (121-123) has been shown to interact with p210$^{bcr/abl}$ (121). Furthermore, antisense oligos directed against *RasGAP* mRNA inhibited the growth of BV173 cells, a Ph^{1}positive cell line (122). P210$^{bcr/abl}$ can be coimmunoprecipitated with antibodies directed against p120GAP (121). It has been demonstrated that expression of p210$^{bcr/abl}$ in fibroblasts results in the activation of p21^{ras} and the inhibition of p120GAP GTPase activating activity as well as an increase in tyrosine phosphorylation of the p120GAP associated proteins, p62 and p190 (123). Taken together these results would suggest that p120GAP's effector function is required for p210$^{bcr/abl}$ mediated transformation.

In closing, the cloning of an SH3 binding protein with RhoGAP activity would suggest that Abl also interacts with signaling through another member of the small G protein family. Since deletion of the SH3 domain leads to activation of its transforming potential one would be inclined to suggest that this interaction affects Abl transforming activity in a negative fashion (57).

D) Crk

The identification of Crk binding sites in the C-terminal portion of Abl suggested that Crk could be a substrate for the Abl tyrosine kinase (56). The observation that Crk-like (CRKL) protein is the major tyrosine phosphorylated protein in Ph1 positive

neutrophils from patients with CML also suggested that activated forms of Abl could interact with Crk gene products (124). The physiological relevance of this interaction came to light when p150$^{c\text{-}abl}$ was shown to be the Crk kinase (125). Feller and colleagues suggest that p150$^{c\text{-}abl}$ specifically phosphorylates tyrosine 221 of Crk, a residue present in the intervening region between the two SH3 domains (see figure 3). They propose that phosphorylation at this site induces the formation of an intramolecular SH2-Ptyr bond. Their own experiments hinted that phosphorylated Crk behaved as a monomer. This intramolecular interaction is reminiscent of the interaction between the phosphorylated tyrosine 514 in p60$^{c\text{-}src}$ which interacts with its own SH2 domain (126). This was later corroborated by NMR studies and hydrodynamic measurements on purified proteins (127). This phosphorylation event potentially modulates the binding activity of the Crk SH2 and SH3 domains. Interestingly, the v-*crk* oncoprotein lacks this tyrosine residue and hence could escape these controls. The predominantly nuclear localization of p150$^{c\text{-}abl}$ would appear to preclude this interaction but in v-*crk* transformed cells a substantial fraction of p150$^{c\text{-}abl}$ appears to relocalize to the plasma membrane where the v-*crk* oncoprotein is located (125). If we consider that Crk mediated transformation is associated with elevated tyrosine kinase activity and the effect of localization of Abl on its tyrosine kinase activity, then it is tempting to speculate that p150$^{c\text{-}abl}$ is an important mediator of Crk transforming activity. The use of mouse embryo fibroblasts from c-*abl* knockout mice could prove useful in answering these questions.

E) PI-3 Kinase

PI-3 kinase has been found to be associated with a number of receptor tyrosine kinases including the polyoma MiddleT/pp60$^{c\text{-}src}$ complex (128). Its pivotal role in mitogenic signal was demonstrated in platelet derived growth factor receptor (PDGFR) mutants where inactivating mutations in PI-3 kinase docking sites were impaired in mitogenic signalling (129). PI-3 kinase products are markedly increased in cells

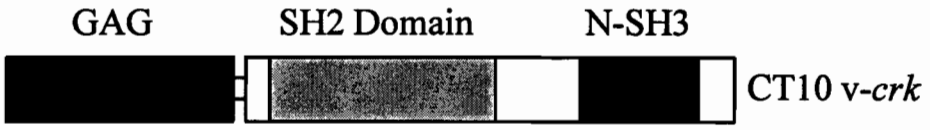
Figure 3. A) Schematic Representation of Crk proteins.

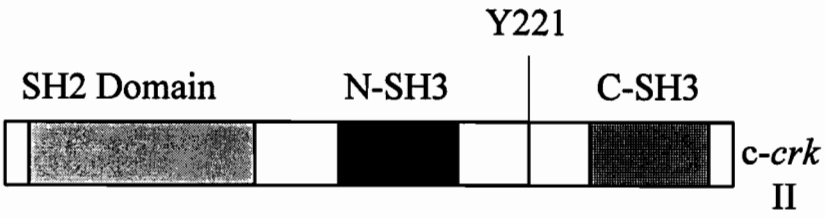
The structure is shown approximately to scale. The gene products of the avian CT10 virus and the chicken c-*crk*II gene are represented: GAG, amino acids derived from the *gag* of avian retroviruses; SH2, the SH2 domain; SH3, the N and C-terminal SH3 domains; Y221, tyrosine residue 221, candidate site for regulatory phoshorylation event.

B) Schematic Representation of the Proposed Intramolecular Crk SH2-Phosphotyrosine 221 Interaction.

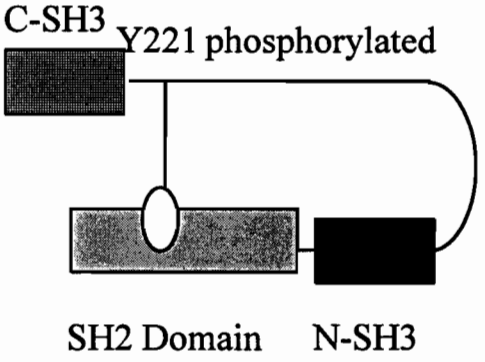
The phosphotyrosine residue at position 221 interacts with the SH2 domain contained in the N-terminal portion of the Crk protein. This intramolecular interaction is believed to block access to the N-terminal SH3 domain, thus affecting Crk signaling. The absence of this tyrosine residue in the v-*crk* would allow it to escape normal cellular controls mediated through the phosphorylation of this residue. P150$^{c\text{-}abl}$ has been proposed to be the Crk kinase (125).

A)


GAG
SH2 Domain
N-SH3
CT10 v-*crk*


Y221
SH2 Domain
N-SH3
C-SH3
c-*crk*
II

B)


C-SH3
Y221 phosphorylated
SH2 Domain
N-SH3

expressing myristoylated activated *abl* proteins (130). Thus, myristylation mutants of p160$^{v\text{-}abl}$ and the activated p150 $^{c\text{-}abl}$IV fail to increase the products of PI-3kinase and transform NIH 3T3 cells even though their association with the latter is not affected. PI-3 kinase possibly interacts with Abl through interaction of PI-3 kinase SH2 domains with phosphotyrosines on Abl. It has been documented that engagement of the PI-3 kinase SH2 domains is sufficient to cause activation (131,132). Alternatively, the PI-3 kinase SH3 domain could interact with the proline rich sequences in Abl (133). The contribution of this cellular signaling molecule to Abl-mediated transformation will require the specific disruption of this interaction.

F) The *cbl* Gene Product

The v-*cbl* gene, for Casitas B-lineage lymphoma, was first identified as the transforming sequence of the Cas NS-1 retroviral genome, which arose from the transduction of c-*cbl* sequences into the Cas-Br-M retrovirus genome (134). The Cas NS-1 retrovirus induces predominantly pre-B cell lymphomas as well as myelogenous leukemias at a much reduced frequency and it also transforms immortalized fibroblasts *in vitro* (134,135). Structural comparisons of the c-*cbl* gene product have yielded little or no information concerning its potential physiological role (135). Finally, much like c-*abl*, overexpression of c-*cbl* does not transform cells (135,136). Activation of c-*cbl* oncogenic potential occurs through the deletion of specific codons. At the protein level this correlates with an increased ability to be phosphorylated on tyrosine. The similarities in the pathological states induced by v-*abl* and v-*cbl* suggested that v-*abl* could cause transformation through the increased phosphorylation of c-*cbl*. To test this hypothesis, the tyrosine phosphorylation status of c-*cbl* in a variety of v-*abl* transformed cells as well as the human K562 cell line, which harbours p210$^{bcr/abl}$, was examined. The authors demonstrated increased tyrosine phosphorylation of the c-*cbl* gene product in these cell lines (135). Moreover, this phenomenon appears specific to activated Abl-transformed cell lines since cell lines of similar lineages transformed by other oncogenic events did

certain cell lines c-*cbl* protein coimmunoprecipitates with activated Abl proteins. This suggests that the c-*cbl* protein is a target of activated Abl proteins. It is speculated that the interaction may be mediated by the Nck adaptor protein since both proteins have been shown to bind to this protein (56,137) and that it appears that the interaction between these two proteins is phosphotyrosine-independent (135). The biological relevance of this interaction will become clear as the role of the c-*cbl* gene product in cellular processes is being determined.

1.1.3.2 Effectors

A) The MYC Connection

The final target of proliferative signals have long been suspected to be transcription factors which are activated by a variety of modifications at the transcriptional or posttranscriptional levels to bring about the mitogenic program.

C-*myc* transcriptional activation has been described for a variety of mitogenic stimuli including growth factor dependent stimulation as well as proliferative signals mediated by activated forms of Abl in a variety of cellular contexts (77,87,138,139). Further evidence of synergism between the c-*myc* protein and Abl has been documented in a number of different experimental systems: 1) tumors arising in *abl* transgenic mouse studies often display high levels of c-*myc* expression suggesting a strong biological selection for such a phenomenon (140); 2) *bcr/abl* shows an increased transforming activity of fibroblasts when v-*myc* or c-*myc* is overexpressed (70,138), furthermore overexpression of c-*myc* can complement mutations in the SH2 domain of p185$^{bcr/abl}$ which impair phosphotyrosine binding and transformation of Rat-1 cells; 3) increases in c-*myc* expression can be seen in myeloid cells expressing v-*abl* (87) as well as in *bcr/abl* transformed growth factor dependent cell lines (77); 4) dominant negative forms of Myc specifically suppressed the transformation of Rat-1 fibroblasts by p160$^{v\text{-}abl}$ and p185$^{bcr/abl}$, as assayed by growth in soft agar (88). Although this firmly establishes a

role for Myc as a downstream effector of Abl oncogenesis, the inability of Myc overexpression to rescue the transformation activity of Grb2 binding site mutant suggests that p185$^{bcr/abl}$ operates through at least two distinct pathways which involve activation of Ras and Myc (70).

1.1.3.3 Regulators

A) The Retinoblastoma Protein, Rb

The retinoblastoma protein is a tumour suppressor gene which regulates progression through the cell cycle (141). A number of proteins have been identified which interact directly with Rb and modulate its growth inhibitory properties (141). Its targets for growth inhibition remain more elusive. Recent studies have shown that Rb and p150$^{c\text{-}abl}$ interact *in vivo* (142). Rb appears to bind p150$^{c\text{-}abl}$ specifically in the ATP binding lobe of its tyrosine kinase domain. *In vitro* translated p150$^{c\text{-}abl}$ kinase activity was inhibited in the presence of a GST-Rb protein. This suggests that Rb regulates p150$^{c\text{-}abl}$ kinase activity in a cell cycle regulated manner. In monitoring p150$^{c\text{-}abl}$ kinase activity in cells overexpressing an epitope tagged p150$^{c\text{-}abl}$ the authors of this study measured an increase in p150$^{c\text{-}abl}$ tyrosine kinase activity as cells progressed to S phase and this correlated with the phosphorylation of Rb and its release from the p150$^{c\text{-}abl}$-RB complex. Furthermore, it was demonstrated that Rb interfered with supertransactivation of a CAT construct by overexpressed p150$^{c\text{-}abl}$ in SAOS-2 cells. Thus, as cells progress through the cell cycle p150$^{c\text{-}abl}$ kinase activity is activated, transactivation activity would be increased due to the disruption of a p150$^{c\text{-}abl}$-Rb complex. This phenomenon would enable proper integration of growth stimulatory (p150$^{c\text{-}abl}$, putatively) and growth inhibitory signals (Rb) (142). However, the relatively low level of p150$^{c\text{-}abl}$ associated with Rb in some cell lines coupled with the mounting evidence that the c-*abl* gene product is a negative regulator of cell growth (12) does not

fit with this model of integration. Thus the biological relevance of this interaction remains unclear.

1.1.3.4 Genetic Modifiers of *Drosophila abl* (*Dabl*)

Simpler genetic systems have proven very useful in identifying the components of signal transduction pathways. This section, reviews studies of the *Drosophila Melanogaster* homolog of *abl*, termed *Dabl*, and the information concerning genetic modifiers of *Dabl* as well as their potential roles in Dabl signal transduction. Please note that for clarity the Dabl protein and locus will be referred to as Abl and *abl*.

D. melanogaster lacking Abl function die as pharate adults or shortly after eclosion with relatively normal external structures with the exception of roughened eyes (143). In a search for genetic enhancers and suppressors of *abl* function hence of substrates, four loci have been identified, three have been characterized at the molecular level. They are termed *disabled*, *enabled* and *prospero* (144-146). *Disabled*, *prospero* and a third undescribed locus were identified by screening ethyl methyl sulfonate (EMS) mutagenized *abl* null animals for mutations which shifted the lethal phase of the *abl* background to an embryonic or larval death. The genetic screen was designed in such a way that disruption of a single copy of these loci would be sufficient to give rise to earlier lethality, thus this effect termed haploinsufficiency dependent on an *abl* mutant (HDA) was also used in the description of these loci (144,146). Conversely, *enabled* was identified in a screen for genetic suppressors of the *abl* loss of function alleles (145).

Although $abl^{-/-}$ embryos have no obvious defects in their nervous system, $abl^{-/-}$ $dab^{+/-}$ fail to form proper axonal connections in their central nervous system (CNS) and an additional copy of either gene restores the axonal architecture. The cellular and subcellular localization of both proteins is compatible with a direct interaction. Furthermore, the Dab protein contains 10 amino acid motifs with sequence similarity to the *abl* autophosphorylation site and is phosphorylated *in vivo* on tyrosine. However, the

authors were unsuccessful in demonstrating a direct interaction between these two proteins (146). A recently identified protein, which is phosphorylated in response to stimulation by colony stimulating factor 1 (CSF-1) of Bac1.2F5 cells, shares significant amino acid similarity to the N-terminal and C-terminal domains of the *disabled* protein (20). The authors of this study suggest that this protein is involved in the signaling cascade originating from the CSF-1 receptor in Bac1.2F5 cells (20).

Prospero had been previously identified as a gene encoding a nuclear protein required for proper differentiation and axonal outgrowth in embryonic neurons (129). Differences in the timing of expression and subcellular localization of the Abl and Pros proteins would appear to preclude a direct interaction (146).

Since a reduction in the level of Enabled (Ena) can suppress the lethal effects of *abl* mutant alleles, Gertler et al. suggest that Ena is serving an opposing function to that of Abl which requires a precise control of their dosages (147). *Abl*$^-$ animals display reduced Ena tyrosine phosphorylation. Furthermore, Ena colocalizes with Abl to the axons in the developing CNS suggesting that a key role for Abl in *Drosophila* development is to regulate Ena function by phosphorylation. The primary amino acid sequence of Ena suggests that it could interact with the Abl SH3 domain since it harbours many proline-rich motifs. *In vitro* studies with recombinant proteins support this hypothesis. However, the authors were unable to demonstrate *in vivo* evidence for this interaction. The exact role of Ena phosphorylation in modulating its activity is still unclear. Finally, the murine counterparts of these gene products have not been identified although the Ena protein shows extensive sequence similarity in some domains with human and canine vasodilator stimulated phosphoproteins (147).

1.2 The *Ras* Oncogene

1.2.1 Introduction

The Ras proteins are part of the superfamily of GTP binding proteins. This family of proteins act as binary switches, alternating between GTP bound or "on" and GDP bound or "off" modes, regulating a host of cellular processes including receptor signaling, cytoskeletal structure and membrane trafficking (references within this section) (148). The first oncogenic variants were identified in the *ras* subfamily.

Four different strains of acutely transforming retroviruses harbour cellular *ras* genes transduced into their retroviral genome, the Harvey and Kirsten rat retroviruses as well as the murine Balb and Rasheed strains (149-152). The *ras* genes were also found to be activated by proviral insertion in avian and mammalian tumours (153) as well as in animal models of carcinogen-induced tumorigenesis (154-157). The link between *ras* activation and human tumours was first recognized when transfection assays for dominant activated oncogenes in human tumor DNA led to the rediscovery of activated *ras* genes (158-161). At this time, it is believed that as many as 30% of all human tumours harbour mutations in *ras* genes (162), making *ras* mutations the most common oncogenic activation associated with carcinogenesis. Finally, germinal studies by Dennis Stacey and his collaborators suggested a pivotal role for $p21^{c\text{-}ras}$ in transformation mediated by certain oncogenes as well as in the response of normal cells to mitogens (102,163). These properties of the *ras* family have fueled a very intense effort to identify its mode of action, its substrates, effectors and modulators. The following review summarizes the findings of these studies.

1.2.2 Ras Structure

This section will focus on the four members of the mammalian Ras family, namely H-ras, N-ras, K-rasA and K-rasB, all of which have been shown to act as

oncoproteins when activated by mutations or overexpressed. The Ras proteins are made up of roughly 190 amino acids which can be divided into three different domains with varying levels of conservation among members of the Ras subfamily. The N-terminal third of the protein is highly conserved while the next 80 amino acids diverge slightly. The C-terminal region is highly variable except for a highly conserved (C)ysteine-(A)liphatic amino acid-(A)liphatic amino acid-(X) any amino acid tail (Figure 4). According to X-ray crystallographic studies the basic backbone of these proteins is arranged in five alpha helices, one six-stranded beta-sheet divided into two three-stranded subsets and 10 interconnecting loops. These studies have yielded some clues to the identity of the critical amino acids involved in nucleotide binding, GTPase activity and effector binding.

The guanine base of GDP or GTP is bound to approximately amino acids 120 and 145 while the phosphate group contacts the protein at amino acids 12-20, 32-35 and 60. Nucleotide binding is partly mediated by amino acids at positions 116,117 and 119. The observation that activating and dominant negative mutations of *ras* often coincide with the precise localization of these domains also supports the structural studies. For instance, activated mammalian *ras* alleles often bear point mutations at positions 12,13,59,61. *In vitro* generated activated alleles also harbour mutations at positions 35,63,116,119. Certain amino acid substitutions (namely the activating ones) impair the nucleotide binding capacity of Ras thus the mutant protein may exchange its GDP for GTP at a higher rate which would obviate the need for a nucleotide exchange factor, a class of proteins which has been shown to play a critical role in coupling receptor signaling to the Ras cascade (see section 1.2.3). Considering the vast excess of GTP over GDP in cells, this leads to a predominantly active form of the Ras variant. Mutations or activating substitutions at positions 12,35 and 61 appear to impair the GTPase activity of Ras. In a model outlined by Grand (164), it appears that these mutations interfere with the

Figure 4. A) Ras protein Structure.

The linear structure is shown approximately to scale: EBD, effector binding domain ; HV, the hypervariable region among members of the Ras subfamily; MA, membrane association encoding the C-A-A-X motif. Open triangles represent activating point mutations, black triangles represent dominant negative mutations. The relative position of the amino acids responsible for binding of the phosphoryl and guanine base of the guanine nucleotide are indicated by black boxes on the appropriate lines. (149,173)

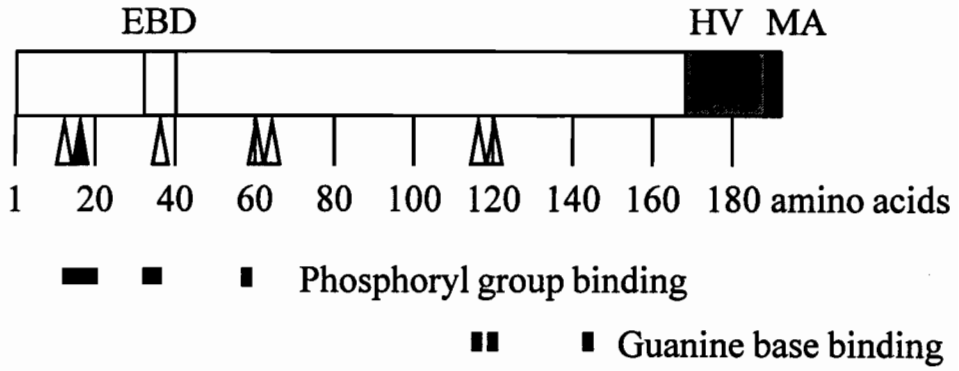
B) The Ras Cycle

The Ras proteins alternate between GDP bound (off) and GTP bound (on) states. Progression through the cycle is affected by : GAP, GTPase activating proteins; GEF, guanine nucleotide exchange factors; GDI, guanine-nucleotide-dissociation-inhibitors which may negatively affect progression through the cycle. Adapted from (148).

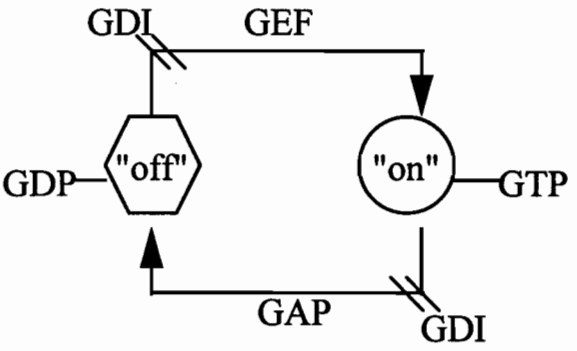
C) Primary Structure of p120GAP

P120GAP consists of : Hydrophobic, hydrophobic N-terminus; SH domains, 2 for SH2 domain, 3 for SH3 domain; PH, pleckstrin homology domain found in many signaling molecules (116-118); GTPase stimulation, its catalytic or GTPAse domain. Adapted from (148).

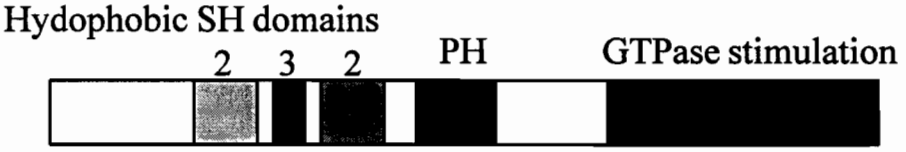
A) Ras structure



B) Ras Cycle



C) p120GAP



followed by 2) cleavage of the C-terminal three amino acids and 3) carboxymethylation of the newly exposed alpha carboxy group of the farnesylated cysteine residue. Some members of the Ras family undergo a further lipid modification by the addition of palmitic acid to cysteine residue(s), a process dubbed palmitoylation. Although farnesylation appears sufficient to confer membrane localization these further modifications appear to improve membrane association and actually enhance transforming activity (171). It should be noted that stretches of basic amino acids can substitute for palmitoylation as is seen for K-rasB (172). Palmitoylation is a dynamic process, the half life of this modification is estimated to be approximately twenty minutes while the half life of the protein is close to twenty four hours (173). Its exact role remains unclear but reversible acylation has been suggested to be a mechanism for modulating the signaling potential of small G proteins as well as the trimeric G proteins (174). Myristylation is a cotranslational modification which is considered stable. The stability of this modification may explain the observation that an exogenous myristylation signal can activate the transforming potential of p21^{ras} (170).

1.2.3 Substrates, Effectors and Modulators of Ras Activity

The simple nature of the Ras proteins belies the complexity of the phenomena they mediate. Ras proteins can affect such varied processes as the differentiation of PC12 and F9 cells into neuronal cells and endodermal cells, respectively, the differentiation of skeletal myoblasts, the mitogenic response of NIH 3T3 to serum derived growth factors and tyrosine kinase mediated oncogenic transformation (163,167,168,175-177). This apparent paradox is being resolved as the identity of some of the Ras regulators/effectors is determined, the *ras* proteins often interact with proteins with more complex structures and activities. It has been suggested that Ras acts as a "turnstile" for a multitude of different signals and that the specification of the cellular response is dictated by the specific proteins interacting with Ras within any cellular context. These proteins either

affect the progression of the Ras cycle or transduce the signal received from the product of this cycle, GTP bound Ras.

1.2.3.1 Modulators of the Ras cycle

Ras proteins are GTPases which alternate between a GTP and GDP bound state. The regulation of this cycle (see figure 4B) (148) displays remarkable complexity and calls into play a variety of activities. These proteins can be divide into four classes: 1) GTPase activating proteins, 2) GTPase inactivating proteins, 3) proteins that lock the GTPase into one state and 4) proteins that affect the intracellular location of the Ras proteins through postranslational modifications. As alluded to previously, GTP is present in excess of GDP in cells, thus, when Ras is nucleotide free it is more likely to bind GTP. The GTP bound form is slowly converted to the GDP bound form through its low intrinsic GTPase activity. The nature of the reaction, hydrolysis of GTP to GDP, renders the cycle unidirectional. GTPase activating proteins (GAPs) stimulate this GTPase activity thus driving the process of inactivating the Ras protein. The exchange of GDP for GTP bound to Ras is catalysed by guanine-nucleotide-exchange factors (GEFs) also known as guanine-nucleotide-releasing proteins (GNRPs) or guanine-nucleotide-dissociation stimulators (GDS) but can also be inhibited by guanine-nucleotide-dissociation inhibitors (GDI). GDI also appear to inhibit GAP activity, although GDI activities specific to Ras proteins have not been identified and will not be discussed.

A) GAPs

GAP activity was first characterized in *Xenopus Laevis* oocyte extracts (178). Subsequently, genes encoding RasGAPs were isolated from human placental (179) and bovine brain (180) cDNA librairies. The structure of the p120GAP protein is quite complex with two N-terminal SH2 domains and one SH3 domain (see figure 4C), a pleckstrin homology domain and a C-terminal GTPase activating catalytic domain (148).

Its modular nature may in fact be responsible for its ambivalent interaction with Ras proteins, acting both as a negative regulator and downstream effector for *ras* proteins.

Its role as negative regulator is demonstrated in the following observations: 1) activated forms of the Ras have lower GTPase activity and are refractory to p120GAP action (181) 2) stimulation of $p21^{ras}$ upon T-cell activation appears to involve decreased GAP activity (182) 3) GAP can suppress c-*ras* mediated transformation (183). In addition, certain mutations in the effector domain of the $p21^{c\text{-}H\text{-}ras}$ decreased p120GAP binding but activated its transforming potential in Rat-2 cells (184).

Conversely, a variety of other observations would appear to support a role for p120GAP as an effector. p120GAP binds through its C-terminal domain to the Ras effector domain (181,185). Oncogenic Ras effector domain mutants which do not bind to p120GAP are transformation defective (181,185). Antibodies directed against the effector domain block p120GAP binding and neutralize Ras activity (178,186). Furthermore, Ras mutants which do not associate with the membrane but still bind GAP tightly inhibit the function of membrane bound Ras and this inhibition is alleviated by addition of GAP (187). It has been suggested that Rap1a, a small G protein part of the Ras-related family, suppresses *ras* transformation by binding tightly to p120GAP and preventing Ras from interacting with its downstream effector (188). It should be noted that a rap1aGAP has been cloned and bears no resemblance to p120GAP, thus, although a competitive model to explain suppression of transformation by Rap1a is still possible it is also plausible that Rap1a signals an antagonistic pathway through Rap1aGAP (189). In *Xenopus* oocytes GAP enhances germinal vesicle breakdown (GVBD) by v-*Ha-ras* and insulin (190,191) and this activity requires the SH3 domain of GAP (192). A Ras-rasGAP complex is involved in uncoupling the muscarinic receptors from the atrial potassium channels (193,194). It has been suggested that the N-terminal portion of p120GAP is sufficient to uncouple the muscarinic receptor from the G_{k+} protein (194). Thus, the interaction with $p21^{ras}$ would induce a conformational change which in turn would activate the SH2-SH3

regions of p120GAP. The SH2 and SH3 domains are attractive candidates for mediating the downstream effects of GAP: SH2 and SH3 domains are involved in protein-protein interaction critical in cell signaling (please see section 1.1.2.2). It was demonstrated that p120GAP interacts with the EGF receptor, the PDGF receptor and pp60$^{v\text{-}src}$ and is phosphorylated by these kinases (195-202). Furthermore, certain GAP associated-proteins, p62 and p190 are also phosphorylated as a result of transformation by activated tyrosine kinases (198,203). One of the potential roles of GAP is to attenuate Rho signaling through its interaction with the p190 protein, which harbours a RhoGAP activity (204). The functional role of p120GAP, or the GAP complexes with p62 and p190, in v-*src* mediated transformation was further ascertained by the demonstration that overexpressing the N-terminus of GAP could rescue the transforming activity of a cytosolic transformation-defective v-*src* and that this transformation process was still dependent on c-*ras* (205). Although, there appears to be a good correlation between p120GAP and GAP associated protein phosphorylation with oncogenic transformation, the requirements for GAP activity for c-*ras* seem very different suggesting that the downstream effectors of oncogenic and normal Ras differ. For instance, mutations in the GAP binding domain that impair GAP binding in oncogenic Ras and reduce transformation activity do not affect normal Ras (206). Also, overexpression of a truncated GAP containing the N-terminal portion, lacking the catalytic domain, inhibits oncogenic Ras transforming activity without affecting the action of normal Ras (207). In short, for mammalian cells GAP effector activity may be limited to activated versions of Ras while its negative regulatory effects would be restricted to the normal *ras* protein.

The gene product for Neurofibromatosis type 1 (NF1), a human genetic disease associated with increased risk of developing certain malignancies including schwanommas was found to contain a RasGTPase domain (208-211). It was proposed that the malignancies arose from improper modulation of Ras activity. This hypothesis gained support with the observation that certain tumour cell lines derived from NF1

patients displayed elevated levels of Ras bound to GTP (212-214). In other tumour cell lines of similar tissular origin but not from NF1 patients, it appears that inactivating mutations in NF1 can occur without affecting cellular GTP-Ras (215). The authors suggest that this may reflect a tumour suppressor activity independent of its RasGTPase activity. In keeping with this hypothesis, overexpression of neurofibromin, the NF1 gene product, in melanoma cell lines deficient for NF1 is growth inhibitory and induces differentiation (216). It affects the proliferation of NIH 3T3 cells (216) and also affects oncogenic transformation by v-*ras* (216,217) which is generally considered to be impervious to GAP activities (178). Neurofibromin differs from p120GAP with respect to several biochemical properties: its GAP-like domain has a 20 fold higher affinity for RasGTP even though its GAP activity is 30 times lower (218) and in contrast to GAP, overexpression of a wild type or mutant GAP-like domain from NF1 can suppress transformation by v-*ras* (216,217,219). Therefore, neurofibromin may provide a growth inhibitory signal as evidenced by the melanoma differentiation studies but it could also sequester RasGTP from some downstream effectors because of its high affinity and further regulate RasGTP by facilitating hydrolysis to GDP through its GAP activity. Its restricted pattern of expression may reflect the cell context specific modulation of Ras and its exact role as either a transducer, competitive inhibitor or negative regulator of RasGTP may be dictated by this cellular context.

B) GEFs

Guanine nucleotide exchange is slower than GTP hydrolysis. Thus, it constitutes the rate limiting step in the Ras cycle (220). The existence of GEFs in *Saccharomyces cerevisae* that can act on mammalian Ras proteins suggests that an evolutionary conserved activity exists for mammalian Ras (221). The identification of the *Drosophila Son of Sevenless* gene product (SOS) as a protein with extensive homology to the yeast *CDC25* protein (222) and its genetic relationship with the *Sevenless* receptor tyrosine kinase pathway suggested it could act as a link between the activation of the Ras signal

transduction pathway by receptor tyrosine kinases. Earlier observations had clearly established a role for the adaptor protein Sem-5/Drk/Grb2 (see figure 5) (104-106,113,223,224) adaptor protein in receptor tyrosine kinase signal transduction. It has now been proven by a number of groups that upon receptor activation Grb2 is recruited to the activated receptor via its SH2 domain and that its SH3 domains engage Sos which harbours proline rich motifs with affinity for these SH3 domains. The relocalized Sos can now interact with membrane bound RasGDP and catalyze GDP/GTP exchange (107-111). Membrane localization of human Sos (hSOS) is sufficient to activate the Ras cascade (summarized in figure 5) and overexpression of membrane bound hSos causes transformation in immortalized fibroblasts (225). These studies underscore the requirement of membrane localization for efficient signaling as well as uncovering a possible negative regulatory interaction in the C-terminal portion of the protein (225). Sos can also interact with another adaptor protein termed Nck, thus modulation of RasGEF may be a common mechanism for signaling which may in turn rely on different adaptor proteins (226). The GEFs are believed to be the targets of the N17 Ras dominant negative mutants which would lock the GEFs into dead-end complexes (227,228). Other GEFs such as the small GTP binding protein guanine-nucleotide-dissociation-stimulator may interact with some members of the Ras family but the physiological relevance of these interactions remains unclear (148). The *vav* protooncogene protein has been shown to stimulate GEF in NIH 3T3 cells and support signal transduction through the Ras-MAP kinase pathway, thus supporting a role for *vav* in ras signaling in hematopoietic cells (60,229). However, more recent data appear to dispute the GEF activity of Vav (230).

