Conditional and Dissociation Mutants of pp60$^{v\text{-}src}$: Tools for Dissecting Transformation and Mitogenesis

by

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This thesis is submitted in part fulfilment of the Degree of Doctor of Philosophy in the University of Glasgow.

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March 1992

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For Dad.

Abstract

The biological and biochemical properties of *ts*LA32 *Src* were examined in established rat fibroblasts. A cell clone expressing this protein (Rat1 f32) was found to be markedly temperature sensitive for morphological transformation and anchorage-independent growth. Rat1 f32 are rendered quiescent by serum deprivation at restrictive temperature; temperature shift in the absence of added serum factors results in cell cycle transit. The conditional nature of the biological phenotypes was not reflected in any examined biochemical parameter: *ts*LA32 *Src* elevated cellular phosphotyrosine at both restrictive and permissive temperatures, and was non-conditionally autophosphorylated. *ts*LA32 *Src in vitro* kinase activity was not measurable by immune-complex assay under conditions where thermolabile and wild-type controls behaved as expected, perhaps through lability of the mutant protein. Published data indicate that *ts*LA32 *Src* remains in the particulate fraction of CEF at restrictive temperature. These data suggest that *in vivo* enzyme activity, autophosphorylation and membrane localization are together insufficient for transformation of Rat1 cells. Non-catalytic domains of pp60$^{v\text{-}src}$ and their possible roles in transformation are discussed.

These properties suggest *ts*LA32 *Src* would be useful in defining early *Src*-responsive events. Preliminary results indicate that very few proteins become differentially labelled with ^{35}S-methionine after temperature shift of quiescent Rat1 f32. Dissociation of transformation parameters would allow dissection of these and other *Src*-responsive events. Dissociation was attempted using a thermolabile kinase defective for morphological transformation through point mutation of the N-terminal glycine. This non-myristylated protein, *ts*LA29A2 *Src*, retained thermolabile *in vitro* kinase properties but was somewhat toxic in Rat1 cells. *ts*LA29A2 was non-mitogenic, and did not induce AP-1 binding activity in temperature-shifted quiescent CEF. Furthermore, *ts*LA29A2 *Src* was non-mitogenic in quiescent CEF under conditions where AP-1 activity was artificially elevated. These data suggest a need for membrane association in the induction of both AP-1 binding activity and other v-*src*-induced events required for mitogenesis.

Acknowledgements

I would like to acknowledge the Medical Research Council and Cancer Research Campaign for supporting this work. Thanks go also to Professor John Wyke for stimulating (and sometimes heated !) discussions which served to simplify a number of ideas during this Ph.D., and to Professor Julius Gordon for fruitful discussions on the nature of kinase assays and rhubarb pies !

Laboratory work, and even the inevitable failures, were made all the more enjoyable by the good-humour, generosity and helpful nature of all those (past, present and administrative) in Research Group 1. Special thanks go to Val Fincham for her guidance in the molecular biology, and Dr Sam Crouch for her help in generating avian retroviral constructs. Latterly, I have been fortunate enough to work closely with Dr Margaret Frame, whose enthusiasm and fresh ideas have been inspirational.

I am deeply indebted to all members of R1, other Beatson staff, and family and friends for their practical help and support during the last six months of 1990, without which this Ph.D. would have been impossible.

Last but not least, I would like to thank the central research staff for their often unrecognised efforts within the Institute; David Tallach for the photography, and Vivien for printing the majority of the figures in this thesis.

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Abbreviations

A_x	absorbance$_{wavelength}$
AP-1	activator protein-1
ATP	adenosine 5' triphosphate
b	base
bp	base pairs
BSA	bovine serum albumin
C	centigrade
cAMP	3',5' cyclic adenosine monophosphate
c-	cellular
CEF	chicken embryo fibroblasts
Ci	Curie
cm	centimetre
cpm	counts per minute
Da	Dalton
DMEM	Dulbecco modified Eagles medium
DNA	deoxyribonucleic acid
dNTP	3' deoxyribonucleoside 5' triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EGF	epidermal growth factor
env	envelope
g	gram
gag	group antigen
GAP	*Ras* GTPase activator protein
G418	geneticin, G418-sulphate
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid
hr	hour
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
IPTG	isopropylthiogalactoside
k	kilo
l	litre
λ	bacteriophage lambda
LTR	long terminal repeat
m	milli
M	molar
mA	milliampere
min	minute
mm	millimetre
mol	mole
n	nano
neo	neomycin resistance marker
nm	nanometre
NP40	Nonidet P40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
phage	bacteriophage
PI3-kinase	phosphatidylinositol 3-kinase
PIPES	Piperazine-N, N'-bis [2-ethanesulphonic acid]
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonylfluoride

PrA	Prague-strain Rous sarcoma virus, subgroup A
PS	phosphoserine
PT	phosphothreonine
PY	phosphotyrosine
RAV	Rous-associated virus
RCAN	replication-competent avian retrovirus, no splice-acceptor
RF	replicative form
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
sec	second
SH	*src*-homology
TBR	tumor-bearing rabbit
TCA	trichloroacetic acid
TEMED	tetramethylenediamine
TLC	thin-layer chromatography
T_m	melting temperature
TPA	see PMA
tricine	N-tris (hydroxymethyl) methylglycine
tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
tRNA	transfer RNA
ts	temperature-sensitive
u	micro
uv	ultraviolet
V	volts
v-	viral
V8	*Staphylococcus aureus* V8 protease
W	watts
v\v	volume for volume
w\v	weight for volume
wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Amino acid one and three letter codes

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine

V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
X	-	Any

Throughout this thesis genes \ alleles are indicated with lower case lettering, *e.g.* v-*src*. Proteins are indicated with upper case lettering, *e.g.* v-*Src*.

Chapter I

I INTRODUCTION

In 1911, Peyton Rous described the propagation of a spindle-celled sarcoma of chickens by means of a cell-free filtrate[1]. This observation provided one of the first clues to the viral etiology of certain forms of cancer, and with the advent of modern tissue culture, enabled a thorough investigation of the mechanisms of action of these agents. The study of transforming retroviruses, of which Rous sarcoma virus (RSV) is just one, and subsequent identification of the genes responsible for induction of the neoplastic state, has shaped our current understanding of the molecular basis of cancer[2]. The realization that these transforming genes, oncogenes, were originally transduced from the host cell genome[3] has revealed genes whose products function in the process of normal cell growth. Hence, the analysis of transforming retroviruses provides a unique opportunity to identify and analyze cellular proteins that play important roles in the regulation of normal and malignant cell growth.

Genetic and biochemical studies using RSV have provided a wealth of data on the relationship between virus expression and cellular transformation. Genetic evidence that a viral gene was required for transformation was provided when several groups in the late 1960s and early 1970s isolated temperature sensitive transformation mutants of RSV[4,5,6]. Further work by Martin and Duesberg[7], and Vogt[8] demonstrated that mutant viruses lacking a particular segment of the wild-type genome were defective for transformation whilst retaining the ability to replicate efficiently. Identification of this sequence, *src*, as the transforming function of RSV, led to the seminal observation that homologous sequences were present in the host cell DNA[3]. To follow shortly was the identification of the *src* gene product, pp60^{src}[9], and the demonstration that the *Src* protein was a unique protein kinase[10,11] with specificity for protein tyrosine residues[12]. These observations opened the door to a great deal of biochemical and genetic experimentation on the role of tyrosine kinase oncogenes and proto-oncogenes in normal and malignant cells.

This introduction will concentrate on the known properties of the *Src* protein, and characteristics of cells expressing this and its mutant derivatives. I will also describe attempts to identify substrates and effector molecules for the kinase activity and appraise the known candidates. This will lead to a description of my approach to this particular problem.

The mechanisms involved in regulating the cell cycle have received much attention recently, such that it is now possible to derive a molecular model for some of the important control points[13,14]. This introduction will outline the available data on *Src*-induced mitogenesis and in particular, will describe an attempt to dissociate the transforming and mitogenic activities of the *Src* protein, since these biological endpoints presumably reflect activity against specific subsets of proteins.

I.1 Transformed Phenotype

Although isolated by virtue of its tumorigenic nature in chickens, much of what is known about RSV and the v-*src* oncogene is the result of *in vitro* studies. Firstly, the target cell for the virus, namely chicken fibroblasts, are easily cultured *in vitro* and secondly, RSV infected tissue culture cells display a number of phenotypic changes that correlate well with *in vivo* tumorigenicity[15]. Changes to the cellular phenotype can be classified loosely into two categories, namely morphological changes and changes in growth control. Collectively, these novel characteristics constitute the tranformed phenotype. Cells transformed by v-*Src in vitro* display a markedly different morphology to parental fibroblasts. Typically, the cells are rounded and very refractile, presumably as a result of underlying changes in the structure of the cytoskeleton and extracellular matrix. These refractile cells have now acquired the ability to overgrow upon reaching confluence, unlike parental fibroblasts which become contact-inhibited. Indeed, against a background monolayer of parental fibroblasts, v-*Src* transformed cells are able to form clumps of disorganised, refractile cells, called foci. Furthermore, individual transformed

cells are able to proliferate and form colonies in semi-solid media, confirming their capacity for anchorage independent growth. v-*Src* transformed fibroblasts are also able to proliferate under conditions where normal cells become arrested, for instance in the presence of low concentrations of serum. This presumably results from changes in expression or activity of growth factors and / or receptors, or alternatively reflects the ability of the tyrosine kinase to replace these functions.

There are a number of metabolic and molecular changes associated with acquisition of the transformed state. For example, glycolysis is stimulated in v-*Src* transformed fibroblasts, leading to rapid acidification of culture medium. At least three glycolytic enzymes are phosphorylated on tyrosine under these conditions[16,17], although these may be adventitious events since these tyrosine-phosphorylated enzymes are neither rate-limiting nor more active. Rather, it appears that this enhanced metabolic activity is a result of increased uptake of extracellular glucose. v-*Src* appears to achieve this end by two distinct mechanisms depending on the cell type studied. Increased transcription of the glucose transporter gene has been reported in transformed rodent fibroblasts[18], whereas in transformed avian cells, v-*Src* apparently increases the net amount of transporter by reducing the rate at which the protein is degraded[19]. Adenylate cyclase activity is reduced rapidly upon v-*Src* expression[20]. The consequent change in cAMP levels may play a role in controlling glucose transport and cell shape, since addition of cAMP analogues to transformed cultures tend to restore these parameters to a more normal condition[21].

Additional changes in gene expression following v-*Src* expression include increased synthesis of plasminogen activator[22], and increased expression of a gene coding for a potential growth regulatory / inflammatory factor[23,24], although the functional significance of the latter is in some doubt. Other workers have identified in cDNA clones, genes whose transcription is increased as temperature-sensitive *Src* exerts its mitogenic and transforming effects in avian fibroblasts[25]. In similar experiments, Durkin and

Whitfield detected six new proteins as quiescent *ts*RSV-infected NRK cells entered cell-cycle and became transformed following shift to the permissive temperature[26,27]. These may represent polypeptides required for the G1 to S transition. Using a rather different approach, other workers have demonstated that the activity of AP-1 responsive promoters is enhanced in cells expressing v-*Src*[28] . Work from this laboratory has shown that increased transcription of c-*fos* and c-*jun* is not an early consequence of inducing cells into cycle using a *ts* v-*Src* mutant, although there is a concomitant increase in the AP-1 binding activity of nuclear extracts prepared under these conditions[29].

To conclude this section on aspects of cellular transformation, it is important to stress that these observations relate primarily to v-*Src* transformed fibroblasts, and that the expression of this oncogene can have very different effects in different cellular backgrounds. For instance, expression in the PC12 pheochromocytoma line results in differentiation rather than transformation[30], whereas differentiation is suppressed by v-*Src* expression in murine glial cells[31]. It is also noteworthy that a subset of mutant proteins transformation defective for fibroblasts are still capable of extending the lifespan of neuroretinal cells in culture[32,33].

I.2 The *Src* Protein

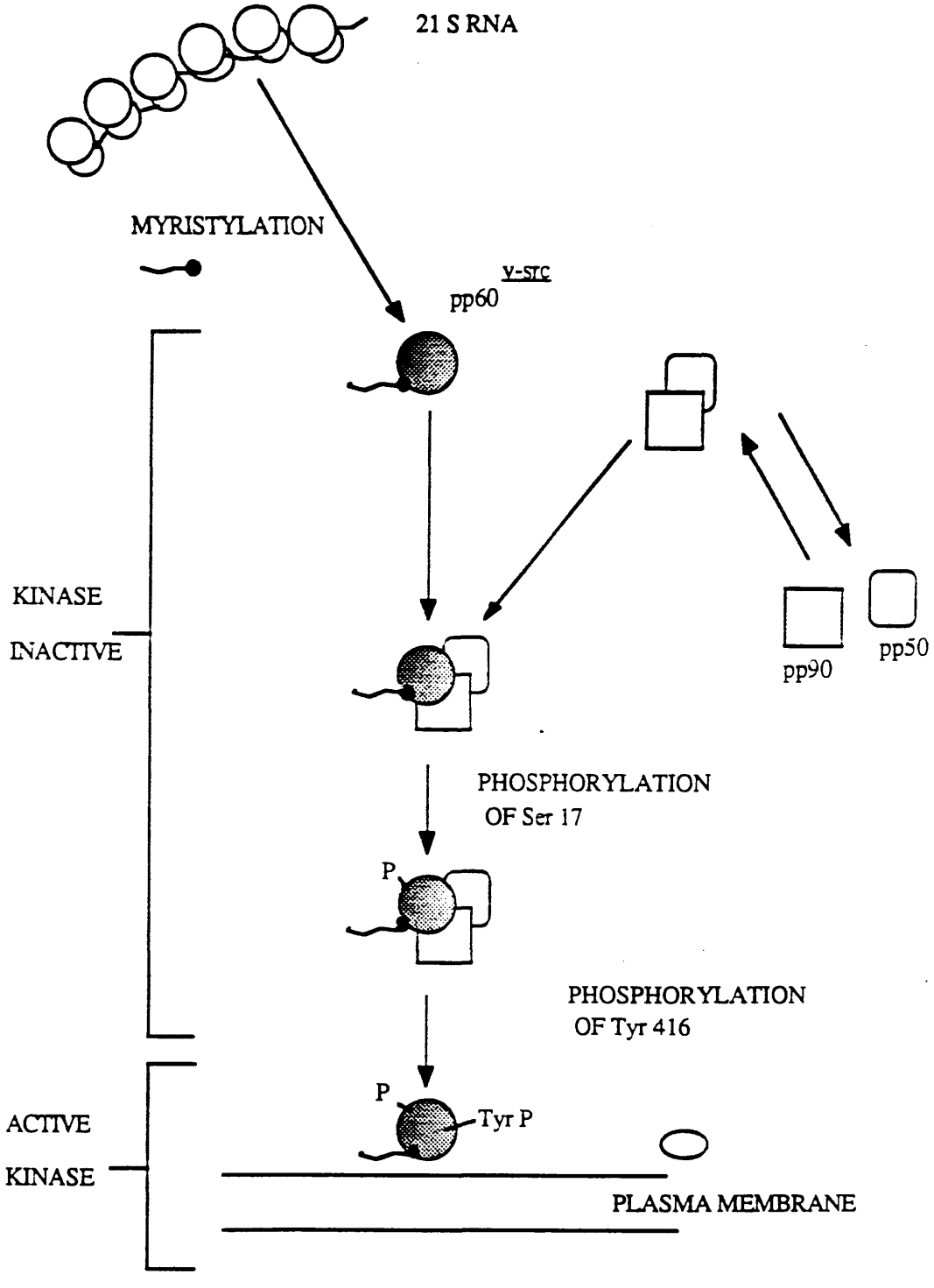
The *src* gene of RSV encodes a 60kDa phosphoprotein, pp60. This protein is plasma-membrane associated by way of non-covalent interaction with a specific receptor protein[34]. Additional populations of *Src* protein are found in the nuclear membrane[35], and in particular within adhesion plaques[36], structures where the cell and substratum are closely opposed. Localization of the protein to the particulate fraction of the cell is an absolute requirement for transforming activity[37]. The only characterised enzymatic activity of pp60 is that of a protein-tyrosine kinase. This is true of highly purified protein from eukaryotic cells[38,39], and also of protein purified from recombinant *E. coli*[40], suggesting that this activity is intrinsic to the protein. Immunoprecipitation of pp60 from