1.2.3.2 The Ras Cascade

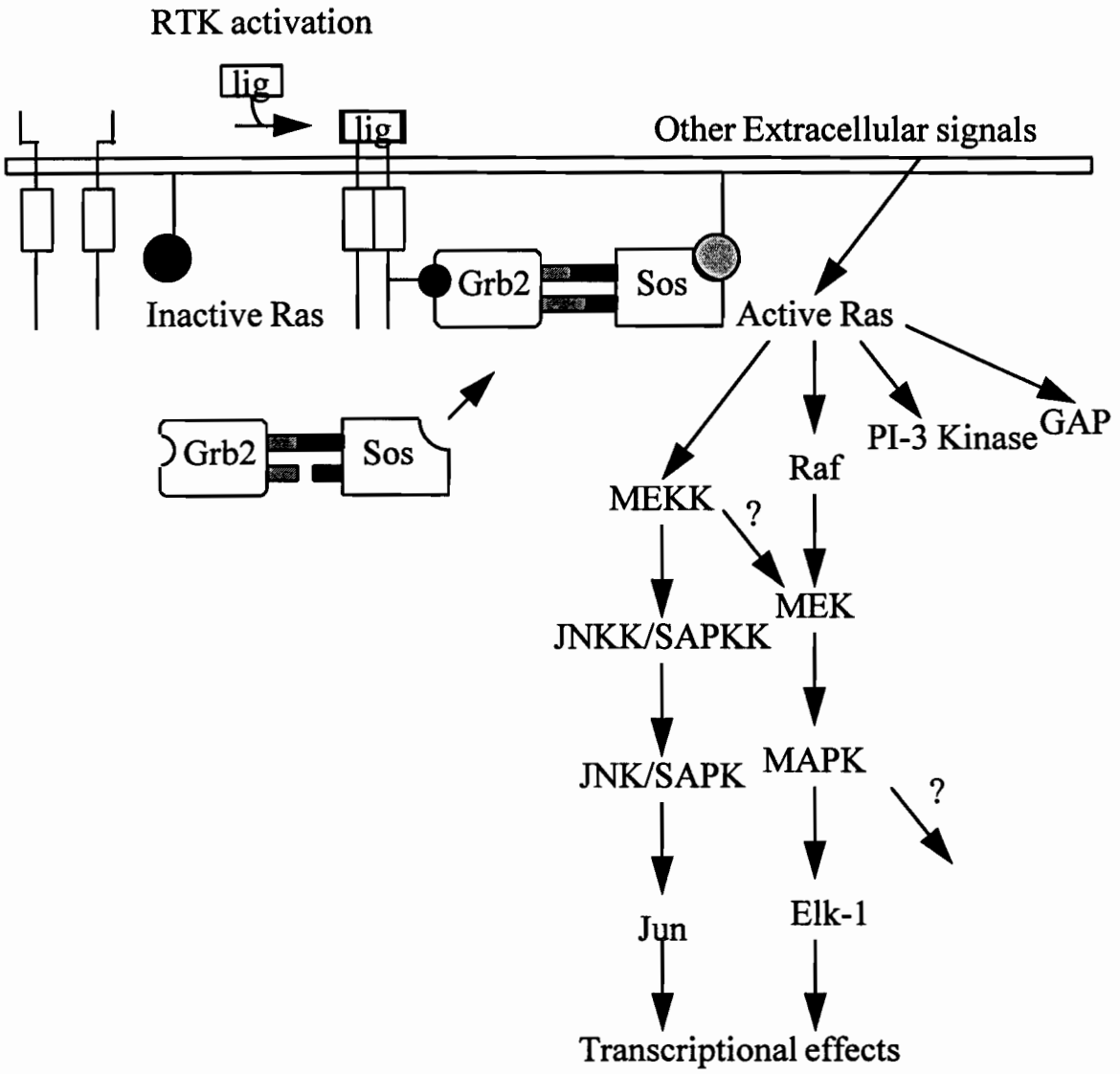
A variety of external signals lead to the activation of extracellular regulated kinases (114). MAP/ERK kinases are regulated by upstream dual specificity kinases termed the MEK kinases for MAPK/ERK kinase. These kinases are also regulated by

upstream phosphorylation events by MEKkinases or MEKKs (Figure 5). The activation of the ERK pathway has been determined to be the critical event in Ras mediated signaling (231,232). The link between Ras and the ERK pathway was uncovered when the gene product of c-*raf*-1 was shown to interact with GTP bound Ras (233-236). Previous studies had established that Raf could act as a MEKkinase (237-239). Ras and Raf interact through the effector binding domain of Ras and the N-terminal regulatory domain of Raf respectively (237,238), thus Raf and RasGAP may compete for binding of the Ras effector domain. Since effector binding domain mutations which lead to decreased transforming activity also result in decreased binding of Raf, it is plausible that reduced transforming activity reflects loss of activation of Raf, not GAP binding. Furthermore, Rap1a can interact *in vitro* with Raf, thus one could suggest that it exerts its Ras transformation suppressor activity through the formation of a non-productive Rap1a/Raf complex (233). It should be noted that interaction of Ras with Raf is not sufficient to activate its kinase activity, it appears that a serum derived signal is required for full activation (240). It has been demonstrated that once Raf is recruited to the membrane it no longer requires Ras to be fully activated. Addition of a membrane targeting sequence to Raf obviates the need for Ras's membrane-anchor activity and gives rise to a fully activated Raf protein (241,242). Recent evidence suggests that Ras may activate a number of different MAP/ERK kinase pathways (243-245). In fact, it has been suggested that Ras activation can lead to both Raf-1 activation of the ERK pathway as well as the activation of MEKK and the JNK (c-Jun specific kinase) pathway (246). The ultimate outcome of both pathways are the modulatory phosphorylations of transcription factors and their concomitant effect on gene expression (247). Finally, Ras may interact with the components of other mitogenic pathways as evidenced by the coimmunoprecipitation of PI-3 kinase with Ras (248). The significance of this interaction is reviewed in the following section.

Figure 5. Diagrammatic Representation of Ras Activation and the Ras Cascade.

Upon ligand induced activation and autophosphorylation, the Grb2-Sos complex is recruited through the Grb2 SH2 domain (open semi-circle). Grb2 and Sos interact via SH3 -proline rich interactions, SH3 domains are indicated by stipled and the proline rich domains of Sos by dark boxes. Sos stimualtes GDP release from Ras and yields an activated Ras. Other intracellular signals result in the activation of Ras and the Ras cascade. Active Ras interacts with a number of putative effectors including GAP, PI-3 kinase, Raf, or MEKKs. The activation of Raf or MEKK leads to the phosphorylation and activation of an ERKK (JNKK,SAPKK,MEK) which in turn phosphorylates and activates an ERK (JNK,SAPK,MAPK). ERKs have a host of substrates, some of which are transcription factors (single examples are shown for simplicity) whose phosphorylation affects their transcriptional activity. Other ERK pathways have been proposed to exist and their dependence on Ras is under investigation (243).

Note that some cytoplasmic tyrosine kinase are believed to activate Ras by recruiting the Grb2-Sos complex through Shc, an adaptor protein. Thus by recruiting Shc and phosphorylating it on tyrosine they can recruit this complex so that it can activate Ras signaling. Raf, MEKK and ERKs are serine/threonine kinase while ERKK are dual specificity kinase phopshorylating also tyrosine residues. Adapted from (107,240).


RTK activation
lig
lig
Other Extracellular signals
Inactive Ras
Grb2
Sos
Active Ras
Grb2
Sos
PI-3 Kinase
GAP
Raf
MEKK
?
MEK
JNKK/SAPKK
JNK/SAPK
MAPK
?
Jun
Elk-1
Transcriptional effects

1.2.3.3 PI-3 kinase

Initial reports that direct binding of the non-catalytic subunit of PI-3 Kinase (p85) to the PDGF receptor was required for Ras activation following PDGF stimulation (59) were supported by the studies of Valius and his colleagues (129). Utilizing addback mutants of the PDGF receptor beta harbouring only the p85 binding site, the authors demonstrated that the mutant PDGF receptor could still activate Ras, as evidenced by increased levels of RasGTP, and mitogenic signaling (129). However, recent reports have suggested that this may only occur in certain cell types (249). This finding coupled with the observation that Nck can bind to the same site as p85, hence could activate Ras through its own interaction with Sos, has cast some doubt on the role of PI-3 kinase as an upstream activator of Ras. Alternatively, other studies have suggested an effector function for PI-3 kinase (250). It was demonstrated that Ras interacts directly with the catalytic subunit of PI-3Kinase, p110, through its effector binding domain. Other members of the Ras family, specifically RalA and Rap2A, do not associate with p110. The interaction does not increase the activity of the p110 kinase, thus one could propose that some coordinate signal may be required for full activation, in a manner similar for Raf activation. The overexpression of the N17 Ras mutant attenuates growth factor induced production of 3'phopshoinositides in PC12 cells and overexpression of Ras proteins results in an increase of these same products. The authors of this study propose that a phosphoprotein-dependent Ras-independent pathway is unaffected by N17 but that the Ras-dependent activation is affected (250). Thus, PI-3 kinase activity can be regulated in a number of ways following growth factor stimulation, the pathways might synergize to cause strong activation of this kinase. However, the relative importance of this phenomenon is unclear as the mitogenic role of the phosphoinositides is still being examined. Nonetheless, these studies underscore the remarkable variety of signals potentially modulated by the Ras protein.

1.2.3.4 Regulation of Ras Activity through Localization

As discussed previously the proper localization of Ras proteins is regulated through a complex series of posttranslational lipid modifications. The enzymes that regulate these processes have proven to be interesting targets for antitumour therapy (251-254). The target of choice has been the farnesyl transferase since this lipid modification is less prevalent in cells than the geranylgeranyl modification, one would expect its inhibition to be less damaging to its cellular environment. A number of approaches have been used to investigate the effect of inhibiting farnesyl transferase (FTase), the enzyme which catalyzes the addition of the farnesyl moiety to the C-terminal cysteine residue: 1) the use of organic compounds which mimic the peptide substrate of the FTase or the farnesyl diphosphate, the lipid substrate of this enzyme, and competitevely inhibit FTase activity 2) the disruption of endogenous FTase activity either through the expression of antisense or dominant inhibitory constructs and 3) the disruption of the isoprenoid biosynthetic pathway. The treatment of cells with isoprenoid synthesis inhibitors, such as lovastatin results in non-specific cytotoxic effects (255). The overexpression of antisense and dominant inhibitory constructs also leads to considerable growth suppression in normal and *ras* transformed fibroblasts (256) probably as a result of general effects on cellular farnesylation. The utilization of peptidomimetic compounds has demonstrated more encouraging results: two independent groups utilizing compounds which mimic the CaaX motif were able to reverse specifically oncogenic *ras* (253,254) and *src* (253) mediated transformation without deleterious effects on normal cell growth (254). These effects on *ras* mediated biological phenomena were extended to *X. Laevis ras*-dependent GVBD where a FTase inhibitor was shown to block solely insulin (Ras-dependent) and not progesterone (Ras-independent) induced GVBD (257). More recent studies examining the mechanism of reversion of *ras* transformed cells following treatment with a similar compound, designated L-739,749, have uncovered a more complex mode of reversion than initially hypothesized (258). The authors of this study

observed that not only does treatment with L-739,749 reduce anchorage independent growth of *ras*-transformed cells but also that a transient treatment is sufficient to rapidly and stably cause morphological reversion of *ras*-transformed cells. In examining the state of Ras protein processing in the treated cells they determined that reversion occurred even though appreciable levels of properly processed Ras existed. In fact, after withdrawal of L-739,749 appearance of properly processed activated Ras protein preceded re-reversion to the transformed morphology. It appears that this compound is capable of inducing in normal as well as *ras* transformed cells rapid and substantial change in the cytoskeletal architecture. It is of interest to note that *raf* transformed cells are resistant to these effects and do not display appreciable changes in cytoskeletal architecture. Thus the authors conclude that the antitumour effect of L-739,749 is not a consequence of altered activated Ras protein processing and that the morphologically reverted state induced in *ras*-transformed cells is not a normal cellular phenotype *per se* since presence of activated Ras after removal of inhibitor is not sufficient to cause transformation. The authors suggest that the reversion phenomenon may involve farnesylated proteins that control the cytoskeletal architecture, such as some of the Rho/Rac protein family members (95,259). This observation suggests an interplay between these two GTPase families in mediating/coordinating the transformation process. In light of these results it is interesting to note that two putative Ras effectors have putative links with rhoGAP proteins, namely PI-3 kinase and GAP binding protein p190.

1.3 Multidrug Resistance (MDR)

Since this thesis focused primarily on the potential role of MDR as a target for transformation mediated by v-*Ha-ras*, this review will not present an exhaustive examination of structure/function studies of the *mdr* proteins. The reader is referred to several excellent reviews for these considerations (260,261)

Typically, MDR arises when cell lines are subjected to selection with a single cytotoxic agent. MDR cells display resistance to a variety of unrelated cytotoxic compounds. Hence, multidrug resistance (MDR) can be defined as the resistance to a broad spectrum of cytotoxic compounds. The drugs most often involved in this phenomenon include vinca alkaloids, anthracyclines, alkylating agents, protein synthesis inhibitors, DNA intercalating agents, all of which share little or no structural similarity not to mention the variety of intracellular components these drugs target (261). Germinal studies by Victor Ling and his collaborators established that a single genetic event could be responsible for this property (263). Furthermore, MDR is a result of the decreased influx and increased efflux of these compounds. Subsequently, the gene product of the *mdr1* gene was shown to be necessary and sufficient for both functions (262). In fact the expression of the *mdr1* gene product, the P-glycoprotein, was later shown to be the most consistent change in MDR cells (263). The *mdr* gene family includes three family members in rodents while only two members exist in humans (264). Two of the members of the mouse *mdr* family, *mdr1b* and *mdr1a* (initially named *mdr1*and *mdr3*, respectively), can independently confer MDR with some specificity (265-267) while mdr2 can not confer the MDR phenotype (268,269). The human *mdr1* gene product shares 88% identity with the mouse *mdr1a* gene product (261) and also confers the MDR phenotype when transfected into cells.

1.3.1 Structure and Function

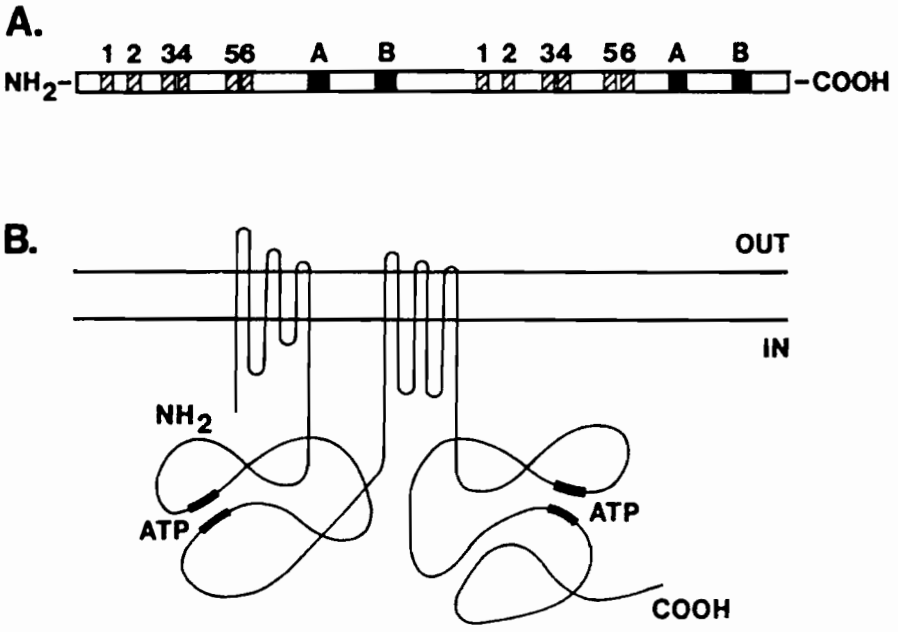
The Mdr proteins are part of a large family of transporter proteins, the ABC transporters, for ATP-binding cassette (for review see 270). The characteristic motif of the ABC transporters is approximately 200 amino acids long and is present in at least 50 proteins of eukaryotic and prokaryotic origin. It should be noted that not all members have transport activity, for instance the peptide elongation factor of yeast EF-3 or the UvrA protein of *E. coli* involved in DNA repair are such members. In humans, other members of this family include the Cystic Fibrosis Transporter protein (CFTR) (271), the gene products odf the candidate genes for Zellweger Syndrome (272) and X-linked adrenal leukodystrophy (ADL) (273) which are believed to encode peroxisomal transporters, and finally two linked genes (Tap1 and 2) associated with transport of peptides into the endoplasmic reticulum for class I antigen presentation (270).

The human cDNA for the *mdr1* gene encodes 1280 amino acids arranged in two similar halves each containing six transmembrane regions and one large intracytoplasmic loop which harbours the ATP binding site (figure 6).(262,274,275). It has been demonstrated to have ATP-dependent transport activity (261,263) as well as the ability to act as a volume-gated chloride channel (276,277). In accordance with the latter activity, it has been suggested that the physiological role of the human Mdr1 is to act as a chloride channel in epithelial cells that do not express the CFTR protein (278). The expression pattern of both of these genes seems complementary. In normal cells it would appear that expression of one of these proteins coincides with the lack of expression of the other. Furthermore, within a single cell type under different conditions a switch in expression can be seen. Thus, it is suggested that these two genes are coordinately regulated since they provide redundant functions. More recent studies suggest that P-gp regulates the activation of cell volume-activated chloride channels hence it is a channel regulator rather than a channel itself (279). It may enhance channel activity but it also confers PKC-sensitivity on these channels (see section 1.3.3)

Figure 6. Diagrammatic Representation of the P-glycoprotein

A. In this linear representation of the protein the stippled boxes represent the six putative transmembrane domains contained within each half of the protein. The dark boxes denoted A and B represent the two regions involved in ATP binding.

B. This schematic represents the proposed structure within the membrane. The two horizantal parallel membranes represent the cell membrane while **out** and **in** denote the extracellular and intracellular spaces respectively. Note the six transmembrane domains in each half of the protein and the folding of the intracellular domains which give rise to the ATP binding domains signified by **ATP**. The N-terminus and C-terminus of the protein are designated by **NH2** and **COOH** respectively. Adapted from (263).


A.
1 2 34 56 A B 1 2 34 56 A B
NH2-
-COOH
B.
OUT
IN
NH2
ATP
ATP
COOH

The steroid transport capabilities of the Mdr1 protein (280) coupled with observations of the substrate capacities of other members of the ABC transporter family has lead some to speculate that Mdr1 may function as a pump for peptide hormones which lack the traditional signal sequence, for instance the fibroblast growth factor (FGF) (260). The expression profile of the murine *mdr1a/b* genes would also support such a hypothesis (281) and the human *mdr1* gene product can transport peptides (282). However, disruption of the murine *mdr1a* gene gives rise to increased sensitivity to ivermectin neurotoxicity as a result of its increased accumulation in the brain and no other obvious defect (283). It is possible that the intact *mdr1b* can complement the loss of *mdr1a*. The authors of this study observed an increase in its expression with respect to tissues in a normal animal. The authors conclude that *mdr1a* is an important component of the blood-brain barrier preventing toxic xenobiotics from entering the brain (283).

The human Mdr2 protein like its murine homolog does not transport drugs of the MDR spectrum. However, murine *mdr2* appears to be involved in the transport of phospholipids into bile (284).

1.3.2 MDR Expression and Cancer

The expression of MDR poses important problems for chemotherapeutic approaches to cancer cell treatment. The expression of MDR follows no clear patterns with respect to tumorigenesis. Although, double minutes (DM) and homogeneous staining regions (HSR) are common karyotypic alterations in MDR cells, increased *mdr* expression in the absence of DNA amplification is often observed in cancer patients and established cell lines (261). Furthermore, DNA amplification does not always give rise to increased expression (285). Hence, the regulation of *mdr* expression is not strictly regulated by gene copy number.

Elevated levels of expression of *mdr* can be found in tumours which originate from tissues that typically express important levels of *mdr*. Conversely, certain

malignancies such as acute and chronic leukemias, non-Hogdkin lymphoma, neuroblastoma, some lung cancers express elevated levels of *mdr* even though the normal tissue or cellular origin has relatively little or no *mdr* expression (285). A number of observations suggest that MDR may contribute to the process of transformation and that its expression may be affected by this process. In childhood neuroblastomas, elevated levels of *mdr* expression at presentation correlates with a poor prognosis which often coincides with failure to respond to chemotherapeutic agents (286). A study of clinical samples of mammary carcinomas has revealed that amplification of *mdr* can occur in the absence of selection with cytotoxic agents (287). It has been reported that P-gp positive invasive colon cancer cells may have an increased potential for dissemination (288). Studies on the human *mdr*1 promoter have suggested that the process of malignant transformation could increase expression of *mdr*1, specifically, overexpression of the c-Ha-*ras* oncogene and or the p53 oncogenic variant of the p53 tumour suppressor gene can activate expression from the *mdr*1 promoter in NIH 3T3 cells (289). Interestingly, transfection of the rat TAP-2 gene into a lymphoma mutant restores its tumorigenic potential by a complex phenomenon that is believed to involve an increased capacity to escape rapid elimination by Natural Killer (NK) cells (290). Taken together, these data suggest that increased expression of the *mdr* gene product, or certain ABC transporter proteins, may contribute to some aspects of tumour progression.

In contrast to these observations, Biedler and Spangler have reported decreased tumorigenecity of cell lines selected in tissue culture for elevated levels of drug resistance: Chinese Hamster lung cells, mouse sarcoma cell lines and human neuroblastoma cell lines displayed reduced tumorigenic potential *in vivo* after *in vitro* selection for very high levels of MDR. It should be noted that the concentrations of drugs used far exceeded physiological doses and that in most cases the revertant cell lines regained properties of the parental lines when maintained in drug-free medium. It is not clear if the changes in tumorigenic potential are directly linked to the changes in MDR

expression or to the effect of its overexpression on the integrity of the plasma membrane or to unrelated phenomenon mediated by the selective agents (291).

1.3.3 Regulation of Mdr Activity

It is quite clear that the glycosylation of the P-glycoprotein plays little or no role in the modulation of its activity : 1) treatment of MDR cells with tunicamycin, a potent inhibitor of protein glycosylation does not affect the MDR phenotype 2)MDR cells can be isolated from lectin-deficient cells which are glycosylation defective 3) finally, there are important differences in the glycosylation sites and states between the human and murine P-glycoproteins, thus this lack of conservation suggests that glycosylation is not required for its function (261,262,275).

Phosphorylation of Pgp was detected in human KB carcinoma cells treated with phorbol ester (292). The phosphorylation sites on P-gp corresponded to the sites detected in *in vitro* assays with protein kinase C (PKC). Thus the increase in Pgp phosphorylation seen in phorbol ester treated KB cells probably corresponds to the phorbol ester mediated activation of PKC and its translocation to the plasma membrane. Furthermore, phorbol ester treatment results in an increase in transport activity of Pgp and a concomitant increase in drug resistance. Thus, these studies suggest that protein kinase C may modulate drug transport. Phosphorylation by PKC also affects the channel regulator activity of Pgp (279). In HeLa cells, which lack endogenous Pgp expression, the transfection of a Pgp expression construct renders the activation of endogenous volume-activated chloride channels sensitive to PKC treatment. Channel activation was decreased after PKC treatment in transfected cells while it is unaffected in control HeLa cells. Furthermore, cells expressing constructs for a Pgp mutant lacking the PKC consensus phosphorylation sites do not display a PKC-dependent inhibition of channel activation. This suggests that the phosphorylated form of Pgp inhibits channel activation(279). Thus, it appears that phosphorylation by PKC is an important regulative modification.

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1.5 Objectives and Rationale

The object of this thesis was to identify the components of the transformation pathways involved in v-*abl* and v-*Ha-ras* mediated oncogenesis. Our laboratory has favored a genetic approach centered around the isolation and characterization of revertants of transformation. Reversion can occur through three distinct phenomenon: 1) the inactivation of the oncogene 2) a mutation in an effector gene or through 3) the activation of an anti-oncogene. We reasoned that by determining the mechanism responsible for reversion, we could identify the critical components of the transformation pathways.

Through the course of our studies we developed a novel technique to isolate revertants of transformation from v-*abl* transformed Rat-1 cells. Our analysis of these revertants revealed that an inactivating mutation in the v-*abl* oncogene was responsible for the reversion process and that this mutated *abl* behaved as a dominant negative, blocking transformation selectively by other oncogenes. Although the analysis of the reversion process did not lead to the direct isolation of a critical signal transducer, the v-*abl* mutant isolated by this process may provide us with some invaluable clues to the important effectors of transformation by *abl* and a number of other oncogenes.

In studying a v-Ha-*ras* revertant cell line we uncovered evidence that suggested that the product of the *mdr* gene could play a role in *ras*-mediated transformation. We chose to investigate the potential role of this gene product in transformation mediated by the v-Ha-*ras* oncogene.

CHAPTER 2: ISOLATION OF A V-*ABL* MUTANT THAT SUPPRESSES TRANSFORMATION BY SEVERAL ONCOGENES.

2.1 Summary

In order to understand the mechanism by which *v-abl* transforms fibroblasts *in vitro* we have designed a novel technique to derive revertants of transformation from *v-abl* transformed Rat-1 cells. We found that the revertant lines display a characteristic pattern of resistance to retransformation by a variety of viral oncogenes : they are resistant to v-*abl*, polyoma middle T, as well as most activated forms of receptor tyrosine kinases with the notable exception of *tpr-met*. Further studies indicated that these lines were susceptible to retransformation by v-*mos*, v-*src*, v-*fes*, v-*Ha-ras*, v-*Ki-ras* and v-*gag-fos-fox*. The revertant lines obtained all contained inactivating mutations in the v-*abl* provirus that could not be complemented by a wild type v-*abl* virus. Our analysis of the mutated protein coupled with our analysis of the mutant genome indicates that a largely truncated form of $p160^{v\text{-}abl}$ can suppress transformation by a variety of oncogenes.

2.2 Introduction

The v-*abl* oncogene was first identified as the transforming gene of the Abelson murine leukemia virus (A-MuLV) (Witte et al., 1978; Reynolds et al.,1978; Witte et al., 1979). The human cellular homolog of *abl* is rearranged in 90-95 % of the Chronic Myelogenous Leukemias (CML) as well as in a lesser propotion of Acute Lymphoblatic Leukemias or (ALL) Acute Myelogenous Leukemias (AML) (Kurzrock et al.,1988). In recent years the search for a molecular answer to *abl*-mediated oncogenesis has led to the identification of a large number of potential substrates, effectors and regulators of Abl function. Some of these include the Syp phosphotyrosine phosphatase, PI-3 kinase, pRb, the C-terminal domain of RNA polymerase II, p120Ras GAP, the so-called adaptor proteins Grb2,Nck,Crk, the Shc proteins, the Abl SH3 binding proteins 3BP1 and 3BP2, and c-*myc* which has been implicated by a large body of evidence as an important mediator of transformation by activated forms of Abl (Tauchi et al.,1994; Varticovski et al., 1991;Welch and Wang,1993; Baskaran et al., 1993; Druker et al., 1992; Skorski et al., 1993; Skorski et al., 1994; Pendergast et al., 1993; Puil et al., 1994; Ren et al., 1994; Feller et al., 1994; Cicchetti et al., 1992; Lugo and Witte, 1989; Rosenbaum et al., 1990; Afar et al., 1994; Sawyers et al., 1992). Some of the targets of Abl have been identified owing to their ability to form stable complexes *in vivo* with Abl. Alternatively, newer technologies such as the two hybrid system have helped in identifying potential substrates without having to rely solely on coimmunoprecipitation. Unfortunately, these techniques do not directly address the physiological relevance of these interactions. Although a combined approach using information from simpler genetic systems may help circumvent this hurdle (Egan and Weinberg, 1993), our group has attempted to identify effectors of transformation through genetic means in a mammalian system. Specifically, we are interested in isolating and characterizing revertants of transformed mammalian cell lines.

The process of reversion can occur through three different mechanisms: 1) through a mutation in the viral oncogene responsible for the transformation in the

recipient cell line, 2) a mutation in an effector gene or 3) through the activation of an anti-oncogene. In order to achieve our goals, we have devised a new technique to isolate revertants from v-*abl* transformed Rat-1 cell lines. The isolation procedure relies on the differential retention of a fluorescent dye by non-transformed and transformed cell lines. The cells isolated by this protocol show characteristics of non-transformed cell lines. Furthermore, the reversion process appears to have affected a critical pathway in transformation mediated by v-*abl* as well as other oncogenes as demonstrated by a variety of assays. It appears that reversion has occurred through an inactivating mutation in the viral oncogene. Interestingly this v-*abl* mutant can apparently suppress transformation by a "wild type" v-*abl* as well as by Polyoma middle T, v-*erbB* and other oncogenes while having no effect on transformation mediated by v-*gag-fos-fox,* v-*mos,* v-*Ha-ras,* v-*Ki-ras* and v-*src*. Our analysis of the v-*abl* mutant protein suggests that a largely truncated version of the wild type protein is capable of suppressing transformation mediated by several other oncogenes. These findings suggest that oncogenes from several different classes share a common pathway that is blocked by the mutant v-*abl* but is unnecessary for transformation by other oncogenes.

2.3 Materials and Methods

Cell culture, Transfection, Infection,Transformation and Agar Assays

Rat-1 cells and derivatives were routinely passaged in Minimal Essential Medium (MEM) supplemented with 10% calf serum, penicillin and streptomycin. The RC1A line was obtained by recloning twice in agar the 1349D8 cell line which is a Rat-1 cell line infected with the rescued amphotropic retrovirus of a pABFBJ (v-*abl*) transfected Rat-1 cell line (Jolicoeur et al., 1991). The AblA cell line was derived by picking a transformed clone from an infection of Rat-1 cells with viral supernatant from the CVII cell line. The R1510R22 and R11164R 24,32,37,46,54 cell lines were derived by infection with retrovirus pseudotyped from the revertant lines T5-10 and T16-4, respectively. Briefly, Rat-1 cells were infected with retroviral supernatant MoMuLV infected T5-10 or T16-4 cell lines. Cells were diluted in order to pick individual clones. 50 and 60 clones were picked at random from each infection. RNA was extracted from these clones and prepared for dot blot analysis (see below). Clones expressing v-*abl* RNA were expanded and Southern analysis with an *abl*-specific probe was performed to ascertain the presence of a mutant v-*abl* proviral insertion.

Viral infections, transfections, foci titrations and agar assays were performed essentially as described (Zarbl et al., 1987) with the following modifications: for titration of the neomycin resistance harbouring retrovirus 0.2 mg/mL G418 was added to the medium right after infection (GIBCO BRL). Plates were stained with 0.2% methylene Blue (Sigma) and darkly stained foci were counted on duplicate plates for every viral dilution. Transfections for transformation assays were carried out using approximately 10 μg of the indicated oncogene bearing plasmid as well as 2 μg of pSV2neo (Southern and Berg, 1982). 48 hours following transfections cells were passaged 1:4 for foci assay and 1:8 for neo selection. Medium was changed every 3-4 days with fresh MEM 10% and antibiotics for the foci assay while medium with G418 (at 0.2 mg/mL) was used for neo selection. The MMTV-NEU (Bouchard et al., 1989) and Tpr-met (Rodrigues and Park,

1991) have been described elsewhere, the *tpr-met* construct was kindly provided by Morag Park (Molecular Oncology Group, Departments of Medicine, Oncology and Biochemistry McGill University).

Cells were plated in soft agar at a density of 1 X 10^4cells per 35 mm plate. For agar assays coupled to infection with transforming retroviruses , 5 X 10^4 cells were infected with 1:5 dilutions (10 plates each) of the appropriate retroviral supernatants using typical infection protocols. 24 hours later cells five plates of each were trypsinized and plated in soft agar as previously described at a cell density of 5 X 10^4cells per 60 mm plate. The others were kept for a focus forming assay in petris.

Retrovirus stocks

Most retroviruses were generated by rescue of transforming retroviruses from previously described cell lines 208mt-R1 (polyoma middle T antigen), FBR-R2 (v-*gag-fos-fox*), 1333Emol also known as pHTV-8 R1 (v-*mos*). The CVII cell line producing an ecotropic helper-free v-*abl* was kindly provided by Naomi Rosenberg. The erbBneo (v-*erbB*) virus was obtained by infecting with a replication competent amphotropic retrovirus a cell line containing the pMLVerbBneo3 construct generously provided to us by J. Michael Bishop. The neofms (v-*fms*) virus was also obtained from J. Michael Bishop.

P12 staining, FacSTAR analysis and sorting of putative revertant lines

P12, also termed P-96, was from Molecular Probes Inc. Stock solution was prepared in anhydrous DMSO (SIGMA). 1 X 10^5 cells were stained for 2 hours in medium containing 20 μM P12 without serum. Cells were washed in complete medium 2 times and then trypsinized, pelleted and resuspended in 1 mL of PBS or medium and filtered through a nylon mesh (70 μm) to separate clumps. Cells were put on ice and analyzed for cell size and fluorescence. The analysis was carried out on a FacSTAR

(Becton Dickinson) utilizing the combined UV lines 351nm and 364nm (Nahas et al., 1987). A dielectric filter was used to exclude scattered laser light from the fluorescence detector. Cell size was determined by forward angle light scatter.

Five 100mm plates containing 1 X 10^6 cells were treated with EMS at a concentration of 5.5mM. Cells were extensively washed in PBS after a 20 hour incubation period. Cells were split 1:3 on day five post-treatment. P12 staining was carried out on day 7 posttreatment as described in previous paragraph. The window for sorting was simply set to select $1X10^4$ cells with the strongest fluorescence intensity out of 2 X 10^6 cells sorted. Cells were plated at 500 cells per 100 mm plate (20 plates) and clones were picked after 14-21 days.

Rescue of Retroviruses

Revertant cell lines were infected with replication competent Moloney murine leukemia virus (MoMuLV). Cells culture supernatants were tested seven days post infection for reverse transcriptase activity as described (Rassart et al., 1988). Positive cell culture supernatants, containing rescued retrovirus, were titrated by focus forming assay as well as for the presence of retroviral RNA (see next section). Rescued retrovirus from the appropriate cell line was used to infect NIH 3T3 cells in rollers and 20 hours post infection Hirt supernatants were collected and tested by Southern hybridization for the presence of unintegrated provirus as described previously (Aziz et al., 1989).

Nucleic Acids Analysis

Genomic DNA was prepared by phenol extraction and ethanol precipitation (Jiang et al., 1994). Hirt DNA was extracted from freshly infected cells as previously described (Hirt, 1967). DNA was digested with restriction endonucleases following the manufacturers recommendations (EcoRI, Pharmacia). DNA fragments were separated on

1% agarose gels and transferred onto Nylon membranes (Amersham) as previously described (Sambrook et al, 1989).