RSV-infected cells yields immune complexes which, in the presence of Mg^{2+} (or Mn^{2+}) and ATP catalyze both the phosphorylation of pp60 itself (autophosphorylation)[41], and / or exogenous substrates[42]. This forms a convenient assay for the kinase activity, and in general this *in vitro* activity reflects the tyrosine kinase activity of pp60 *in vivo*. That the tyrosine-specific kinase activity of pp60 is necessary for initiation and maintenance of the transformed phenotype can be deduced from two experimental observations. Firstly, transformation by RSV is accompanied by a dramatic increase in the level of cellular phosphotyrosine[43], and secondly, mutations in the v-*src* gene that inactivate the *in vivo* kinase activity are invariably defective for transformation[44,45,46]. These results provide compelling evidence that the tyrosine kinase activity of pp60 is indispensable for transformation. However the mechanism by which tyrosine phosphorylation mediates the events leading to cellular transformation is unclear.

I.3 Biogenesis and Post-translational Modification of pp60

pp60 is translated from a 21S mRNA on cytosolic polyribosomes before transit to the cell periphery[47,48]. A diagram showing the proposed biosynthetic route of pp60 is shown in figure I.1. The precise temporal sequence of events is not known, and hence this model should be viewed as a framework only. Cotranslationally, the initiating methionine at position 1 is cleaved and glycine 2 is myristylated. The latter modification will be discussed in detail below (see section I.5). Despite intimate association with the plasma membrane, pp60 appears not to transit via the endoplasmic reticulum and Golgi apparatus[49], suggesting a novel transport mechanism is required to localize the protein correctly within the cell. One candidate for this transport function is a tri-molecular complex formed between pp60 and two cellular phosphoproteins of 50 and 90kDa shortly after pp60 synthesis[50]. The kinetics of association and dissociation of pp60 with this complex are consistent with the kinetics of appearance of the protein at the inner surface of the plasma membrane[51]. However, some mutant pp60

Figure I.1


21 S RNA
MYRISTYLATION
v-src
pp60
KINASE
INACTIVE
pp90
pp50
PHOSPHORYLATION
OF Ser 17
P
PHOSPHORYLATION
OF Tyr 416
P
Tyr P
ACTIVE
KINASE
PLASMA MEMBRANE

proteins are able to localize correctly in the absence of detectable complex formation[52], while the product of another *src* family member, v-*Fgr*, associates with pp50 and pp90 in a very stable complex[53]. In addition, this complex is undetectable in cells overexpressing the c-*Src* protein[54] (S. Courtneidge, personal communication). These observations argue against this tri-molecular complex being the only transport mechanism for the *Src* protein. Indeed, other lines of evidence suggest that the complex could play an entirely different role in removing partially denatured forms of pp60 from the cell. Firstly, the 90kDa host cell protein is a member of the heat-shock protein family[50], and secondly some temperature-sensitive mutants of pp60 show an increased level of complex formation, particularly at restrictive temperature[55]. One can also postulate other functions for this complex either in protecting the cell from cytosolic protein tyrosine kinase activity, since complexed pp60 is essentially kinase inactive *in vitro*[56], or in protecting the *Src* protein from proteolysis after synthesis. There is no firm evidence to substantiate these hypotheses.

pp60 contains two major sites of phosphorylation : tyrosine 416, a proposed autophosphorylation site[57] and serine 17, a site of phosphorylation by protein kinase A[58]. The stoichiometry of phosphorylation of these residues is approximately 30 and 60% respectively[59]. pp60 found in the tri-molecular complex described above is phosphorylated only on serine 17, full kinase activation and tyrosine 416 autophosphorylation occurring only after release from this complex[56]. pp60 can also be phosphorylated at serine residues 12 and 48 in CEF in response to pharmacological agents that increase the activity of protein kinase C[60]. These residues are normally unphosphorylated, and some work suggests that serine 48 may not be a target for protein kinase C in mammalian cells[61]. The functional significance of the serine phosphorylation events is unclear since mutation of these residues fails to compromise the transforming activity of the protein, and has only modest effects on kinase activity[62,63]. However, since $pp60^{v\text{-}src}$ is typically highly overexpressed, subtle changes in localization or activity

cannot be ruled out. Indeed, the activity of the cellular homologue, chicken pp60$^{c\text{-}src}$ appears to be stimulated in part by phosphorylation of two N-terminal threonine residues, 34 and 46, and serine 72[64]. Human pp60$^{c\text{-}src}$ is also subject to mitosis specific phosphorylation[65]. In both cases, these modifications are present only in mitotic cells, disappearing within 15min of release from nocodazole arrest, in parallel with cytokinesis[66]. Interestingly, all three sites are located within the consensus sequence charged-polar-Ser\Thr(P)-Pro-X-basic, closely related to the sites in histone H1 that are phosphorylated by p34^{cdc2} during mitosis[67]. The stringent temporal correlation suggests that pp60$^{c\text{-}src}$ may play a role in regulating passage through mitosis, although the survival of mice[68] and cell lines[69] lacking endogenous c-*src* indicate that pp60$^{c\text{-}src}$ is not essential for mitosis. N-terminal tyrosine (and possibly serine) phosphorylation of pp60$^{c\text{-}src}$ also occurs in response to stimulation of fibroblasts with PDGF, resulting in a modest increase in the kinase activity of the protein[70,71]. Recent results have indicated that PDGF stimulates the association of pp60$^{c\text{-}src}$, and other *src*-family members, with the PDGF receptor, and that these associated proteins have an elevated *in vitro* kinase activity[72]. The role of *src*-homology (SH) domains in the redistribution of *src*-family kinases and other signalling molecules in response to growth factors and transformation is discussed below (section I.6).

C-terminal tyrosine phosphorylation appears to play an important role in conferring oncogenicity in *src* family members, at least *in vitro*. All activated *src* family kinases are phosphorylated *in vivo* at a tyrosine in the catalytic domain. This includes the viral transforming proteins, chimaeras with their N-terminal three-quarters coded by v-*src*, c-*Src* proteins with altered C-termini, c-*Src* proteins complexed with polyoma middle T antigen, and overexpressed p56^{lck} from LSTRA cells[73]. This tyrosine is also the primary site of phosphorylation when purified *src* family kinases are incubated with ATP *in vitro*, suggesting that the reaction is autocatalytic[57]. This reaction is believed to be intermolecular rather than intramolecular[74]. Direct evidence that tyrosine 416

phosphorylation augments activity has recently been obtained. Incubation of fibroblasts with *ortho*-vanadate, a protein-tyrosine phosphatase inhibitor, causes pp60$^{c\text{-}src}$ to become extensively phosphorylated at tyrosine 416, and pp60 purified from these cells has an elevated *in vitro* kinase activity[75]. In v-*Src*, though tyrosine 416 may be dispensable for cell transformation *in vitro*[63,76] (but see ref. 77), its loss has a clear effect in experimental animals. Mouse cells transformed by a substitution mutant containing phenylalanine at position 416 are tumorigenic in nude mice but essentially non-tumorigenic in immunocompetent syngeneic animals[78]. The biochemical basis for this observation is not understood. It is interesting that the v-*Src* protein encoded by this mutant contains reduced but significant phosphotyrosine, indicating that other sites of tyrosine phosphorylation exist in the protein. Some published data support this idea and suggest that these sites are tightly regulated *in vivo* by protein phosphatases[79]. As with the other sites of post-translational phosphorylation, establishing a functional role has been difficult. Indeed, one should always bear in mind that these sites may no longer play any regulatory role due to the presence of additional mutations within the viral protein.

The c-*src* product is also tightly regulated by C-terminal tyrosine phosphorylation. pp60$^{c\text{-}src}$ is phosphorylated to high stoichiometry on tyrosine 527 and low stoichiometry on tyrosine 416 in unsynchronised cells, this correlating with a relatively low *in vitro* kinase activity[73]. Dephosphorylation of tyrosine 527[80] or mutation of this residue to phenylalanine or serine[81,82,83,84] markedly stimulates both kinase activity and phosphorylation of tyrosine 416. Tyrosine 527, which has been deleted in v-*Src*, may play the major role in negatively regulating pp60$^{c\text{-}src}$ *in vivo*. A kinase capable of catalysing the specific phosphorylation of tyrosine 527 has been purified[85] and recently cloned[86] although the phosphatase responsible for reversing this modification is presently unknown. As noted, the kinase activity of pp60$^{c\text{-}src}$ can be increased by prior treatment of the cells with vanadate, an inhibitor of protein tyrosine phosphatases[75]. The increase in kinase activity correlates with an increase in tyrosine 416 phosphorylation, the level of

tyrosine 527 phosphorylation remaining high. Although suggesting that kinase activity can be regulated independently through phosphorylation of tyrosine 416 or tyrosine 527 under conditions where the action of protein tyrosine phosphatases has been reduced, there is no direct evidence that this plays a physiological role in regulating pp60$^{c\text{-}src}$ kinase activity. Available data tentatively suggest that physiological activation of the c-*Src* protein, for instance at mitosis, is accompanied by a small decrease in the stoichiometry of phosphorylation of tyrosine 527[87], in addition to the appearance of N-terminal serine and threonine phosporylation[64,65].

In summary, it appears that the deletion of tyrosine 527 in v-*Src* is largely responsible for the increased enzymatic and transforming activity of this protein. Other phosphorylation sites appear to play a relatively minor role in determining v-*Src* kinase and transforming activity. However, v-*Src* is typically overexpressed, and subtle effects of modification to an active population of molecules may go un-noticed.

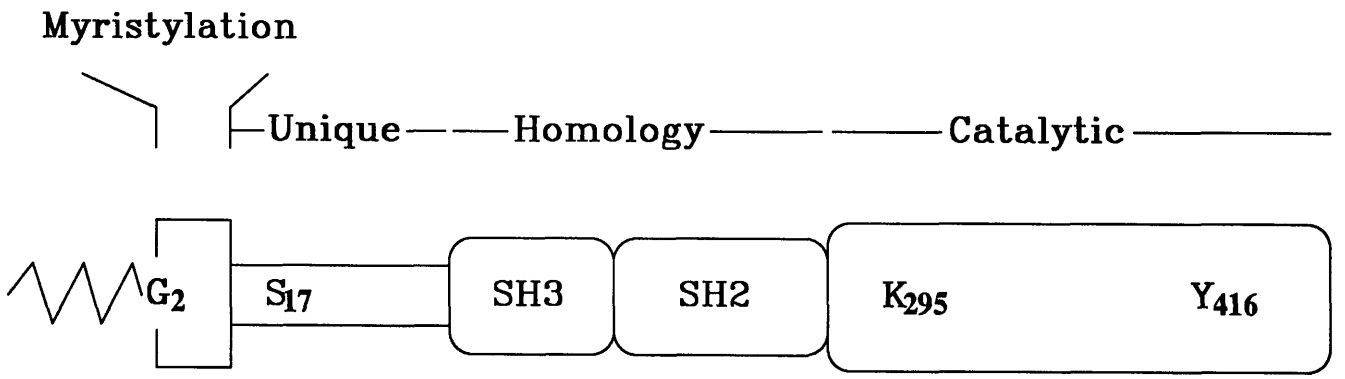
I.4 Domain Structure of pp60

In vitro mutagenesis techniques have facilitated attempts to define important structural and functional motifs within the *Src* protein. Together with analysis of biologically selected and naturally occurring variants, this approach has resulted in a division of the protein into a linear series of domains. It is important to stress that this is a gross oversimplification, since the protein is probably globular in nature and widely separated residues are able to interact to form functional structures. Indeed, single point mutations can have pleiotropic effects on pp60 function. The term "domain" is used in its most general sense. The domain structure of pp60 will be discussed within this context. The model referred to is outlined in figure I.2, and since extensive reviews[55,88] are available on this subject, only the major features will be discussed.

Figure I.2

Domain Structure of pp60$^{v\text{-}src}$

Indicated residues are referred to in the text.



The catalytic activity of pp60 resides in a 30kDa proteolytic fragment from the C-terminus. This fragment exhibits protein-tyrosine kinase activity *in vitro*[89], and also shares amino acid sequence homology with other members of the *src* family, with receptor tyrosine kinases[90], and, to a limited extent serine / threonine kinases[91]. Mutations within the catalytic domain usually have effects on kinase activity and transforming potential.

Myristylation of pp60 and subsequent membrane binding is mediated in part by an amino-terminal domain consisting of the first 7-10 amino acids of pp60[92]. As discussed below, this domain contains the site of myristylation and additional residues required for recognition by the fatty-acyl transferase. Additional N- and C-terminal sequences also play a role in ensuring membrane association without directly influencing myristylation[52,93,94].

The remaining amino terminal sequences comprise what is termed the "modulatory region" of the *Src* protein. This region of pp60 contains the major site of serine phosphorylation of the enzyme (serine 17), as well as sequences that exert positive and negative effects on the activity of pp60. The region encompassing residues 18 to 83 is quite divergent within the *src* family of tyrosine kinases, and has been termed the "unique domain"[73]. One hypothesis is that this region may dictate functional interactions of the cellular *src* family members with non-tyrosine kinase receptor molecules, increasing the kinase activity of pp60$^{c\text{-}src}$. This interaction would be redundant in the case of v-*Src*, a constitutive tyrosine kinase. Indeed, the T cell product of the c-*lck* gene has been shown to associate non-covalently with both CD4[95,96] and CD8[95,97], *lck* residues 15-23 being essential for these interactions[98]. These complexes appear to be functional, since antibody-mediated cross linking of CD4 leads to rapid kinase activation of associated pp56^{lck}[99]. Association with cell surface receptors \ glycoproteins is not unique to pp56^{lck}. Several *src*-related tyrosine kinases associate with cell surface receptors \ glycoproteins : pp60^{fyn} with the T-cell receptor[100], pp56^{blk}, pp56^{lyn} and pp60^{fyn} with

the B-cell receptor[101], pp56^{lyn} and pp62$^{c\text{-}yes}$ with the IgE receptor[102] and pp60^{fyn}, pp62$^{c\text{-}yes}$ and pp56^{lyn} with the platelet glycoprotein CD36[103].