Total RNA was extracted from cell lines by the Method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA was separated on 1% Agarose-formaldehyde gel and transfered to Nylon membranes (Amersham).

For viral RNA studies, five mL of cell culture supernatant containing rescued retrovirus was centrifuged at 15000rpm to pellet virions. Pelleted virions were subjected to the same RNA extraction protocol and virion RNA was dot blotted with a Scheiler and Schuell dot blot apparatus, according to manufacturer's recommendations, onto a Nylon membrane.

For the screening of Rat-1 clones infected with rescued retrovirus from the T5-10 and T16-4 cell lines, a rapid extraction method was used. Briefly, cells were rinsed with PBS and harvested by scraping in 1 mL of PBS and pelleted by centrifugation in a microfuge for 30 seconds. Cell pellets were resuspended in 45μL of cold 10mM Tris pH 7.0, 10mM EDTA. 5μL of 5% NP40 was added to lyse the cells and incubated on ice for 5 minutes. An additional 5 μL of NP40 was added prior to a 2.5 minute spin in the microfuge at maximal speed. 50μL of the supernatant was mixed with 30μL of 20XSSC and 20 μL of a 37%(w/v) formaldehyde solution (Fisher). Samples were incubated at 60°C for 15 minutes prior to dot blotting onto a nylon membrane (Hybond N, Amersham) soaked in blotting buffer, 50%H_2O:30% 20XSSC:20% 37% formaldehyde. Hybridization procedure was as previously described (Jiang et al.1994). The *abl* specific probe is a 1.8 kb SacI- HindIII fragment from the plasmid pAB160 (Latt et al. 1983).

Protein Analysis and Antisera

Cells were metabolically labeled with ^{35}S methionine (>1000Ci/mmol) and immunoprecipitation was performed as described previously (Huang and Jolicoeur, 1990) with a minor modification. Protein-A sepharose bridged to a Rabbit antiGoat antiserum

was used to collect the immunoprecipitates. Bridging was accomplished by preincubating protein-A Sepharose CL-4B (Pharmacia) for 1-2 hours with Rabbit anti-goat Immunoglobin antiserum (Jackson Immnumologicals Laboratories) and then washing several times with RIPA buffer to remove unbound antiserum.

Western blot analysis was carried out as previously described (Balsalobre and Jolicoeur, 1995)

Goat anti-MuLVp12 and goat anti-MuLVp15 sera were obtained from the Program and Logistics, Viral Oncology, National Cancer Institute, Bethesda, Md. Anti-pEX-5 antibody was harvested according to established protocols (Harlow and Lane; 1988) from hybridoma line 24-21-20-2 (Schiff-Maker et al, 1986) a generous gift from Dr. Naomi Rosenberg, Tufts University, Boston, Ma.

Tumorigenicity *in vivo*

CD1 nu/nu mice (Charles River Laboratories) were injected subcutaneously at 2 sites with $1x10^7$ cells of the appropriate cell line. Mice were sacrificed after 8 weeks and autopsies of the injection sites performed.

Cloning and Sequencing of the Mutant ABL

Genomic DNA was prepared from the revertant line T5-10, digested with EcoRI, fractionated by sucrose density centrifugation as previously described (Lemay and Jolicoeur,1984; Villemur et al., 1987). A fraction containing fragments of 7 to 12 kbp was ethanol precipitated. The DNA was used to clone into EMBL4 phage arms according to manufacturer's recommendations (Stratagene). The phage insert was sublconed into pBR322 and pSV2neo yielding plasmids $ABL^{mut5-10}$ and $pSVABL^{mut5-10}$. The pBR322 subclone was used as a double-stranded substrate for the Sanger dideoxynucleotide sequencing method (Sanger et al.,1980). Primers were derived for the region of interest by consulting the published sequence (Reddy et al.,1983): primer 98

CGGTATGAAGGGAGG [1952-1966], primer 99 CGCACCGACATCACC [2171-2185], primer 100 GAACCACCATTCTAC [2378-2392], primer 101 GTGAAGGTGGCTGAT [2584-2598], primer 103 CCCTAGCAGCTGCAC [2365-2351], primer 104 ACCGTAGATAGTGGG [2157-2123], primer 105 CTCACTCTCCCGCAC [1921-1907], primer 106 CATCGTTCTGTGTTG [1042-1056], primer 107 CAAGGTCTTTTCACC [1291-1305], primer 108 CGCTCATCGACCTAC [1551-1565], primer 157 GTAGGTCGATGAGCG [1565-1551]. pABFBJ (Jolicoeur et al., 1991) contains the $p160^{v\text{-}abl}$ sequences until the BamHI site where a BglII-HindIII fragment from the FBJ-2 clone (Curran et al., 1982), containing 3' LTR and flanking genomic sequences, was inserted by standard cloning methods (Sambrook et al., 1989). Since the retrovirus had gone through at least one round of replication both Long Terminal Repeats (LTR) were FBJ-2 derived. Thus, we constructed a composite map using the GCG program with the following sequences: msjmusv [101-573]; mlapro [527-1855]; mlamlva [5-3818] and msjmusv [3097-4126]. The initiation codon and termination codons for $p160^{v\text{-}abl}$ are thus located at positions 1094 and 4255 respectively of this composite map.

2.4 Results

Rat-1 and *v-abl* Transformed Rat-1 cells, Have Different Spectra for P12, a Fluorescent Dye.

Previous work from our laboratory had shown that differences in Rhodamine 123 retention could be used to enrich for revertants of *v-fos* transformed cells (Zarbl et al., 1987). Unfortunately, Rhodamine 123 proved limited in its applicability since only *v-fos* transformed Rat-1 cells had a significantly different pattern of retention that could easily be exploited using Fluorescence Activated Cell Sorting (FACS) (Zarbl et al.,1987; unpublished results). Thus, we set about to find another combination of *v-onc* transformed cell line and fluorescent dye that would yield such an exploitable pattern. Our studies led us to the following combination: P12, a fluorescent fatty acid analog and *v-abl* transformed Rat-1 cell lines. In figure 1, we show the difference in fluorescent spectra between *v-abl* transformed cells (RC1A) and Rat-1 cells. This difference would serve to separate revertants of transformation from transformed cells.

Isolation Scheme of Revertants

We hypothesized that revertants of transformation would have a fluorescent spectra similar to that of the untransformed parental Rat-1 cells. Thus, we treated with the mutagen ethyl methyl sulfonate (EMS) the v-*abl* transformed cells, RC1A, to increase the frequency of reversion and sorted cells in a Rat-1 specific window (figure 2). The separated pool was then plated at low density in order to isolate clones. The pool represented approximately 0.5% of the total number of cells sorted. Our second criteria for selection of revertants was a flat morphology in tissue culture. Therefore clones which exhibited a flat morphology, as shown in figure 3 were picked and characterized further. Over 95% of the clones present exhibited a flat morphology. We chose five lines for further study, they are designated T3-8, T5-10,T12-6, T16-4 and T19-7.

Growth Characteristics of Revertants: Revertants Have Characteristics of non transformed Cells.

All five putative revertant cell lines failed to form foci at confluence maintaining a uniform monolayer with a morphology similar to that of the parental non-transformed Rat-1 cells (data not shown). A more stringent assay for transformation (growth in agar) was also performed. Control normal Rat-1 cells as well as the five revertant cell lines assayed did not form foci while the parental v-*abl* transformed formed large colonies in agar with a plating efficiency ranging between 5 and 8%. No foci were detected in plates containing as many as $1x10^4$ revertant cells. Furthermore, none of the revertants gave rise to tumours in nude mice while the transformed RC1A line gave rise to large tumours 6-8 weeks post-inoculation (data summarized in Table 1). Therefore, it appears that the flat clones are revertants of transformation.

Flat Clones are Derived from *v-abl* Transformed Cells (RC1A): Southern and Northern Analyses of Putative Revertants.

To ascertain that the revertants arose from the parental transformed cells and not from contaminating non-transformed cells, we performed a Southern analysis. As shown by restriction analysis with an *abl*- specific probe, the revertant clones have the same v-*abl* integrated genome as the transformed parental RC1A cells (fig.4). This confirms that the revertant lines were derived from RC1A cells. Furthermore, they still express the v-*abl* mRNA as evidenced by the 6.1kb band detected by Northern blot analysis (figure 5). Note that the proto-oncogene c-*abl* is detected by this probe both at the DNA and RNA levels, although the mRNA levels for c-*abl* are inferior to those of its viral counterpart.

The Revertants Exhibit a Specific Pattern of Resistance to Retransformation by *v-abl* as well as Other Oncogenes.

We assessed the ability of the revertants to resist to transformation by a variety of oncogenes. For this purpose we infected the revertant clones with oncogene harbouring retroviruses. Our initial tests used a simple focus forming assay to determine the susceptibility of these cell lines. Interestingly, a significant decrease in the number of foci with respect to the number detected in Rat-1 cells was detected after infection of the revertants with retroviruses harbouring v-*abl*, v-*erbB* and polyoma middle T. For other oncogenes such as v-*gag-fos-fox*, v-*mos*, v-*Ha-ras* and v-*Ki-ras*, no significant difference in the number of foci induced in the revertant lines with respect to the number in Rat-1 cells was seen. An example of assays for viruses that induce and do not induce foci growth are shown in Table 2. Similar results were also obtained with a more stringent assay for transformation: in this assay, resistance to some oncogenes (v-*abl*, v-*erbB* and polyoma *middle T*) translated into a lower plating efficiency in agar of pools of infected cells (Table 3). Note that the revertant clones still retained the ability to form comparable numbers of large colonies in agar when infected with v-*Ha-ras*. Thus the lack of foci in some assays does not reflect an inherent inability to form foci nor does it reflect a decreased infectability. The latter possibility was tested further with an infection with a neomycin resistance harbouring retrovirus. Revertant cell lines showed comparable numbers of G418 resistant colonies to that of Rat-1 cells (data not shown).

These results indicate that the resistance to retransformation is selective. Thus, the genetic event responsible for the reversion of the v-*abl* transformed cells also affects the transformation process mediated by different but not all classes of oncogenes. This in turn suggests that certain elements of the transformation process are shared by different classes of oncogenes. The data from a series of transformation assays (summarized in Table 4), suggests that v-*abl*, v-*erbB*, *polyoma middle T*, v-*fms* and *neu* share a common

element in their transformation pathways as indicated by their inability to retransform the revertant lines.

The Mechanism of Reversion Is Virus Borne.

To determine whether the reversion was caused by a mutation in the v-*abl* oncogene or in some cellular gene(s) essential for the transformation process, we did a rescue experiment. After three separate trials we were unable to detect any transforming activity by v-*abl* virions "rescued" in supernatants from the revertants (data not shown). Analysis of the RNA genome present in the pseudotyped virions shows that the v-*abl* genome was indeed encapsidated (fig.6). Furthermore, Southern analysis of Hirt supernatants from newly infected cells (16 hours post infection) revealed that the cells were infected by v-*abl* harbouring retroviruses and that the genome was reverse transcribed (fig.6). Therefore, these results suggest that the v-*abl* genome has been mutated in the revertant cell lines and has become transformation defective. Furthermore, this inactivating mutation in the v-*abl* oncogene would not only be responsible for reversion, but may also suppress transformation by v-*abl* as well as by other oncogenes. Thus the mutated v-*abl* would behave as a dominant negative mutant.

The Reversion Phenotype is Induced by a Viral Mutation and not by a Mutation in a Cellular Gene.

Our inability to retransform the revertant lines with a wild type v-*abl* coupled with the observation that the revertant produces a transformation defective v-*abl* virus (Table 2 and fig.6) suggested to us that the mutated v-*abl* was acting as a dominant negative. In order to ascertain that no accessory/independent cellular mutation was contributing to the suppression phenotype, the mutant virus from two revertant cell lines T5-10 and T16-4 was rescued by infecting with helper Moloney murine leukemia virus (MoMuLV) and used to infect Rat-1 cells. Clones were picked from each infection at random and tested

for high levels of v-*abl* RNA expression by a rapid RNA extraction protocol. Putative positives were tested by Southern analysis to confirm the integration of a v-*abl* retrovirus (data not shown). One non-transformed clone, clone R1510R 22, was obtained from infection with the T5-10 viruses, while five non-transformed clones were obtained from infection with the T16-4 viruses, clones R1164R 24,32,37,46,54. The ability of these clones to resist retransformation after infection with v-*abl*, v-*erbB* oncogenic retroviruses or after transfection with *neu* or *tpr-met* DNA constructs was assessed in a focus forming assay. The data are summarized in Table 5. Since the resistance to retransformation is transmitted by infection with the mutant virus, we conclude that the suppression of transformation is encoded by the v-*abl* mutant derived from our revertant lines. Thus it behaves as a dominant negative mutant.

The v-*abl* Mutant Protein is a largely Truncated version of the Wild-Type p160$^{v\text{-}abl}$.

Our analyses of the *v-abl* DNA and RNA present in the revertant lines had not revealed any considerable deletions or rearrangements of the v-*abl* genome (figures 4 and 5). Other groups had reported dominant negative cytoplasmic tyrosine kinase with point mutations in the tyrosine kinase domain rendering it inactive (Anderson et al, 1993; Levin et al.,1993; Devary et al.,1992). We set about to determine the structure of the v-*abl* mutant oncoprotein. For this purpose, revertant cells harbouring the mutant virus, the revertant lines T16-4 and T5-10, Rat-1 cells and wild type *v-abl* (AblA) transformed Rat-1 cells were metabolically labelled with ^{35}S-methionine. Lysates from these cells were subjected to immunoprecipitation with a goat polyclonal serum directed against the p15 (MA) *gag* moiety present at the N-terminus of the p160$^{v\text{-}abl}$. A distinctive protein product of approximately 50 kDa was immunoprecipitated from the revertant lines. The absence of a wild type species of 160 kDa suggested to us that the mutant *v-abl* encodes a truncated version of the wild type protein (Figure 7, lanes designated 5-10 and 16-4).

Subsequent analyses by Western blot with the anti- p15 antiserum as well as anti-p12, an epitope C-terminal with respect to p15 but still present in the *gag* moiety, also detected this novel ~50 kDa species (data not shown). Western blotting with an antibody directed against a C-terminal epitope, 24-21-20-2 (Schiff-Maker et al., 1986), fail to detect $p160^{v\text{-}abl}$ in lysates from the revertant lines (data not shown). These results suggested that a large portion of $p160^{v\text{-}abl}$, potentially C-terminal to the *gag* moiety, has been deleted. This 50kDa species was detected in the five revertant lines tested. Thus, it appears that all the revertant lines originated from a common predecessor expressing this truncated protein.

The *v-abl* Mutant Genome has a Small Deletion which Causes Premature Termination of the Open Reading Frame.

In order to clone and sequence the mutant genome, we constructed a genomic lambda phage library from one of the revertant cell lines, T5-10. The library was screened with an *abl*-specific probe and one clone was obtained. The purified phage DNA was then used to subclone the provirus into plasmid vectors. The observation that the mRNA present in the revertant lines was detected by a probe which detects a region of the v-*abl* sequence coding for the last 225 C-terminal amino acids as well as 3' untranslated region, coupled with our data from the Western analysis suggested to us that a small deletion or point mutation may cause premature termination of the open reading frame. Thus, we derived sequencing primers to cover a region extending up to 1.8 kb downstream of the initiation codon. We determined that a 2bp deletion at nucleotides 2248-2249 of our composite map (refer to Materials and Methods for generation of this map) causes a frameshift in the open reading frame. The resulting open reading frame terminates at codon 416. Therefore, the last 28 amino acids of this protein are frameshift encoded. A search of SWISSPROT protein sequences did not reveal any significant homology between these 28 amino acids and sequences contained within this database. The

estimated molecular weight of the protein product would be 46kDa which is lower than our observations. A preliminary phosphoamino acid analysis (data not shown) has revealed that this may be accounted for by phosphorylation of serine and threonine residues. We also detected five point mutations in the wild type and the mutant virus with respect to the published sequences. These were all located in the *gag* encoding sequences. Four out of five mutations did not result in an amino acid substitution. The point mutation at position 1748 of our composite map results in the substitution of an alanine residue for a threonine residue but this mutation is present in the wild type virus and does not appear to impact on its transforming activity (data not shown). Thus, the mutant protein would consist essentially of the *gag* moiety and the SH2 domain while most of the tyrosine kinase domain and the entire C-terminal domain is deleted. Figure 8 shows a schematic representation of our sequencing data.

The Cloned Retrovirus Suppresses Transformation

To ascertain that the retrovirus was sufficient to suppress transformation, we transfected the cloned mutant viral genome into Rat-1 cells. Clones expressing various levels of the mutant protein were subjected to a focus forming assay with v-*mos* and v-*abl* harbouring retroviruses. It should be noted that Western blot analysis of the transfectants revealed the presence of a protein of identical size as the one in the revertant lines, providing further proof that the cloned retrovirus codes for a truncated v-*abl* protein. Clones 392-19 and 392-27 as well as clone RIP5-1 demonstrated significant resistance to retransformation by v-*abl* in two separate trials. These two series of clones were derived from separate transfections. Interestingly, clone 392-27 did show a moderate resistance to retransformation by v-*mos* as evidenced by the five-fold decrease in the number of foci induced by v-*mos* infection in two separate trials (Table 6). In comparing the levels of expression of the mutant protein in these clones, we observe that clones expressing levels comparable to the revertant line displayed resistance. This finding suggests that the

mutant's ability to block transformation is dosage sensitive. It is also tempting to speculate that very high levels of expression may impede transformation by the second class of oncogenes defined by our assays on the revertant lines. Our preliminary experiments with chimeric retroviruses expressing the mutant virus with a selectable marker, which consistently (in our hands) produce less protein than the cloned mutant, appear to support this finding. The dosage sensitivity of the transformation block is currently under investigation.

2.5 Discussion

We have successfully devised a new technique to isolate revertants from v-*abl* transformed Rat-1 cells. By mutagenizing *v-abl* transformed cells with EMS and then selecting cells that show fluorescence intensities specific to Rat-1 cells after P12 staining, we were able to pick flat clones that exhibited properties of non-transformed cells. The proportion of revertants obtained, 1 per 200, is much larger than the anticipated 1 per 10^5 (Zarbl et al., 1987). This large proportion of reversion may reflect some increased propensity for reversion of rat fibroblast cell lines transformed with the v-*abl* oncogene, this phenomenon having been reported by another group (Oka et al., 1990). Alternatively, certain steps of our EMS treatment protocol may have impacted on our selection for revertants. Other groups have previously reported the isolation of revertants of v-*fos* transformation by successive rounds of washing with a cation deficient PBS (Wisdom and Verma, 1990). In washing the cells extensively following EMS treatment we may have selected against v-*abl* transformed cells. Finally, keeping in mind the reported cytotoxic and growth inhibitory effects of v-*abl* transformation on fibroblasts (Goff et al., 1982; Renshaw et al., 1992), the recovery period following the treatment may have further enriched the culture for revertants. All five lines were picked from different plates following sorting and low density plating. The observation that all five harbour the same mutant protein suggests that they are identical and that one very successful revertant clone may have been overrepresented prior to sorting. It should be noted that prior to mutagenesis the cell lines had been recloned twice. Thus, it is unlikely that the selection process had taken place prior to our treatment. The two base pair deletion detected in the cloned retrovirus is not the typical mutation expected with the EMS treatment. However, there is evidence that EMS can cause such mutations (reviewed Sega G.A,1984)

Our data suggests that an inactivating mutation in the v-*abl* is not only responsible for the reversion phenomenon but that the truncated protein encoded by this mutant may suppress transformation by wild type v-*abl*. The revertant cell lines show a distinct

pattern of resistance to retransformation by a variety of other viral oncogenes: they are resistant to v-*abl*, polyoma middle T, as well as most activated homologs of receptor tyrosine kinases with the notable exception of *tpr-met*. Furthermore, they are susceptible to a wide scope of transforming oncogenes such as the serine/threonine kinase v-*mos*, the cytoplasmic tyrosine kinase *v-src*, two forms of activated v-*ras*, and a nuclear oncogene v-*fos*. Interestingly, polyoma Middle T which has been shown to bind to and activate pp60$^{c\text{-}src}$ (Cantley et al., 1991) is also affected by the mutant protein. Thus, it is tempting to speculate that the mutant blocks an early event in signal transduction that is common to activated receptor tyrosine kinases, v-*abl* and potentially c-*src*.

To our knowledge this constitutes the first demonstration of a dominant negative mutant of v-*abl*. Dominant negative mutations of oncogenes have been described for a variety oncogenes and proto-oncogenes from many classes including nuclear oncogenes (Ransone et al.,1990; Lloyd et al.,1991), receptor tyrosine kinases (Ueno et al., 1991; Kashles et al., 1991), small G proteins (Farnsworth et al., 1991; Szeberenyi etal., 1990; Cai et al., 1990), cytoplasmic tyrosine kinases (Hirai and Varmus, 1990; Anderson et al, 1993; Levin et al.,1993; Devary et al.,1992), serine/threonine kinases (Kolch et al.,1991) and dual specificity kinases (Cowley et al., 1994). Dominant negative forms of anti-oncogenes have also been described although their effects lead to a different outcome (Milner and Medcalf, 1991). In some of these examples the block in transformation can extend to several different classes of oncogenes (Ransone et al, 1990; Cowley et al, 1994). However, to our knowledge the v-*abl* mutant described here constitutes the only example of a dominant negative cytoplasmic tyrosine kinase which suppresses transformation by other classes of oncogenes. It should also be noted that the blocking action of the mutant protein does not appear to seriously impede cell growth.

Dominant negative proteins are believed to exert their effect through several different mechanisms that are not mutually exclusive (Herskowitz, 1987). One such mechanism is the formation of inactive dimers: truncated forms of the PDGFb receptor

(lacking the kinase domain) interfere with normal mitogen induced signaling by forming dimers with normal PDGFb receptors thus leading to receptor dimers that cannot fully autophosphorylate therefore are not activated upon ligand binding (Ueno et al., 1991). A similar mechanism has been proposed for some EGF receptor variants (Kashles et al.,1991). For the v-*abl* mutant, it has been suggested that the *gag* portion may allow the protein to dimerize (Luban et al., 1992). However, although this is an attractive explanation for the block of v-*abl* transformation it can not account for the suppression of transformation by oncogenes lacking this *gag* moiety (middle T for instance). In other cases it is postulated that the dominant negative form impedes the function of its normal counterpart by interacting with and titrating out crucial components in the signal transduction pathway. For example *c-jun* mutants (lacking their activation domain) may inhibit the binding of transcription factors to *jun* motifs or form inactive homo or heterodimers with other transcription factors (Ransone et al., 1990). Our sequence analysis has revealed that the mutant *v-abl* encodes a largely truncated form of the wild type protein. This mutant contains only the *gag* and SH2 portions of the wild type protein. It is now well established that the SH2 domain binds phosphotyrosine residues and that it acts in mediating protein-protein interactions critical for cellular signaling (for recent reviews Cohen et al., 1995; Pawson, 1995). Thus the structure of the mutant would suggest that it suppresses transformation by sequestering a vital component of the transformation pathway via its SH2 domain. There is some similarity in the structure of the mutant Abl with the SHC adaptor proteins (Pelicci et al., 1992). However, unlike the SHC proteins overexpression of the mutant does not lead to transformation. This may reflect the unique properties of the Abl SH2 domain. All SH2 domains share a common affinity for tyrosine phosphorylated peptides but detailed studies (Songyang et al., 1993) have shown that it may be possible to group SH2 domains according to their relative affinity for specific phosphopeptides. It should be noted that the *abl* SH2 may also bind to phosphoserine or threonine (Pendergast et al., 1991; Muller et al., 1992). The Abl SH2

has been demonstrated to be essential for transformation (Mayer and Baltimore, 1994) by activated forms of Abl. In contrast, certain reports suggest that the Abl SH2 may serve a negative regulatory function within the c-*abl* protein (Muller et al., 1993). Some have suggested that the cytoplasmic localization of the activated Abl proteins may have contributed to the uncoupling of the negative regulatory effect of the Abl SH2 domain from its ability to elicit a transforming signal (Muller et al., 1993). Our studies indicate that the truncation may have reactivated this negative regulatory function. Some preliminary studies on the localization of the mutant suggest that it is still associated with the plasma membrane (unpublished data). Mayer and Baltimore had shown that other SH2 domains can effectively substitute for the Abl SH2 in an activated form of $p150^{c\text{-}abl}$. It will be interesting to determine if other SH2 domains can substitute for the Abl SH2 in the mutant protein. It is noteworthy that no specific substrate for the *abl* SH2 domain has been reported. Although it has been shown to bind *in vitro* with the EGF receptor (Zhu et al., 1993) and a number of potential substrates have been identified through a search in the protein sequence data bases (Songyang et al., 1993), a physiological target remains elusive. The mutant described in these studies may provide us with a unique opportunity to identify this critical component of the transformation pathway which interacts with the Abl SH2 domain and is potentially a critical mediator of transformation by a number of other oncogenes. Finally, we can not exclude the possibility that the *gag* moiety is necessary for the mutant's activity.

The *gag* moiety is a remnant of the recombination event that gave rise to the *v-abl* genome (for review on structure Risser and Holland, 1989). The role of *gag* in transformation in fibroblast appears minimal since large deletions covering almost all *gag* encoded amino acids do not affect the fibroblast transforming ability of the virus. However, in lymphoid cells these residues may be essential for protein stability (Prywes et al., 1985). It is believed that the major role of the *gag* moiety is to localize the *v-abl* gene product to the membrane through its myristylation signal contained at its N-

terminus (Risser and Holland, 1989). However, these observations do not necesssarily preclude some other contribution of the *gag* moiety to the mutant's action since the phenotype assayed is quite the opposite of cellular transformation. Recent evidence suggests that a protein with sequence similarities with retroviral *gag* proteins may be part of a microfilament-associated signal transduction particle (Juang et al., 1994). Also, the etiological agent of Murine Acquired Immunodeficiency Syndrome (MAIDS) has been demonstrated to be a defective retrovirus which codes solely for a p60*gag* polyprotein with extensive homology with the Murine Leukemia viruses *gag* proteins (Aziz et al., 1989). Thus a potential role for the *gag* coding portion of the mutant *v-abl* protein should not be ruled out and is presently being assessed.

In closing the mutant we isolated seems to define a common step in a tumorigenic pathway utilized by a number of different oncogenes. A molecular dissection of the mutant protein and the identification of its target could ultimately lead to the design of specific blockers of transformation.

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2.7 Figures and Tables

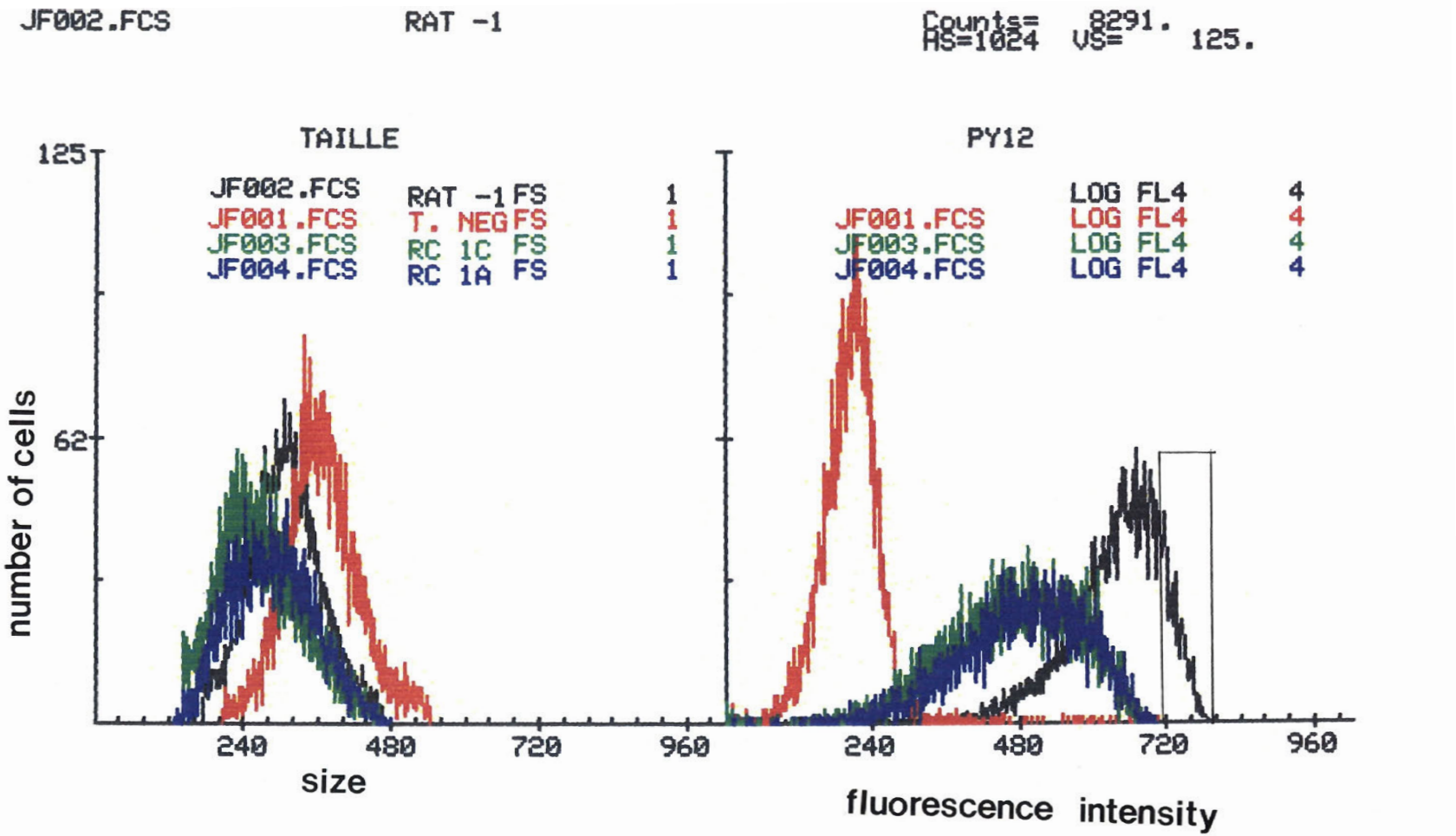
Figure 1. Flow cytometric analysis of Rat-1 and v-*abl* transformed Rat-1 cells treated with P12.

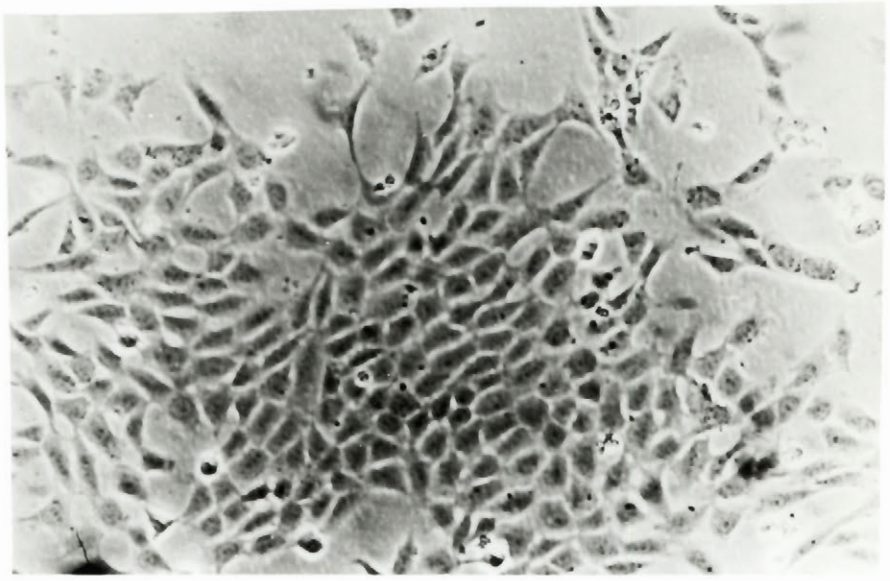
Staining procedure, cell processing and FACstar analysis are described in Materials and Methods.

(A) Cell sizing. Approximately $1X10^5$ were analyzed. Cell size was determined by forward light scatter for treated and untreated cells utilizing a FACStar. Untreated Rat-1 cells are in red, P12 stained Rat-1 in black, two stained v-*abl* transformed Rat-1 cell lines, RC1A and RC1C are in blue and in green, respectively.

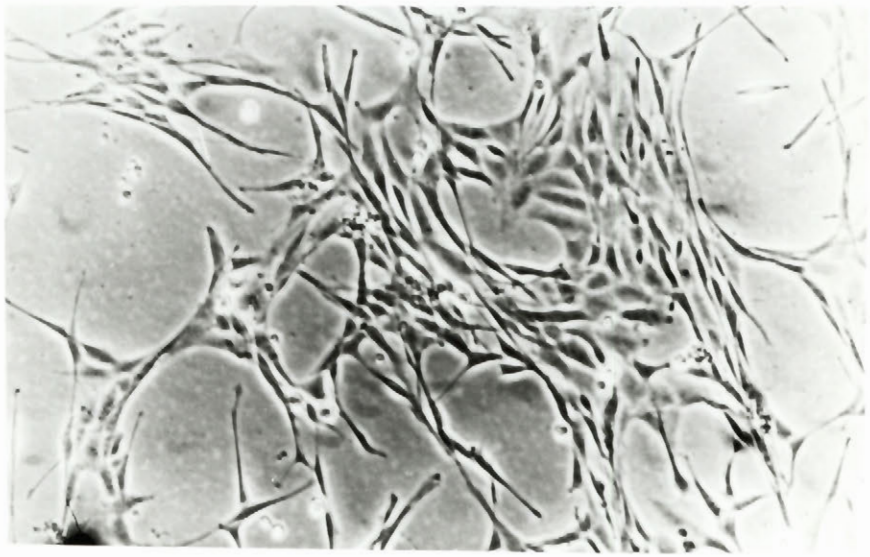
(B) Analysis of P12 fluorescence spectra.

The same samples were analyzed for fluorescence. Designation for each curve is as described in (A). The boxed area represents a section that appears Rat-1 specific.

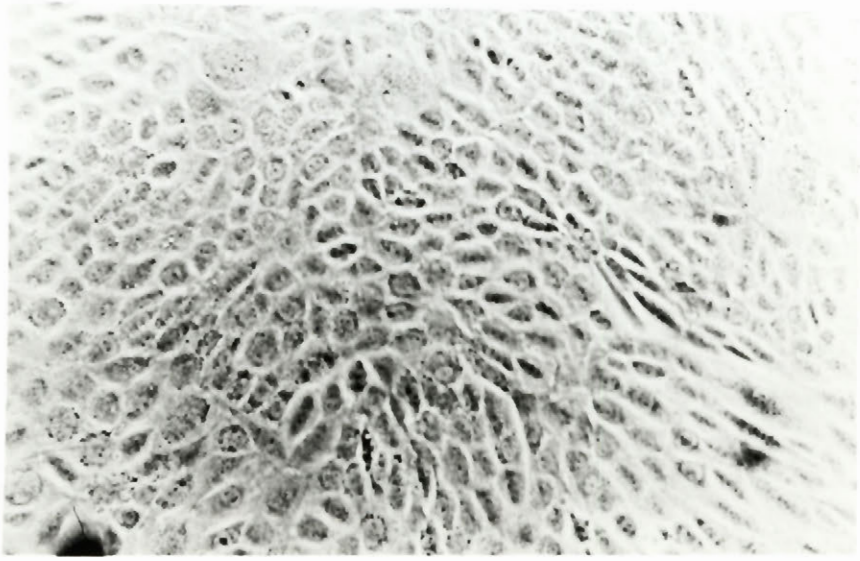

JF002.FCS
RAT -1
Counts= 8291.
HS=1024 VS= 125.
TAILLE
PY12
125
62
number of cells
JF002.FCS RAT -1 FS 1
JF001.FCS T. NEG FS 1
JF003.FCS RC 1C FS 1
JF004.FCS RC 1A FS 1
JF001.FCS LOG FL4 4
JF003.FCS LOG FL4 4
JF004.FCS LOG FL4 4
240
480
720
960
size
fluorescence intensity



a



b



c

Figure 4. Southern analysis of putative revertants.

Genomic DNA was extracted from cell lines of interest and 20 μg were digested with EcoRI. Putative revertants are cell lines T3-8, T5-10, T12-6, T16-4, T19-7 (lanes 1-5,respectively). Rat-1 cells are represented in lane 6 while the parental v-*abl* transformed Rat-1 cells, RC1A, are in lane 7. The *abl* apecific probe used (see Materials and Methods) recognizes a band corresponding to c-*abl*. The v-*abl* genome is present only in the putative revertants and in RC1A cells. Lane MW contains ^{32}P-labeled molecular weight markers.

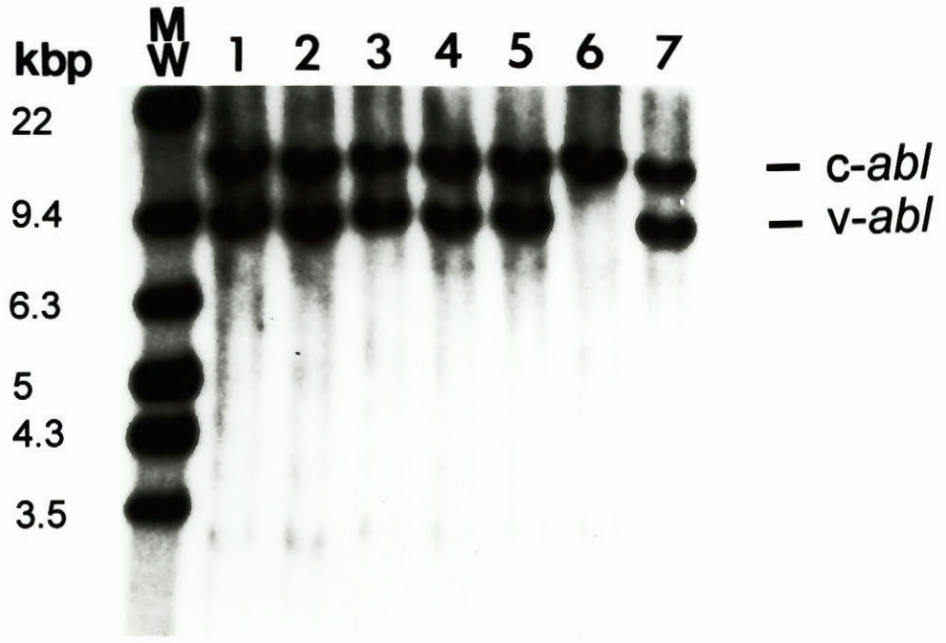

kbp
MW
1
2
3
4
5
6
7
22
9.4
6.3
5
4.3
3.5
c-abl
v-abl

Figure 5. Northern blot analysis of putative revertants.

A) 15 micrograms of total RNA was loaded on the gel, transferred on nylon membranes and hybridized with the *abl*-specific probe. RNAs from revertants T3-8, T5-10, T12-6, T16-4, T19-7 were loaded into lanes 1-5 respectively, while Lane 6 and 7 contained RNA from normal Rat 1 and parental v-*abl*-transformed, RCIA cells. Again, c-*abl* is detected by this probe, its location with respect to the v-*abl* mRNA is indicated. The sizes (kb) of molecular weight standards are indicated at left.

B) The same filter was washed and rehybridized with an actin probe to control for transfer efficiency and equal loading. Lane designation same as in panel A.

A

B

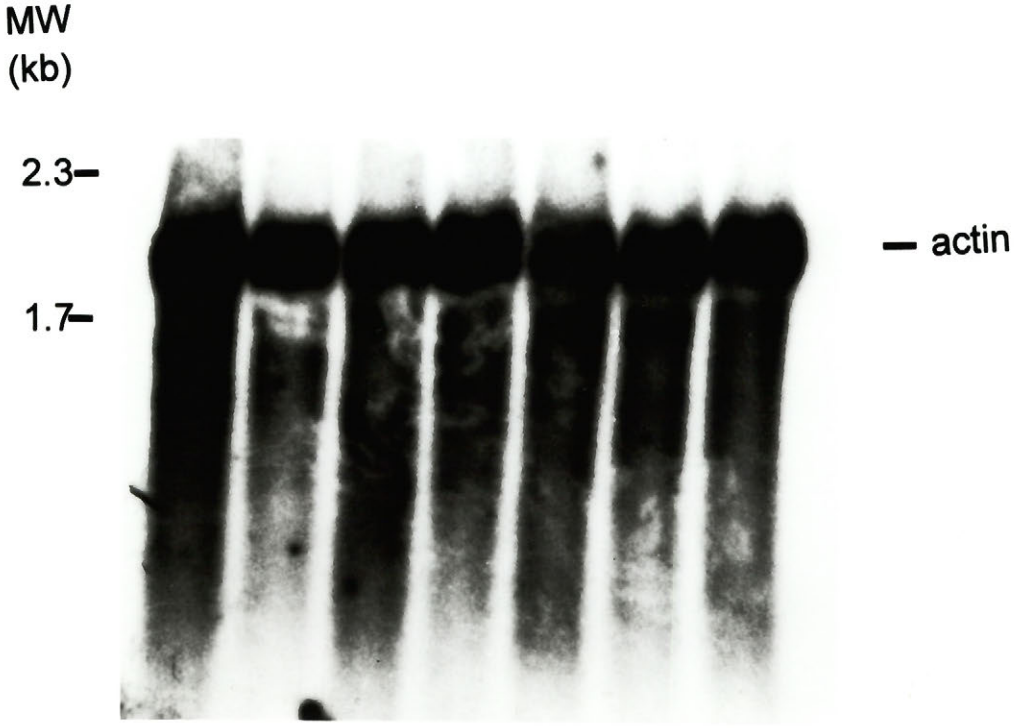


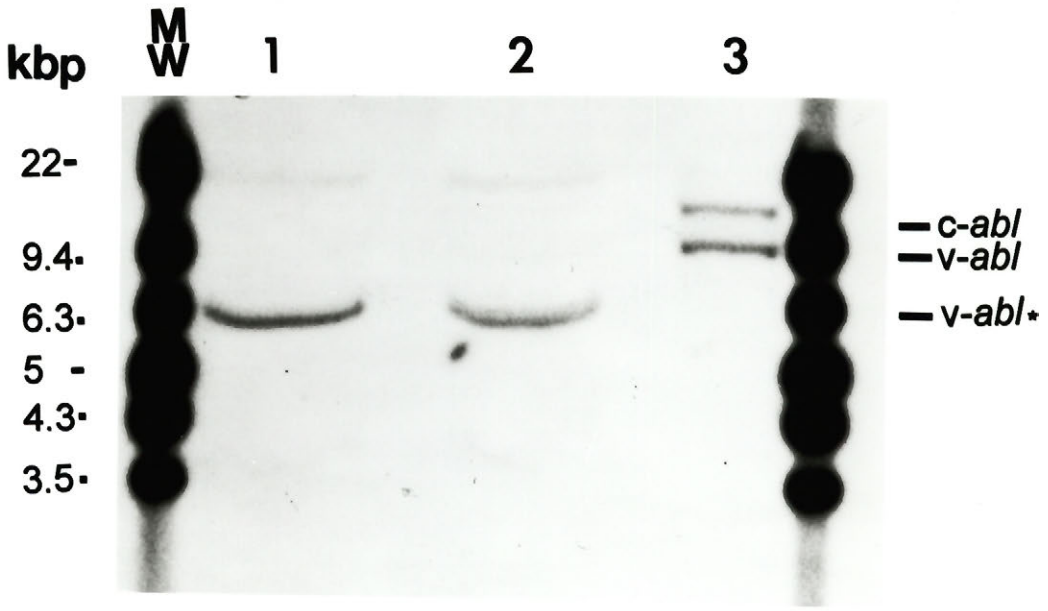
Figure 6. Characterization of virions produced by revertant cell lines.

(A) Hirt analysis of newly-infected cells.

The supernatant obtained (see below and Materials and Methods) from T5-10 revertant cells was used to infect NIH 3T3 cells (~ 12 X 10^6 cells). As a positive control a virus harbouring a transforming *v-abl*, CVII, was used to infect NIH/3T3 cells in similar conditions. 16 hours post-infection, Hirt supernatant DNA was extracted from the infected cells and analyzed by Southern analysis using the *abl*-specific probe. Hirt-extracted DNA from cells infected with Abelson virus of revertant T5-10 (lane 1) or with control infectious Abelson CVII virus (lane 2). In lane 3, control EcoRI cleaved genomic DNA from the T5-10 cell line was loaded. Abelson viral DNA (6.5 kbp) designated as v-*abl** was detected in both Hirt supernatants. C-*abl* and v-*abl* bands in the genomic sample are also indicated.

(B) Dot blot analysis of retroviral RNA. Revertant cell lines were infected with a replication competent retrovirus, a Moloney murine leukemia virus (MoMuLV) for rescue. Cells were then routinely passaged for two weeks. After verifying the rA.dT for the presence of virions in the supernatant with reverse transcriptase activity we collected and filtered supernatants for RNA extraction. Extracted RNA was loaded on a dot blot and probed with the ^{32}P-labeled *abl*-specific probe. The negative control is a supernatant from cells infected with MoMuLV (dot 7) while the positive control is a transforming *v-abl* supernatant 1349D8 (dot 8). Rescued viral RNAs from lines T3-8, T5-10, T12-6, T16-4, T19-7 as well as parental transformed RC1A cells were blotted in dots 1-7. A strong signal is detected in all revertant cell lines.

A



B

Figure 7. Immunoprecipitation of v-*abl* mutant

Cells were metabolically labeled with ^{35}S-methionine and lysed in RIPA buffer. Lysates from normal Rat-1 (lane1), *v-abl* transformed Rat-1 cells, Abl A (lane 2), revertant lines T5-10 (denoted 5-10) T16-4 (denoted 16-4) were immunoprecipitated with a polyclonal goat antiserum directed at the p15 epitope of retroviral *gag* antigen. Arrows indicate the presence of the wild type p160$^{v\text{-}abl}$ in the AblA cell line (w.t.v-*abl* 160kDa) and the presence of the truncated form in both revertant lines (mutant ~50kDa). The size (kDa) of molecular weight standards (Sigma) are indicated at left.

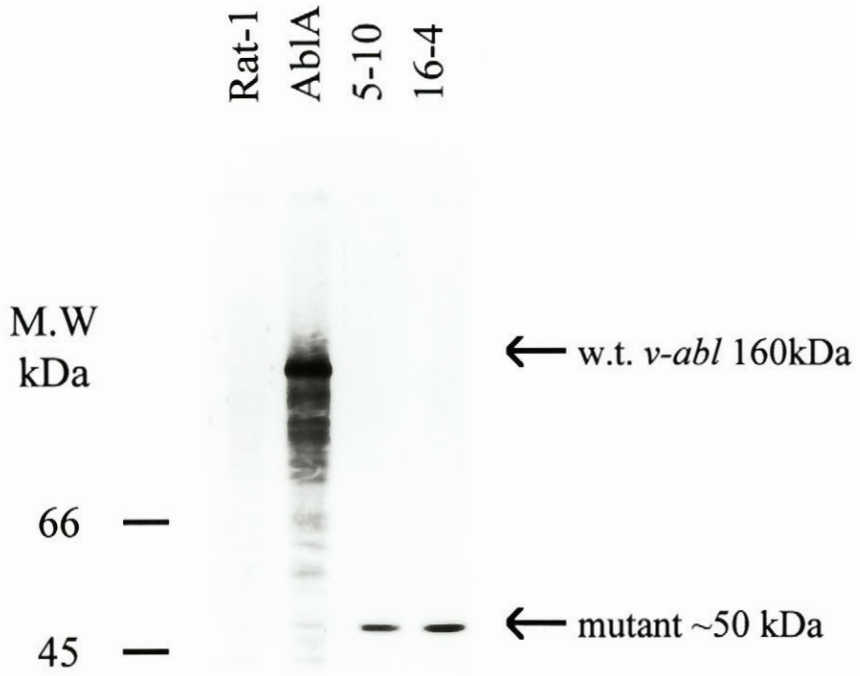

Rat-1
AblA
5-10
16-4
M.W
kDa
66
45
← w.t. v-abl 160kDa
← mutant ~50 kDa

Figure 8. Comparison of the nucleotide and amino acid sequence of the cloned v-*abl* mutant with the wild type v-*abl*