From residue 84 and extending to the N-terminal boundary of the catalytic domain (residue 280), significant sequence homology is evident among the non-receptor tyrosine kinases. This "homology region" was initially designated the SH2 (*src* homology 2) region based on sequence comparisons of the v-*fps*, v-*abl* and v-*src* genes[104]. This region also exhibits striking similarity with v-*crk*[105], an oncogene whose product appears not to be a tyrosine kinase, phospholipase Cγ[106], phosphatidylinositol 3 kinase-associated protein p85[107,108,109] and the *Ras* GTPase activating protein GAP[110]. This region has been subdivided on the basis of the biological properties of mutations within this region and the homologies with v-*crk*, phospholipase Cγ, phosphatidylinositol 3 kinase-associated p85 and GAP.

I.5 Myristylation Domain

The transforming activity of the v-*Src* product is absolutely dependent on association with the particulate fraction of the cell[92,111]. Presumably, membrane association of the normal cellular product is also essential for proper function[112]. Unlike most membrane proteins pp60 is synthesized on cytosolic polysomes[47,48,113] and sequence analysis reveals that the protein lacks a conventional hydrophobic signal sequence[114]. The interaction of pp60 with cellular membranes requires covalent modification of an amino terminal glycine residue with myristic acid[52,115,116,117]. The importance of myristylation has been clearly demonstrated by mutational analysis of the N-terminal sequences of pp60[92,118]. These studies, together with gene fusion experiments[119], have demonstrated that the first seven residues of pp60 may contain the minimal recognition sequence for the fatty-acyl transferase, and that glycine 2 is the site of acylation[111,120]. A basic residue at position 7 is critical for myristylation of *Src* : replacement of lysine 7 by aspartate but

not by arginine, greatly reduced myristylation, membrane association and transforming activity of pp60[121].

Though essential for membrane localization of the *Src* protein, recent biochemical evidence suggests that myristylation of pp60 is not itself sufficient for proper insertion of pp60 in cellular membranes. Using *in vitro* reconstitution experiments, Resh has shown that stable association of pp60 with membrane vesicles requires both myristylation of pp60 and also the presence of cellular proteins in the reconstitution mix[122]. These results suggest that cellular proteins are involved in the stable insertion of pp60 in cellular membranes. Subsequent work from the same group has implicated a 32kDa host cell protein in stimulating association of pp60 with purified plasma membranes[123]. This cellular protein appears to be an integral membrane protein, and available data suggest it may be acting as a specific receptor for myristylated *Src* proteins[124].

Analysis of several mutant *Src* proteins indicates that structural features distant from the myristylation domain also play a role in ensuring stable membrane association (reviewed in ref. 125). A number of *ts* mutants, for example the pp60 of *ts*LA29 RSV, exhibit a reduction in membrane binding particularly at the non-permissive temperature despite full myristylation[93]. Since the critical mutation in *ts*LA29 *Src* is at residue 507, it has been suggested that membrane association at the non-permissive temperature is influenced by a defect in transit to the membrane[94].

In summary, both genetic and biochemical experiments support the notion that myristylation of the *Src* protein requires a domain spanning the first seven residues of pp60. However, the association of myristylated pp60 with cellular membranes is more complex, and requires the interaction of other pp60 sequences and at least one cellular membrane protein.

I.6 Homology Domain

Analysis of mutant *Src* proteins suggests that the homology domain plays a role in modulating the function of both the viral and cellular *Src* proteins. The properties of individual mutants with structural alterations in this region, and the noted homology between sequences within this domain and other cellular and viral proteins, suggests that the homology domain can be further subdivided into at least two regions, SH2 and SH3[126].

Immediately N-terminal to the catalytic domain is a sequence of approximately 100 residues which is present in proteins encoded by v-*src*, v-*fps* and v-*abl*[104]. The overall sequence identity in this SH2 domain is approximately 30%, although the more N-terminal 45 residues are especially conserved. Most of the evidence relating to the function of this domain in cytoplasmic protein tyrosine kinases has been gleaned using oncogenic proteins with cellular transformation as a biological endpoint. Since the SH2 domain can be removed from v-*Src*, v-*Fps* or v-*Abl* proteins without destoying kinase activity, it is clearly unnecessary for this catalytic function[126]. Nonetheless, both circumstantial and direct genetic evidence suggest that the SH2 domain is important for efficient fibroblast transformation. This domain is retained in all non-receptor tyrosine kinase oncoproteins[104,127,128] and mutations within this domain of pp60 render the protein entirely, conditionally or partially defective for transformation[77,104,129,130,131,132,133]. Mutations within the highly conserved N-terminal sequences of this domain can abolish v-*Src* and v-*Fps* transforming activity. In v-*Fps*, these mutations reduce the specific kinase activity of the protein, suggesting that the SH2 domain might act in *cis* with the kinase domain to modify catalytic activity[104]. Similar mutations in v-*Src* have a less pronounced effect on kinase activity, but appear to destabilize the protein following its arrival at the plasma membrane[133]. Both v-*fps* and v-*src* have yielded SH2 domain mutants which are temperature sensitive for transformation in chick embryo fibroblasts.

The decrease in the kinase activity at the non-permissive temperature is again more marked for v-*Fps* mutants than for v-*Src* mutants[129,131,133].

In addition to associating with the kinase domain, the SH2 domain also interacts in *trans* with a cellular factor or factors[104,130,131,132]. Informative in this respect is the observation that several mutants of v-*Fps* and v-*Src* with lesions in the SH2 domain are host-dependent in their transforming activity[77,131,134,135,136]. This can be explained if the mutant proteins fail to bind a critical SH2 ligand in the non-permissive (usually rat) host. In elegant experiments Pawson and colleagues have shown that cellular factors bind to the SH2 domain. Using a *trp*E-SH2 fusion protein, they were able to collect complexes containing a cellular protein of 62kDa[137]. A protein of identical molecular weight is found in immunoprecipitates of the *Ras* GTPase activator protein, GAP[138], and in immunoprecipitates of v-*Fps*[139]. The binding of the pp60 SH2 domain to a cellular protein might positively regulate the activity or stability of the adjacent kinase domain, or allow access to important phosphorylation targets. The SH2 domain may have multiple functions by virtue of its interactions with the kinase domain and host cell factors.

The *Src* and *Abl* proteins share an additional non-catalytic domain (SH3) of approximately fifty residues directly N-terminal to SH2[105,106]. Mutant v-*Src* proteins lacking the SH3 motif are able to transform chick embryo fibroblasts[130], and indeed it is possible to oncogenically activate c-*Src* proteins by amino acid substitutions and deletions in the SH3 region[140]. These observations imply that the SH3 domain in the c-*Src* protein may have an inhibitory effect on oncogenic potential. SH3 does appear to play a positive role in the morphological changes induced by v-*Src* in transformed chick embryo fibroblasts. A number of v-*Src* mutants that induce a fusiform transformed morphology have deletions or amino acid substitutions in the SH3 domain[77,130,141,142,143]. These observations can be incorporated into a model in which SH3, like SH2, forms a binding site for cellular ligands that regulate and \ or effect *Src* function. SH2 and SH3 apparently have quite different, and perhaps opposing effects.

In addition to the cytoplasmic protein tyrosine kinases, recent data have demonstrated that at least nine cellular polypeptides and a retroviral oncoprotein contain sequence elements similar to SH3 and \ or SH2[73,74,75,144]. These are discussed below.

Phospholipase C-γ (PLC-γ) cleaves phosphatidylinositol 4,5 *bis*phosphate to yield the two second messangers: diacylglycerol, which activates protein kinase C, and inositol 1,4,5 *tris*phosphate, which raises intracellular calcium ion concentration. The turnover of inositol phospholipid is a rapid and important result of stimulation of cells with various mitogenic agents (reviewed in refs. 145,146,147), and there is some evidence that this turnover may be enhanced in v-*Src* transformed cells[148,149,150,151,152]. The functional relevance of these observations is not clear. The SH2 and SH3 elements in PLC-γ are not required for catalytic activity, are not found in other PLC isozymes and are therefore presumed to be regulatory[153,154]. Stimulation of phospholipase C activity in response to polypeptide mitogens appears to require the intrinsic tyrosine kinase activity of the receptors since kinase defective mutants that bind ligand fail to stimulate hydrolysis of inositol phospholipids[155,156,157]. Both platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) induce association of PLC-γ isozyme 1 with their respective receptors and stimulate the phosphorylation of PLC-γ1 on tyrosine residues by the receptor kinase and on serine residues by an unknown protein kinase[158,159,160]. Nishibe *et al.* have demonstrated an increase in the catalytic activity of PLC-γ1 following stimulation of cells with EGF[161]. This effect is mediated in part through tyrosine phosphorylation of PLC-γ1[161]. However, the stimulation of phospholipase C activity is not essential for PDGF-dependent mitogenesis[162], and colony stimulating factor 1 (CSF-1) does not stimulate association of PLC activity with its cognate tyrosine kinase receptor[163].

Phosphatidylinositol 3-kinase (PI3-kinase) was the first of this group of proteins containing *src*-homology regions to be found associated with a tyrosine kinase[164,165]. Subsequent purification by conventional means has demonstrated that the enzyme is a

tightly complexed heterodimer of 85 and 110kDa subunits[166]. Molecular cloning of the p85 subunit revealed the presence of an SH3 and two SH2 domains[107,108,109], the latter being implicated in binding of this subunit to other cellular proteins. The catalytic activity of PI3-kinase appears to reside in the 110kDa subunit, suggesting that the 85kDa subunit may direct protein-protein interactions[107,109,166]. Phosphatidylinositol, phosphatidylinositol 4-phosphate and diacylglycerol have been shown to be phosphorylated by activities that co-purify with pp60$^{v\text{-}src}$[167]. Phosphatidylinositol kinase activity has also been demonstrated in immunoprecipitates of pp68$^{v\text{-}ros}$[168]. Since these activities were not easily separated from and were inhibited in parallel with tyrosine kinase activity, they were originally believed to be intrinsic activities of the same polypeptide. Phosphatidylinositol kinase activity was also found associated with the polyoma middle t-pp60$^{c\text{-}src}$ complex, and was shown to be separable from the complex in high salt and detergent solutions[165]. Subsequent results indicated that this enzyme was distinct from the major phosphatidylinositol 4-kinase activity in fibroblasts[165], and that it phosphorylated the D-3 position of the inositol ring[169]. Association of this enzyme with the middle t-pp60$^{c\text{-}src}$ complex appears to correlate strongly with transformation of 3T3 fibroblasts[170], although not absolutely since a point mutant of middle t, 248m, which complexes with both tyrosine and lipid kinase activities, is transformation defective[171]. However, there appears to be absolute correlation in this system between elevation of the lipid products of PI 3-kinase and cellular transformation *in vivo*[172]. Mutants of pp60$^{v\text{-}src}$[173] and of pp130$^{gag\text{-}abl}$[174] that fail to associate with phosphatidylinositol 3-kinase fail to transform, whereas transforming mutants of pp60$^{c\text{-}src}$ are able to associate with the lipid kinase activity[175,176]. Furthermore, Hanafusa and colleagues have demonstrated that PI3-kinase or an associated protein is phosphorylated on tyrosine by growth factor stimulation or in cells transformed with tyrosine kinase oncogenes and that phosphorylation is required for the association of PI3-kinase and pp60$^{v\text{-}src}$[177]. Neither tyrosine phosphorylation nor kinase activity of pp60 is required for binding[177].

Interestingly, PI3-kinase activity is co-precipitated with mutants of v-*Src* defective for myristylation, suggesting that neither prior membrane localisation nor transformation are required for association with pp60[173]. These data indicate that association of PI3-kinase with pp60 is itself insufficient to elicit transformation, although when one considers the membrane localization of substrates for this enzyme, this is not surprising. This prompts speculation that the absolute requirement for membrane localization of pp60 for transformation need not imply the existence of critical membrane targets for the kinase activity of *Src* : an alternative hypothesis would be that membrane association of the v-*Src* product allows recruitment of other molecules to the appropriate membrane environment[163]. The requirement for pp60 tyrosine kinase activity could be incorporated into this model : it might favour interaction with these signalling molecules either by direct phosphorylation or through conformational change induced upon activation of the kinase, or alternatively prevent dissociation once the complex has formed.

All studied receptor-type tyrosine kinases also associate with a phosphatidylinositol 3-kinase activity upon ligand binding[163]. Binding of PI3-kinase is best characterised for the PDGF receptor. Association of p85 and PI3-kinase activity can be detected within one minute of stimulation of fibroblasts with PDGF[164,165]. Association of PI3-kinase with the receptor requires the kinase insert domain containing one of the autophosphorylation sites, tyrosine 751[178]. Deletion of the kinase insert[179] domain or mutation of tyrosine 751 to phenylalanine[180] dramatically reduces recruitment of PI3-kinase following PDGF stimulation. Binding of PI3-kinase to the PDGF receptor is dependent on the tyrosine kinase activity of the receptor[178,180]. Deletion of the kinase insert domain abolishes ligand induced DNA synthesis, suggesting that association of PI3-kinase with the receptor is required for the mitogenic activity of PDGF[178]. It is proposed that PI3-kinase binds to autophosphorylated receptors through the SH2 domains of its p85 subunit, and that tyrosine 751 forms part of the binding site for this enzyme (reviewed in ref. 163). Increased tyrosine (and serine) phosphorylation of PI3-kinase

occurs following association with the activated PDGF receptor[164], though at present it remains unclear whether the lipid kinase activity is modulated by this modification. It may be that recruitment to the plasma membrane, where inositol lipid substrates reside is all that is required. However, the stoichiometry of tyrosine phosphorylation of PI3-kinase in response to PDGF suggests an important role, perhaps in recruiting other SH2-containing proteins to the receptor. The ubiquitous association of PI3-kinase with activated tyrosine kinases suggest an evolutionary link, and furthermore implies important functions for the novel lipid products of this enzyme[163].

Functional $p21^{ras}$ is essential for the transduction of signals from the protein tyrosine kinases of growth factor receptors[181] and transforming oncogenes[182]. Biological activity of the *Ras* proteins is regulated by binding of guanine nucleotides and also by the intrinsic GTPase activity of the protein (reviewed in refs. 183,184). $p21^{ras}$-GTP has been demonstrated to be the biologically active form of *Ras* proteins in mammalian cells[185,186]. Activated *Ras* proteins are transforming, suggesting that they exist mainly in the GTP-bound form[185]. *In vivo* measurements demonstrate that the rate of GTP hydrolysis is greater in cells expressing the normal protein than in those expressing the activated mutants, consistent with this hypothesis[185]. However, *in vitro* measurement of GTPase activity revealed that the intrinsic catalytic activities of normal and activated protein were similar, implicating a cellular factor in modulating *in vivo* activity[185]. This GTPase activator protein (GAP) has been purified and cloned[110]. The GAP sequence contains 2 SH2 domains[110] and a recently identified SH3 domain[187] in the N-terminal region of the protein, with the catalytic activity residing in the C-terminal 344 amino acids[188]. The SH2 and SH3 domains are believed to play a role in protein-protein interactions[137] (see below) and are not required for catalytic activity[188]. Purified preparations of GAP stimulate hydrolysis of GTP bound to normal *Ras*, but not when bound to activated *Ras*, accounting for the observed *in vivo* difference in GTP hydrolysis[185]. The catalytic activity of GAP suggests a role in negative regulation of *Ras* activity, and hence modulation of

this activity could control *Ras* activity. However, since GAP is found in great excess of *Ras*[189], it is possible that GAP has other functions in addition to downregulating $p21^{ras}$ activity. Recent evidence suggests that GAP can act as a signal effector in addition to its role as a signal terminator. The region of $p21^{ras}$ to which GAP binds *in vitro*, the effector binding region, is important for *Ras* function. Point mutations within the effector binding region of v-Ha-*Ras* that destroy biological (transforming) activity also prevent GAP mediated stimulation of normal *Ras*[190,191]. This suggests that GAP binding is essential for $p21^{ras}$ function, *i.e.* GAP itself may be part of the effector complex. The best evidence for an effector function of GAP was obtained in an unexpected fashion. K^+ channels in isolated atrial membranes are controlled by G_k after activation of muscarinic receptors. Addition of recombinant GAP or $p21^{ras}$ blocks the current flow through activated K^+ channels[192]. The effect of recombinant GAP is dependent on endogenous $p21^{ras}$, and *vice versa*. Yatani *et al.* conclude that p21.GTP-GAP is responsible for blocking the channels[192]. Since this effect could be overcome by binding GTPγS to G_k, it is likely that GAP acts directly by inhibiting coupling of G_k to the activated muscarinic receptors or indirectly by inhibiting association of $G_{k\alpha}$ to its βγ subunits[192]. Since βγ subunits are often shared amongst G proteins, this observation may have some bearing on the downregulation of G protein dependent phosphatidylinositol lipid turnover in growth-factor stimulated v-*ras*-transformed cells[193,194].

GAP is phosphorylated on tyrosine in response to EGF[138], PDGF[195,196], CSF-1[197] and the tyrosine kinase oncogene products of v-*src*[138] and v-*fps*[138]. In addition GAP associated proteins of 62 and 190kDa become phosphorylated on tyrosine in cells transformed by protein-tyrosine kinase type oncogenes[138,196]. One of the GAP SH2 domains is required for binding of p62[137]. Approximately 10% of GAP becomes associated with the PDGF receptor upon stimulation with ligand, though the receptor sequence involved is not completely clear[196]. (Phosphorylation of receptor tyrosine 751, the autophosphorylation site implicated in PI3-kinase binding is not primarily responsible

for this association[196]). Perhaps association with the PDGF receptor blocks the ability of GAP to downregulate p21.GTP, leading to a mitogenic signal. In support of this notion, it has been demonstrated that much of the GAP in stimulated cells is complexed to p190, and in this form is relatively inactive in stimulating p21 GTPase activity[198]. However, inhibition of p21.GTP hydrolysis is unlikely to be the whole story given the relative abundance of GAP and the evidence for an effector function of the p21.GTP-GAP complex. An alternative idea is that p62 \ p190 \ GAP transduces a mitogenic signal in complex with p21.GTP. Excess GAP would terminate signalling by competition, *i.e.* GAP may act as both an effector and inhibitor of $p21^{ras}$[199]. One weakness in this model is the failure to detect association of p62 or p190 with GAP in v-*Ras* transformed cells[163].

At present there is no evidence to suggest that GAP catalytic activity is modulated by tyrosine phosphorylation. However, Ha-*Ras* GTPase activity is regulated *in vitro* by phospholipids and some fatty acids released upon growth factor stimulation[200]. This observation is intriguing given the co-recruitment of PI3-kinase and PLC-γ1 to activated PDGF receptors. *In vivo* regulation of GAP activity has been observed in activated T cells. Downward *et al.*[201] have observed a large and very rapid increase in the fraction of GTP-bound $p21^{ras}$ upon stimulation of T cells, apparently through a protein kinase C mediated inhibition of GTPase activity. Direct phosphorylation of GAP has not been observed, leading the authors to speculate that the activity of a GAP inhibitor might be modulated through protein kinase C[201]. These observations are interesting in view of the dissociation of morphological transformation and mitogenesis in *ras*-transformed fibroblasts chronically treated with phorbol ester[202,203,204].

Data from a number of groups indicate that GAP is unlikely to be the sole *Ras* target. Intact GAP or the catalytic C-terminus of GAP suppresses c-*Ras* but not v-*Ras* transformation of NIH 3T3 cells upon co-transfection, and is able to reverse established c-*Ras* transformation, consistent with its proposed function as negtive regulator of $p21^{ras}$ activity[205]. Notably, GAP does not augment c-*ras* transformation in this system,

indicating that it is not the sole downstream target of $p21^{ras}$[205]. However, these experiments do not rule out the possibility that GAP functions in conjunction with other proteins downstream of $p21^{ras}$. Since the transforming and mitogenic activities of v-*Src* are dependent on functional *Ras*[182], and GAP co-precipitates with v-*Src*[206], similar experiments to address the inhibitory activity of GAP were undertaken in v-*Src* transformed cells. Although inhibition or reversion of transformation was observed, the morphology of the revertant clones suggested that these cells were still partially transformed[207,208].

In addition to negative regulation by GTPase activator proteins such as GAP, $p21^{ras}$ is also subject to control at the level of nucleotide exchange. Three groups have reported partial purification of a protein or proteins capable of stimulating guanine nucleotide exchange on *Ras* proteins, including transforming and effector mutants that are insensitive to the action of GAP[209,210,211]. These activities may be equivalent to the yeast CDC25 product that appears to promote nucleotide exchange on $p29^{RAS}$[212,213]. Elegant work by Rubin and colleagues has demonstrated that a putative activator of *Ras* guanine nucleotide exchange performs a crucial role in signalling by the Drosophila *Sevenless* protein tyrosine kinase[214,215]. In the absence of functional *Sevenless*, each omnatidium of the Drosophila compound eye lacks a particular photoreceptor cell, the R7 cell. Using a temperature sensitive variant of *Sevenless*, the workers were able to use the development of the R7 cell as a genetic screen to find downstream effectors of this receptor-type tyrosine kinase. Two genes identified by this procedure encode proteins with homology to *Ras* and the yeast activator of *Ras* guanine nucleotide exchange, CDC25[214]. These data suggest that *Sevenless* may enhance signalling through *Ras* by stimulating exchange of bound GDP for GTP, though alternative mechanisms (inhibition of GAP for example) have not been ruled out[214]. Subsequent work from this group indicates that activated *Ras1* can substitute for *Sevenless* function under conditions where the *Sevenless* ligand, *Boss*, is absent[215].

The v-*Crk* product of the CT10 virus is a 47KDa *Gag-Crk* fusion protein[105]. Sequence analysis indicates that this protein has no significant homology with any known catalytic domain, and hence v-*Crk* is believed to transform via a modulation of endogenous enzyme activity[105]. Indeed, cells transformed with v-*Crk* contain elevated levels of protein phosphotyrosine[105], much of which is concentrated in three cellular proteins that co-precipitate with p47$^{gag\text{-}crk}$[105,216,217]. Immunoprecipitates of p47 formed with anti-*Gag* antiserum contain both serine \ threonine and tyrosine kinase activities[105,216]. Hanafusa and colleagues have proposed that p47 forms a ternary complex with both kinases and substrates, and that this complex favours tyrosine phosphorylation of specific proteins either by activating kinase activity or by protecting phosphorylated substrates from the action of protein tyrosine phosphatases[216].

Association of tyrosine phosphorylated proteins with p47$^{gag\text{-}crk}$ is dependent on a number of structural features. Mutation of either SH2 or SH3 domains reduces or destroys transforming ability[218,219]. Deletion of N-terminal *Gag* sequences leads to relocation and partial loss of transforming activity[218]. In all cases there is an absolute correlation between transformation and elevation of cellular phosphotyrosine[218]. SH2 domains appear to bind to conformational determinants that are dependent on tyrosine phosphorylation rather than phosphotyrosine itself, since competitor phosphotyrosine decreases but does not abolish binding[217].

Bacterially expressed SH2 domains have been used by a number of groups to demonstrate specific binding of tyrosine phosphorylated proteins. Pawson and colleagues have demonstrated *in vitro* binding of p62 to the N-terminal SH2 domain of GAP, and binding of unidentified proteins p130 and p70 to v-*Crk* SH2 domains in *trp*E fusion proteins[137]. This group has also demonstrated binding of GAP and PLC-γ1 SH2 domains to the PDGF and EGF receptor in lysates of stimulated cells[137,220,221]. Other workers have described the association of two tyrosine phosphorylated proteins, p110 and p130 that associate with activated pp60$^{c\text{-}src}$ proteins in chicken cells[222]. Binding is dependent on

an intact pp60 SH2 domain, and also on the presence of activated kinase[223]. Notably, fusiform SH3 domain mutants fail to associate with p110, and indeed p110 is not detectably tyrosine phosphorylated in these cells[223]. It is apparent that different SH2 domains show different binding specificities *in vitro* and presumably *in vivo*[126,137,163]. Indeed, high-level expression of the GAP *src*-homolgy region is non-transforming, in contrast to the transforming effect of the *src*-homology region of *Crk*.

Three recent reports document the isolation of SH2-containing sequences encoding a protein tyrosine phosphatase, PTP1C[224], and a protein serine \ threonine kinase, *Akt*[225] or *Rac*[226]. PTP1C contains two N-terminal SH2 domains, which in isolation can bind to a variety of phosphotyrosine-containing proteins, including the activated EGF receptor[224]. Although complex formation between PTP1C and activated receptors has not been observed *in vivo*, it is tempting to speculate that this enzyme is representative of a class of phosphatases involved in regulating (down-regulating ?) the activity of signal complexes formed in response to ligand binding. Alternatively, signal tranduction may consist of independent parallel pathways of tyrosine phosphorylation and dephosphorylation, regulated by kinases and phosphatases respectively.

The AKT8 virus, the only acute transforming retrovirus isolated from a rodent T cell lymphoma, encodes a serine \ threonine kinase, v-*Akt*, that contains sequences resembling an SH2 domain[225]. $p105^{v\text{-}akt}$ is a *Gag-Akt* fusion protein that demonstrates significant catalytic domain homology with protein kinase A and protein kinase C[225]. Both v-*Akt* and the cellular homologue, c-*Akt* or c-*Rac*[226], have limited sequence homology with previously described SH2 domains, lacking approximately 20 amino acids found in the C-terminal region of other SH2 domains and a number of highly conserved SH2 residues[225]. Although suggestive of a link between tyrosine and serine \ threonine kinases, the functional significance of this SH2-like domain remains to be established. At present there is no evidence to indicate that this protein or isolated *Akt*-

SH2 domains form specific complexes with tyrosine phosphorylated proteins *in vitro* or *in vivo*.

In addition to the second messenger and oncoprotein molecules described above, there are a number of actin-binding proteins that contain SH2 or SH3 domains. Tensin, an actin-binding component of focal contacts and other submembranous cytoskeletal structures, contains a single SH2 domain that, in the form of a glutathione S transferase fusion proten is capable of binding to tyrosine phosphorylated proteins *in vitro*[227]. Increased tyrosine phosphorylation of tensin is evident in *Src*-transformed cells[227], suggesting that by possessing both actin-binding and phosphotyrosyl-protein binding activities and being itself a substrate for tyrosine kinases, tensin may link signal transduction pathways with the cytoskeleton. SH3 (but not SH2)-like sequences are found in the actin-binding proteins myosin and spectrin, and in a yeast cytoskeletal protein[228,229]. Although the functional significance of SH3 domains is not clear, it is possible that these domains are important in localizing proteins to the cytoskeleton. Two experimental observations are consistent with this hypothesis. First, deletion of SH3 from the c-*Abl* protein eliminates association with actin filaments and results in transformation[230,231,232]. Second, the SH3 domain appears to determine morphological phenotype in *Src*-transformed cells[77,130,141,142,143].

It is apparent that SH2 domains play a potentially important role in promoting complex formation between molecules involved in signal transduction. Extracellular ligand binding to its cognate receptor leads to rapid tyrosine phosphorylation and recruitment of specific SH2-containing proteins, allowing co-ordinate regulation of enzyme activity in response to growth factor stimulation. Thus, a primary role of tyrosine phosphorylation may be to promote relocation of cytosolic enzymes to concentrations of substrate molecules, rather than (or in addition to) modulating enzyme activity by allosteric mechanisms. The spectrum of proteins recruited to specific receptors is perhaps dependent on the sequence surrounding the tyrosine autophosphorylation sites, specific

autophosphorylation sites binding specific SH2-containing proteins. Signalling from this complex could be terminated in a number of ways. Firstly, receptor internalisation and degradation of signalling molecules could lead to signal termination. Secondly, taking GAP as an example, excess unmodified protein might compete for the effector molecules leading to signal termination. Thirdly, SH2-containing protein tyrosine phosphatases (for example PTP1C) might be recruited to activated receptors, perhaps leading to dephosphorylation of the receptor and \ or secondary signalling molecules, thereby disrupting established complexes. Available data prompt speculation that activated non-receptor tyrosine kinases also promote recruitment of specific tyrosine phosphorylated and SH2-containing proteins, the resulting complex resembling a ligand-stimulated receptor. Given the tyrosine kinase activity of v-*Src* is constitutive, it is plausible that the signal complexes formed are stable, and hence mitogenic and tranforming signals are generated continuously. One weakness with this model is that mutation of the pp60 autophosphorylation site appears not to prevent transformation *in vitro* in mouse fibroblasts[76] (but see ref. 77). However, as noted previously, this tyrosine 416 mutant retains detectable phosphotyrosine at other unidentified sites[76,78], perhaps allowing association with SH2-containing ligands. Other tyrosine phosphorylated signalling molecules might associate with pp60 through its SH2 domain. This model is consistent with the dependence of *Src*-transformation on proper membrane localization : mutants defective for membrane association might form similar signal complexes but these would be unable to act upon appropriate substrates, such as membrane lipids. One prediction of this model is that appropriate SH2-domain mutants of pp60 might lead to dissociation of transformation parameters, such as cell morphology and growth control.