The sequence of the pBR322 subclone containing the entire retroviral genome of the T5-10 cell line was determined. The mutant sequence is indicated. Differences in the published sequence are indicated above the string. Note that all the single base pair mutations with respect to the published sequence were also found in the wild type (transformation competent) virus. Our analysis revealed that the mutant had sustained a 2 bp deletion indicated by the gap in the string at codon 388 or position 2258-2259 of our composite map, for the generation of this map please refer to Materials and Methods. This gave rise to a frameshift (28 amino acids are encoded by this frameshift) and premature termination at codon 416. The resulting protein is depicted on the following page. It is comprised of the entire *gag* portion of the *v-abl* wild type but only extends slightly pass the SH2 domain. Therefore it lacks the entire tyrosine kinase domain and C-terminal domain of the wild type.

```
                                                  T
1094  ATGGGCCAGACTGTTACCACTCCCTTAAGTTTGACCTTAGGTCACTGGAAAGATGTCGAG   1153
      M  G  Q  T  V  T  T  P  L  S  L  T  L  G  H  W  K  D  V  E

                            T
1154  CGGATCGCTCACAACCAGTCGGTAGATGTCAAGAAGAGACGTTGGGTTACCTTCTGCTCT   1213
      R  I  A  H  N  Q  S  V  D  V  K  K  R  R  W  V  T  F  C  S

                     C
1214  GCAGAATGGCCAACTTTTAACGTCGGATGGCCGCGAGACGGCACCTTTAACCGAGACCTC   1273
      A  E  W  P  T  F  N  V  G  W  P  R  D  G  T  F  N  R  D  L

1274  ATCACCCAGGTTAAGATCAAGGTCTTTTCACCTGGCCCGCATGGACACCCAGACCAGGTC   1333
      I  T  Q  V  K  I  K  V  F  S  P  G  P  H  G  H  P  D  Q  V

1334  CCCTACATCGTGACCTGGGAAGCCTTGGCTTTTGACCCCCCTCCCTGGGTCAAGCCCTTT   1393
      P  Y  I  V  T  W  E  A  L  A  F  D  P  P  P  W  V  K  P  F

1394  GTACACCCTAAGCCTCCGCCTCCTCTTCCTCCATCCGCCCCGTCTCTCCCCCTTGAACCT   1453
      V  H  P  K  P  P  P  P  L  P  P  S  A  P  S  L  P  L  E  P

1454  CCTCTTTCGACCCCGCCTCGATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGGCGCC   1513
      P  L  S  T  P  P  R  S  S  L  Y  P  A  L  T  P  S  L  G  A

1514  AAACCTAAACCTCAAGTTCTTTCTGACAGTGGGGGGCCGCTCATCGACCTACTTACAGAA   1573
      K  P  K  P  Q  V  L  S  D  S  G  G  P  L  I  D  L  L  T  E

1574  GACCCCCCGCCTTATAGGGACCCAAGACCACCCCCTTCCGACAGGGACGGAAATGGTGGA   1633
      D  P  P  P  Y  R  D  P  R  P  P  P  S  D  R  D  G  N  G  G

1634  GAAGCGACCCCTGCGGGAGAGGCACCGGACCCCTCCCCAATGGCATCTCGCCTGCGTGGG   1693
      E  A  T  P  A  G  E  A  P  D  P  S  P  M  A  S  R  L  R  G

                  A                                          A
1694  AGACGGGAGCCCCCTGTGGCCGACTCCACTACCTCGCAGGCATTCCCCCTCCGCGCAGGA   1753
      R  R  E  P  P  V  A  D  S  T  T  S  Q  A  F  P  L  R  A  G


1754  GGAAACGGACAGCTTCAATACTGGCCGTTCTCCTCTTCTGACCTTTACATCACCCCCGTC   1813
      G  N  G  Q  L  Q  Y  W  P  F  S  S  S  D  L  Y  I  T  P  V

1814  AACAGCCTGGAGAAACATTCCTGGTATCATGGCCCTGTATCTCGGAATGCTGCTGAGTAT   1873
      N  S  L  E  K  H  S  W  Y  H  G  P  V  S  R  N  A  A  E  Y

1874  CTGCTGAGCAGCGGAATCAACGGCAGCTTCTTAGTGCGGGAGAGTGAGAGTAGCCCTGGC   1933
      L  L  S  S  G  I  N  G  S  F  L  V  R  E  S  E  S  S  P  G

1934  CAGAGATCCATCTCGCTGCGGTATGAAGGGAGGGTGTACCACTACAGGATCAACACTGCC   1993
      Q  R  S  I  S  L  R  Y  E  G  R  V  Y  H  Y  R  I  N  T  A

1994  TCTGATGGCAAGCTGTACGTGTCCTCCGAGAGCCGCTTCAACACTCTGGCTGAGTTAGTT   2053
      S  D  G  K  L  Y  V  S  S  E  S  R  F  N  T  L  A  E  L  V

2054  CACCATCACTCCACGGTGGCTGATGGCCTCATCACCACACTCCACTACCCAGCTCCCAAG   2113
      H  H  H  S  T  V  A  D  G  L  I  T  T  L  H  Y  P  A  P  K

2114  CGCAACAAGCCCACTATCTACGGTGTGTCCCCCAACTACGACAAGTGGGAAATGGAGCGC   2173
      R  N  K  P  T  I  Y  G  V  S  P  N  Y  D  K  W  E  M  E  R

2174  ACCGACATCACCATGAAGCACAAGTTGGGTGGAGGCCAGTACGGGGAGGTGTACGAGGGC   2233
      T  D  I  T  M  K  H  K  L  G  G  G  Q  Y  G  E  V  Y  E  G

                              GT
2234  GTTTGGAAGAAGTACAGCCTCACT..GGCCGTGAAGACCTTGAAGGAGGACACCATGGAG   2293
      V  W  K  K  Y  S  L  T   G  R  E  D  L  E  G  G  H  H  G  G

2294  GTGGAGGAGTTCCTGAAGGAAGCGGCGGTGATGAAGGAGATCAAACACCCTAA  2346
        G  G  V  P  E  G  S  G  G  D  E  G  D  Q  T  P  *
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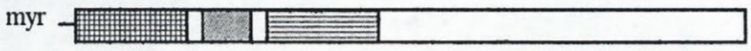


v-*abl* p160

v-abl mutant ~53kDa

Legend

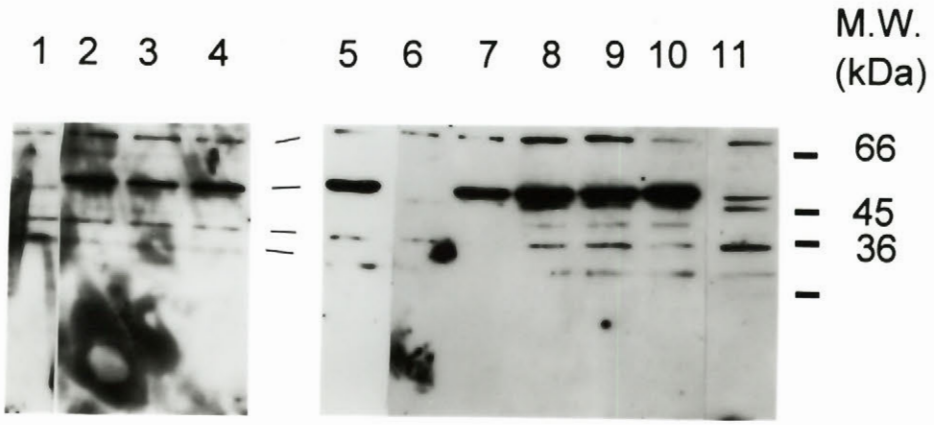
M-MuLV *gag* :

SH2 domain :

Tyrosine Kinase (SH1) :

Frame shift encoded amino acids :

Figure 9. Western blot analysis of Rat-1 clones transfected with the mutant viral genome. Extracts were prepared from Rat-1 clones expressing the mutant virus. These were derived by transfection with the cloned mutant v-*abl* DNA, $ABL^{mut5-10}$ DNA for 392 clones or $pSVABL^{mut5-10}$ for the RIP clones. 50 micrograms of protein were loaded in the following manner: lane 1 and 6: clone 392-1; lane 2: clone 392-3; lane 3: clone 392-4; lane 4: 392-6; lane 5: clone 392-19; lane 7: clone 392-26; lane 8: clone 392-27; lane 9: clone RIP5-1; lane 10: clone RIP5-7; lane 11: T5-10. Note that the extract from T5-10 was harvested under suboptimal conditions and does not adequately reflect the usually higher levels of expression of the mutant in this cell line (data not shown), it was used strictly as a reference for the migration of the mutant protein.


1 2 3 4
5 6 7 8 9 10 11
M.W.
(kDa)
66
45
36

	Morphology	Growth in Agar	Tumorigenecity *in vivo*	DNA v-*abl*	RNA v-*abl*
T3-8	Flat	-	-	+	+
T5-10	Flat	-	-	+	+
T12-6	Flat	-	-	+	+
T16-4	Flat	-	-	+	+
T19-7	Flat	-	-	+	+
Rat-1	Flat	-	-	-	-
RC1A	Refringent	+	+	+	+

Table 1. Growth and Molecular Characteristics of Revertants Cell Lines.
(A).Morphology. Cells were observed at confluence in tissue culture dishes in MEM+10%C.S.. Flat designates a cell line that displays an organized monolayer in these conditions which translates into a non-refringent aspect under phase contrast microscopy at low magnification. Refringent indicates that a cell line displays a refringent aspect under such microscopy conditions as well as a disorganized monolayer under these culture conditions.
(B) Growth in Agar; Assay for Anchorage Independent Growth.
2 plates were seeded for each cell line. (-) signifies a number of foci that is equal or less than that found in Rat-1 and/or the absence of foci larger than that found in Rat-1. (+) signifies the presence of foci that are significantly larger than those found in Rat-1 and present in greater numbers as exemplified by RC1A.
(C) Tumorigenicity *in vivo*.
30-40 day old female nu/nu CD1 mice were injected subcutaneously at 2 different sites. This was repeated with another mouse for each line. Mice were then checked every week for the presence of tumours. (+) signifies the presence of tumours of 1 cm or more in diameter while (-) signifies the absence of tumours (less than 2mm in diameter) after 8 weeks. In the case of RC1A, tumours appeared 5-6 weeks post-injection. No tumours or masses were found after 8 weeks in all revertant cell lines.
(D) DNA analysis. (+) Denotes the presence and (-) the absence of a v-*abl* insertion similar in size to that of RC1A as shown in figure 4.
(E) RNA analysis. (+) Denotes the presence and (-) the absence of a v-*abl* mRNA as evidenced in figure 5.

Viral dilution	control	1:5	1:100	1:1000
T3-8	0	>100	25.5	2.5
T5-10	0	n.d	19.5	0
T12-6	0	>100	n.d.	0[a]
T16-4	0	>100	13.5	0.5
T19-7	0	>60	7.5	0[a]
Rat-1	0	>100	22	4

A) FBR virus, *v-gag-fos-fox* oncogene.

a= duplicate lost to contamination

n.d.= both plates lost so undetermined.

Viral dilution	control	1:5	1:100	1:1000
T3-8	0	0	0	0
T5-10	0	1	0	0
T12-6	0	0	0	0
T16-4	0	0.5	0	0
T19-7	0	0	0	0
Rat-1	0	33	2.5	0

B)208mt virus, Polyoma middle T antigen oncogene.

Table 2. Foci assay of revertants with middle T and *v-fos* retroviruses.

Cells were seeded at $5x10^4$ in duplicate 60mm plates for each dilution the day before infection. Cells were infected with 0.2 mL of the appropriate dilution of virus in MEM+10%C.S. with polybrene. Once at confluence the cells were fed twice a week with MEM+5%C.S.. After 4 weeks foci were counted. The control column refers to uninfected 60mm plates. These represent typical examples of retroviruses that transform (*v-fos*) and do not retransform (Polyoma middle T) the revertant clones.

v-*onc*	v-Ha-*ras*			Middle T			v-abl			v-erbB		
Foci size	XL	L	M	XL	L	M	L	M	S	L	M	S
Rat-1	29 (6)	51	47	23 (6)	10	0.5	1.7 (5)	6	6	4 (4)	16	29
T5-10	25 (4)	45	46	3 (7)	2	0.4	0.3 (6)	0.7	0.3	0.7 (3)	1	2
T16-4	23 (5)	50	44	0 (6)	0.7	0.5	0 (4)	0	1.2	0 (2)	1.5	1

Table 3. Resistance to retransformation assayed by agar growth.

60 mm petris were seeded with $5x10^4$ cells for each cell line and infected with 1:5 and 1:10 dilutions of the appropriate virus. Four petris infected with each virus of interest at a 1:5 dilutions were trypsinized the next day and $1x10^5$ cells from each plate were seeded at $5x10^4$ cells per 60mm agar plates as described in table 1. A focus forming assay in petris was conducted in parallel with the remaining infected cell lines (for procedure refer to legend to table 2). The size standards were set according to the range found in Rat-1 agar clones for all the viruses assayed in this table. Thus XL= extra large, L=large, M=medium and S=small. The numbers indicated are the average number of foci (per plate) belonging to each size category for a given cell line and retroviral infection. The number of agar plates utilized to derive this average is indicated in parentheses.

	Retroviral Stock Reference Number					
	1349D5	CVII	208mt	ERB-B	neofms	MMTV neu[b]
	Oncogene					
	v-*abl*[a]	v-*abl*[a]	*PyMT*[a]	v-*erbB*[a]	v-*fms*	*neu*
T3-8	R	R	R	R	R	N.D.
T5-10	R	R	R	R	R	R
T12-6	R	R	R	R	R	N.D.
T16-4	R	R	R	R	R	N.D.
T19-7	R	R	R	R	R	N.D.
Rat-1	S	S	S	S	S	S

	Retroviral Stock Reference Number					
	MPSRC	1338a7	Ka31	1333Emol	FBR	tpr-met[b]
	Oncogene					
	v-*src*	v-Ha-*ras*[a]	v-Ki-*ras*	v-*mos*	v-*gag-fos-fox*	*tpr-met*
T3-8	S	S	S	S	S	N.D.
T5-10	S	S	S	S	S	S
T12-6	S	S	S	S	S	N.D.
T16-4	S	S	S	S	S	N.D.
T19-7	S	S	S	S	S	N.D.
Rat-1	S	S	S	S	S	S

Table 4. Summary of retransformation assays of revertants by various oncogenes.

Assays were carried out as described in previous pages. S=susceptible, R=resistant, N.D.=not determined.a=assays were done both in agar and in petris for these retrovirus stocks. Note that for v-*abl* and v-Ha-*ras* assays in agar (re:Table 3) were done with other stocks. Other oncogenes were not assayed by titration of foci in agar

b=assay carried by transfection: briefly, cells were transfected with the same calcium phosphate precipitate and allowed to grow to confluence. Foci were counted after 4 weeks.

Line no.	Origin of pseudotype	Resistance to retransformation	Number of Integrations	Susceptibility to retransformation
R1510R 22	T5-10	*v-abl* *v-erbB* *neu*	1	*tpr-met*
R1164R 24	T16-4	*v-abl*	1	n.d.
R1164R 32	T16-4	*v-abl* *neu*	1	*tpr-met*
R1164R 37	T16-4	*v-abl*	1	n.d.
R1164R 46	T16-4	*v-abl* *neu*	2	*tpr-met*
R1164R 54	T16-4	*v-abl*	2	n.d.
T5-10	n.a.	*v-abl* *neu*	1	*tpr-met*
T16-4	n.a.	*v-abl* *neu*	1	*tpr-met*
Rat-1	n.a.	n.a.	0	n.a.

Table 5. Resistance to retransformation is virus borne: characteristics of Rat-1 clones infected with pseudotyped mutant v-abl.

Rat-1 cells were infected with pseudotyped retrovirus from revertant lines T5-10 and T16-4 to obtain mutant v-*abl* expressing cell lines. One clone was derived from the T5-10 pseudotype infection while 5 clones were obtained from the T16-4 pseudotype infection. These clones were assayed for resistance to transformation by several oncogenes in order to ascertain that the resistance phenotype observed in the "parental" revertant lines was caused by the mutated *v-abl.*

Line no.= Designation of clonal line

Origin of pseudotype= Origin of retroviral supernatant used to derive this line.

Resistance or susceptibility to retransformation= A focus forming assay was carried out. Resistance was defined as ten fold or greater decrease in the number of foci (with suitable corrections for each cell line's infectability and transfectability) with respect to controls while susceptibility was defined as a decrease inferior to five fold. Assays were either with retroviruses (v-*abl*, *v-erbB*) or by transfection (*neu, tpr-met*).

Number of integrations= number of proviral v-*abl* genomes as revealed by Southern Analysis.

	Transforming Retrovirus Titers			
	v-*mos* ($X10^4$ ffu)		v-*abl* ($X10^4$ ffu)	
	Trial 1	Trial 2	Trial 1	Trial 2
392-1	9.8	29	3.4	6.0
392-3	26	8.9	3.3	4.6
392-4	27	2.6	6.2	<.1
392-6	13	13.1	4.3	n.d.
392-12	31	n.d.	3.9	n.d.
392-19	3.1	n.d.	<.13	<.13
392-26	23	22	4.7	n.d.
392-27	1.7	4.3	<.11	<.06
RIP5-1[a]	9.5	.4	<.05	<.05
RIP5-7[a]	1.3	n.d.	<.05	n.d.

Table 6. The cloned mutant *abl* blocks transformation: analysis of transformation susceptibility of Rat-1 clones transfected with the mutant viral genome

Rat-1 clones expressing the mutant virus were derived by transfection with the cloned mutant v-*abl* DNA, $ABL^{mut5\text{-}10}$ DNA for 392 clones or $pSVABL^{mut5\text{-}10}$ for the RIP clones. Clonal lines were infected with oncogenic retroviruses CVII (v-*abl*) and the 1333Emol (v-*mos*). Data from two separate trials for each oncogenic retrovirus are presented. To control for infectability clones were infected with the N2 virus expressing the neo resistance gene. The titer of the N2 virus was used to normalize for the calculation of the titer. Focus forming assays were carried out as described previously (Zarbl et al, 1987).

a= since the transfectant DNA also has a neo resistance gene relative titers were not normalized for these two cell lines. Note the 392-1 (and 392-12) clone does not express detectable levels of the mutant protein by Western analysis and serves as a positive control for these assays.

b= titers preceded by < indicated that no foci were detectable at the highest concentration of virus utilized. These values represent the lower limit of detection (corrected for infectability) for these particular in each trial.

In order to determine the mode of action of the mutant *abl* we undertook a molecular dissection of the mutant protein. We also sought to determine if the mutant could interfere with other cellular signalling processes that were not intrinsically linked to transformation. The following chapter details the results of our preliminary attempts aimed at identifying the mode of action of the mutant.

CHAPTER 3 ADDENDUM TO CHAPTER 2: STUDY OF THE EFFECT OF THE V-*ABL* MUTANT ON DIFFERENT CELLULAR SIGNALING PROCESSES.

3.1 Summary

In order to determine the mode of action of the mutant isolated in our previous studies, we generated a series of variants of the mutant to assess the relative contribution of each of the remaining domains in the resistance to retransformation. As a natural extension of this work we were also interested in determining if the mutant affected signal transduction in other well characterized signal transduction pathways such as PC12 neuronal differentiation as well as B and T cell receptor complex signaling and a proliferative pathway in a factor dependent cell line.

The second avenue of research we undertook focused on the isolation of a putative substrate for the *abl* mutant. We used an *in vitro* assay utilizing recombinant mutant *abl* protein as a an immobilized substrate for affinity chromatography (GST pull down) of cell lysates.

In studying the variants, we found that the *abl* mutant/neo chimeric virus we constructed for the purposes of these studies was unable to block transformation. This inability to block transformation may reflect an inadequate expression level and suggests that the transformation block is dosage sensitive. The results of our analyses with signaling in other cellular context revealed that the mutant can affect PC12 neuronal differentiation. Our observations are detailed in the following chapter.

3.2 Materials and Methods

Cell lines

Rat-1 cells and derivatives were maintained in MEM (GibcoBRL) supplemented with 10% calf serum (HYCLONE) and penicillin/streptomycin. PC12 cells were obtained from the ATCC and maintained in RPMI1640 (GibcoBRL) supplemented with 10% heat-inactivated fetal bovine serum (FBSI) and 5% horse serum (Gibco). For neomycin resistance selection during titration, G418 (GibcoBRL) was added to a final concentration of 400μg/mL. For puromycin selection of transfectants with the inducible *abl* construct pL7/ABL$^{mut5\text{-}10}$, puromycin (Sigma) was added to a final concentration of 4μg/mL. BiKR3 and WEHI-3B cell lines were maintained as previously described (Tsubata et al., 1993). Bac1.2F5 cells (Schwarzbaum et al., 1984; Morgan et al., 1987) were grown in DMEM supplemented with 10% FBSI and 20% L-cell conditioned medium as a source of CSF-1. Following infection with neomycin resistance encoding retroviruses, Bac1.2F5 were selected in complete medium supplemented with 400μg/mL G418.

T cell Receptor Signaling Assay

The assays with the BiKR3 cells were carried out in the following manner: approximately 1X10^6 BiKR3 cells were incubated with 3 mL of virus (SAM 12 for the mutant virus, and N2 for the neo control) in complete medium with polybrene at 2μg/mL overnight. Cells were precipitated and resuspended in complete medium and plated at 10^3 cells per well in 96 well plates in complete medium containing 750μg/mL G418. After complete selection, pools of G418 resistant Bi cells were tested for expression of TCR by staining with FITC conjugated anti-TCR antibody, H57FITC, followed by FACS analysis according to standard protocols (Kubo et al., 1989). For TCR activation, pools of G418 resistant/TCR+ BiKR3 pools were seeded at 5X10^4 cells per well in a 96 well plate precoated with the H57 antibody as previously described (Kubo et al., 1989). After an overnight incubation at 37^{o}C, plates were centrifuged and the supernatant was diluted

serially in four fold dilutions in complete medium. $5X10^3$ CTLL-2 cells, an IL-2 indicator cell line, were seeded in these dilutions and incubated for 48 hours at 37°C, at which time 10μCi of H^3-Thymidine was added to each well. After a 16 hour pulse, the pools were washed and harvested and thymidine incorporation was measured.

B Cell Receptor Signaling and PC12 Neuronal Differentiation

WEHI-3B stimulation and PC12 cells neuronal differentiation assays were conducted esssentially as previously described (Tsubata et al., 1993; Hempstead et al., 1994).

Plasmids and Constructs

The constructs were made according to established protocols (Sambrook et al., 1989). The chimeric *abl* mutant virus containing a SV40 promoter neo resistance cassette was constructed in the following manner: 1) a BamHI-EcoRI fragment containing the SV40 neo cassette of pLJ (obtained from R.J. Mulligan,) was subcloned into PSL1180 2) a HindIII fragment containing the 3' Long Terminal Repeat (LTR) of pABFBJ (Jolicoeur et al., 1991) was subcloned into pBR322 (pAB/FBJ-LTR3'). To construct the complete retrovirus an Eco RI-HindIII fragment from the cloned mutant retrovirus, ABL $^{mut5-10}$, was ligated to 1) the SV40 neo cassettte excised by a BstXI-EcoRI digest via a HindIII-BstXI linker, 2) a EcoRI-XbaI fragment from pAB/FBJ-LTR3' and 3) the pJRD184 vector digested with EcoRI-XbaI (Heuterspreute et al., 1985). This construct is referred to as ABL $^{mut5-10/neo}$. This construct serves as the basic backbone for the variants of the *abl* mutant.

Construction of the myristylation variant:

The myristylation mutant was constructed by PCR directed mutagenesis: two mutagenic primers were synthesized to create a point mutation at nucleotide position 1098 of our composite map (please refer to chapter 2, figure 8) resulting in the

substitution of a G for a C and Gly→Ala substitution at the amino acid level. This substitution had been demonstrated previously to abolish N-terminal myristylation and membrane association in murine leukemia viruses (MuLVs) (Rein et al., 1986). The primers spanning the mutation had the following sequence, the base substitution is underlined: 5'AAAATATGGCCCAGACTGTTAC3' (oligo 142,sense) and 5'TAACAGTCTGGGCCATATTTTC3' (oligo 140, antisense). Primers 139 5'GCGGACCCGTGGTGGAAC3' (position 783-800 of the composite map) and 141 5'CCGTCTCGCGGCCATCCG3' (position 1254-1237) were used for amplification. Briefly, in the first round of synthesis, 10ng of ABL $^{mut5-10}$ was amplified using primer pairs 139-140 and 141-142 in two separate reactions. The PCR reaction was subjected to three cycles with an annealing temperature of 50°C, then further amplified for 22 cycles at 57°C. The resulting PCR products were gel purified, mixed in equimolar quantities and amplified with primers 139 and 142 for three cycles with an annealing temperature of 55°C and for a further 22 cycles at 57°C. The resulting PCR product was digested with PstI, gel purified and ligated to PstI digested pGEM4 (Promega) to make ABL-MYR$^-$/A. The mutation was verified by sequencing utilizing the Sanger method as previously described (Aziz et al., 1989). ABL-MYR$^-$/A was digested with PstI, the PstI fragment was subcloned into ABL $^{mut5-10}$ /K-S 5'(2.3) which was constructed by ligating a KpnI-SacI fragment containing a portion of the 5' long terminal repeat (LTR) and the *abl* mutant ORF from ABL $^{mut5-10}$ into a KpnI-SacI digested pSL1180 vector (Pharmacia). This construct is termed ABL-MYR$^-$/B. To obtain a myristylation variant *abl*/neo chimeric virus, a KpnI-BstEII fragment from ABL-MYR$^-$/B was ligated to a BstEII-KpnI fragment from ABL $^{mut5-10/neo}$ containing the SV40 neo cassette and a portion of the 3'LTR and a KpnI-KpnI fragment from this same plasmid containing 5' and 3' LTR sequences as well as vector.

Construction of the SH2 deleted variant:

The SH2 deleted variant was constructed in the following fashion: a KpnI-XhoI fragment from ABL $^{mut5-10}$ was cloned into the pJRD184 vector cut with KpnI and XhoI, this construct was named ABL $^{mut5-10}$/K-XhoI(5'). A partial HincII digest was performed and the fragment of interest was gel purified. This fragment was ligated to the amber stop oligo, oligo 153 5'CTAGTCTAGACTAG3', which inserts stop codons in all three reading frames as well as one XbaI site at position 1813 of our composite map. A KpnI-XhoI fragment from this construct, ABL $^{mut5-10}$/K-XhoI(5')/153, was ligated to KpnI-XhoI and XhoI-XhoI fragments from ABL $^{mut5-10/neo}$ to create the deleted SH2 variant of the *abl* mutant/neo chimera.

Construction of the *gag* deleted variant:

The *gag* deleted variant was constructed by digesting the ABL $^{mut5-10}$/K-XhoI(5') construct with BstEII and HincII which eliminates the intervening sequence which encodes for the *gag* portion of the protein. These two ends were ligated by using the following pair of oligos 5'GTTACCCCGGTC3' and 5'GACCGGG3' for an in frame fusion. A KpnI -XhoI fragment from this construct, ABL $^{mut5-10}$/ΔGAG-A, was ligated to the KpnI-XhoI and XhoI-XhoI described in the previous paragraph to yield the deleted *gag* variant of the *abl* mutant/neo chimera, ABL $^{mut5-10}$/ΔGAG-B.

Construction of the inducible *abl* mutant:

The inducible construct was made by digesting pL7CAT (Baim et al., 1991) with StuI and HpaI and inserting a SmaI fragment from ABL $^{mut5-10/neo}$ which contains the entire open reading frame of the *abl* mutant. This plasmid was designated pL7/ABL $^{mut5-10}$. The pSV2puro plasmid is a derivative of pSV2neo (Southern and Berg, 1982) and has a puromycin resistance encoding gene under the control of the SV40 promoter (Jolicoeur and Laganiere; unpublished results). The pHβALAP267 was described previously (Baim et al., 1991).

The GST fusion construct was obtained by ligating a BstEII(blunted)-SmaI fragment from ABL $^{mut5-10}$ /K-S 5'(2.3) into a SmaI digested pGEX-3X to yield ABL $^{mut5-10}$/Bs-Sm. Sequence analysis revealed that a portion of the BstEII (the GGT portion) was deleted during the blunting reaction but the frame is not disrupted.

Transfections and Generation of Retroviral Stocks

Transfections on Rat-1 cells to produce a Rat -1 cell line expressing the inducible *abl* mutant clone were carried out using lipofectin (Gibco BRL) according to the manufacturer's recommendations. PL7/ABL $^{mut5-10}$, pHβALAP267 (5μg each) and pSV2puro (1μg) were cotransfected into Rat-1 cells plated the previous day at a density of $3.5X10^5$ cells per 60mm tisue culture plate. 48 hours after transfection, cells were split into eight 100mm tissue culture plates. 24 hours after plating, the media was changed with normal medium containing 4μg/mL puromycin. The medium was changed every 3-4 days. 40 clones were picked and expanded. To test for expression, 90-95% confluent duplicate plates of each clone were incubated for 10 hours in medium with or without 1mM IPTG, a concentration that had been shown to be maximal for induction in previous experiments (Balsalobre and Jolicoeur; 1995). Cells were harvested after the 10 hour incubation and RNA was extracted using an NP40 extraction protocol (see below).

Retroviral stocks for the variants were generated by transient transfection into BOSC23 cells, kindly provided by Dr. Warren S. Pear, Department of Biology MIT, as previously described (Pear et al; 1993). For the mutant *abl*/neo a stable transfectant was also derived by transfecting the Ψ2 packaging cell line with the ABL$^{mut5-10}$/neo construct as described in the previous paragraph. Ψ2 cells were selected in DME 5% calf serum with 400μg/ml G418. A clone producing the desired virus, SAM12, was further expanded. Viral supernatant from transient transfections on BOSC 23 cells for all the viruses (including the mutant *abl*/neo) were used to infect the amphotropic packaging cell

line GP+*env*Am 12 (Markowitz et al., 1988) and pools of G418 selected cells from these infections were used to generate the amphotropic stocks of viruses .

NP-40 RNA Extraction

Briefly, cells were rinsed with PBS and harvested by scraping in 1 mL of PBS and pelleted by centrifugation in a microfuge for 30 seconds. Cell pellets were resuspended in 45μL of cold 10mM Tris pH 7.0, 10mM EDTA. Cells were lysed by addition of 5μL of 5% NP40 and incubation on ice for 5 minutes. An additional 5 μL of NP40 was added prior to a 2.5 minute spin in microfuge at 15 000 rpm. 50μL of the supernatant was mixed with 30μL of 20XSSC and 20 μL of a 37%(w/v) formaldehyde solution (Fisher). Samples were incubated at 60°C for 15 minutes prior to dot blotting onto a nylon membrane (Hybond N, Amersham) soaked in blotting buffer, 50%H_2O:30% 20XSSC:20% 37% formaldehyde. The filter was hybridized with an *abl* specific probe which was the 1.8kb SmaI fragment used to construct pL7/ABL $^{mut5\text{-}10}$ (see previous paragraph).

Anti-sera, Western Blot analysis

Cell lysates were prepared and separated by SDS-PAGE on 7.5%-15% acrylamide gradient gels as previously described (Balsalobre and Jolicoeur, 1995). Protein concentrations of lysates were determined by microBCA assays (Pierce). The antiphosphotyrosine antibody was obtained from UBI (#05-321) and the manufacturer's protocol was used in western analysis. Anti-*gag* antisera were described previously (Houle and Jolicoeur, 1995). The secondary antibodies were from DAKO and used according to manufacturer's recommendations.

Affinity Chromatography with the Abl Mutant Protein

The fusion protein produced from ABL $^{mut5\text{-}10}$/Bs-Sm was prepared by Sarkosyl extraction (Lavoie, 1995) with the following modifications: the bacteria, Y1090, (Sambrook et al, 1989) were incubated at 25°C during induction which was carried out with 100μM IPTG for four hours. The control GST protein was produced from pGEX-3X transformed DH5α (Sambrook et al, 1989). Glutathione agarose was used according to the manufacturer's recommendations (Sigma). 500μL of packed slurry was incubated with approximately 270μg of the appropriate fusion protein for 30 minutes on ice. The slurry was washed twice in HNTG buffer (20 mM Hepes [pH7.5], 200mM NaCl, 0.1% Triton X-100, 10% glycerol, 1mM sodium orthovanadate; Koch et al., 1992).