I.7 Catalytic Domain

The catalytic domains of protein kinases are approximately 30kDa in size. Single subunit protein kinases normally contain N-terminal regulatory sequences, although these

are usually dispensable for catalytic activity and indeed are believed to play regulatory roles (see I.6). The catalytic domains of the protein kinase family contain short amino acid stretches that presumably determine the amino acid (serine \ threonine or tyrosine) substrate specificity of the enzymes. In a recent study by Hanks *et al.*[91], the catalytic domains of 65 protein kinases were compared, and areas of homology identified. The catalytic domains consist of regions of high and low conservation, with 11 subdomains being relatively highly conserved. The authors suggest that these highly conserved regions are important for catalytic function either through direct involvement in the mechanism of catalysis, or indirectly through contributing to the formation of the active site. Significant stretches of unique sequence are found in receptor tyrosine kinases, these kinase insert domains playing a role in recruitment of specific proteins to activated receptor molecules (see I.6).

Highly conserved residues are believed to participate in catalysis. Nine residues are conserved in all 65 sequences examined, these being : glycine 276, lysine 295, glutamate 310, aspartate 386, asparagine 391, aspartate 404, glycine 406, glutamate 432 and arginine 506 (pp60 residues according to Schwartz *et al.*[114]). Another five residues are conserved in 64 of the 65 sequences analysed. The Gly-X-Gly-X-X-Gly motif found in subdomain I near to the catalytic domain N-terminus is found in many nucleotide binding proteins as well as protein kinases, and is believed to form part of the ATP binding site. Other residues involved in Mg^{2+}-ATP binding are found in subdomains VI and VII (aspartate 386, asparagine 391, aspartate 404, phenylalanine 405 and glycine 406).

Conserved subdomain II contains an invariant lysine (295) that appears to be directly involved in the phosphotransfer reaction, possibly mediating proton transfer. All substituents at this position in v-*Src* result in loss of kinase and transforming activity[45,46].

The central core of the catalytic domain consists of subdomains VI to IX. Subdomain VIII contains the consensus triplet Ala-Pro-Glu, a conserved feature often

mentioned as a key protein kinase catalytic domain indicator. Mutagenesis studies have demonstrated that each residue of the Ala-Pro-Glu (430-432) sequence is required for catalytic activity of v-*Src*[44,233]. Studies on other kinases have shown that this sequence may be very close to the catalytic site, and indeed sites of autophosphorylation found in many protein tyrosine kinases lie within 20 amino acids of this consensus triplet. The role of autophosphorylation is discussed in sections I.3 and I.6. Subdomains VI and VIII are of additional interest in that they contain residues that are specifically conserved in either the protein serine \ threonine or the protein tyrosine kinases and as such may play a role in recognition of the correct hydroxyamino acid. The most striking indicator of amino acid specificity is found in subdomain VI lying between the invariant residues corresponding to *Src* aspartate 386 and asparagine 391; two of the residues implicated in ATP binding. The consensus sequence Asp-Leu-Lys-Pro-Glu-Asn in this region is a strong indicator of serine \ threonine specificity, whereas the protein tyrosine kinase consensus is either Asp-Leu-Arg-Ala-Ala-Asn or Asp-Leu-Ala-Ala-Arg-Asn. Another such region is found immediately to the N-terminal side of the Ala-Pro-Glu sequence and is highly conserved in the protein tyrosine kinases with a more limited conservation among the protein serine \ threonine kinases.

The possible functions of residues in conserved subdomains III, IV, V, IX, X and XI are less well understood.

Mutants temperature sensitive for transformation *in vivo* have proven valuable in many studies of *src* gene function (reviewed in refs. 55,88,234. Molecular cloning and DNA sequence analysis has revealed that the majority of temperature sensitive mutants contain lesions in the catalytic domain, and in some cases the specific mutations responsible for the conditional phenotype have been defined. Single point mutations confer temperature sensitivity on a number of mutant v-*Src* proteins, including *ts*LA24 (arginine 480 to histidine)[235], *ts*LA29 (proline 507 to alanine)[94] and *ts*LA31 (glycine 478 to aspartate)[235], whereas two mutations are required for the temperature sensitivity of

*ts*NY68[236]. Three general features have emerged concerning the lesions involved in temperature sensitivity of the biologically selected kinase domain mutants. One, lesions throughout the kinase domain, spanning amino acids 325-503, can influence expression of the temperature sensitive phenotype. Two, although many lesions coincide with highly conserved sequences among protein kinases and some are predicted to have significant effects on protein secondary structure, this is not the case for all lesions in the kinase domain that have a profound effect on *Src* protein function. Three, analysis of these mutants has revealed many long range interactions between distant residues. For example, revertants of both *ts*LA24 and *ts*LA31 that transform at high temperatures contain second site mutations at either amino acid 377 or 492[235], and a wild-type revertant of *ts*LA29 encodes an additional mutation at amino acid 427[94]. These data suggest an interaction between the compensating mutations and the original temperature sensitive lesions.

The studies described above of non-conditional and conditional mutants encoding lesions in the *Src* kinase domain provide strong support for the hypothesis that tyrosine kinase activity is essential for v-*src*-induced cell transformation.

I.8 *In vivo Src* substrates

A longstanding interest in the mechanism of transformation of v-*src* has resulted in numerous attempts to define important substrates for the tyrosine kinase activity of pp60. This has proven difficult because protein phosphotyrosine represents less than 1% of the total phosphohydroxyamino acid present, even in v-*Src* transformed cells[12], and the kinase activity of pp60 is rather promiscuous, resulting in phosphorylation of numerous adventitious substrates[88]. Thus, by conventional labelling techniques, important targets have been difficult to pinpoint. Some problems created by the low relative abundance of protein phosphotyrosine have been circumvented using anti-phosphotyrosine antisera[237,238,239,240], since these can enable detection of phosphotyrosine in proteins

containing a large excess of phosphoserine and \ or phosphothreonine. The major shortcoming with this approach is the selective nature of the antiserum : not all antiphosphotyrosine antisera react with the same proteins. Another approach using monoclonal antibodies to specific tyrosine phosphorylated proteins has been developed by Kanner and colleagues[241]. Using antiphosphotyrosine affinity columns, these workers purified tyrosine phosphorylated proteins present in chick embryo fibroblasts expressing activated mutants of c-*Src*. These proteins were used to develop monoclonal antibodies specific to known tyrosine phosphoproteins of 110, 120 and 130kDa and several previously undescribed proteins. Subsequent work by the same group, discussed below, has indicated that two of these proteins form complexes with pp60 in addition to being tyrosine phosphorylated in cells expressing pp60[222,223].

The majority of studies designed to define important substrates for the tyrosine kinase activity of pp60 have made a comparison between fully transformed and normal cells. There are a number of problems with this approach. Firstly, tyrosine phosphorylation events involved in initiating transformation can no longer be identified as early events, and indeed these proteins may no longer be tyrosine phosphorylated in the fully transformed cell. Secondly, although a number of the tyrosine phosphorylated proteins present in the fully transformed cells may be required for maintenance of this state, the majority of tyrosine phosphoproteins might be either adventitious substrates of the promiscuous kinase activity or the result of a cellular response to transformation. This renders discrimination of important targets from secondary or adventitious events difficult. Superimposed on these problems are growth state effects : *Src*-transformed cells may proliferate more rapidly than their normal counterparts, and the mechanisms controlling this partially serum-independent growth might differ from those controlling the growth of normal cells. In particular, tyrosine phosphorylation events regulating transit from one stage of the cell cycle to another might be very difficult to detect in an asynchronous culture. Hence, examination of steady-state transformed cells generally

precludes identification of kinase substrates important in particular facets of transformation.

Some of these problems can be overcome by using temperature sensitve kinase mutants. Since the ATP pool can be labelled in the absence of pp60 kinase activity, this enables analysis of very early events following temperature shift. Furthermore, cells expressing a number of these mutants can be rendered quiescent in a phenotypically normal state at restrictive temperature, a shift to permissive temperature stimulating these cells to exit G0 and proceed through mitosis[26,29,242,243]. This approach has the potential to reduce growth state effects and to facilitate identification of important tyrosine phosphorylation-dependent control points within the cycle of *Src* stimulated cells. However, some features of these mutants introduce other problems. These mutants are often temperature sensitive for membrane localization as well as for kinase activity[234]. This change in subcellular localization together with rapid reactivation of kinase activity against all substrates following temperature shift leads to phosphorylation of many adventitious targets. These problems could be resolved using mutant proteins that are temperature sensitive for transformation, whilst still retaining kinase activity and membrane localization at restrictive temperature. If cells expressing these mutants can be rendered quiescent at restrictive temperature, the number of adventitious phosphorylation events seen upon temperature shift might be reduced. This may facilitate identification of relevant early events. This approach might also be used to identify proteins whose synthesis is required for, or is a result of the transforming activity of pp60. The use of a putative minimal deviation mutant in searching for proteins whose synthesis or phosphate content is modulated in response to activating the transforming and mitogenic activities of pp60 forms the basis of part of this Ph.D., and will be discussed below (see I.13).

I.9 Cytosolic Substrates

A number of cytosolic proteins are phosphorylated on tyrosine in v-*Src* transformed cells. Brief descriptions of these proteins are given below, but it should be noted that the phosphorylation of none of these correlates invariably with transformation *in vitro*.

I.9.1 p50

The 50kDa member of the previously described tri-molecular complex (see I.3) becomes phosphorylated on tyrosine in v-*Src* transformed avian cells[12]. Although phosphorylation of this protein appears to be the result of pp60 kinase activity rather than a consequence of transformation, tyrosine phosphorylation of this protein is not observed in RSV-transformed mammalian cells[50]. Hence phosphorylation of this protein appears not to be essential for transformation.

I.9.2 pp42 \ MAP kinase

The microtubule-associated or mitogen-activated protein kinases (MAP kinases) are a family of protein serine \ threonine kinases which respond to growth factor stimulation (reviewed in refs. 244,245,246). MAP kinases are believed to play an important role in mitotic and meiotic division in eukaryotic cells[245,247,248,249,250,251]. This kinase family is also implicated in the nerve growth factor-induced differentiation of PC12 cells[252]. The best characterised member of this family is pp42; the following discussion will focus on this enzyme, although it seems likely that other family members will play similar roles. Activity *in vitro* is absolutely dependent on both threonine and tyrosine phosphorylation of the enzyme, dephosphorylation of either threonine 13 or tyrosine 15 resulting in a complete loss of kinase activity[253]. The rapid activation of this enzyme in response to purified mitogens suggests that pp42 might act as a switch kinase, receiving signals from tyrosine kinases and converting this information into serine \ threonine phosphorylation of target proteins. Integration of threonine and tyrosine

phosphorylation, found in MAP kinases and in p34^{cdc2}[254], presumably allows "fail-safe" control of activity. The rapid kinetics of activation of MAP kinase following challenge of cells with polypeptide mitogens prompts speculation that tyrosine phosphorylation of the enzyme is catalysed by activated growth factor receptors, and that pp42 is a target for the activity of these peripheral enzymes. A number of observations indicate that this may be an oversimplification. It has been noted that treatment of fibroblasts in culture with the protein kinase C agonist TPA results in both threonine and unexpectedly, tyrosine phosphorylation of pp42[255,256]. Since phosphorylation of receptor kinases by protein kinase C is generally inhibitory[257,258], the tyrosine phosphorylation of pp42 is unlikely to result from receptor kinase activity under these conditions. Recent work by Cohen and colleagues[252] and Ahn *et al.*[259] has described activities capable of reactivating dephosphorylated MAP kinase *in vitro* by stimulating both the tyrosine and threonine phosphorylation of p42[252]. Both the threonine kinase and tyrosine kinase activities towards dephosphorylated p42 appear to co-purify through a number of chromatography steps, suggesting (but not proving) that both activities might be intrinsic to a single protein[252]. There have been reports recently of kinases with dual specificity for protein serine \ threonine and tyrosine residues[260,261], though the significance of these observations remains unproven. The MAP kinase kinase itself appears to be dependent on serine \ threonine phosphorylation, suggesting the existence of a kinase kinase kinase[252]. Although providing a possible explanation for the ability of TPA to promote tyrosine phosphorylation of MAP kinase, this model argues against pp42 being a direct target for activated receptors. Independent control of MAP kinase threonine kinase and MAP kinase tyrosine kinase activities would be required *in vivo* if dual phosphorylation of MAP kinase were to provide a fail-safe mechanism for activation of this enzyme. At present these apparent kinase activities cannot be distinguished from activities that stimulate autophosphorylation by MAP kinase. Indeed, the regulatory phosphorylation sites at threonine 13 and tyrosine 15[262] lie close to the Ala-Pro-Glu consensus sequence

mentioned in I.7, a region containing autophosphorylation sites in a number of protein kinases and furthermore, a number of recent reports indicate that MAP kinase purified from recombinant *E. coli* is able to autophosphorylate on tyrosine and threonine, albeit extremely slowly, and to the author's knowledge, to an unspecified stoichiometry[263,264,265]. Ahn *et al.* have proposed that MAP kinase activators might stimulate markedly the rate and extent of MAP kinase autophosphorylation in response to mitogens, leading to activation of enzyme activity[266].

Tyrosine phosphorylation of MAP kinase occurs in response to a number of mitogenic agents, including pp60[17,245,255,267]. However, reproducible tyrosine phosphorylation of this protein in *Src*-transformed mammalian cells has been difficult to achieve, and only one group has suggested that enzyme activity is enhanced in this situation[268]. Dephosphorylated pp42 is a poor substrate for purified pp60 *in vitro*[163], suggesting that tyrosine phosphorylation of this protein in *Src*-transformed cells might be indirect.

Given the proposed role of this family of kinases in regulating the response of cells to mitogens, growth state effects may have a profound influence on the phosphorylation state of this protein. Studies on the tyrosine phosphorylation of this putative switch kinase would be facilitated in synchronous populations of cells stimulated to transform and re-enter cycle by reactivation of a conditional *Src* mutant.

Pulverer *et al.* have recently reported that purified MAP kinases can phosphorylate c-*Jun* protein on some of the N-terminal serine residues that are utilised *in vivo*[269]. Phosphorylation of these residues is reported to correlate with increased transactivation activity as measured by transient assays[269,270,271]. These data are interesting in view of two observations : there is a rapid increase in AP-1 binding activity in nuclear extracts following temperature shift of cells expressing conditional *Src* mutants[29], and transcription from AP-1 responsive reporter elements is enhanced in *Src*

transformed cells[28,272]. The role of MAP kinases in mediating these effects would be amenable to study in the temperature shift system outlined above.

I.9.3 Glycolytic Enzymes

Three soluble, non rate-limiting glycolytic enzymes (enolase, phosphoglycerate mutase and lactate dehydrogenase) are phosphorylated on tyrosine in v-*Src* transformed cells[16,17]. As is the case with p50, the phosphorylation of none of these proteins correlates well with cellular transformation.

I.9.4 PI3-Kinase

Tyrosine phosphorylation and association of this enzyme with activated tyrosine kinases has been described in detail in section I.6. Tyrosine phosphorylation of this enzyme is required for association with pp60[177], though neither tyrosine phosphorylation nor association with pp60 correlate absolutely with transformation[173]. This may be due to the location of the substrates for this enzyme : mutants of pp60 defective for transformation through cytosolic localisation still associate with PI3-kinase activity[173], but it is presumed that this complex is non-functional given the absence of inositol phospholipids in the cytosol. In view of the ubiquitous association of this enzyme with activated tyrosine kinase receptors (see I.6), PI3-kinase is a potentially important substrate for all mitogenic tyrosine kinases, including pp60.

I.10 Cytoskeletal and Extracellular Matrix Proteins

Since pp60 associates with the cytoskeleton, particularly at sites of cell-substratum contact, it is not surprising that several cytoskeletal proteins are phosphorylated on tyrosine in v-*Src* transformed cells (reviewed in refs. 273,274). Presumably tyrosine phosphorylation of these proteins is involved in the induction of morphological changes observed upon transformation.

I.10.1 Vinculin

A fraction of this 130kDa protein is localized in adhesion plaques, as is pp60. Approximately 1% of the vinculin in *Src* transformed cells becomes phosphorylated on tyrosine[275], although whether the adhesion plaque fraction displays a higher stoichiometry of phosphorylation is unknown. The relationship of vinculin phosphorylation to transformation has been examined in cells infected with a wide variety of *Src* mutants[276,277] but no relationship between the degree of vinculin phosphorylation and any measured aspect of transformation was discernible.

I.10.2 Integrin

The integrins are a complex of heterodimers with multiple affinities for extracellular matrix proteins such as fibronectin, vitronectin and laminin. On the cytoplasmic face of the plasma membrane, this glycoprotein complex appears to associate with several cytoskeletal components including fibulin and talin. In turn, talin interacts with another cytoskeletal protein, vinculin, and possibly the actin bundling protein, α-actinin (reviewed in refs. 274,278,279). Since fibronectin loss is a result of *Src* transformation[280], and may also play a role in inducing morphological changes[273], this link between the extracellular matrix and the cytoskeleton is an obvious candidate for a functional target. Some data from Kellie and his colleagues suggests that integrin phosphorylation may be a relevant event in *Src* transformation[281].

I.10.3 Talin

Talin is a protein that appears to interact with both integrin and other cytoskeletal components. This protein, like vinculin, is phosphorylated to low stoichiometry in *Src* transformed cells[282]. However, this tyrosine phosphorylation does not correlate well with transformation[273,274,282].

There are also a number of other unidentified cytoskeletal proteins which become phosphorylated on tyrosine in v-*Src* transformed cells. Glenney and co-workers have

demonstrated that a 22kDa membrane \ cytoskeletal protein becomes phosphorylated on tyrosine in chick embryo fibroblasts expressing transforming v-*src* genes[283], although phosphotyrosine in this protein is reduced by 95% in cells expressing kinase active but transformation defective pp60[284]. There are no data to suggest that the cytoskeletal SH3-containing proteins described in I.6 are tyrosine phosphorylated. If these proteins were to associate with pp60, as proposed in an section I.6, one might expect them to be targets for the kinase activity of the *Src* protein.

I.11 Calcium Binding Proteins

Two calcium binding proteins, calpactin I[285,286] and calmodulin[287], are phosphorylated on tyrosine in *Src* transformed cells. Calpactin I is phosphorylated to low stoichiometry despite co-localizing with pp60, and this phosphorylation correlates poorly with any aspect of transformation[288]. Calmodulin contains labile phosphotyrosine at sites adjacent to the Ca^{2+} binding site[287]. The functional relevance of this phosphorylation has yet to be evaluated, although the central importance of calmodulin in regulating cellular responses to calcium indicates that this would be a worthwhile area for investigation.

I.12 Membrane Proteins

Since membrane association of pp60 is essential for transformation *in vitro*, it is assumed that important substrates reside in this fraction of the cell. A number of tyrosine phosphorylated proteins have been identified in membrane preparations, some of these correlating well with transformation.

I.12.1 Epidermal Growth Factor Receptor

Rapid tyrosine phosphorylation of the EGF receptor occurs upon *Src* transformation[289]. The stoichiometry of phosphorylation approaches that achieved with saturating levels of EGF[289]. However, variant cells which lack the EGF receptor can still be transformed by v-*Src*, indicating that the receptor is not a necessary target for *Src*

transformation[289]. Cells overexpressing the c-*Src* protein are hyper-responsive to EGF[290], and this enhanced responsiveness is dependent on intact c-*Src* myristylation, homology and kinase domains[112]. The role of the EGF receptor in v-*Src* stimulated mitogenesis has not been investigated, although phosphorylation of this receptor could be indicative of a class of membrane targets whose phosphorylation might induce alterations in growth control. One idea is that pp60-mediated phosphorylation of, and \ or association with the EGF receptor, or another tyrosine kinase receptor, promotes assembly of the signalling complexes described in section I.6.

I.12.2 Insulin-like Growth Factor 1 Receptor

Identified as a glycoprotein whose tyrosine phosphorylation correlates tightly with transformation using a battery of diverse *Src* mutants[291], Weber and colleagues have demonstrated that the IGF-1 receptor is constitutively phosphorylated on tyrosine in *Src* transformed cells[292]. pp60 appears to synergise with ligand, leading to elevated receptor autophosphorylation[292]. This suggests a mechanism whereby *Src* could override the requirement for external growth factors, enhanced signalling resulting from intracellular interactions[292]. The association of signalling molecules with the activated IGF-1 receptor is not well characterised, although one report suggests that PI3-kinase activity is detectable[163].

I.12.3 *Ras* GTPase activating Protein GAP

In cells expressing a temperature sensitive mutant of pp60, GAP-associated p62 becomes tyrosine phosphorylated very rapidly following shift to the permissive temperature, with phosphorylation of p190 and GAP occurring more slowly[138]. In addition, the proportion of $p21^{ras}$ in the GTP bound form increases following expression of v-*Src*[293], although there is no evidence to suggest that this results from a reduction in the GTPase activity of the GAP protein. In Rat2 cells expressing a non-transforming SH2

domain mutant of v-*Fps*, tyrosine phosphorylation of p62 and p124 is missing[139]. p124 might be GAP. GAP and its associated cellular proteins would appear to be candidates for functional substrates of pp60.

I.12.4 Unidentified Membrane Proteins

A number of groups have demonstrated that tyrosine phosphorylation of membrane proteins in the 110-150kDa range correlates with transformation *in vitro*. Reynolds *et al.* identified a protein of 120kDa whose tyrosine phosphorylation correlates well with transformation using a battery of c-*src* derived mutants[294]. This protein is not tyrosine phosphorylated in cells expressing non-myristylated pp60[294], although it is phosphorylated in cells transformed by other tyrosine kinases[294]. Linder and Burr[295] identified particulate fraction proteins of 115-130kDa whose tyrosine phosphorylation mimicked that of the 120kDa protein identified by Reynolds *et al*[294]. Both groups confirmed that these proteins were neither integrin nor vinculin.

Wendler and Boschelli[296] also identified a group of membrane proteins in the 120-150kDa size range which became tyrosine phosphorylated in cells expressing wild type v-*Src*. The tyrosine phosphorylation of this group of putative substrates was reduced in cells expressing partially transforming SH2 domain mutants of v-*Src*[296]. However, it is difficult to correlate tyrosine phosphorylation of this group of proteins with transformation, since in addition to being partially defective for transformation, the SH2 domain mutants employed displayed reduced kinase activity[296]. Furthermore, a number of the mutants employed in this study demonstrated transforming properties only after extended periods in tissue culture; the correlation between substrate phosphorylation and *Src* lesion is therefore tenuous.

Lau[297] has identified a tyrosine-phosphorylated protein of 120kDa in immunoprecipitates prepared using antisera to pp60. This protein does not co-migrate

with the protein identified by Reynolds *et al*[294]., and is not vinculin[298]. Peptide mapping and glucosamine labelling experiments suggest that the 120kDa protein is not integrin[297].

Tyrosine phosphorylated proteins of 110 and 130kDa are associated with activated c-*Src* variants in chicken embryo fibroblasts[222] but neither association with pp60 nor tyrosine phosphorylation are sufficient for transformation since tyrosine phosphorylated p110 and p130 co-precipitates with non-myristylated, kinase-active pp60[223]. Kanner *et al.* have generated monoclonal antibodies to p110 and p130 which have enabled a more thorough characterisation of these proteins and their interactions with pp60[241]. Several structural and functional prerequisites appear necessary but perhaps not sufficient for complex formation. Activation of the c-*Src* tyrosine kinase activity is required : uninfected chick embryo fibroblasts do not contain detectable complexes between p130, p110 and endogenous pp60$^{c\text{-}src}$, and mutations which inactivate *Src* kinase activity block both tyrosine phosphorylation and association with pp60[223]. Stable complex formation also requires the structural integrity of part of the SH2 domain of *Src*[223]. Mutations within the SH3 domain significantly reduce the association of p110 with pp60[223]. The association of p110 with pp60 in transformed cells appears to correlate with the redistribution of p110 to focal adhesion plaques and the plasma membrane[223]. Cells expressing fusiform SH3 domain mutants of *Src* contain p110 that is neither tyrosine phosphorylated nor associated with pp60[223]. These observations suggest that tyrosine phosphorylation and complex formation with membrane associated pp60 are required for wild-type transformation.

It is clear that a number of potentially relevant substrates residing in the particulate fraction have been identified in *Src* transformed cells. The identification of these proteins, and in particular the generation of specific antisera, will facilitate further study.

I.13 *ts*LA32 *Src*, A Minimal Deviation Mutant

Many of the problems encountered in searching for relevant targets of pp60 tyrosine kinase activity may be circumvented using mutants which differ only minimally from the wild type protein in their biochemical properties. The protein encoded by *ts*LA32 *src* is believed to be one such mutant. Stoker *et al.*[299] have demonstrated that chick embryo fibroblasts (CEF) expressing this protein are temperature sensitive for transformation by a number of criteria. Firstly, these cells have a normal morphology at restrictive temperature and a fusiform transformed morphology at permissive temperature. CEF infected with *ts*LA32 RSV are temperature sensitive for focus formation on a monolayer of normal CEF, and are temperature sensitive for anchorage independent growth. These *in vitro* parameters of transformation are believed to correlate well with *in vivo* tumorigenicity[15]. Hexose uptake, a physiological measure of transformation, is increased in *ts*LA32 RSV infected cells at permissive temperature, although this enhanced transport is not as striking as that induced by the wild-type protein[142]. This mutant retains elevated kinase activity at restrictive temperature, both *in vitro* and *in vivo*[299]. Association of this mutant protein with the particulate fraction of the cell is largely independent of growth temperature[93]. These data indicate that the transformation defect in *ts*LA32 *Src* is subtle in nature, and that transformation by this mutant is unlikely to result from a gross change in kinase activity or subcellular localisation upon shift to permissive temperature. The *ts*LA32 kinase may fail to associate with or phosphorylate one or more crucial protein substrates at restrictive temperature, thus precluding transformation. However many substrates will be phosphorylated at restrictive temperature, and upon shift of these cells to the permissive temperature, functionally relevant phosphorylation events should be identifiable. This should also enable identification of a group of proteins whose tyrosine phosphorylation at restrictive temperature is insufficient to elicit transformation. If cells expressing this mutant protein can be rendered quiescent at restrictive temperature, the number of

adventitious phosphorylation events seen should be reduced further. This system should enable a search for novel targets of the tyrosine kinase activity, and an assessment of the relevance of previously identified candidate target proteins. Similar arguments apply to the identification of proteins whose synthesis or turnover is modulated as quiescent fibroblasts are stimulated to transform and re-enter cycle by activating a thermolabile *Src* mutant.

The presence of membrane-associated, kinase active pp60 in cells infected but not transformed by *ts*LA32 RSV at restrictive temperature prompts other interpretations. It is plausible that a subtle change in subcellular localisation of the kinase and its associated proteins occurs following temperature shift, and this brings pre-existing pp60-associated signalling molecules into contact with their substrates. This model suggests that new tyrosine phosphorylation events would not be observed immediately following temperature shift, or if such events were detected, that these might be adventitious consequences of a subtle change in localisation. However, one would predict a rapid increase in the cellular levels of products generated by the pp60-associated signalling enzymes.

The proposed function of SH2 domains in binding tyrosine phosphorylated proteins suggests another potential mechanism whereby the association of signalling molecules with *ts*LA32 pp60 might be affected by growth temperature. Subtle changes in the stoichiometry of autophosphorylation of the active population of pp60 in response to temperature might lead to quantitative changes in the association of specific signalling molecules. In this regard it is intriguing that one of the mutations in *ts*LA32 *Src* lies three residues downstream from the autophosphorylation site. Subtle changes in the stoichiometry of autophosphorylation might be difficult to detect since the protein is overexpressed.

The three mutations in *ts*LA32 *src* have been identified (Stoker, unpublished data[142]), two being located in the kinase domain of the protein at positions 300 (glycine to

valine) and 419 (arginine to glutamine), the third being located within the SH3 domain at position 107 (arginine to proline). The temperature sensitive characteristics of this protein in chick embryo fibroblasts are determined by the catalytic domain mutation at position 300 (V. Fincham, unpublished data). The consequences of the other mutations are unclear, though the presence of a mutation in the SH3 domain is interesting in view of the fusiform morphology of chick embryo fibroblasts expressing this mutant[299]. The mutation at position 419 is close to the autophosphorylation site at tyrosine 416.

Previous work from this laboratory demonstrated that Rat1 cells expressing a temperature sensitive kinase mutant of v-*src* could be rendered quiescent by serum deprivation at restrictive temperature, and that these cells could re-enter cycle upon shift to permissive temperature in the absence of added factors[29]. (This is also the case in CEF expressing *ts src* mutants[242,243]). With this in mind and given the practical difficulties of working with primary cultures of chick embryo fibroblasts, it was decided to express the *ts*LA32 *src* gene in established Rat1 fibroblasts. In light of the problems associated with Rous sarcoma virus expression in rodent cells[300], it was decided to employ a Moloney murine leukaemia virus-based vector[77,301] to express this mutant. The majority of data presented in this thesis concern the generation and characterisation of a clonal Rat1 line expressing *ts*LA32 *Src*, Rat1 f32. These studies were facilitated by generation of clonal lines expressing wild-type Prague A v-*Src* and a well characterised temperature sensitive mutant, *ts*LA29 *Src*[29,93,94,299]. The biological and biochemical properties of the mutant proteins are described in chapters III to VI. Preliminary results obtained by two-dimensional gel electrophoresis of lysates from quiescent Rat1 f32 cells shifted to permissive temperature are presented in chapter VII.