^{35}S labelled lysates were prepared according to established protocols (Harlow and Lane, 1988) for immunoprecipitation except that the cells were lysed in PLC-LB (Koch et al., 1992) buffer at 200mM NaCL and 0.5% DOC (Fisher). The lysates were cleared by ultracentrifugation at 30 000 rpm for 20 minutes in a TL-100 rotor (Beckmann). The lysates were split into two equal portions which were incubated for 90 minutes on ice with either 50μL of packed slurry prebound to GST or GST-*abl* mut. After the incubation the slurry was spun down and washed several times with HNTG with protease inhibitors (aprotinin, leupeptin, pepstatin, TLCK, EDTA). The final pellet was resuspended in 50μL 2XSDS loading buffer and 25μL of each was loaded onto a gel and processed as previously described (Sambrook et al., 1989). The gel was transferred to a PVDF membrane (Millipore) and exposed.

3.3 Results

Generation of a ABL Mutant/NEO Chimeric Virus and Variants

In order to assess the contribution of the remaining domains to the *abl* mutant to its transformation blocking activity, and potential effects in other signaling pathways, we generated SH2 and *gag* deleted variants of the *abl* mutant. Furthermore, we also generated a myristylation signal defective variant to determine if the localization of the mutant affected its ability to block transformation. Previous studies have underscored the importance of the myristylation signal in directing membrane association and the proper localization of *gag* proteins (Rein et al; 1986). We believed that these variants could provide some insights into the potential targets of the *abl* mutant. Figure 1a is a schematic representation of the gene products produced by these variants. Stocks of chimeric retroviruses were generated by transient transfection of BOSC 23 cells. These viral supernatants were used to infect Rat-1 cells. Pools of neomycin resistant clones were analysed to determine if the chimeric retroviruses allowed for expression of the expected variant protein. Western blot analysis was conducted on cell lysates from these pools, as shown in figure 2. The *gag* deleted variant was not detectable with the antibodies used (data not shown) due to the large deletion of *gag* amino acids. As expected the myristylation mutant gave rise to a gene product of identical size to that of the mutant since the loss of the myristoyl group does not significantly affect its migration. The deleted SH2 variant was detectable after prolonged exposure only with one of the *gag* directed antiserum, α-p12, which we have observed to be more sensitive than the α-p15 (unpublished results). The myristylation defective infected pool was further analysed by cell fractionation to determine if the amino acid substitution introduced by PCR-directed mutagenesis resulted in a predominantly cytosolic localization as expected. For this purpose, myristylation defective mutant and mutant *abl* infected pools were fractionated and the relative proportion of each species in S100 and P100 fractions compared. As demonstrated in figure 2C the proportion of cytosol localized mutant Abl versus

membrane associated Abl appears to be inverted in the cells infected with the myristylation-defective variant such that the most intense signal is found in the S100 or cytosolic fraction. The amount of protein loaded in each lane was rigorously controlled. This result confirms that this mutation affects the cellular localization of the mutant Abl.

The Ability of the Mutant Abl to Block Transformation Is Dosage-Sensitive

We proceeded to determine if these chimeric and variant retroviruses could block transformation. Our assays are summarized in table 1. Foci appearing in duplicate plates were counted and the average number is indicated in the appropriate box. Note that in previous experiments expression of the *abl* mutant in a different construct had been shown to suppress dramatically transformation by v-*erbB* and Polyoma middle T while having little or no effect on v-Ha-*ras* mediated transformation. The pool of cells infected with the mutant *abl*/neo chimeric retroviruses does not resist to retransformation by v-*abl* and Polyoma middle T (chapter 2). A preliminary Western analysis of this pool of cells, shown in figure 3, reveals that the quantity of mutant Abl present in this pool is inferior to that of the revertant cell line 5-10 from which the mutant v-*abl* was originally cloned (refer to chapter 2). This analysis was repeated and similar results were obtained. These results suggest that the ability of mutant Abl to block transformation is dosage sensitive which is supported by other observations with cells transfected with the cloned mutant virus (chapter 2). The extra bands that appear in the mutant infected pools are proteolytic cleavage products of *gag* precursor proteins of a replication competent helper virus which arose spontaneously in our Ψ2 packaging cell line (SAM12). The presence of this helper virus should not affect the ability of the mutant Abl to block transformation: cell lines derived from infection with rescued v-*abl* mutant virus (chapter 2) display an identical pattern of resistance to retransformation as the revertant cell lines from which this virus was derived even though they contain helper virus.

As an alternative approach, we placed the *abl* mutant ORF under the control of an IPTG inducible promoter. Rat-1 cells were transfected with the pL7/ABL$^{mut5\text{-}10}$ construct as well as a selectable marker and a construct expressing constitutively the LAP267 IPTG-inducible activator protein. After selection clones were analysed for inducible expression of the transfected gene. Ten clones were found to have the desired expression profiles at the mRNA level (data not shown). All were subjected to Western analysis but none displayed comparable levels of Abl mutant expression (data not shown) at concentrations of IPTG previously shown to induce maximal expression (Balsalobre and Jolicoeur, 1995). As expected, even the highest expressor fails to block transformation (data not shown). We proceeded to study the effect of the mutant Abl in other cellular contexts with these chimeric viruses even though these constructs do not afford sufficient levels of mutant protein to block transformation.

Effects of The Mutant Abl in Growth Factor, B and T cell Receptor Complex Signaling and PC12 Differentiation.

In an effort to identify the substrates of the mutant *abl* protein, we studied the effect of the mutant's expression on other cellular processes with well-defined signaling pathways. Our initial findings that the mutant could block transformation by activated receptor tyrosine kinases prompted us to determine its effect on signaling through receptor tyrosine kinases involved in the survival and differentiation of established cell lines. Thus we looked at the effect of the expression of the mutant Abl in 1) Bac1.2F5 cells, which require the addition of CSF-1 and concomitant activation of the c-*fms* receptor tyrosine kinase in order to proliferate, 2) PC12 cells, which survive serum starvation and differentiate into neurons in the presence of Nerve Growth Factor (NGF). NGF signaling occurs through the *trkA* protooncogene, a member of the receptor tyrosine kinase family (Barbacid; 1995).

We also investigated the effects of the mutant Abl on signaling through the B-cell receptor and T-cell receptor complexes by antibody induced crosslinking in WEHI-3B cells and Bi cells respectively. Our experiments with the mutant *abl*/neo chimeric virus demonstrated virtually no effect on three of the assay systems evaluated.

Firstly, we reasoned that if the mutant blocked cell signaling through the c-*fms* receptor tyrosine kinase, the CSF-1 dependent Bac1.2F5 cell line should not be able to tolerate the expression of the mutant Abl. To assay this possibility, we infected Rat-1 cells and Bac1.2F5 cells with the mutant *abl*/neo chimeric retrovirus (8694Aam12) and a neomycin resistance encoding retrovirus, N2. If the mutant v-*abl* causes cell growth arrest or death, we would expect that the number of neo^r clones in Bac1.F5 cells infected with the chimera to be smaller than in a comparable infection of Rat-1 cells where the mutant Abl has little or no effect on cell growth. The infection with the N2 virus serves as indicator of the relative infectability of these cell lines. After selection with G418, the number of neo resistant (neo^r) colonies in both the Rat-1 and Bac1.F5 infected cells were counted. The results are summarized in table 2. There is very little difference in the relative titer of these retroviruses in Bac1.2F5 versus Rat-1 cells, indicating that the chimera has little or no apparent effect on signaling through the c-*fms* pathway. It is also possible that the level of protein afforded by the neo chimera is not sufficient to efficiently block this pathway.

Secondly, the B-cell receptor complex signaling pathway was assayed by crosslinking surface IgM with anti-IgM antibodies, and measuring the level of radioactive thymidine uptake. Under these conditions WEHI-3B cells undergo apoptosis with increasing amounts of crosslinking and thus have reduced thymidine uptake. Pools of mutant *abl*/neo infected cells and control N2 infected cells were subjected to this analysis. As evidenced in figure 4a, there is no marked decrease or enhancement of the response to the anti-IgM antibody. A similar trial with increasing amounts of antibody also fails to

uncover a potentiating or inhibiting effect (figure 4b). Thus, the mutant Abl does not appear to affect this pathway.

Thirdly, in the T-cell receptor signaling assay, Bi cells are treated with anti TCR antibody to stimulate IL-2 production. The supernatant from these cells is used to feed an IL-2 dependent cell line which is pulsed with tritiated thymidine. The level of incorporation of tritiated thymidine reflects the level of IL-2 production in Bi cells, thus the stimulation along this pathway. Two independent pools of neo chimera infected cells and a number of N2 derived pools were derived. The cells were tested for their level of surface TCR expression. Pools with the proper level of expression were treated with anti TCR and the supernatant was harvested. Two separate pools of the mutant *abl*/neo infected cells gave opposite responses: in one of these pools IL-2 production was dramatically decreased while in the other there was little or no effect on IL-2 production (data not shown).

The infection of PC12 cells yielded surprising results. In deriving neo resistant clones from PC12 we noticed that we obtained far fewer clones than in an identical infection with an N2 viral stock of similar titer. If we compare (table 3) the apparent titer of the SAM12 (mutant *abl*/neo) and the N2 viruses on PC12 to that obtained on Rat-1 cells, we observe a 10 fold lower ratio with the SAM12 virus. Thus it appears that a significant percentage of SAM12 infected PC12 fail to form colonies. Furthermore, under microscopic examination the clones appear darker and flatter than the parental cell line or the N2 clones. Other groups have suggested that this altered morphology represents an early response to NGF differentiation signals (Stephens et al, 1994) Clones were expanded and their ability to undergo neuronal differentiation was examined. The less refringent morphology of the SAM12 clones appeared to suggest an increased susceptibility to the differentiative process. Thus the ability of these cells (and control N2 infected cells) to undergo differentiation was measured over a wide range of NGF concentrations in low serum conditions. It appears that the SAM 12 expressing clones can

undergo a substantial amount of neuronal differentiation at NGF concentrations of 5ng/mL which is ten fold lower than the usual concentration required for normal PC12 cells or N2 infected cells (figure 5). Furthermore, the mutant expressing cells often display sprouting of numerous small axons (data not shown). Thus, this suggests that the expression of the mutant protein facilitates neuronal outgrowth and possibly differentiation.

The Search for Effectors of the v-*abl* Mutant's Action: Detection of a Putative 60 kDa Candidate.

A recombinant GST-mutant *abl* fusion protein coupled to glutathione agarose was used to precipitate proteins which interacted with the mutant protein. We incubated ^{35}S-methionine labelled lysates from 1) Rat-1 cells or 2) Rat-1 cells harbouring the mutant (5-10) or 3) Rat-1 cells harbouring the wild type v-*abl* (AblA) with GST-mutant *abl* fusion protein absorbed to glutathione agarose. In a control experiment the lysates were incubated with GST protein absorbed to glutathione agarose. The data is presented in figure 6A. We observed specific ^{35}S labeled bands only in the lysates from cell lines 5-10 and AblA precipitated with the GST-mutant *abl* fusion protein. This suggests that either the presence of an *abl* protein in the cells affects the availability of these interacting proteins after lysis, or that in these lysates, specific complexes are formed and the recombinant *abl* displaces the mutant and wild type v-*abl* in these structures. Alternatively, the smaller species may represent degradation products of the larger protein of 60kDa, even though protease inhibitors were used throughout experimentation. Interestingly, when we blot for the presence of phosphotyrosine in these precipitates (figure 6B) we do not detect these specific bands in the lysate from a cell containing the mutant. After prolonged exposure only faint bands appear which do not comigrate with the ^{35}S labelled proteins. Furthermore, this faint reactivity is also seen in the Rat-1 lysate precipitated with GST-mutant *abl*. In contrast, in the lysate from v-*abl* transformed cell

precipitated with the GST-mutant *abl*, we observe three predominant bands. The most intense signal is seen for a protein migrating at approximately 60 kDa where one the ^{35}S labelled substrate was detected. Thus, this putative substrate reacts with the antiphosphotyrosine antisera. The other bands in the v-*abl* lane may reflect the presence of transformation specific substrates or spurious binding to the recombinant GST-mutant *abl* due to the generalized increase in phosphotyrosine in the v-*abl* transformed cells. These results suggest that the mutant protein can interact with these putative signal transducers in a phosphotyrosine independent manner and that the 60kDa substrate is usually phosphorylated on tyrosine in v-*abl* transformed cells.

3.4 Discussion

Although the initial aims of these experiments were not achieved, these studies suggest that the dosage of the mutant protein is critical for its transformation suppression activity. Our inability to observe a measurable effect in other cell signaling contexts may also be the result of insufficient expression. The determination of the contribution of each of the remaining domains to the mutant protein's suppression activity must await the development of expression constructs with reproducibly higher levels of expression.

However, our results suggest that the mutant Abl levels afforded by the neo chimeric virus is sufficient to affect neuronal differentiation in the PC12 cell line. It is tempting to speculate that the observed phenotype in PC12 cells requires a lower threshold of expression. It is also noteworthy that the estimated number of *trkA* molecules in PC12 is low, some estimates suggesting that only 10000 molecules are present per cell (Hempstead et al., 1994). This may also explain the dramatic effects of the mutant Abl on PC12 growth. NGF stimulation in low serum conditions rescues PC12 cells from apoptosis (Batistatou et al., 1993). The effect of the mutant appears to be increased neuronal outgrowth in low NGF concentrations which would appear to reflect decreased apoptosis. In contrast, in high serum conditions the decreased titer of neo^r clones observed following infection with the mutant expressing virus would appear to suggest decreased proliferation. The darker and flatter morphology of the few clones obtained also suggest a predifferentiative state (Stephens et al., 1994). These results lead us to speculate that the mutant stimulates differentiation. Interestingly, our observations parallel those reported by an other group (Hempstead et al., 1994) who studied the effect of v-*crk* expression on PC12 cells. PC12 cells expressing v-*crk* displayed a slight flattening of the cell body as well as increased sensitivity to NGF induced neurite outgrowth. The v-*crk* oncogene resembles the *abl* mutant in that it is composed of a *gag* moiety as well as an SH2 domain although it also contains an SH3 domain (Reichman et al., 1992). The authors suggest that the v-*crk* gene product may interact directly with the

*trk*A receptor and potentiate signaling through this pathway. Furthermore, the overexpression of v-*crk* in PC12 cells renders them capable of differentiation upon stimulation with EGF which usually elicits a proliferative response in PC12 cells. Our preliminary experiments suggest that the expression of the Abl mutant does not affect the proliferative response to EGF nor does it give rise to EGF-dependent differentiation (data not shown). It is not clear if the same pathways are involved in fibroblast-transformation and PC12 differentiation, nonetheless previous studies with *ras* and dominant negative forms of *ras* as well as v-src and MAPKK (Bar-Sagi and Feramisco; 1985; Noda et al; 1985; Szeberenyi et al; 1990; Alema et al; 1985; Cowley et al; 1994) have suggested that similar pathways are involved. Thus, the substrate(s) of the mutant Abl could potentially play a role in two opposing phenomena i.e. differentiation and transformation. Our *in vitro* experiments have provided us with some clues as to the possible nature of this substrate and the potential mode of action of the mutant in blocking transformation.

Our ability to precipitate specifically three metabolically labelled proteins from cells expressing the mutant and wild type v-*abl* proteins suggest the existence of a multicomponent complex in these lines. Furthermore, the binding of substrates to the mutant may not require phosphotyrosine as demonstrated by the lack of immunoreactivity of the complexed proteins with the phosphotyrosine specific antibodies. The increased level of phosphorylation on the 60kDa subunit of this putative complex in extracts from cells infected with the wild type v-*abl* might reflect increased phosphorylation following recruitment into this complex. Thus, it is tempting to speculate that in cells harbouring the mutant this substrate is sequestered and not adequately modified (tyrosine phosphorylation) thus preventing signal transduction through an oncogenic pathway. Our analysis will eventually extend to cells with the variants of the mutant to determine if the presence of this complex correlates with the resistance phenotype and also to determine which domain of the mutant is critical for complex formation. Other groups have observed phosphotyrosine independent binding of proteins to the Abl SH2 domain

although this interaction is believed to be much weaker (Pendergast et al., 1991; Muller et al., 1992). The lack of a requirement for tyrosine phosphorylation also suggests that a genetic system such as the two hybrid system (Phizicky and Fields, 1995) may be amenable to identifying substrates for the mutant.

Acknowledgements

I am indebted to Steven Klein and Aurelio Balsalobre for deriving the Wehi-3b pools and the Bi pools, respectively. I wish to acknowledge Normand Beaulieu for expert and invaluable technical assistance with the analysis in the PC12 cells and Benoit Laganiere for assistance with the retrovirus constructs.

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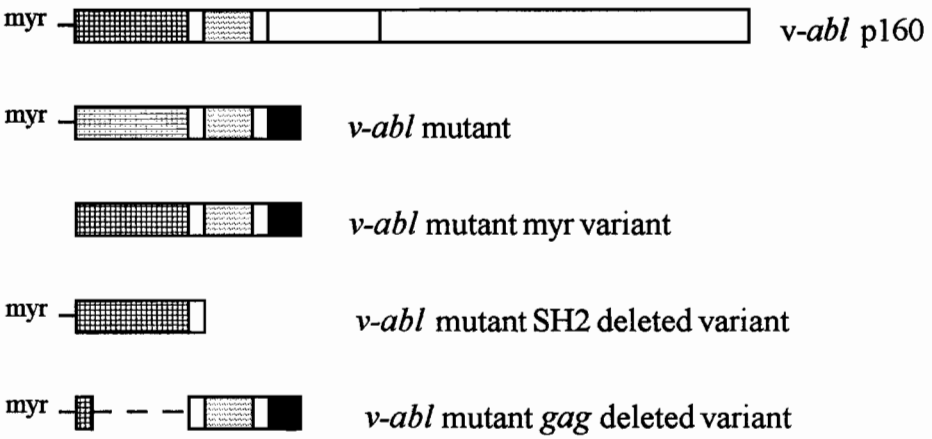
3.6 Figures and Tables

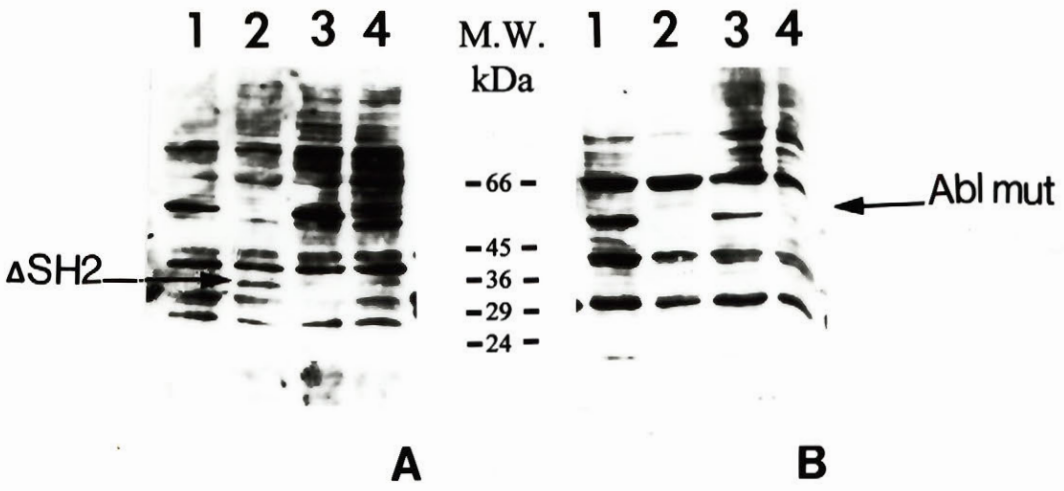
Figure 1. Schematic representation of the mutant Abl and its variants.

The gene products encoded by the mutant and the variants are presented to scale except for the myristyl group (myr-) present on all proteins save for the myristylation-defective variant. Note that the mutant encodes a largely truncated form of the p160$^{v\text{-}abl}$. The *gag* encoded amino acids present in the mutant (and wild type v-*abl*) are comprised of the entire p15(MA), p12 and 20-21 aminoacids of p30(CA) *gag* from the Moloney Murine Leukemia virus (MoMuLV *gag*). The SH2 domain is approximately 100 amino acids long. It is absent in the SH2 deleted variant due to premature termination of the ORF. Finally *gag* deleted variant lacks some 200 amino acids of the *gag* due to an in frame deletion.



Legend

M-MuLV *gag* :
SH2 domain :
Tyrosine Kinase (SH1) :
Frame shift encoded amino acids :


1 2 3 4
ΔSH2
M.W.
kDa
-66-
-45-
-36-
-29-
-24-
1 2 3 4
Abl mut
A
B

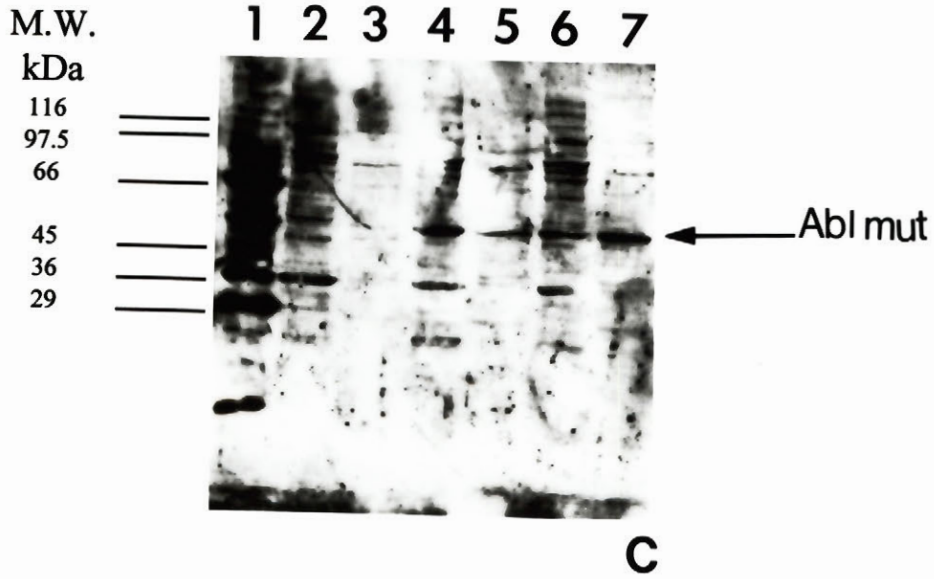

M.W.
kDa
116
97.5
66
45
36
29
1 2 3 4 5 6 7
Abl mut
C

Figure 2. The mutant *abl*/neo chimeric virus, the myristylation-lacking variant and SH2 deleted variant express the expected gene products.

Extracts were prepared from pools of neo resistant Rat-1 clones from infections with the mutant, myristylation minus, SH2 deleted viruses as well as N2 virus (neomycin resistance encoding) as a negative control. Approximately 50 μg of total protein from pools of mutant *abl*/neo chimera infected Rat-1 cells (lane 1 panels A,B); myristylation defective variant infected Rat-1 (lane 3, panels A,B); SH2 deleted variant infected Rat-1 cells (lane 2, panels A,B) and N2 infected Rat-1 cells (lane 4, panels A,B) were loaded onto a gel and transferred for Western blot analysis.. Panels A and B represent the result of Western blot analysis with anti-p12 and anti-p15 antisera, respectively. Molecular weight markers are indicated in the middle.

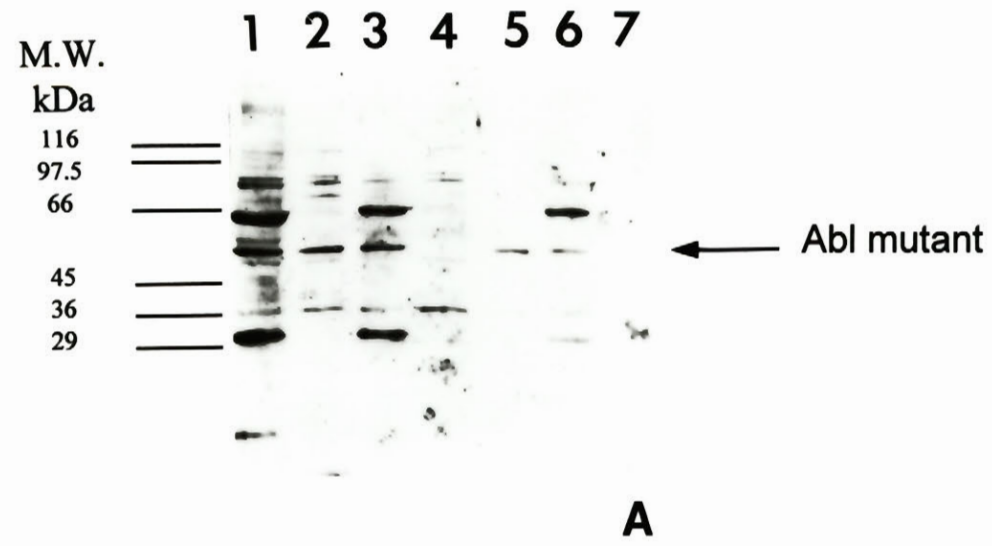
Figure 2 C. The myristylation-defective variant does not associate with membranes.

S-100(cytosolic) and P-100(particulate) fractions were prepared from pools of G418 resistant Rat-1 clones from infections with the mutant and the myristylation-defective variant. Fractions from uninfected Rat-1 cells were also prepared and a total protein lysate from a Rat-1 clone infected with rescued mutant R1164R37 (chapter 2) was used as a positive control. Approximately 50 μg of each of these fractions were loaded onto a gel in the following order: lane1, total lysate from R1164R37; lane 2, 3: P-100 and S-100 fractions from Rat-1 uninfected; lanes 4, 5: P-100 and S-100 fractions from mutant infected Rat-1 pool; lanes 6, 7: P-100 and S-100 fractions from myristylation-defective infected Rat-1 pool.

Figure 3. The mutant *abl*/neo chimeric virus produces less Abl mutant protein.

Protein extracts were prepared from a pool of SAM 12 (mutant *abl*/neo) infected Rat-1 cells, the D2 pool. These extracts were used in a comparative Western blot analysis with the anti-p12 antiserum. Decreasing amounts of protein extracts from a revertant line 16-4 (chapter 1), resistant to retransformation and expressing the original mutant *abl* from which the chimera was derived, and the D2 pool were loaded in each lane. In panel A, lanes 1, 3, 6 contained respectively 50, 10 and 5 μg of total protein from cell line D2 (see table 1); lanes 2, 5, 7 contained respectively 10, 5 and 1 μg of total protein from cell line 16-4 . Lane 4 contained 10 μg of total protein from Rat-1 cells as a negative control. In panel B, This analysis was repeated. the lane designations are as follows: lanes 2, 3, 6, 8 contained 50, 25, 12.5 and 10 μg of total protein from cell line D2; lanes 1, 4, 5, 7 contained 50, 25, 12.5 and 10 μg of total protein from the revertant line 16-4.

For both panels, the molecular weights standards are indicated to the left of the gel and the position of the mutant Abl is indicated by an arrow.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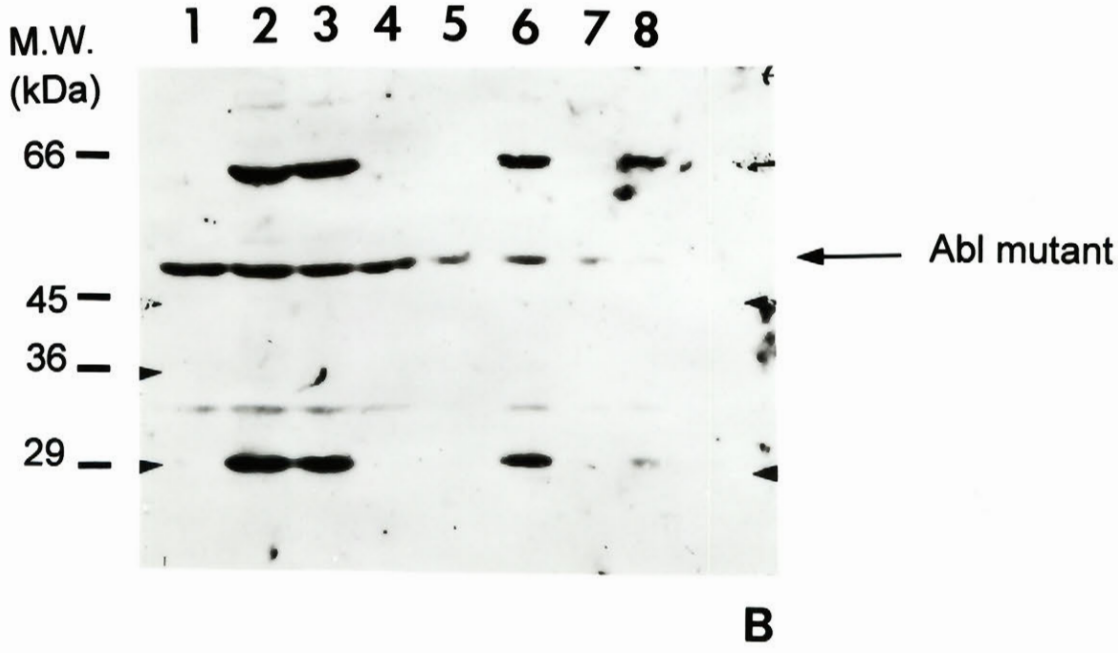
M.W.
kDa
116
97.5
66
45
36
29
Abl mutant

A


1 2 3 4 5 6 7 8
M.W.
(kDa)
66
45
36
29
Abl mutant

B

Figure 4. The mutant *abl* does not affect B-cell receptor signaling in WEHI-3B cells. Pools of G418 resistant WEHI-3B cells from infections with 1) the mutant *abl*/neo virus, designated as Wehi/PS12, and 2) the N2 designated as Wehi/N2. Uninfected and unselected cells were added as a control (Wehi). In panel A, the concentration of antibody in the +gam assay was 10μg/mL (white colums). In panel B on the following page, increasing amounts of antiserum were used and are indicated in the legend (0-10μg/mL). Tritiated thymidine uptake values represent the average of triplicates for each set. Error bars represent the standard error of the mean.

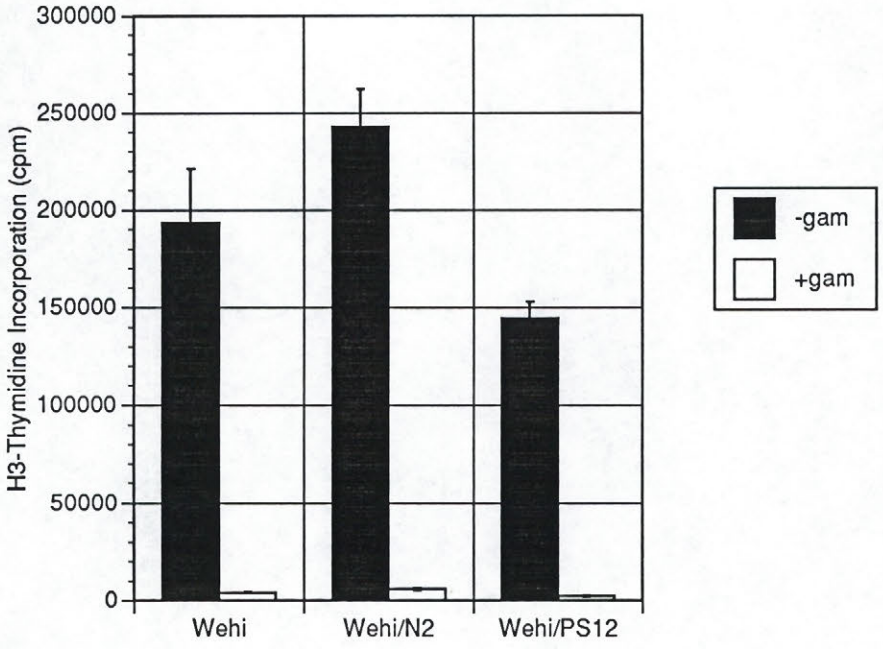

300000
250000
200000
150000
100000
50000
0
H3-Thymidine Incorporation (cpm)
Wehi
Wehi/N2
Wehi/PS12
-gam
+gam

Figure 4 a

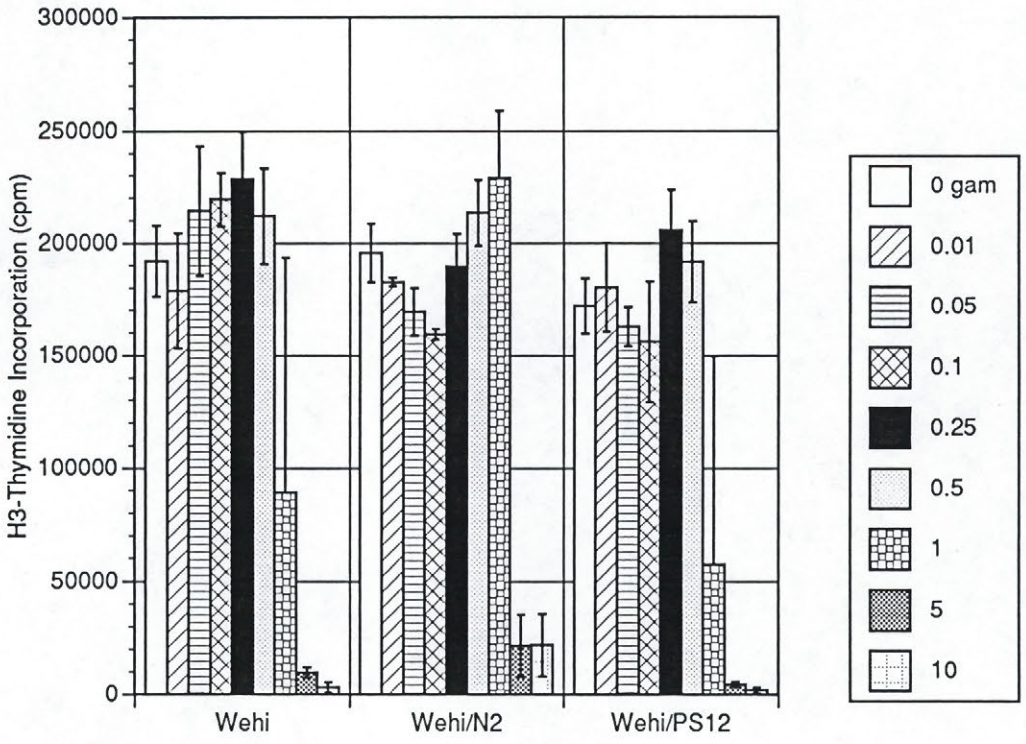
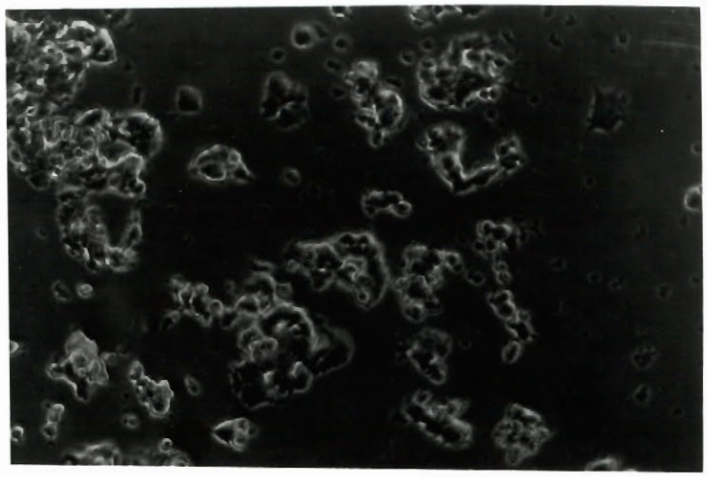

300000
250000
200000
150000
100000
50000
0
H3-Thymidine Incorporation (cpm)
Wehi
Wehi/N2
Wehi/PS12
0 gam
0.01
0.05
0.1
0.25
0.5
1
5
10

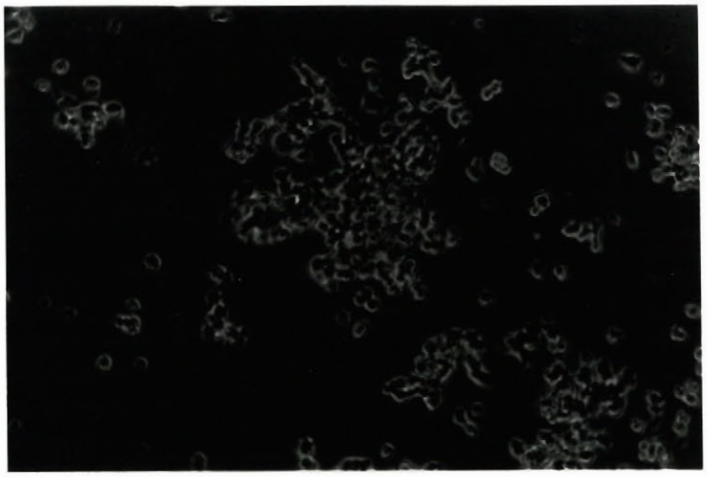
Figure 4 b

Figure 5. The mutant *abl* affects neuronal differentiation in PC12 cells.

Cell lines were derived by infection with the SAM12 (mutant *abl*/neo) and N2 retroviruses. A neuronal differentiation assay was carried out as previously described (Hempstead et al., 1994) at a concentration of 5ng/mL of NGF. Panels a, b, c are representative fields of neuronal outgrowths from three different N2 infected clones, Y2N2-1, -6, -7 respectively, after a week long induction. Panels d, e, f are representative fields from SAM12 infected PC12 cell lines, 1752 NB-12, -13, -19 respectively, under the same induction conditions. Magnification 100X.



a



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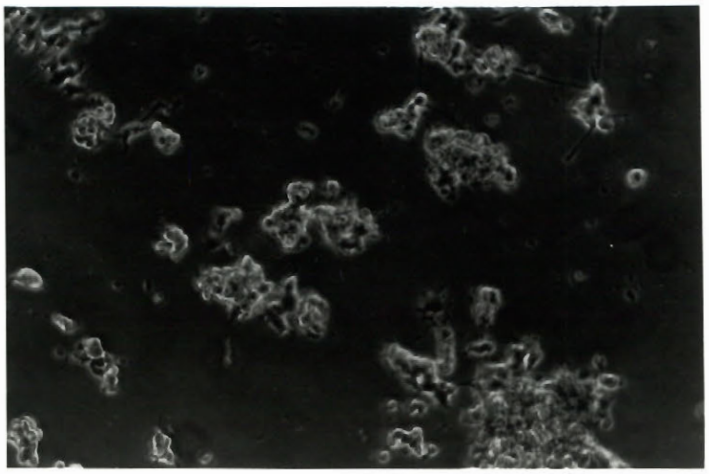
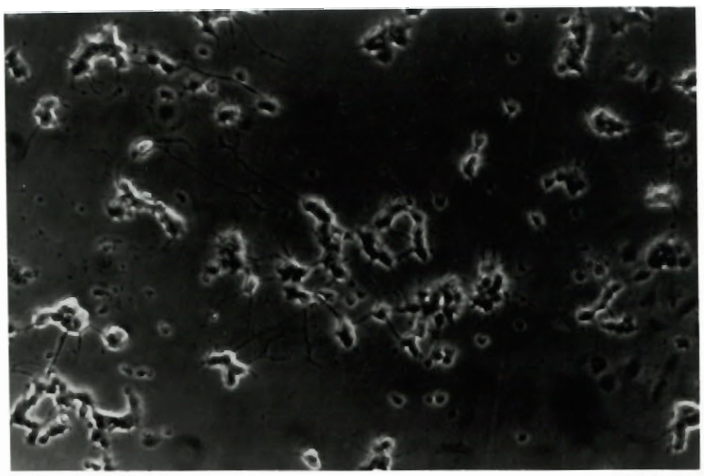
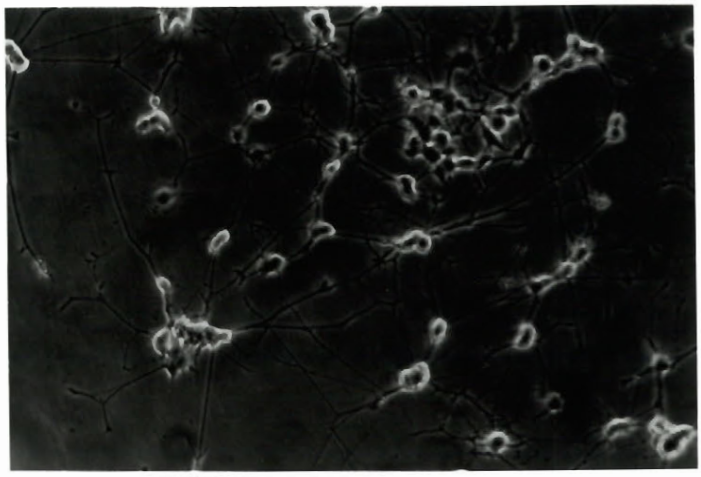


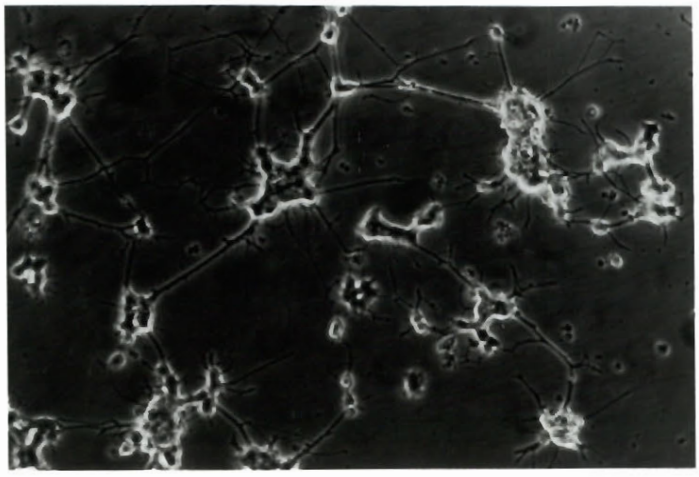
c



d



e



f

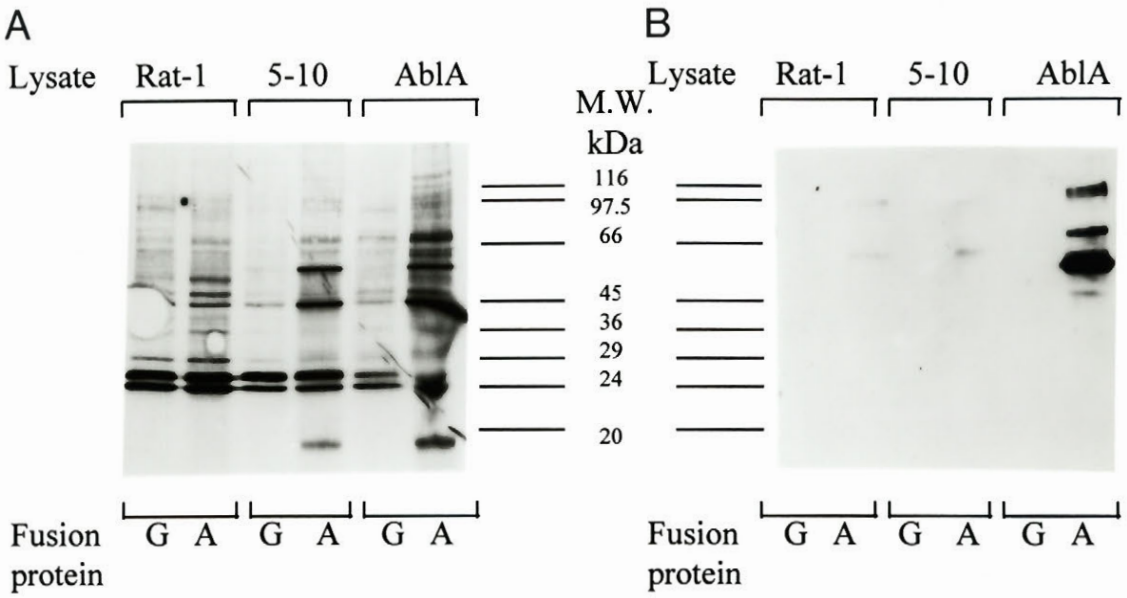
Figure 6A. Substrates of the mutant *abl* protein.

Briefly, ^{35}S-methionine labeled lysates (in PLC-LB) from Rat-1 cells (lanes1, 2), the revertant line T5-10 (lanes 3, 4) and the *v-abl*-transformed Rat-1 cell line, AblA (lanes 5, 6) were incubated with either recombinant GST bound to GSH-agarose (lanes 1, 3, 5) or GST-r*abl* mutant (lanes 2,4,6) for 1 hour on ice. After 3-5 washes in HNTG buffer the GSH- agarose was pelleted and SDS loading buffer was added. The samples were run on a 7.5-15% gradient SDS PAGE . The gel was blotted onto PVDF and exposed to film. Molecular weight markers are indicated in the middle of this figure.

Predominant bands that do not appear in the Rat-1 control have apparent molecular weights of 15, 45, and 60 kDa.

Figure 6B. Interaction of the mutant v-*abl* and with its putative substrates does not require phosphotyrosine.

The membrane from panel A was subjected to Western blot analysis with a monoclonal mouse antibody to phosphotyrosine (UBI #05-321) and the secondary antibody was a horseradish peroxidase conjugated antimouse immunoglobulin antibody. Some very light bands are detected in the Rat-1 and T5-10 lysates precipitated with the recombinant *abl* protein and these bands comigrate. Additional bands are seen in the AblA sample precipitated with the GST-r*abl* mutant, one of which appears to comigrate with the 60kDa ^{35}S-labelled substrate.