I.14 v-*Src* and Mitogenesis

Converging with this interest in substrates of pp60 tyrosine kinase activity is our interest in the mitogenic properties of this transforming protein. As stated above, v-*Src*

transformation is accompanied by changes in the growth properties of fibroblasts, leading to a degree of serum independence. This property of v-*Src* has been studied in this and other laboratories with temperature sensitive mutants[26,27,29,242,243]. Early work in this group employed chick embryo fibroblasts infected with *ts*LA24 RSV[242]. Quiescent cultures incorporate labelled thymidine after approximately 5-7hrs at permissive temperature, with incorporation peaking at approximately 15hrs post temperature shift (J. Bell, unpublished data). The brief duration of G1 in chick embryo fibroblasts coupled with problems in obtaining stationary cultures (J. Wyke, personal communication) precluded a more thorough investigation of the requirements for pp60 in this response. These problems have been overcome in a Rat1 cell system. Welham *et al.*[29] have demonstrated that Rat1 *ts*LA29 cells can be rendered quiescent at restrictive temperature as morphologically normal cells. Quiescent cultures can be stimulated to transit G1, synthesise DNA and divide by addition of serum at restrictive temperature, or by shifting to permissive temperature in the absence of added factors[29]. Temperature shift and serum are not synergistic in this assay, indicating that both mitogens might function through the same intermediates[29]. Activation of thermolabile pp60 appears sufficient to initiate all events required for mitosis in these cells. Furthermore, since the G1 phase of the cycle extends for 16-20hrs in Rat1 cells, this system is amenable to detailed analysis. In experiments where the thermolabile pp60 is activated for periods of time before the cells are restored to restrictive temperature, the ability of these cells to enter S phase and mitosis appears to be dependent on them having passed a commitment point 4-6hrs prior to the onset of S phase (A.W. Wyke, personal communication). Preliminary data indicate that cells shifted to permissive temperature for periods shorter than that required to commit them to S phase appear to reset when returned to restrictive temperature, such that a second shift to permissive temperature does not result in more rapid onset of DNA synthesis. This result indicates that hypothetical early G1 events required for entry into S phase are not stable, perhaps implying the existence of short-lived molecular species

rather than stable cellular changes (*e.g.* cell mass). The simplest interpretation of these data is that pp60 activity is required throughout G1 until cells have passed the late commitment point, though it is not possible at present to rule out the existence of two or more discrete commitment points. Indeed, similar experiments reported by Durkin and Whitfield using *ts Src*-transformed NRK cells indicate that a short incubation at permissive temperature is sufficient to propel these cells into S phase, but not into mitosis[26]. Mitosis does however occur if cells are incubated at permissive temperature until S phase has started[26]. These results imply the existence of a second point in G1 or S at which pp60 is required to permit further cell cycle progression.

One rapid consequence of activating the mitogenic and transforming properties of *ts*LA29 in quiescent Rat1 cells is the induction in nuclear extracts of activities capable of binding consensus AP-1 oligonucleotides[29]. The induction of these activities occurs in the absence of detectable increases in c-*fos* and c-*jun* transcripts[29], and is insensitive to protein synthesis inhibition (A. W. Wyke, unpublished data). These results indicate that induction of AP-1 binding activity is regulated at a post-translational level upon activation of the thermolabile *Src* mutant. This laboratory and others have obtained data that suggest AP-1 transactivation activity is also increased in response to *Src* transformation[28,272]. Efforts are being made to correlate these activity changes with specific modifications, in particular phosphorylation, of the proteins involved (A. Catling and M. Frame, unpublished data). Interesting in this regard is the published data demonstrating that MAP kinase family members can phosphorylate c-*Jun* on N-terminal serine residues that are phosphorylated *in vivo*[269]. Pulverer *et al.*[269] and Binetruy *et al.*[270] demonstrate that enhanced phosphorylation of these c-*Jun* residues occurs in response to TPA and activated *Ras*, and is said to correlate with an increased transactivation activity of the protein. However, it is apparent that c-*Jun* from TPA stimulated U937 cells generates a number of other tryptic phosphopeptides in addition to those identified by Pulverer *et al.*[269], and indeed peptides with similar electrophoretic and chromatographic

properties can be derived from c-*Jun* phosphorylated *in vitro* with pp54 MAP kinase (A. Catling, E. Black, D. Gillespie and J. Woodgett, unpublished data). Interestingly, pp42, later identified as MAP kinase, was found by virtue of its tyrosine phosphorylation in *Src*-transformed cells, and may be a functional target for the tyrosine kinase activity of pp60 (see section I.9.2). We are at present mapping c-*Jun* phosphorylation sites in quiescent cells stimulated to divide by reactivation of a thermolabile *Src* mutant (A. Catling and M. Frame, unpublished). The post-translational modification of *Fos* in response to transformation by *Src* has not been examined, though it seems likely that this will also play a role in regulating AP-1 activity in response to pp60 kinase activity.

Since in these systems transformation accompanies mitogenesis, it is difficult to correlate molecular events with a biological endpoint. Studies by Hanafusa and his colleagues indicated that non-myristylated, and thereby non-transforming, *Src* proteins were able to prolong the lifespan of chick embryo neuroretinal cells in culture[32,33], suggesting that the transforming and mitogenic properties of pp60 might be dissociable. Similar mutants increased the saturation density of chick embryo fibroblasts[37], though the mitogenic activity was not assayed more directly in this study. A more comprehensive discussion of the properties of myristylation defective *Src* proteins is given in chapter VIII. In an effort to dissociate the mitogenic and transforming activities of pp60, I have constructed a myristylation defective protein based on the temperature sensitive mutant *ts*LA29 *Src*. One predicts that this mutant protein should retain thermolabile kinase properties, whilst being non-conditionally defective for membrane association and transformation. The biochemical and mitogenic properties of this mutant have been asssayed directly in quiescent Rat1 and chick embryo fibroblasts and the results of these experiments are presented in chapter VIII.

Each results chapter of this thesis consists of a brief introduction, a description of the experiments performed and the results obtained. Furthermore, each chapter contains a thorough discussion of the data obtained, factors affecting the interpretation of these data

and suggestions for further work. Chapter IX is a brief general discussion of the results obtained in the context of this field, though for discussion of individual experiments one should consult the appropriate results chapter.

Chapter II

II METHODS

II.A. CELL CULTURE AND TRANSFECTION

II.A.1 Cell Culture

Rat1 cells[302] and derivatives were maintained as stock cultures in Dulbecco modified Eagles medium (DMEM) supplemented with 5% newborn calf serum, 2mM glutamine, 37.5ug\ml penicillin and 100ug\ml streptomycin (Rat1 growth medium) in a humidified atmosphere containing 5% (v\v) CO_2. Stock cultures of Rat1, Rat1 f, Rat1 f29, Rat1 f32 and Rat1 fPrA, and two clones expressing low levels of 29A2 pp60, Rat1 f29A2 G and H, were maintained at the permissive temperature of 35°C. Rat1 f29A2 3B cells were maintained at the restrictive temperature of 40°C. (See chapters III and VIII for a description of these clones). Stock cultures were subcloned at a ratio of 1:10 every 3 days, as follows. Growth medium was aspirated from the monolayer, and the cells covered with 0.25% trypsin in CT buffer (see Materials). The majority of the trypsin solution was removed and the monolayers incubated at room temperature or 37°C until the cells could be detached by gentle agitation. Detached cells were resuspended in growth medium and replated at appropriate density. Cultures were generally maintained at the appropriate temperature for at least two days prior to use. This ensured that the cells had acquired the appropriate phenotype.

Quiescent cultures were prepared as follows. Cells were plated at approximately $7x10^3$ \ cm^2 in growth medium and grown at restrictive temperature for approximately two days. Medium was aspirated, and the subconfluent monolayers were washed with an equivalent volume of serum-free DMEM. Cells were refed DMEM supplemented with 0.2% newborn calf serum, 2mM glutamine and antibiotics at 2ml \ 35mm dish or 5ml \ 60mm dish (~0.2ml \ cm^2), and incubated for a further 40-48hrs at restrictive temperature[29]. This rendered Rat1 cells and Rat1 cells expressing *ts src* mutants quiescent.

Chick embryo fibroblasts (CEF) were prepared as described[303]. Cultures were maintained in DMEM supplemented with 10% tryptose phosphate broth, 2mM glutamine, 5% newborn calf serum, 1% heat-inactivated (2hrs at 56°C) chicken serum

and antibiotics (chick cell medium) in humidified incubators containing 5% (v\v) CO_2. To minimise plating losses due to transformation, stock cultures of infected CEF were maintained at restrictive temperature (41°C). Cells were subcultured approximately 1:5 every 2-3 days and were maintained in culture for no more than four weeks. Subculturing was performed as described for Rat1 cell cultures except cells were resuspended in chick cell medium. Quiescent cultures of CEF were prepared essentially as described for Rat1 cells, except cultures were maintained at 41°C.

II.A.2 Cell Storage

Stocks of cells used in this work were maintained in liquid nitrogen. Rat1 cells and derivatives were trypsinised and resuspended in DMEM lacking glutamine and antibiotics but supplemented with 20% fetal calf serum and 7.5% dimethylsulphoxide. Suspensions were aliquoted in 1-2ml Nunc cryotubes and frozen well insulated at -70°C. One to two days later, frozen aliquots were stored in liquid nitrogen. Chick embryo fibroblasts were stored in a similar fashion except storage medium was fetal calf serum supplemented with 7.5% dimethylsulphoxide.

II.A.3 Transfection and G418 selection of Rat1 cells

Transfection of Rat1 cells was generally carried out using Lipofectin reagent according to manufacturers instructions. Briefly, $5x10^5$ Rat1 cells were plated in a 60mm dish at permissive temperature one to two days prior to transfection. The following manipulations were carried out in the wells of a polystyrene 96 well plate. 1ug fpGV-1-based plasmid DNA[301] (see chapter III) was diluted to 50ul in sterile distilled water. In a separate well, 30ug of Lipofectin reagent was diluted to 50ul with sterile distilled water. The diluted DNA and Lipofectin were combined, mixed gently and incubated for fifteen minutes at room temperature. (Mock-transfection mixes lacking DNA were prepared in parallel to act as controls during subsequent G418 selection). During this incubation period, cells were washed twice with serum-free DMEM supplemented with 2mM glutamine and antibiotics, and refed 3mls of the same medium.

The DNA-Lipofectin mixture was added dropwise to the cells with swirling, and transfected cultures incubated for 6hrs at permissive temperature. An equal volume of DMEM supplemented with 10% newborn calf serum, 2mM glutamine and antibiotics was added and the dishes incubated at permissive temperature for a further 24-48hrs. Cultures were then trypsinised and replated in T175 flasks. G418 was added the following day to a final concentration of 1mg \ ml, and selection effected for 14 to 21 days at permissive temperature. G418-containing medium was replaced every 3-4 days. This resulted in the death of mock-transfected cultures and the appearance of 100-300 colonies in transfected flasks. In some experiments, it was found necessary to carry out transfection and selection at restrictive temperature. The method used was otherwise identical.

Occasional transfections were carried out using the calcium phosphate co-precipitation method[304]. Rat1 cells were plated at $5x10^5$ \ 90mm dish and incubated overnight at permissive temperature. The precipitate was formed as follows:

a) 440ul of TE buffer (10mM tris-HCl, 1mM EDTA, pH 8.0) and 50ul of 2.5M $CaCl_2$ were added to a sterile plastic bijou.

b) 10ug of fpGV-1 based plasmid DNA in 10ul TE buffer was added to the bijou and mixed gently.

c) the mixture from b) was added to 500ul of 2xHBS (46mM HEPES, 280mM NaCl, 1.3mM $Na_2HPO_4.2H_2O$, pH 7.1) in a sterile plastic bijou and mixed gently. The precipitate was allowed to form by incubation at room temperature for 30min.

Fresh medium was added to the cells, and the precipitate added dropwise with swirling. Transfections were incubated overnight at permissive temperature in an humidified atmosphere with 5% (v\v) CO_2. The following day, transfected cultures were subcultured at a ratio of 1:10 and incubated overnight. G418-selection was carried out as described above.

II.A.4 Cloning Of Rat1 Derivatives

Well separated colonies were simultaneously scraped and collected using drawn Pasteur pipettes. Collected colonies were replated in 6 well plates and grown further to allow immunoblot analysis, single-cell cloning and, if necessary, liquid nitrogen storage. Typically, 8-12 colonies from 4-5 independent transfections were analyzed and cloned. Clonal lines were generated by plating 50-100 cells in 96 well plates. Positive wells were trypsinised and cells expanded for immunoblot analysis and storage. Care was taken to avoid harvesting colonies resulting from more than one founder cell. Colonies and clonal lines were maintained in normal growth medium lacking G418.

II.A.5 Transfection of Chick Embryo Fibroblasts

Secondary CEF were plated at approximately $5x10^5$ \ T25 flask and incubated overnight at 41°C. Calcium phosphate-DNA co-precipitates[304,305] were formed as follows :

a) 10ug of RCAN based plasmid DNA[306] (see chapter III) was mixed with 240ul 0.2xSSC (20xSSC stock is 175.3g NaCl, 88.2g trisodium citrate per liter, pH 7.0) in a sterile plastic bijou

b) 28ul of 2.5M $CaCl_2$ was added and mixed gently

c) 250ul of 2xHEBS (2xHEBS is 274mM NaCl, 10mM KCl, 1.4mM $Na_2HPO_4.7H_2O$, 11mM glucose, 38mM HEPES, pH 7.1) was added and the mixture incubated at room temperature for 30min.

The resulting precipitate was added to the cells, and the flasks incubated at 41°C for 4hr. Medium was aspirated and cells washed with 3ml chick cell growth medium. Cells were then shocked for 4min at room temperature in 1ml of HEBS-glycerol (1xHEBS with 15% glycerol). Cultures were then washed briefly in 5ml chick cell growth medium before refeeding and further incubation at 41°C. Cultures typically reached confluence 4-7 days post-transfection whereupon they were subcultured at a ratio of 1:5. Uniform infection, as judged by Western blot and morphology at permissive temperature, was achieved after 1-3 passages.

II.A.6 Soft Agar Assays

Soft agar assays were used as an indicator of transformation in Rat1 transfectants[307]. 40ml of 2.5% Difco bacto-agar in a 200ml Duran bottle was melted in a microwave and cooled to 44°C. 40ml of 2xDMEM (see below) and 20ml newborn calf serum was added to the cooled agar, and the volume made up to 200ml with 100ml 1xDMEM. The resulting 0.5% agar solution was maintained at 44°C for pouring (~5ml \ 60mm plate). Base plates were allowed to set at room temperature or 4°C. The required number of cells (10^4 Rat1 *src* cells, 10^5 Rat1 or Rat1 f cells) were resuspended in 0.5ml Rat1 growth medium and mixed in a plastic universal with 1ml cooled (~37°C) base agar. A base plate was carefully overlayed with this soft agar \ cell mix and allowed to set at room temperature. Soft agar assays were typically performed in triplicate, and colonies were counted after 14 days incubation at the appropriate temperature.