G=GST
A=GST-mutant abl

Tables

A)*Ras* focus forming assay

Sample	Control	1:10	1:100
Rat-1	0	47	3.5
D2	0	65	8

B) MiddleT focus forming assay

Sample	Control	1:10	1:100	1:1000
Rat-1	0	40	5.5	0.5
D2	0	42	4	0.5

C) *ErbB* focus forming assay

Sample	Control	1:10	1:100	1:1000
Rat-1	0	tmc	31	2
D2	0	tmc	40	6

Table 1. Expression of the mutant *abl*/neo chimeric virus fails to block transformation.

Rat-1 cells infected with the SAM12 virus were selected with G418 and the resulting clones were pooled and expanded to form the D2 pool. The D2 pool and control Rat-1 cells were infecting with transforming retroviruses harbouring the following oncogenes: v-Ha-*ras*, v-*erbB* and polyoma middle T, according to previously published protocols. The designation in the first row of every table indicates the dilution of the transforming virus used during infection. The control column indicates the number of foci present following mock infection and thus corresponds to the spontaneous occurence of foci. The infectability of each line was tested by infection with a puromycin resistance encoding retrovirus and subsequent selection in puromycin.

tmc= too many to count, the foci are too tightly clustered to permit proper enumeration.

Virus Stock	Titer on Rat-1[1]	Titer on Bac1.2F5[1]	Ratio tit. Bac1.2F5/tit. Rat-1
AmphoN2	$2.9X10^5$	$7.5X10^4$	0.26
8694A am12P2	900	600	0.67
8694A am12P3	1000	360	0.36

Table 2. The mutant *abl*/neo chimeric virus does not affect the growth of Bac1.2F5 cells. Bac1.2 F5 and Rat-1 cells were infected with an amphotropic stock of the N2 virus encoding a neomycin resistance gene and viral supernatant from two separate (G418 selected) pools of GP+*env*Am 12 cells infected with mutant *abl*/neo chimeric virus from a transient transfection on BOSC23 cells (please refer to Materials and Methods).

[1]After selection in complete medium containing G418, neomycin resistant colonies were visualized by staining with methylene blue and the titer, neo^r colonies forming units/mL virus, determined.

Virus Stock	Titer on Rat-1	Titer on PC12	Ratio tit. PC12/tit. Rat-1
Ψ2N2	2.5×10^5	1.5×10^3	0.006
SAM12	2.1×10^5	1.3×10^2	0.0006

Table 3. The mutant *abl* virus affects the growth of PC12 cells.

Rat-1 cells and PC12 cells were infected with the mutant *abl*/neo chimeric virus from the stable transfectant SAM12 and a Ψ2 stock of the N2 virus. After selection with G418, titers were determined as described in Table 2 except that the plates were not stained with methylene blue to visualize the colonies. Thus, the titers represent the number of neo^r colonies/mL of retrovirus.

We were interested in identifying components of the *ras* transformation pathway in an epithelial cell context. In order to achieve this goal, we employed a similar strategy to that detailed in chapter 2. Thus, we attempted to isolate revertants from v-Ha-*ras* transformed Mink cells.

CHAPTER 4 THE ISOLATION OF REVERTANTS OF TRANSFORMATION FROM V-HA-*RAS* TRANSFORMED MINK CELLS.

4.1 Summary

In order to understand the mechanism by which v-Ha-*ras* transforms epithelial cell lines *in vitro* we have devised a technique to isolate revertants of transformation from v-Ha-*ras* transformed Mink cells. We successfully isolated one revertant cell line. The expression of the *mdr* gene in the parental cell line is significantly lower than in the parental transformed cell line. Furthermore, the revertant line no longer harbors the amplification of the *mdr* locus of the transformed parent. In order to assess the contribution of *mdr* expression to v-Ha-*ras* transformation, we utilized two distinct approaches: 1) we overexpressed the murine *mdr1b* gene to determine if it can act as an oncogene in this cellular context and 2) we overexpressed an antisense cDNA to block expression of endogenous *mdr* in transformed cells. Our results do not support a role for *mdr* expression in *ras*-mediated transformation.

4.2 Introduction

Studies have implicated activated *ras* alleles in 30 % of all human tumours (Bos et al., 1989). Our laboratory has been interested in studying the mechanism by which the activated *ras* mediates the oncogenic process in an epithelial cell line. We have favored a genetic approach which centers around the isolation and characterization of revertants of *v-Ha-ras* transformed mink epithelial cells.

Previous studies have reported the differential retention of a supravital dye, rhodamine 123 (rho 123), in transformed versus non-transformed mink cells (Johnson et al., 1982; Haynes and Downing, 1988; Kato and Sherr, 1991). We used this property to select for revertants in a mutagenized population of v-Ha-*ras* transformed Mink cells. In the present study, we report the isolation of one revertant cell line, from v-Ha-*ras* transformed mink cells, using an isolation technique which relies on differential rho 123 retention and fluorescence activated cell sorting technology. These revertant cells have the desired growth characteristics of non-transformed cells. Through the course of our studies we determined that the transformed parental line contained an amplified *mdr* locus even though the cells had not been selected for resistance to chemotherapeutic agents. Interestingly, the revertant line isolated by this method demonstrated reduced amplification of the *mdr* locus as well as reduced expression and multidrug resistance (MDR) activity. Other groups have suggested that regulation of *mdr* expression may play a contributive role to malignant transformation (Weinstein et al., 1991;Chin et al., 1992; Lonn et al.,1992). These reports coupled with our own observations prompted us to determine if *mdr* plays an important role in v-*Ha-ras* mediated transformation. We show that murine *mdr1b* overexpression is not sufficient to transform cells and that inhibiting MDR does not prevent v-*Ha-ras* transformation.

4.3 Materials and Methods

Cell Lines, transfections , infections, growth in agar and somatic hybrids

Mink cells and derivatives were routinely passaged in DMEM (GibcoBRL) supplemented with 5% calf serum (Hyclone) and penicillin/streptomycin. The transformed cell line 1532N was passaged in medium containing 15-20% calf serum. The transformed cell line was derived from the infection of normal mink cells with rescued pp7(v-Ha-*ras*) retrovirus which was obtained from the infection of a Rat-1 cell line, transfected with the pp7 plasmid, with an amphotropic replication competent helper virus (Zarbl et al., 1987). These cells as well as the revertant cell line produce amphotropic helper and pp7 amphotropic pseudotype constitutively.

Assays for growth in agar and the derivation of cell lines carrying each selectable marker for the somatic hybrids were done essentially as described previously except that fusions were passaged into eight 100mm plates for selection and that individual clones from every fusion were picked and expanded before plating in agar at $2X10^4$ cells per 35 mm plate (Zarbl et al., 1987).

Rhodamine 123 staining, FACs analysis and isolation of revertants

Typically, $1 X 10^5$ cells were plated 24 hours before treatment into 60 mm plates. Cells were stained for 30-45 minutes in complete medium containing 10μg/mL Rhodamine 123 (Sigma) and incubated at 37C. Cells were washed in PBS twice and then incubated at 37C for approximately four hours. The medium was changed every hour during this time. Cells were then trypsinized, pelleted and resuspended in 1 mL of PBS or medium and filtered through a nylon mesh (70 μm) to separate clumps. Cells were put on ice and analyzed for cell size and fluorescence. The analysis was carried out on a FacSTAR (Becton Dickinson) as previously described (Zarbl et al.,1987). Cell size was determined by forward angle light scatter.

For the isolation of revertants, $1X10^6$ 1532N cells were plated per 100 mm plate 24 hours prior to treatment with ethyl methyl sulfonate (EMS). Cells were incubated with EMS for a period of 22 hours at a concentration of 5.5mM EMS in complete medium. After this time cells were thoroughly rinsed and allowed to recover for a week. Cells were processed for rho123 staining as described in the previous paragraph.

Vincristine Resistance

$1X10^5$ mink or *v-Ha-ras* transformed (1532N) cells were seeded in 15 plates for each cell line. The next day cells were fed fresh medium containing varying concentrations of vincristine (0-50ng/mL). After 7 days the cells were trypsinized and counted with a hemocytometer. In the clonogenic assay $1X10^4$ cells were seeded per 60mm plate in triplicate for each concentration of vincristine. The next day the cells were fed fresh medium with the various concentrations of vincristine. The cells were then incubated for 10 days. The medium was not replaced during this time. The number of clones was determined by staining the cells with methylene blue (Sigma) and counting the darkly staining clones.

Viral Stocks

Viral stocks of the sense and antisense expressing retroviral vectors were generated by transfection of the appropriate construct into BOSC 23 cell lines according to established protocols (Pear et al., 1993). The retroviral supernatant obtained in this fashion was used to infect AM12G+env (Markowitz et al., 1988), an amphotropic packaging cell line, and the cells were subjected to puromycin selection at 2μg/mL puromycin (Sigma). Retroviral supernatant from a pool of infected clones and used to infect Mink cells chronically infected with a replication competent xenotropic virus. Puromycin resistant clones were selected and clones producing the highest titer (tested by titration with puromycin selection) were expanded and the supernatant from these clones

was used to infect Mink and 1532N cells according to previously described protocols (Zarbl et al., 1987). For selection 1532N cells were subjected to selection at 10μg/mL puromycin while the Mink cells were selected at 2μg/mL. The v-Ha-*ras* retrovirus was harvested from 1532N.

Plasmids and Constructs

The complete murine mouse *mdr1b* cDNA was excised by an EcoRI digest of pMT2K, graciously provided by Dr. Philippe Gros (Gros et al., 1986a,b) and cloned into the pBABEpuro vector (Morgenstern and Land, 1990) in both orientations. This fragment was also used as a probe for Southern and Northern analyses (see below).

Southern and Northern Blot Analysis

High molecular weight DNA was prepared by phenol extraction and ethanol precipitation (Jiang et al., 1994). DNA was digested with restriction endonucleases following the manufacturers recommendations (Pharmacia EcoRI). DNA fragments were separated on 1% agarose gels and transferred onto Nylon membranes (Amersham) as previously described (Sambrook et al., 1989).

Total RNA was extracted from cell lines by the Method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA was separated on 1% Agarose-formaldehyde gel and transfered to Nylon membranes (Amersham). Hybridization procedure was as previously described (Jiang et al., 1994). The complete murine *mdr1b* cDNA (see above) was used as a probe. The actin probe was described elsewhere (Tremblay et al., 1992). The *ras* probe was obtained from Dr. Helmut Zarbl, Fred Hutchison Cancer Center, Seattle, WA, USA.

4.4 Results

Mink cells and *v-Ha-ras* transformed mink cells differ in their pattern of Rhodamine 123 retention.

Previous studies had shown that established epithelial cell lines accumulate rhodamine 123 (rho 123), a supravital dye, at remarkably high levels. Our lab and others have shown that oncogene transformed mink cells display decreased rho 123 retention (Johnson et al., 1982; Haynes and Downing, 1988; Kato and Sherr, 1991). As evidenced in Figure 1, the difference in rho 123 retention between non-tranformed mink and *v*-Ha-*ras* transformed mink cells (1532N) can span three orders of magnitude. The *v*-Ha-*ras* transformed mink cells, 1532N cells, retain very little rho 123 as illustrated by a very low fluorescence intensity. Interestingly, the decreased rho 123 retention in *v*-Ha-*ras* transformed cells correlates with an increased resistance to vincristine (Fig.2), a cytotoxic agent that is part of the MDR spectrum (Gottesman and Pastan, 1993). The 1532N cells are practically unsusceptible to vincristine's toxic effects even at levels (50ng/mL) that significantly exceed the lethal dosage for mink cells (10ng/mL). Note that the increased cell numbers (greater than 100%) at lower concentrations can be accounted for by the tendency of 1532N cells to detach at high cell density. Consequently the quick wash step preceeding trypsinization may have resulted in the loss of some cells and an underestimation of the number of cells in the control. Resistance to vincristine as well as rho 123 clearance in mammalian cells has been shown to be conferred by the P-glycoprotein encoded by the multidrug resistance gene (*mdr*) (Chaudhary and Roninson, 1991; Weaver et al., 1991; Neyfakh, 1988).

To determine if the vincristine resistance and the low rho 123 retention seen in *v-Ha-ras* transformed cells were caused by an increase in *mdr* gene expression, the MDR RNA levels were measured in these cells by Northern blot analysis (Fig.3). The *v*-Ha-*ras* transformed mink cells (1532N) showed a very significant increase in *mdr* RNA levels compared with non-transformed mink cells, probably sufficient to account for the

increased resistance to vincristine in these cells. The increased *mdr* RNA levels could result from the activation of *mdr* transcription, as has been reported by other groups (Chin et al., 1992). In addition, amplification of the *mdr* gene itself has been reported frequently in cells selected for resistance to drugs from the MDR spectrum. Although gene amplification does not always lead to overexpression of *mdr*, we decided to determine if the *mdr* locus was amplified in the transformed cells. By Southern analysis, we detected amplification of the *mdr* locus in the *v*-Ha-*ras* transformed clone, 1532N. (Fig. 4). This transformed clone was never subjected to selection with cytotoxic drugs but was subcloned several times to obtain a highly transformed clone. It should be noted that we did not observe DNA amplification of the *mdr* locus in a number of other v-Ha-*ras* mink cell lines (data not shown). Thus, this is not a hallmark of this transformation process. However, all lines v-Ha-*ras* transformed lines display significantly higher levels of vincristine resistance than the normal cell lines and little or no retention of rho 123. Thus, these results clearly show that *v-Ha-ras* transformation induced a marked increase in the *mdr* gene expression leading to an active efflux of rho 123 and vincristine out of these cells.

Isolation of revertants from *v-Ha-ras* transformed mink cells, 1532N.

We hypothesized that the putative revertants of v-Ha-*ras* transformed mink cells (1532N) would exhibit a rho 123 retention pattern similar to that of Mink cells. To increase the frequency of reversion of 1532N cells, they were treated with ethyl methyl sulfonate (EMS) and allowed to recover before sorting on the FACStar. We gated the mutagenized population in order to recover cells that displayed high rho 123 retention, a pattern similar to the non-transformed mink cells (Fig.5.). The six mutagenized plates contained a total of $7.2X10^6$ cells. Approximately, $2.5X10^6$ cells were analyzed and $1.2X10^3$ cells were found to have the desired retention spectra. Cells were plated at $2X10^3$ cells per 100mm plate in order to recover isolated clones. The v-Ha-*ras*

transformed mink cells have a refringent morphology in culture and tend to form disorganized arrays of cells. Therefore, as a second criteria for selecting putative revertants, clones that were less refringent and more organized were picked. One putative revertant, KS1-4, was further characterized. Our initial analysis revealed that this clone still contained transformed cells: the pattern of rho 123 retention clearly showed two very distinct and distant peaks in fluorescence intensity (data not shown). Furthermore, a vincristine kill curve of this line was biphasic (data not shown). For these reasons we recloned these cells by low density plating followed by rho 123 coloration and selection of cells with high fluorescence. Revertant line KS1-4O was obtained by this method and constituted a single uniform population of cells as judged by rho 123 retention and FACS analysis. This cell line was further characterized.

KS1-4O displays some growth characteristics of non-transformed Mink cells.

We analyzed KS1-4O revertant cells for anchorage independent growth and tumorigenecity *in vivo*. As summarized in Table 1, these cells do not form colonies in agar (figure 6). However, filtered supernatants of KS1-4O contained focus forming retroviruses at a lower titer than the transformed parent. Thus the *v-Ha-ras* retrovirus is intact (further proof is provided in following paragraphs) We were unable to assay tumorigenecity *in vivo* since both the revertant and transformed line shed a large quantity of transforming retrovirus which effectively transforms cells surrounding the injection site thus giving rise to mouse borne tumors. Nonetheless, KS1-4O has lost the ability to grow in soft agar thus, by this criterion has reverted to a non-transformed phenotype.

The KS1-4O revertant clone arose from the parental transformed 1532N cells but differs in its expression of *mdr*.

In order to verify that KS1-4O cells arose from the parental transformed 1532N cells, we performed Southern analysis and looked for common bands of proviral

integration using a *ras* specific probe. As demonstrated in Figure 7, KS1-4O DNA had the same proviral integration sites as 1532N, indicating that this clone originated from the transformed parental line and not from contaminating non-transformed cells. As expected, the KS1-4O cells display a rho 123 fluorescence spectrum similar to that of Mink cells (Fig. 8). In addition, the MDR RNA levels were dramatically decreased in these revertant cells, as compared to the parental transformed cells (Fig. 9A), while the v-Ha-*ras* transcript was still expressed at a comparable level to that of the transformed 1532N line (Fig. 9B). Interestingly, the KS1-4O has also lost the DNA amplification of the *mdr* locus (Fig. 10) initially present in the transformed cell line.

Together these results indicate that KS1-4O cells displays characteristics of non-transformed cells despite the expression of an active v-Ha-*ras* oncogene.

Overexpression of *mdr* does not transform Mink cells.

The reversion process occured concomitantly with a loss of the *mdr* gene expression suggesting that the two events may be functionally related. Thus, we decided to investigate the potential contribution of *mdr* expression to the transformation process. In order to assess the contribution of *mdr* to the transformation process, we cloned the murine *mdr1b* cDNA into a retroviral expression vector in both orientations. We introduced the chimeric retroviruses into Mink cells by infection and selected for puromycin resistance and vincristine resistance. We hoped to determine if *mdr* expression is sufficient to transform Mink cells. We selected vincristine resistant clones at concentrations of vincristine (5ng/mL) which kill most normal mink cells but still have relatively little or no effect on the growth of v-Ha-*ras* transformed cells (figure 2). The clones obtained in this fashion were subjected to an agar growth assay. Only one of the 13 clones infected with the +sense construct (MkSE12) displayed appreciable growth in agar (Table 2). Thus it appears that overexpression of *mdr* at this level is not sufficient to cause transformation.

Overexpression of an antisense *mdr* does not block v-Ha-ras-mediated transformation

After determining that *mdr1b* overexpression was not sufficient to cause transformation, we were interested in determining if blocking the expression of *mdr* would be sufficient to block transformation. Hence, if overexpression of *mdr* is a necessary event in v-Ha-*ras* mediated transformation. The antisense retrovirus was tested for its ability to block vincristine resistance in 1532N. To do so we compared the number of puromycin resistant clones of 1532N cells infected with a high titer of antisense expressing retrovirus and subjected to selection with puromycin or puromycin and increasing doses of vincristine. The number of puro^r clones decreases substantially at 5 and 10ng/mL (data not shown) while in previous assays uninfected 1532N cells remain unaffected at these concentrations of vincristine (figure 2). Our experiments with other v-*Ha-ras* transformed cell line had suggested that the level of vincristine resistance in 1532N was unusually high and that typically, v-*a-ras* transformed cells were considerably more susceptible to vincristine levels of 10 ng/mL. In fact, only 10% of cells from another v-Ha-*ras* transformed Mink cell line survived at this level in a clonogenic assay (data not shown). Therefore, we derived Mink clones overexpressing the antisense *mdr* cDNA in the retroviral vector described above, and proceeded to infect them with a transforming retrovirus harbouring the v-Ha-*ras* oncogene. We surmised that the average transformed clone derived in this fashion would have a sufficient amount of antisense cDNA expression to effectively block *mdr* expression. The results are summarized in table 3. None of the clones tested (MAS) showed a significant decrease in the number of foci with respect to control lines. In fact, at the lowest dilution of transforming retrovirus all clones tested had a large number of foci that was indistinguishable from the control lines infected with the puro vector (MkV) or the + sense (MkSE) construct discussed in the previous paragraph Therefore, *mdr* overexpression does not appear to be a necessary event in v-Ha-*ras* mediated transformation.

The reversion may have occured through two independent genetic events.

Reversion can occur through three different mechanisms including inactivation of the oncogene, mutation in an effector gene or activation of an antioncogene. Since the revertant line produces transforming retrovirus we can discount the first possibility. To differentiate between the two last options we made somatic hybrids with the revertant line and the transformed or normal cell lines. The resulting somatic hybrids were plated in agar and scored for anchorage independent growth as an assay for transformation (table 4). Unfortunately, our results do not provide us with a clear answer: the non-transformed phenotype of the hybrids with the normal cells suggests that the reversion phenomenon is dominant while the phenotype of the hybrids with the transformed cells suggests that the reversion phenomenon is recessive. These observations lead us to believe that reversion occured through two seperate genetic events.

4.5 Discussion

To study the mechanism of v-Ha-*ras* mediated transformation in epithelial cells, we have successfully isolated a revertant cell line from v-Ha-*ras* transformed mink cells. The KS1-4O revertant does not grow in agar. Reversion appears to have occured through at least two separate genetic events, as evidenced by our sudies with somatic hybrids. The amplification of the *mdr* locus in the parental cell line in the absence of drug selection may suggest that the parental line is genetically unstable. Other groups have suggested that expression of an activated Ha-*ras* can contribute to genetic instability (Denko et al., 1994). Thus, the EMS treatment coupled with an underlying genetic instability due to *ras* transformation may have given rise to the revertant line. It should be noted that the amplification of the *mdr* locus was not repeatedly detected in other independently derived clones (our unpublished results). Nonetheles, all *ras*-transformed clones displayed increased expression of the *mdr* gene and Pgp activity. Therefore, it appears that the amplification of the *mdr* locus is not a typical event in this transformation process but its increased expression does correlate with v-Ha-*ras* expression. Alternatively, the amplification of the *mdr* locus which occured in the absence of selection with chemotherapeutic drugs of the *mdr* spectrum may reflect a selective growth advantage for a highly tumorigenic clone. Recent evidence suggests that *mdr* gene expression is a target for *ras* mediated tranformation (Chin et al., 1992 ; as well as the data presented here). The expression of the *mdr* gene is increased in mink cells transformed by some defective oncogenic retroviruses (Johnson et al., 1982; Haynes and Downing, 1988; Kato and Sherr, 1991). This increased expression translates into decreased rho 123 retention and increased resistance to vincristine. Our ability to isolate revertants of v-Ha-*ras* transformed cells by selecting cells with increased rho 123 retention and decreased vincristine resistance (reflecting *mdr* gene expression and activity) shows that a breakdown in the transformation process mediated by v-Ha-*ras* occurs simultaneously with a loss of the *ras*'s ability to activate *mdr* gene expression. The revertant also displays

less DNA amplification of the *mdr* gene than the transformed parental cell line. Our observations suggested a possible role for *mdr* gene expression in this transformation process. Other groups had reported a putative link between transformation and *mdr* expression, for instance, in childhood neuroblastomas elevated levels of *mdr* expression at presentation correlates with a poor prognosis as a result of increase resistance to chemotherapeutic agents (Chan et al., 1991). In a study of clinical samples of mammary carcinomas, amplification of the *mdr* locus has been rported in the absence of selection with cytotoxic agents of the MDR spectrum (Lonn et al., 1992). Finally, it has been reported that P-gP positive invasive colon cancer cells may have an increased potential for dissemination (Weinstein et al., 1991). Interestingly, another member of the ABC transporter family the TAP-2 gene, has been shown to restore the tumorigenic potential of a lymphoma mutant line (Franksson et al., 1993) by enabling it to escape rapid elimination by Natural Killer (NK) cells.

We decided to determine if *mdr* plays a role in the tumorigenic process. Our studies with the sense construct suggests that the overexpression of the mouse *mdr1b* gene to levels confering vincristine resistance at levels comparable to that of the typical *ras*-transformed Mink cells is not sufficient to cause transformation in Mink cells. Furthermore, the overexpression of an antisense mouse *mdr1b* at levels which reduced vincristine resistance in *ras*-transformed cells to Mink cell levels, did not adversely affect transformation. These findings suggest that *mdr* expression is not necessary for transformation and that its overexpression reflects *ras* mediated changes in the transcriptional machinery. Alternatively, the level of Mink *mdr* gene expression required for maintaining transformation may be inferior to that detectable by our vincristine resistance assay. Thus, drug efflux may not adequately reflect its true physiological or transforming function. One possible function would be in the modulation of chloride channel activity. The human *mdr1* gene product appears to modulate the activity of volume-gated chloride channels (Hardy et al., 1995). The ability of the *mdr1* protein to

modulate these channels is affected by phosphorylation of Pgp by protein kinase C (PKC) (Hardy, et al., 1995). Interestingly, phosphorylation by PKC is also believed to increase Pgp transport activity (Chambers et al., 1990).

We have not identified the number of *mdr* genes in Mink cells.There are two *mdr* genes in humans while mouse and rat cells have three *mdr* genes (for review Gottesman and Pastan, 1993). The probe used in this study was the complete cDNA of the murine *mdr1b* gene and it detected what appeared to be a single species. Previous studies had shown that a smaller probe contained within this sequence was capable of detecting all three members of the family (Croop et al., 1989). It is possible that the two or three members of the Mink family produce mRNA species that could not be resolved in our electrophoresis conditions. Thus, the single band may in fact be mink *mdr1a/b,2* mRNAs. The increase in vincristine resistance would suggest that the *mdr1a/b* homologs were present but we can not rule out the possibility that the mink homolog to the murine *mdr2* gene was also expressed. Since the activity of the *mdr2* gene product is poorly understood, although recent reports suggest a role for it in phospholipid transport into bile (Smit et al.,1993), we can not rule out that the *mdr2* gene is actually the transformation effector gene nor can we be sure that we effectively diminished its activity with our antisense construct.

In conclusion, our studies indicate that overexpression of the murine *mdr1b* gene is not sufficient to transform Mink cells. Furthermore, decreased drug efflux by Pgp in mink cells does not seriously impede transformation by the v-Ha-*ras*. Nonetheless, *ras*-transformation correlates with increases in *mdr* expression and a breakdown in the transformation process translates into decreased *mdr* expression. This study further supports a direct effect of *ras*-mediated transformation on *mdr* gene expression in a specific cellular context: the increased *mdr* expression could have serious consequences on the course of treatment of such tumours. Finally, the *ras*-transformed Mink cells and the revertants isolated by the protocol detailed in this paper could provide valuable

insight into the ERK kinase cascades involved in *ras* transformation. The dramatic effects on *mdr* gene expression seen in these cells may result from the modification of the transcription factors which are the targets of these cascades (review Davis, 1994) .

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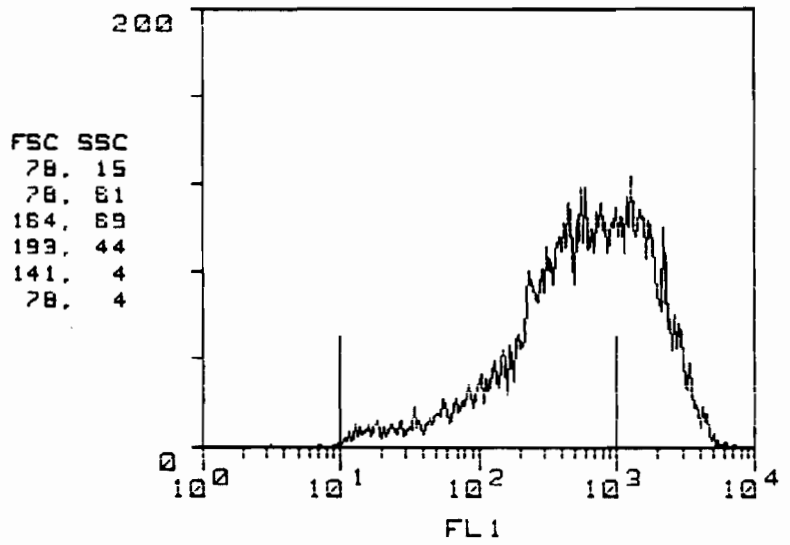
4.7 Figures and Tables

Figure 1. Fluorescence Spectra of Cells Treated with Rhodamine 123.

$1x10^5$ cells were seeded the day before. Cells were incubated for 30-45 minutes in complete medium containing 1mg/mL Rhodamine 123 and then washed every hour with PBS. After 4 hours the cells were trypsinized, spun down at 250g and resuspended in complete medium. The analysis was performed on FACScan using parameters described by Zarbl et al., 1987. Panel A) represents non-transformed Mink cells and panel B) v-Ha-*ras* transformed Mink cells, 1532N.

Sample : MINK 004
Cytometer: FACSCAN



Sample : 1532N1605 003
Cytometer: FACSCAN

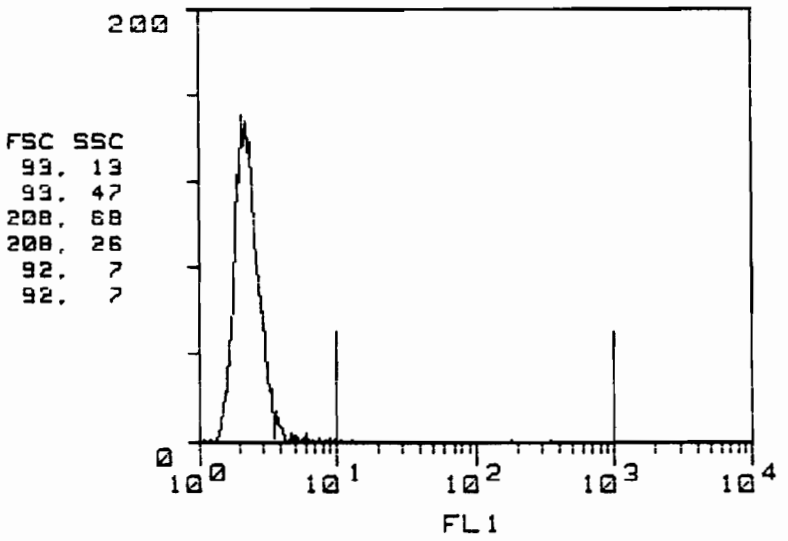


Figure 2. Rhodamine Retention Inversely Correlates with Resistance to Vincristine.

$1X10^5$ mink or *v-Ha-ras* transformed (1532N) cells were seeded in 15 plates for each cell line. The next day cells were fed fresh medium containing varying concentrations of vincristine (0-50ng/mL). After 7 days the cells were trypsinized and counted. The average number of cells for each set of four plates, representing each dilution are expressed as a percentage of the average found in the control plates (without vincristine). Error bars represent the standard deviation.

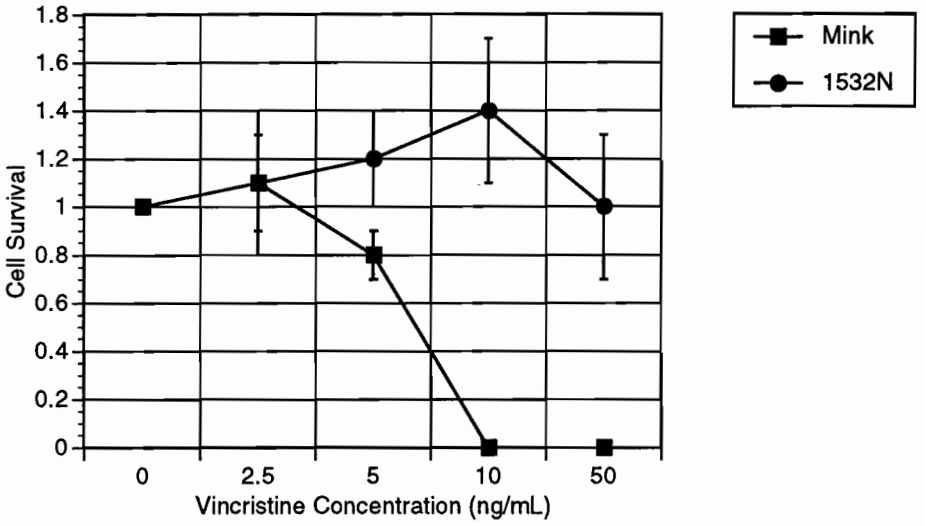
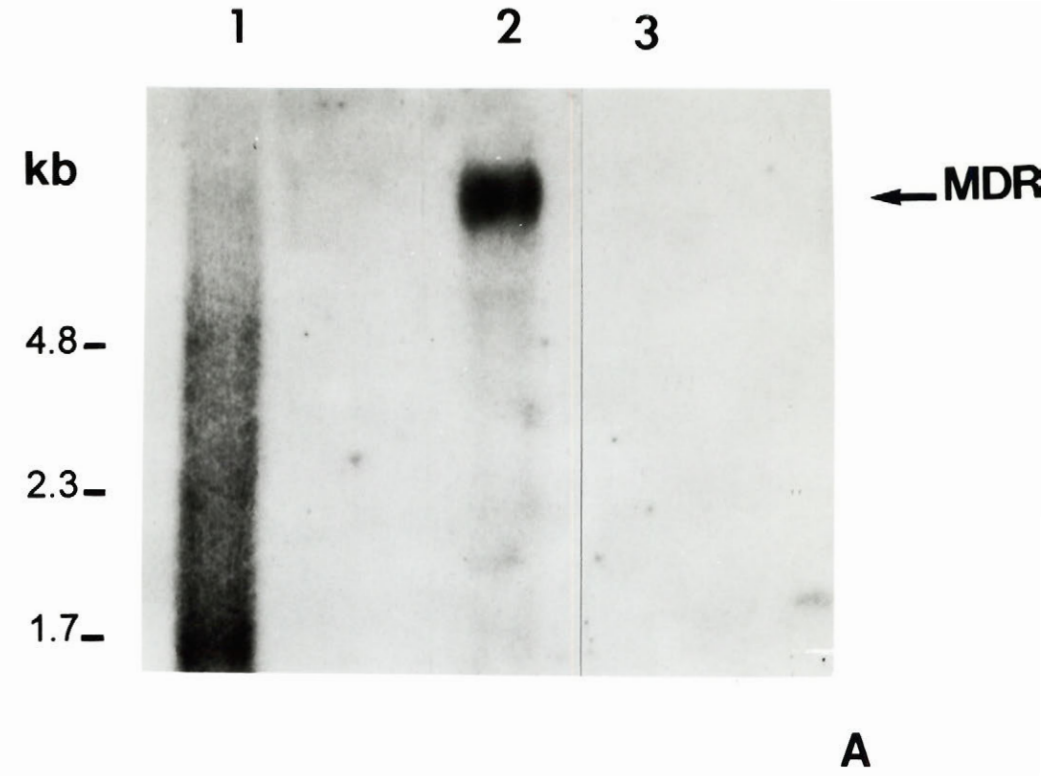
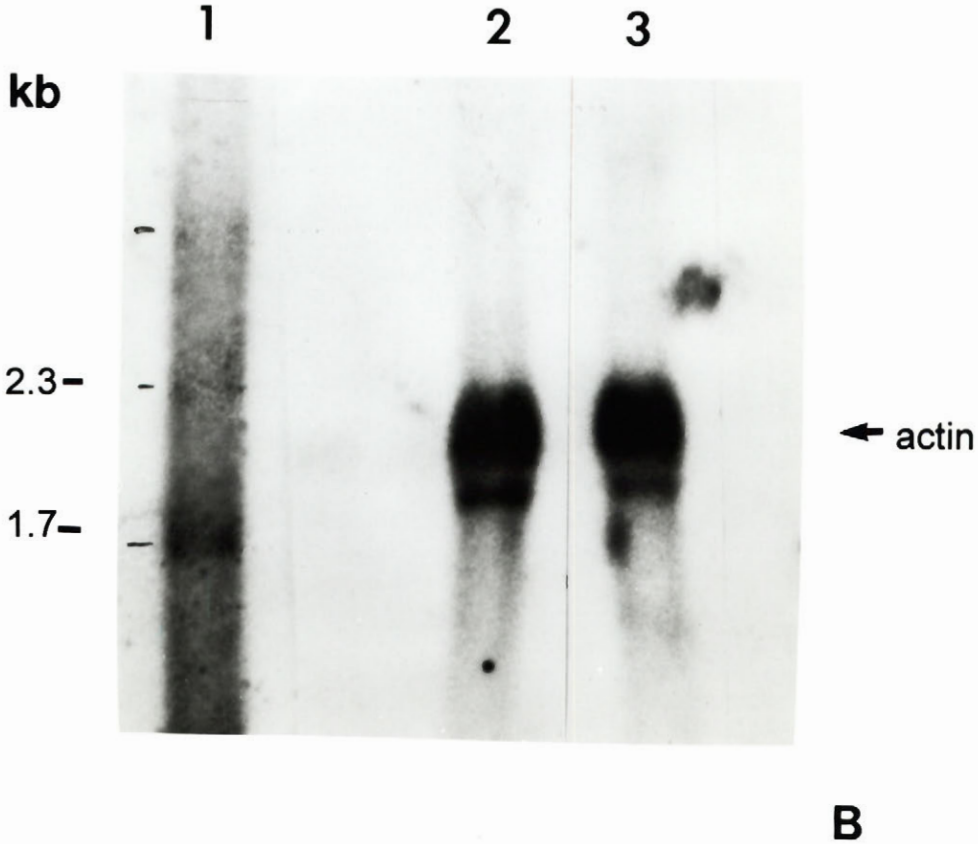

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cell Survival
0
2.5
5
10
50
Vincristine Concentration (ng/mL)
Mink
1532N

Figure 3. Northern Analysis of *mdr* Gene in Normal and v-Ha-*ras* Transformed (1532N) Cells.

Total RNA was extracted from 1532N and mink cells and 20ug were loaded and blotted with the mouse *mdr1b* cDNA probe . The lower portion depicts the same blot probed with an actin probe to control for loading and transfer of the samples. Lane 1, marker ; lane 2, *v-Ha-ras* transformed cells 1532N; lane 3, normal mink cells.



A

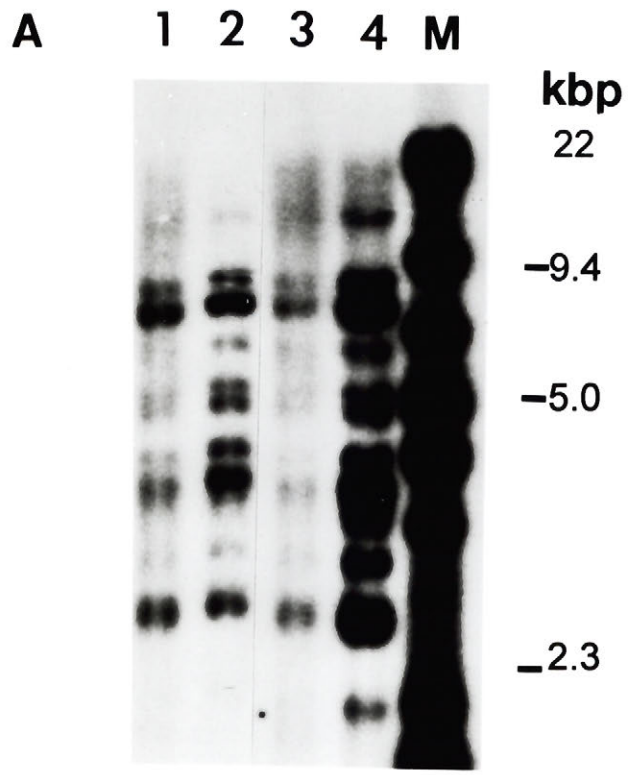


B

Figure 4. Southern Analysis of the *mdr* Gene in Normal and v-Ha-*ras* Transformed (1532N) Cells.

A) Genomic DNA was extracted from the appropriate lines and digested with EcoRI. The probe used for blotting is derived from a mouse cDNA clone of the *mdr1b* gene. Thus, lower stringency hybridization conditions were used. Lanes 1,3: 20ug of digested mink DNA; lanes 2,4: 5ug and 20ug respectively of 1532N DNA. M is the ^{32}P labeled molecular weight markers.

B) The blot was reblotted with a mouse *ras* probe and the protooncogene specific restriction fragment served to verify the relative amounts of DNA loaded in each lane. The viral integrations bands also ascertained that 1532N is infected with *v-Ha-ras* harbouring retroviruses.


A
1 2 3 4 M
kbp
22
9.4
5.0
2.3

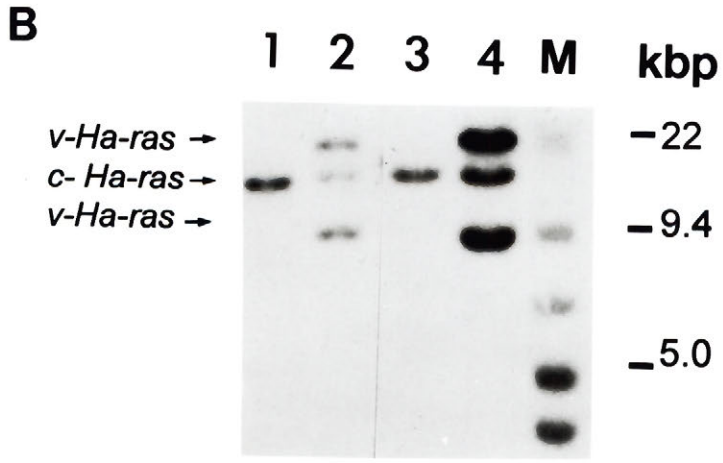

B
1 2 3 4 M
kbp
v-Ha-ras
c- Ha-ras
v-Ha-ras
22
9.4
5.0

Figure 5. Sorting Spectrum of Mutagenized v-Ha-*ras* transformed (1532N) cells.

Six 100mm plates were seeded with 1 X 10^6 1532N cells. The following day the cells were plated in medium containing EMS at a concentration of 5.5 mM for 20 hours. After extensive washing cells were allowed to recover for 7 days. At this point the cells were processed for rho 123 staining and pooled. Approximately 2.4x10^6 cells were screened and 0.5 % were isolated in the gated window. The *low* curve are non-mutagenized 1532N cells, the *high* curve are non-mutagenized mink cells while *sort* represents the mutagenized population of 1532N. All cells with a fluorescence intensity equal or greater to the *lower sort limit* were isolated and plated at low density for cloning.

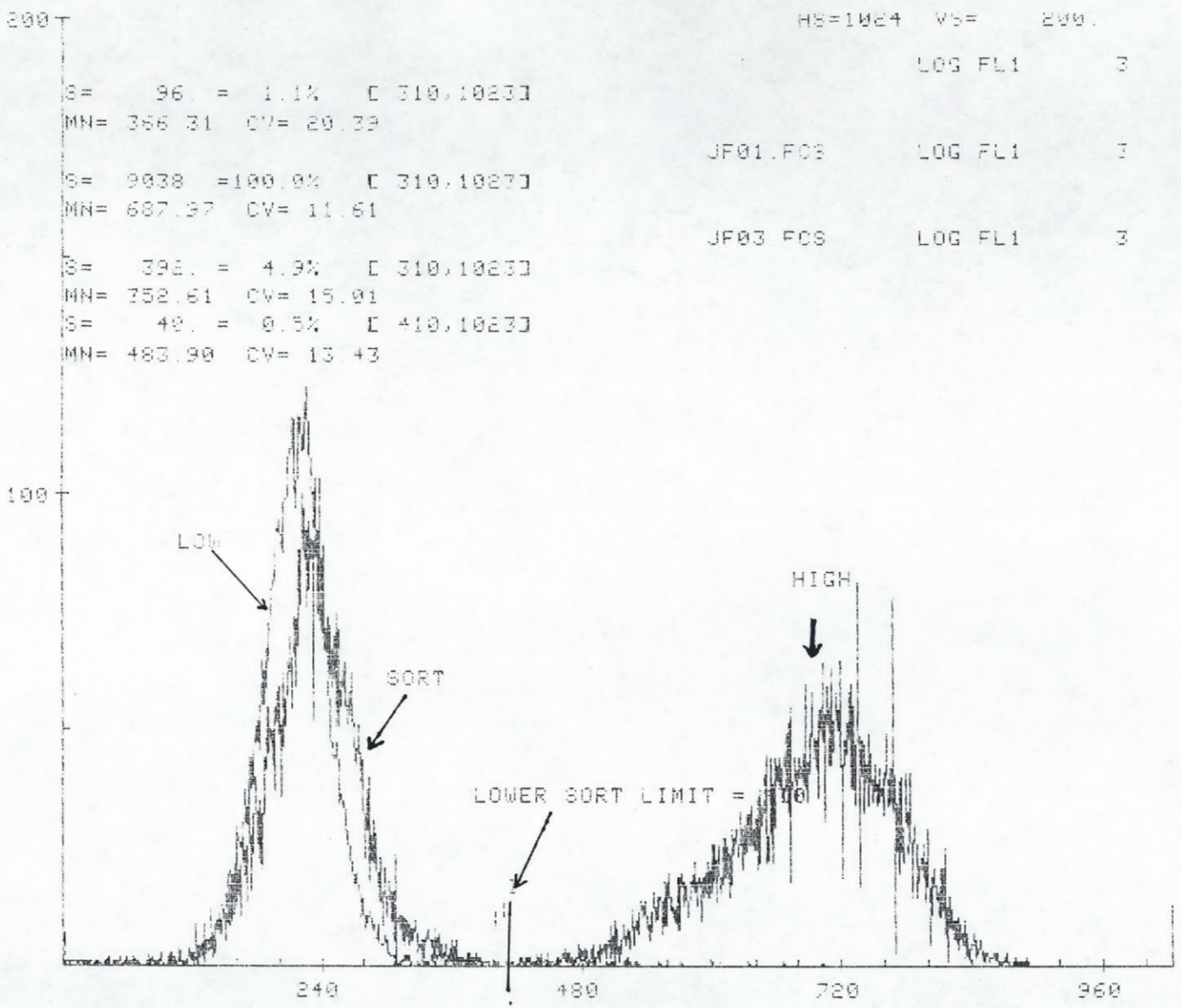
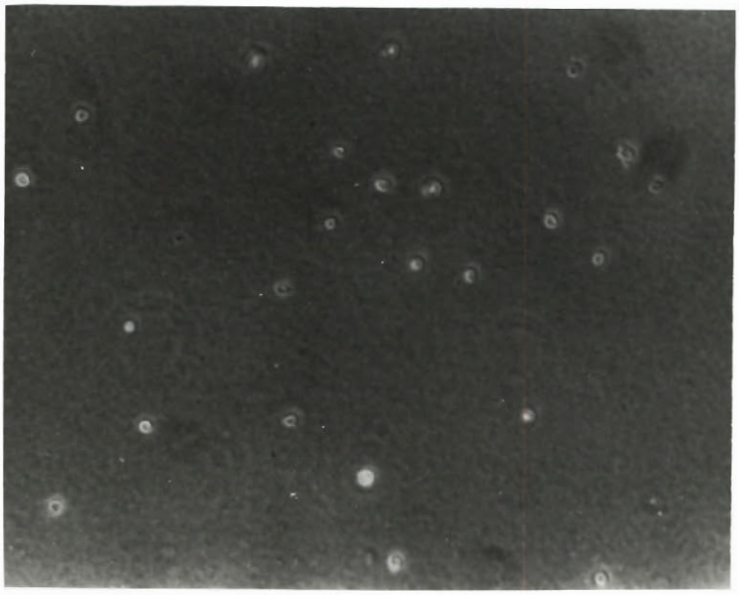
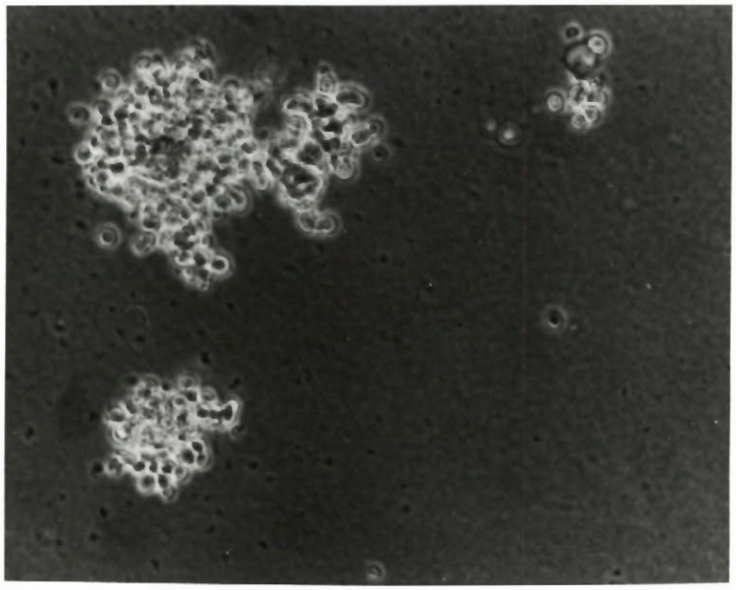

200
100
HS=1024 VS= 200.
LOG FL1 3
JF01.FCS LOG FL1 3
JF03 FCS LOG FL1 3
S= 96. = 1.1% [310,1023]
MN= 366.31 CV= 20.39
S= 9038 =100.0% [310,1027]
MN= 687.37 CV= 11.61
S= 396. = 4.3% [310,1023]
MN= 752.61 CV= 15.01
S= 49. = 0.5% [410,1023]
MN= 483.90 CV= 13.43
LOW
SORT
HIGH
LOWER SORT LIMIT = 400
240
480
720
960

Figure 6. Growth in Agar of Mink, 1532N and Revertant KS1-4O.

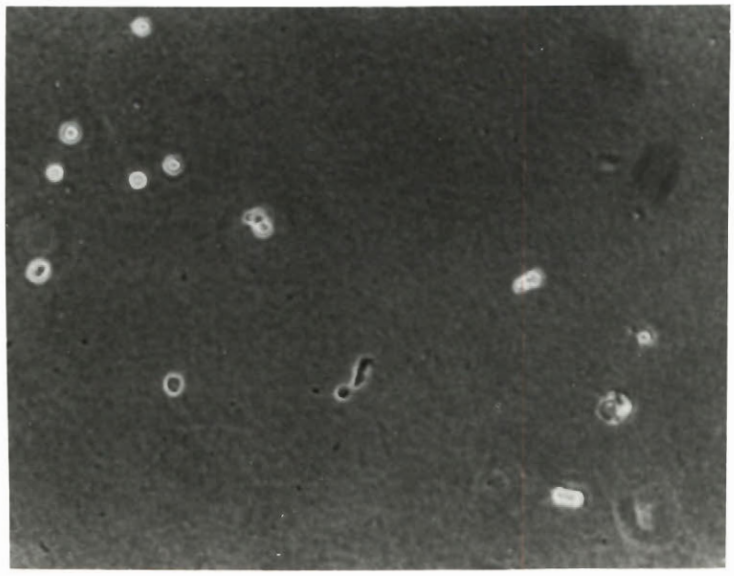
Cells were plated in semisolid media and growth in agar was scored after two weeks. Pictures represent a typical field at a 100X magnification of A) Mink, B) 1532N and C) KS1-4O cells.



A



B



C

Figure 7. Southern Analysis of the Ha-*ras* Gene.

Genomic DNA was extracted and 20 ug was digested with EcoRI and loaded onto a gel. The blot was probed with a *ras* specific probe. The revertant KS1-4O arose from the transformed parent 1532N (see text) since they share the same integration pattern. Lane 1, ^{32}P-lambda marker ; lanes 1532N(20) and 1532N(10) contained 20ug and 10ug of digested 1532N DNA. The lanes designated by Mink and KS1-4O contained 20ug of digested DNA from from the appropriate cell line.

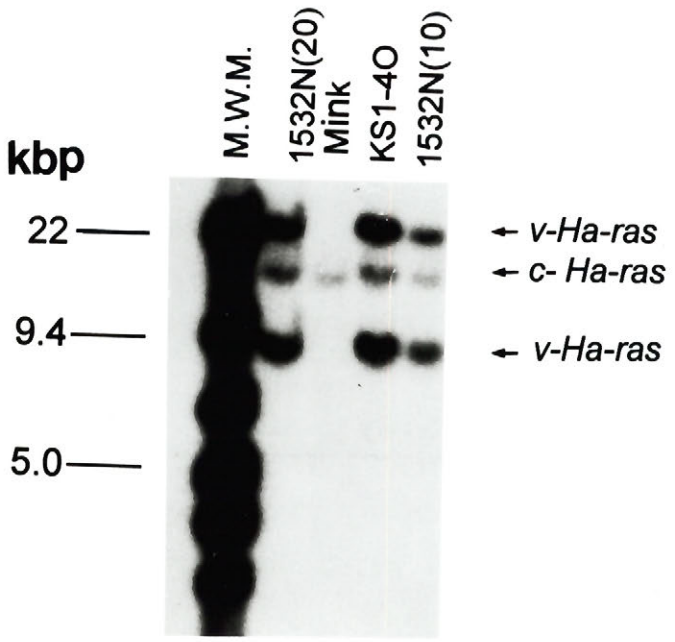

kbp
M.W.M.
1532N(20) Mink
KS1-4O
1532N(10)
22
9.4
5.0
v-Ha-ras
c- Ha-ras
v-Ha-ras

Figure 8. Analysis of Rho 123 Fluorescence Spectrum of KS1-4O.

Cells were processed as described in the legend to Figure 1. The spectrum is similar to that of the normal Mink cells shown in Figure 1.

Sample : KS1-40 010
Cytometer: FACSCAN

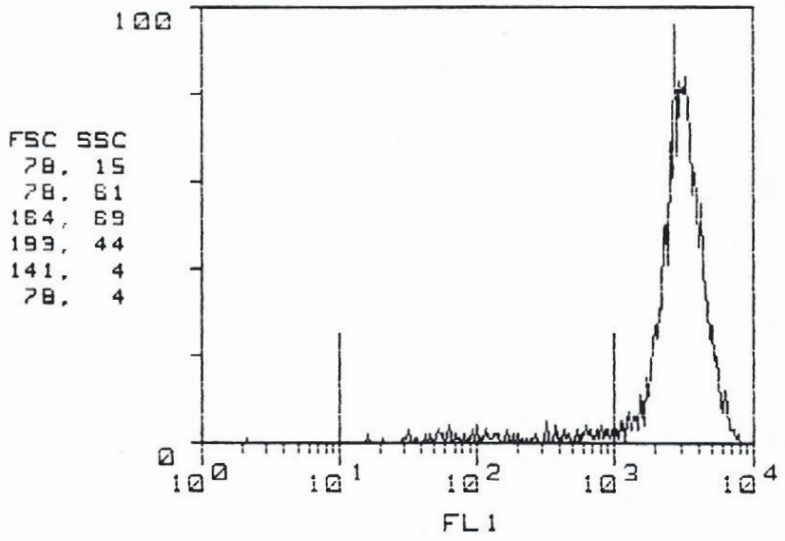


Figure 9. Northern Blot Analysis of *mdr* Expression.

15ug of total RNA from each cell line, 1532N, Mink and KS1-4O was loaded onto a gel, transferred to a Nylon membrane and hybridized with a A) *ras*-specific or an B) *mdr1b*-specific probe. The revertant KS1-4O has little MDR RNA but a comparable amount of *ras* RNA with respect to the transformed parent 1532N.

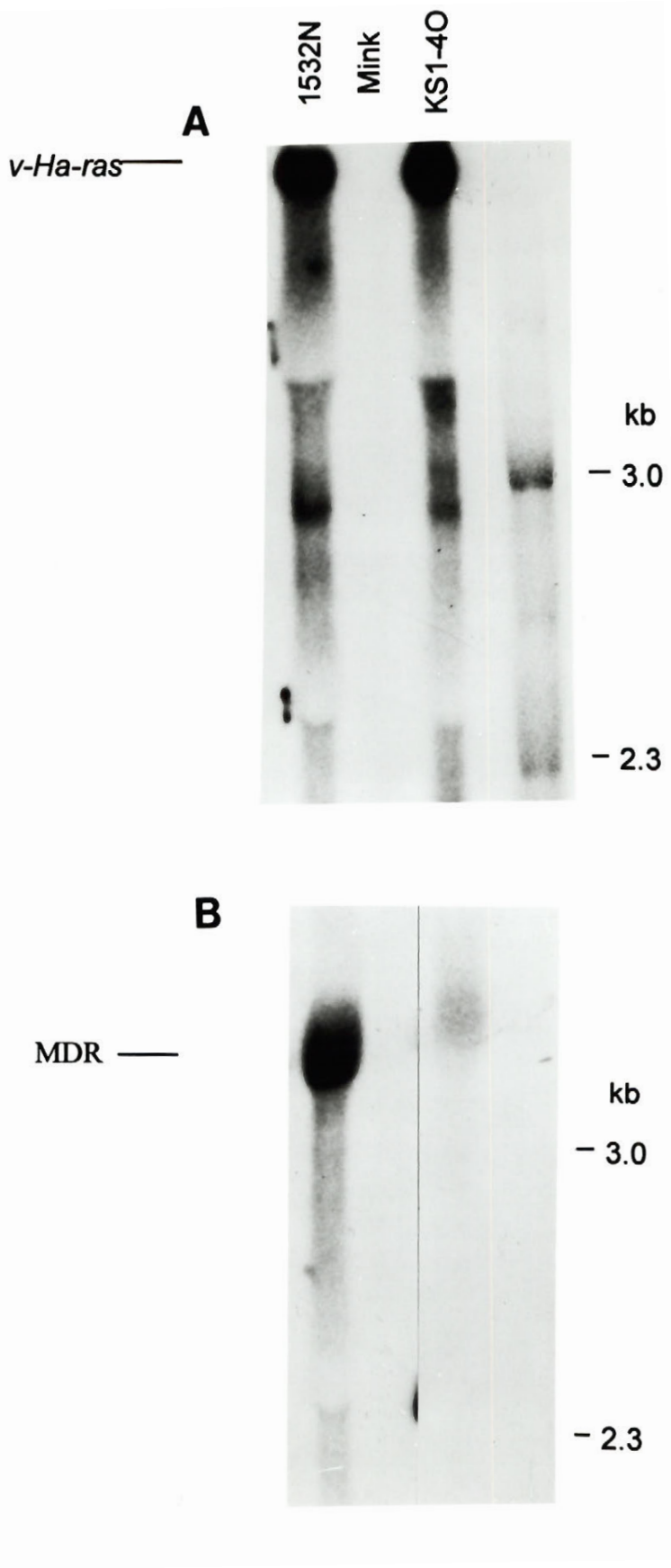
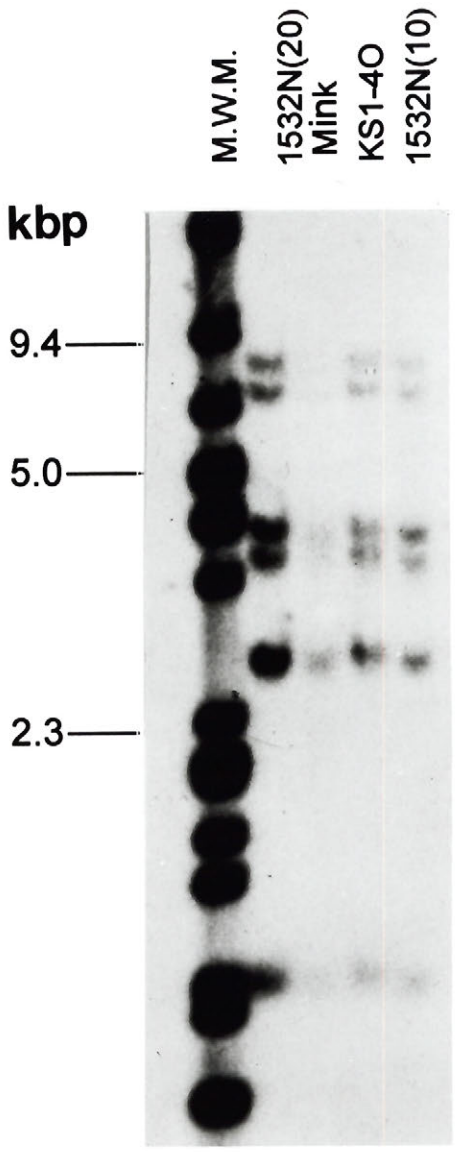

1532N
Mink
KS1-4O
A
v-Ha-ras
kb
3.0
2.3
B
MDR
kb
3.0
2.3

Figure 10. Southern Analysis of the *mdr* Gene.

EcoRI digested genomic DNAs were loaded on a gel, transferred and hybridized with the *mdr1b* mouse probe. The blot utilized in figure 7 was used for this analysis thus the c-Ha-*ras* band detected in this previous analysis served as a control for proper transfer and loading. Lane designations are the same as in figure 7.


M.W.M.
1532N(20)
Mink
KS1-4O
1532N(10)
kbp
9.4
5.0
2.3

Cell line	Tumorigenicity *in vivo*	Growth in Agar	Rescue
Mink	no tumours	-	n.d.
KS1-40	4 weeks	-	+
1532N	2 weeks	+	+

Table 1. KS1-40 Cells Have Some Growth Characteristics of Non-Transformed Mink Cells.

A) Tumorigenicity *in vivo.*

30-40 day old female nu/nu CD1 mice were injected subcutaneously at 2 different sites. 1 site was injected with $1x10^6$ cells the other with $1x10^7$. This was done with 2 mice for each line. The length of time shown corresponds to the amount of time required for the appearance of palpable masses to appear (approx. 0.3 cm in diameter). For both 1532N and revertant KS1-4O large tumours of more than 1 cm in diameter were present two weeks after the initial appearance of the smaller masses.

B) Growth in agar; assay for anchorage independent growth.

$2x10^4$ cells were seeded in a top layer containing 0.3% noble agar and DME+20% Calf serum over a bottom layer containing 0.5% noble agar covering the surface of a 35mm petri plate.

-, no growth of colonies in agar, only single cells; +, growth of colonies

C) Rescue of oncogenic retroviruses.

The 1532N produces an amphotropic virus that also serves to pseudotype the *v-Ha-ras* defective retrovirus transforming this cell line. The revertant KS1-4O cells produce the same retroviruses. Supernatants from these lines were harvested and filtered when cells were 90-95% confluent. These supernatants were titrated by infecting Rat-1 cells at different viral dilutions. Rat-1 cells were allowed to grow to confluence foci that appeared on the confluent monolayer were counted.

phosphotyrosine independent. It was reported that the Abl SH2 domain can interact with other proteins in a phosphotyrosine (Pendergast et al; 1991; Muller et al; 1992). This particular interaction is believed to be mediated by phosphoserine or phoshothreonine motifs (Pendergast et al; 1991; Muller et al; 1992). The weaker affinity of the Abl SH2 domain for these motifs compared to phosphotyrosine containing peptides led the authors to suggest that these interactions may be involved in the initial recruitment of the substrate to the kinase. Our data suggest that the initial interaction between the substrate and the kinase may have to be more stable, stronger than that afforded by these interactions. Interestingly, other cytoplasmic tyrosine kinases, particularly of the Src family, appear to utilize their SH3 domains for the initial recruitment of substrates while the SH2 domain may serve to stabilize the interaction between kinase and substrate once the substrate is modified (Richard et al; 1995). This difference may explain the ability of the activated cytoplasmic tyrosine kinases to transform cells that contain the mutant v-*abl*. This two-tiered interaction offers a teleological explanation for the presence of adjoining SH2 and SH3 domains in a number of signal transducers. At this point, we can not rule out that the *gag* moiety in the v-*abl* protein plays some role in this process. Undoubtedly, the variants of the mutant we described in previous chapters will help define the contribution of each domain to the mutant's action.

B) Blocking access of an effector to the activated Receptor tyrosine kinase

Alternatively, the presence of an overexpressed SH2 domain could interfere with signaling by competition for phosphopeptide binding sites. Receptor tyrosine kinases appear to be largely unspecific *in vitro* (Zhou et al; 1995). The SH2 domains of certain kinase substrates serve to recruit them to the activated kinases where they are activated through phosphorylation events or through the change in subcellular localization that results from their association (Rodrigues and Park; 1995). Thus, the Abl mutant could interfere with the modification or activation of a crucial component of the transforming cascade by blocking access to the kinase. Several *in vitro* studies have uncovered some

putative interactions between the Abl SH2 domain and the activated EGF receptor (Zhu et al., 1994a; Zhu et al., 1994b). However, the site of interaction appears underphosphorylated *in vivo* and its physiological role is unclear. Hence, the Abl SH2 does not appear to compete for a phosphopeptide binding site with any of the known signal transducers which associate with the activated EGF receptor. Our studies in PC12 cells may actually offer clues to which particular mode of action is employed by the mutant. In light of previous studies with transforming proteins (Alema et al., 1985; Bar-Sagi and Feramisco., 1985; Noda et al., 1985; Cowley et al., 1994; Hempstead et al., 1994), the potentiating effect of the mutant is difficult to reconcile with its ability to block transformation.

However, several experiments involving protein tyrosine kinase inhibitors may offer a reasonable hypothesis to explain this apparent paradox. In one study, genistein, a protein tyrosine kinase inhibitor was shown to potentiate NGF-induced neurite outgrowth in PC12 cells (Miller et al., 1993). The authors observed that the growth cone of untreated cells exhibited more extensive remodeling of their lamellipodia and filopodia (Miller et al., 1993). Thus, it appears that tyrosine phosphorylation may serve as a negative regulatory signal in neurite outgrowth. Further experiments have demonstrated that tyrosine phosphorylation may play a significant role in neurite retraction in response to environmental stimuli (Smalheiser, 1993). We have observed in mutant expressing PC12 cells not only a larger number of cells with neurites but also a higher frequency of sprouting cell bodies with ten or more neurites after NGF treatment (Houle et al., unpublished results). It is tempting to speculate that the inhibition of phosphorylation of a specific substrate could give rise to the potentiation phenomenon in mutant expressing cells. Therefore, a single molecular mechanism could elicit transformation suppression in one cellular context and potentiation of neurite outgrowth in an other.

A number of issues need to be addressed concerning the effect of the mutant on PC12 growth and differentiation: 1) Does expression of the mutant induce differentiation

in the absence of NGF? 2) Does it induce cell death? Is it programmed cell death (PCD) ? PC12 cells undergo PCD in the absence of growth factors and NGF (Batistatou et al;1993). 3) Does the mutant interfere with proliferative signals in complete medium in PC12 and give rise to a predifferentiative state that could be rescued from apoptosis by addition of NGF? 4) Is this neurite outgrowth a true differentiation or is it, as suggested by the studies with the inhibitors of tyrosine phosphorylation, an unregulated outgrowth with few if any of the hallmarks of NGF-induced differentiation in normal PC12 cells? The inducible construct described in chapter 3 will aid substantially in clarifying these issues. This construct would also enable the generation of a cell line where mutant Abl dosage could be modulated and its effect on transformation by downstream oncogenes studied.

In order to determine the mode of action of the mutant, cell lines can be derived that harbour both the mutant and the wild type v-*abl* to determine if the presence of the mutant affects the phosphorylation status of the putative substrate. Using this line, the status of certain Abl signal transducers such as the SAPK could also be analysed. The identification of the putative substrate sequestered by the mutant could aid in determining the mode of action of the mutant as well as substantiating the role of this target in transformation as well as other cellular processes. Presumably, this would require a higher affinity interaction between the mutant protein and the substrate than postulated by the weak interaction of the Abl SH2 domain with a non-phosphotyrosine containing target (Pendergast et al., 1991; Muller et al., 1992). Since the interaction appears phosphotyrosine-independent, we have utilized the two-hybrid system to identify interacting proteins. At this time we have several candidates and it appears that at least one binds specifically to the Abl SH2 domain (Cool and Jolicoeur, unpublished results). Its role in the transformation process will be examined. Alternatively, we could used a large-scale purification approach utilizing the recombinant mutant *abl* as an affinity matrix to isolate this substrate (Phizincky and Fields, 1995). However, the inherent

difficulties in producing large amounts of the GST-rAbl mutant (Houle, Beaulieu and Jolicoeur; unpublished results) may preclude such an effort. Finally, the analyses with the variants of the mutant will be repeated to ascertain the role of the remaining domains in the cellular processes the mutant affects. More subtle point mutations in the SH2 domain which block phosphotyrosine binding will also be studied. Again, we can not rule out that the *gag* moiety plays a role in recruiting these putative substrates.

There is an increasing body of evidence which suggests that *gag* proteins may not only play a role in virion assembly but also in cellular signaling and pathogenesis: 1) a recently identified gene present in a microfilament-associated signal transduction particle has extensive sequence similarity with retroviral *gag* proteins (Juang et al., 1994); 2) the retroviral genome of the Murine Acquired Immunodeficiency Syndrome (MAIDS) virus, encodes a p60 protein composed solely of *gag* amino acids (Aziz et al., 1989). The constructs presented in chapter 3 will prove very useful in determining the contribution of the *gag* moiety to the mutant's activity. Studies of *gag* deletions in the transforming v-*abl* suggest that the role of the *gag* moiety in transformation may be limited to proper protein localization and stabilization effects in specific cellular contexts (Prywes et al., 1983).

The Use of the Mutant Abl for Blocking Tumour Growth

The ability of the mutant v-*abl* to block transformation without adversely affecting cell growth could provide us with an ideal therapeutic agent for treating certain types of tumours. To determine the feasibility of such an approach, we have attempted to derive a transgenic animal model but have failed to isolate animals expressing detectable levels of the mutant mRNA (Houle and Jolicoeur, unpublished observations). At this time it is unclear if expression of the mutant is compatible with development.

5.3 MDR and *ras*-Transformation

We attempted to isolate revertants from *ras*-transformed Mink cells based on the well documented observation that transformed mink cells have decreased retention of the supravital dye rhodamine 123 (Kato and Sherr, 1991; chapter 4). Decreased rhodamine retention reflects increased drug efflux mediated by the P-glycoprotein (data presented in chapter 4). The revertant line we isolated utilizing this scheme possessed the expected *mdr* expression characteristics. It also lacked the marked amplification of the *mdr* locus present in the parental transformed cell line. This finding suggested that the amplification of the *mdr* locus in the parental line could reflect some selective advantage for transformation. We addressed the possibility that *mdr1b* was an effector of *ras*-transformation through a number of approaches.

Overexpression of *mdr1b* in the Mink Cell Line is not Transforming

We reasoned that if the stimulation of MDR activity was a vital requirement for *ras* transformation then its overexpression could lead to transformation. Our studies indicated that increases in drug efflux activity as measured by vincristine resistance was not sufficient to cause transformation. However, it is possible that overexpression of the *mdr* protein is not sufficient to render it transforming. A number of cellular counterparts to viral oncogenes such as c-*abl* (Muller et al., 1993) require more than just overexpression to become transforming. The Mdr protein is phosphorylated and its activity is potentially modulated by this process (Chambers et al., 1990; Hardy et al., 1995; Germann et al., 1995). We did not examine the protein levels nor the phosphorylation status of Mdr in the mink clones. We never achieved levels of vincristine resistance that paralleled the transformed parent although this particular clone may have had an unusually high level of resistance (Houle and Jolicoeur; unpublished results). Our constructs afforded vincristine resistance at levels comparable to that of other (Ras-transformed) clones derived independently, yet we can not rule out some effect of Ras

mediated transformation at the post-translational level. In an alternative approach we sought to determine if the overexpression of an antisense cDNA to MDR could revert *ras*-transformed cells to a non-transformed phenotype. Our observations revealed that overexpression of the antisense construct was not sufficient to block transformation. These observations suggested that MDR was not a necessary phenomenon in *ras*-transformation.

These studies do not rule out some contribution to the transformed phenotype by the gene products of the *mdr* gene family for the following reasons. Firstly, our assays measured vincristine resistance but the physiological activity of the *mdr* gene products remains to be elucidated. Some recent work has suggested that Mdr may regulate chloride channel potentiation (Hardy et al., 1995). Another member of the murine *mdr* family, *mdr 2*, may be involved in the efflux of toxic chemicals across the blood-brain barrier. Thus, the vincristine resistance assay may not adequately reflect the inactivation of a relevant activity for cellular transformation. Secondly, two murine *mdr* genes are responsible for drug efflux (*mdr1a* and *mdr1b*) and confer distinct drug-resistance phenotypes (Devault and Gros, 1990). Thus, it is possible that the vincristine resistance assay did not assess the relevant drug efflux activity for transformation. Since the murine *mdr1* genes are highly homologous and the homology between the human and mouse genes can run as high as 88%, we are confident that the failure of the overexpression of the antisense *mdr1b* cDNA to block transformation was not due to a lack of sequence identity between the murine and mink genes. The number of mink genes is unknown at this time. The existence of murine *mdr* gene-specific probes may help resolve this issue.

Finally, a number of studies have suggested that overexpression of *mdr* may be a necessary step in progression of tumours. Thus, the inactivation of the gene product may have little effect on growth in agar (as we assayed) but may hinder progression of the disease *in vivo*.

MDR Promoter Activity as a Tool for Studying *ras*-induced Changes in Transcriptional Activity

The increase in MDR activity parallels transformation in the Mink cell line. The rapid increase in MDR activity following infection with the v-Ha-*ras* transforming retrovirus suggests that Ras signaling affects directly *mdr* promoter activity. Interestingly, *ras*-mediated transformation affects *mdr* expression in a number of different cellular contexts (Chin et al, 1992; Gaboury and Jolicoeur; unpublished results). Furthermore, mutant p53 proteins also affect MDR1 promoter activity, leading some to speculate that this promoter may be specifically activated during tumour progression since many cells acquire mutations in both p53 and *ras* during the later stages of tumorigenesis (Chin et al., 1992). The study of clinical samples has suggested that increased MDR expression may be necessary for progression (Chan et al., 1991; Weinstein et al., 1991; Lonn et al., 1992). The amplification of the *mdr* locus in the parental *ras*-transformed mink cell line does suggest that the cell line is genetically unstable. As discussed previously, this may result as a consequence of *ras*-transformation (Denko et al., 1994) or from mutations in the p53 gene which may occur frequently in established cell lines (Harvey and Levine, 1991; Ulrich et al., 1992; Donehower et al., 1992; Finlay, 1992). The status of p53 alleles was not assessed in the Mink cell lines. The finding that mutations in genes associated with tumour progression significantly affect the expression of the *mdr* genes suggests that treatment of tumours with chemotherapeutic agents which result in selection for cells with increased MDR activity may in fact lead to selection of cells in later stages of tumorigenesis.

Analysis of the murine *mdr1b* promoter reveals the presence of potential recognition sequences for the Sp1 and AP-1 transcription factors as well as some homology with an hepatitis B virus enhancer element (Raymond an Gros, 1990). Oncogenic Ras stimulates phosphorylation of the Jun activation domain by the Jun specific kinase (JNK) which in turn leads to the activation of c-Jun activity (Binetruy et

al., 1991). c-Jun can form homo or hetero-dimers with other members of the Jun family or the Fos family of proteins thus leading to the active AP-1 transcription factor. AP-1 has been shown to recognize a number of sequence elements including the 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) and in concert with the c-*ets*-1 transcritption factor the *ras* responsive element or RRE (Angel and Karin, 1991; Hill and Treisman, 1995). Ras-transformation also results transiently in increased c-*fos* promoter activity. This increase is believed to be mediated by the serum responsive factor (SRF) which recognizes a serum responsive element (SRE) in the c-*fos* promoter (Hill and Treisman, 1995). It is believed that Ras activates the ERK/MAP kinase cascade which results in the phosphorylation and activation of the *elk*-1 protein, a component of the SRF (Hill and Treisman, 1995). Ras mediated transformation not only leads to transcriptional activation but also repression. As transcriptional activation is thought to be a necessary event in unregulated cell proliferation, the need to repress the expression of transformation impeding genes is also critical. Ras transformation leads to repression of a number of cytokeletal protein encoding genes (Leavitt et al., 1985; Herrlich and Ponta, 1989; Kumar and Chang, 1992; Rodriguez-Fernandez et al., 1992; Gluck et al., 1993; Kim et al., 1994). The importance of this phenomenon is further underscored by the observation that overexpression of some of these genes targeted for repression reverts cellular transformation mediated by Ras (Rodriguez-Fernandez et al., 1992; Gluck et al., 1993). The sequence elements involved in this repression are similar to those involved in activation, namely the SREs, although the transcription factors mediating repression may be different (Bushel et al., 1995). The rapid and persistent transcriptional activation of the *mdr* promoter by Ras may be useful in dissecting the roles of Ras effectors in Ras signaling. In closing, considering the importance of the targets of Ras-mediated transcriptional activation and repression, it is surprising that the *mdr* gene, which apparently has a Ras-sensitive promoter, does not play a detectable role in transformation.

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