2xDMEM

10xDMEM	20ml
200mM glutamine	2ml
100mM pyruvate	2ml
7.5% $NaHCO_3$	10ml
1M HEPES	4ml
Sterile distilled H_2O	58ml
+ antibiotics	

II.B. PROTEIN CHEMISTRY AND METABOLIC LABELLING

II.B.1.1 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to resolve proteins according to their apparent molecular weight. Gels were subject to electrophoresis essentially according to Laemlli[308] using a Biorad Protean II apparatus. Typical gels were 10% with respect to acrylamide, although the percentage cross-linker used varied with the technique being used : for Western blots and immunoprecipitations, a stock solution containing 29.2% (w\v) acrylamide, 0.8% (w\v) *bis*-acrylamide was used, whereas for *in vitro* kinase assay gels, the stock solution used

was 29.625% (w\v) acrylamide, 0.375% (w\v) *bis*-acrylamide. The latter ratio of acrylamide and *bis*-acrylamide was found to improve resolution in the 40-60kDa region of the gel. Resolving gels were prepared by mixing the following (for two gels):

stock acrylamide \ *bis*-acrylamide	35ml
1.5M tris-HCl, pH 8.8	39.2ml
distilled H_2O	30.5ml
10% SDS	1.05ml
10% ammonium persulphate	0.35ml
TEMED	0.065ml

The gel mix was pipetted between the gel plates and overlayed with water-saturated butan-2-ol. After polymerisation had occurred (0.5-1hr) the overlay was removed and the top of the resolving gel washed extensively with distilled water. Excess liquid was removed using Whatman 3MM or filter paper, and the comb was inserted before the stacking gel was poured. The stacking gel consisted of (for two gels):

30% acrylamide \ *bis*-acrylamide	8.35ml
0.5M tris-HCl, pH 6.8	6.25ml
distilled H_2O	30.2ml
10% SDS	0.5ml
10% ammonium persulphate	0.25ml
TEMED	0.05ml

The stacking gel was allowed to polymerise (~30min) before assembly into the tank. Both reservoirs were filled with running buffer (5x stock, g \ l tris base, 15, glycine, 72, SDS, 5 in distilled water, ~pH 8.3). Wells were flushed extensively with running buffer, and bubbles removed from the lower surface of the gel plates before the samples were loaded.

Protein samples were denatured in sample buffer (50mM tris-HCl, pH 6.8, 30% glycerol, 2.5% SDS, 2% β-mercaptoethanol) by heating at 95-100^{o}C for 3-7min. Total cellular proteins were extracted by the addition of sample buffer directly to the tissue culture plate (approximately 0.5ml \ 60mm dish). Lysates were sonicated to shear any

genomic DNA present. For Western analysis, 50ul of lysate from a confluent 60mm dish of Rat1 or CEF cells was resolved together with an equivalent amount (by Coomassie brilliant blue staining) of protein from the other cultures of interest. Proteins purified by immunoprecipitation were resuspended and denatured in an equivalent volume of 2x sample buffer.

II.B.1.2 TCA-Precipitation of Labelled Proteins

To ensure consistent spot intensity between individual 2-D gels, equivalent amounts of TCA-precipitable radioactivity were loaded on each gel. Typically, 10ul of labelled lysate was mixed with 100ul of a 1mg \ ml solution of BSA in distilled water in a precooled 1.5ml eppendorf. 1ml of ice-cold 10% TCA was added, mixed and the tube incubated on ice for 30min. The precipitate was collected on a Whatman GF-C filter and washed extensively with cold 10% TCA. Filters were washed with 95% ethanol, dried thoroughly and counted in 10ml Ecoscint.

II.B.1.3 Protein Estimation

Protein concentration in cell lysates was estimated using the Micro BCA assay marketed by Pierce. Protein concentration was normalised prior to pp60 immunoprecipitation. 60mm dishes were lysed in 1ml NP40 buffer (see section II.B.4.1). 24ul of lysate was diluted to a total of 1.2ml in distilled water, and 1ml of this diluted lysate was mixed with an equal volume of the assay reagent. Lysis buffer blanks were included in all assays. Micro BCA assay reagent was prepared according to manufacturers' instructions. Reactions were incubated at 60^{o}C for 30min, and cooled to room temperature prior to reading at 562nm (1cm path length). Protein concentration was estimated by comparison to a standard curve constructed using dilutions of bovine serum albumin under the same assay conditions.

II.B.2 Metabolic Labelling

II.B.2.1 Tritiated-thymidine Labelling

Pulse labelling with tritiated thymidine was used to estimate DNA synthesis in quiescent cultures stimulated to proliferate in response to serum or temperature shift[29]. Quiescent cultures of Rat1 derivatives or infected CEF in 35mm dishes were prepared as described in II.A.1. Cells were stimulated to proliferate by addition of newborn calf serum to 5% (v \ v) at restrictive temperature or by shift to permissive temperature in the absence of added factors. At appropriate intervals, 2uCi of ^{3}H methylthymidine (20uCi \ ml in PBS) was added, and incubation continued for 1hr. Dishes were then removed to ice and the labelling medium aspirated. Monolayers were washed with 2ml ice-cold PBS, then precipitated *in situ* with 2x2ml ice-cold 5% TCA on ice for 2min. Precipitates were washed twice with 2ml ice-cold methanol and air-dried. Precipitated material was dissolved by rocking in 0.5ml 0.02M NaOH for 30min at room temperature. Incorporation of labelled thymidine into precipitable material was measured by liquid scintillation counting of 50ul dissolved material in 5ml Ecoscint.

II.B.2 2 Methionine Labelling

II.B.2.2.1 Immunoprecipitation of pp60

Growing cultures of Rat1, Rat1 f29, Rat1 f32 and Rat1 fPrA at permissive temperature were labelled in T25 flasks as follows. Culture medium was aspirated and cells washed once with 5ml \ flask of serum-free DMEM supplemented with 2mM glutamine and antibiotics but lacking methionine. Cultures were then incubated for 1hr in 2ml \ flask of the same medium before the addition of 300uCi of ^{35}S-methionine. Labelling was for 3hrs. Immunoprecipitates were prepared as described for *in vitro* kinase assays (II.B.4.1) with the exception that lysates were incubated with MAb327[309] for 4hrs. Immune complexes were resolved by SDS-PAGE and labelled proteins were detected by fluorography of the dried gel.

II.B.2.2.2 Methionine Labelling for 2D-gel Electrophoresis

Quiescent cultures of Rat1 and Rat1 f32 were prepared in 35mm dishes or in 24 well plates as described in II.A.1. In some experiments, pulse labelling was used in an effort to identify proteins whose synthesis was modulated by activating *ts*LA32 *Src*. Cultures in 35mm dishes were shifted to permissive temperature for 0 (i.e. labelled at 40^{o}C), 1, 4, 7 or 24hrs before labelling. Cultures were quickly washed with 2ml \ dish serum-free DMEM supplemented with 2mM glutamine and antibiotics but lacking methionine, and incubated for 30min in 1ml \ dish of the same medium at the appropriate temperature. 200uCi of ^{35}S-methionine was added to each dish and labelling effected for 30min at the appropriate temperature. Labelled cells were washed briefly in PBS and lysed *in situ* in 450ul 2D-gel sample buffer (9.9M urea, 4% NP40, 100mM DTT, 2.2% ampholytes pH 3-10, 2D-gel optimised).

Other experiments were undertaken in parallel with ^{32}P-orthophosphate labelling. These experiments were designed to distinguish between dephosphorylation or proteolysis of specific phosphate labelled proteins whose ^{32}P content decreased in response to temperature shift. For these experiments, Rat1 and Rat1 f32 cells were grown and rendered quiescent in 24 well plates as described. Cultures were starved of methionine for 30min before labelling with 200uCi \ ml ^{35}S-methionine in methionine-free, serum-free DMEM supplemented with 2mM glutamine and antibiotics. Labelling was for a total of 4hr, during which time cultures were shifted to permissive temperature for 0, 15, 30, 60 or 120min. Cultures were placed on ice and washed twice with ice-cold PBS before lysis *in situ* with 100ul \ well 2-D gel sample buffer. In these experiments, 500,000 cpm of TCA-precipitable material was resolved for each sample. 2D-gel electrophoresis was expertly performed by Lynn McGarry using a Millipore Investigator 2D-gel system. Labelled proteins were detected by fluorography at -70^{o}C for 27-32 days for the pulse-labelling experiments or for 3-14 days for the 4hr labelling experiments.

II.B.2.3 Orthophosphate Labelling

II.B.2.3.1 Phosphoamino acid Analysis of Whole Cell Lysates

Phosphoamino acid analysis was carried out according to the method of Cooper *et al.*[310] Briefly, cultures of Rat1, Rat1 f29, Rat1 f32 and Rat1 fPrA were maintained in exponential growth for at least two days prior to labelling with carrier-free ^{32}P-orthophosphate. Cells in T25 flasks were washed twice in phosphate-free DMEM supplemented with 2mM glutamine, antibiotics and 5% dialyzed newborn calf serum. (Serum was dialyzed overnight at 4°C against sterile distilled water). Cells were then incubated for 1hr in 2ml \ flask of the same medium at the appropriate temperature before addition of label to 1mCi \ ml. Labelling was for 18-20hrs at the appropriate temperature. Cultures were removed to ice and washed twice with ice-cold PBS before lysis in 1ml of modified RIPA buffer (10mM tris-HCl, pH 7.0, 150mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 2mM EDTA, 10mM sodium pyrophosphate, 10mM β-glycerophosphate, 10uM sodium *ortho*vanadate, 2mM PMSF and ~1ug \ ml aprotinin). Cells were incubated on ice for 10min, then scraped using disposable cell scrapers. Flasks were left for an additional 10min to solubilize adherent structures completely before scraping and transfer to screw-cap eppendorf tubes. Lysates were centrifuged at 14,000 rpm for 20min at 4°C in a Sorvall SS34 rotor. 300ul of supernatant was carefully removed to a fresh tube containing 400ul of NTE (10mM tris-HCl, pH 7.5, 100mM NaCl, 1mM EDTA) and 400ul NTE-saturated phenol at room temperature. Tubes were capped tightly and vortexed for 30sec, before centrifugation for 1min in a bench top microcentrifuge at room temperature. The aqueous layer was discarded, and the phenol phase extracted with a further 800ul NTE. The phenol layer and interface material was transferred to a 30ml Corex tube, to which was added 13ml water and 2ml 100% TCA. After careful thorough mixing, the tubes were placed on ice for 1hr. Precipitated material was collected by centrifugation at 8000 rpm for 10min at 4°C in a Sorvall SS34 rotor, and the supernatant decanted from the pellet of precipitated protein and detergent. The pellet was extracted with 5ml chloroform \ methanol (2:1) at room temperature, and the protein precipitate collected with a 10min centrifugation as before

but at room temperature. Air-dried pellets were dissolved in 200ul 6M HCl and transferred to Wheaton vials with 2x100ul 6M HCl washes. Vials were sealed and hydrolysis carried out for 2hrs at 110°C. Samples were allowed to cool to room temperature before all liquid was collected in the bottom of the vials. The hydrolysate was diluted with an equal volume of distilled water and dried under vacuum over solid NaOH and P_2O_5. Dried residues were washed twice by redissolving in 200ul distilled water and drying under vacuum. Residues were finally redissolved in 30ul pH 1.9 buffer (pH 1.9 buffer is 45:156:1799 98-100% formic acid:glacial acetic acid:water) and stored at -20°C. Approximately 30-50% was loaded on a single plate together with unlabelled standards as outlined in section II.B.2.3.3.

II.B.2.3.2 Phosphoamino acid Analysis of Individual Proteins

Proteins were resolved by SDS-PAGE and located by autoradiography of the fixed or unfixed dried gel. Gel slices were excised and proteins eluted essentially as described by Beemon and Hunter[311]. Briefly, the backing paper was scraped from the dried gel slices and the bands cut into small pieces. These pieces were allowed to swell in a small volume of 50mM NH_4HCO_3, 0.1% SDS before Dounce homogenisation. More buffer was added as necessary. The homogenate, together with washes was transferred to a plastic tube (Falcon 2051) and made 5% with respect to β-mercaptoethanol. The homogenate was boiled for 5min and then shaken overnight at 37°C. Gel fragments were pelleted by centrifugation at 10,000rpm for 10min at room temperature in a Sorvall HB4 rotor, and the supernatant transferred to a fresh tube. The gel fragments were washed with a volume of buffer half that used originally for 2hr at 37°C. The gel fragments were pelleted again and the second supernatant pooled with the first. RNase A was added to 10ug \ ml, mixed thoroughly, and the solution was made 20% in TCA. Proteins were precipitated for 1-4hrs on ice and collected by centrifugation for 15min at 10,000 rpm in a Sorvall HB4 rotor at 4°C. The tube was drained thoroughly by inversion, and the pellet was washed twice with ethanol at -20°C with the same

centrifugation conditions. The dried pellet was resuspended in 6M HCl and hydrolyzed as described above (II.B.2.3.1).

II.B.2.3.3 Thin Layer Separation of Phosphoamino acids

Acid hydrolysates from phosphate-labelled cells or individual proteins were separated according to Cooper *et al.*[310] Dried hydrolysates were dissolved in pH 1.9 buffer and spotted in 0.3-0.5ul aliquots onto a 20x20cm cellulose thin layer plate (Kodak, 100um). 0.5ug of each unlabelled phosphoamino acid (phosphoserine, phosphotyrosine and phosphothreonine) was loaded on each origin to serve as markers following electrophoresis. Hydrolysates were resolved by electrophoresis at pH 1.9 towards the anode at a constant 700V for 2hr. After thorough drying, the plates were rotated and electrophoresis carried out at pH 3.5 towards the anode at a constant 600V for 1hr 40min. (pH 3.5 buffer is 10:100:1890 pyridine:glacial acetic acid:water). The thin layer plate was maintained below 10^{o}C during electrophoresis using a block cooled with tap water at approximately 1l \ min. Following electrophoresis the plate was dried thoroughly before standards were visualised using ninhydrin (0.2% in ethanol, developed at 80^{o}C). Dried plates were exposed to X-ray film.

A quantitative estimate of the abundance of individual phosphoamino acids was required after separation of total cell hydrolysates. After autoradiography, the areas of cellulose containing the ninhydrin stained standards, together with appropriate background areas were simultaneously scraped and collected using a glass wool-plugged blue tip attached to a vacuum line. The collected material was expelled into a scintillation vial containing 10ml of Ecoscint and vortexed vigorously before counting for 10min. Abundance is expressed as a percentage of total radioactivity incorporated into the three phosphohydroxyamino acids.

II.B.2.4 Orthophosphate Labelling for 2D-Gel Electrophoresis

Quiescent cultures of Rat1 and Rat1 f32 were prepared as described in II.A.1 in 35mm dishes or 24 well plates. Cultures were washed in phosphate-free DMEM

supplemented with 2mM glutamine and antibiotics and starved of phosphate for 30min in the same medium. Carrier-free ^{32}P-orthophosphate was added to 0.5-1mCi \ ml and labelling effected for a total of 4hr. Cultures were shifted to permissive temperature for 0, 15, 30, 60 or 120min during this period. After labelling, cells were placed on ice and washed twice with ice-cold PBS before lysis in 2-D gel sample buffer (see section II.B.2.2.2, 100ul per well or 0.5ml per T25 flask). Approximately 75,000 or 500,000 cpm of TCA-precipitable material from each well or flask respectively was resolved by 2-D gel electrophoresis as described above (II.B.2.2.2). Fixed, dried gels were exposed to film for 2-7 days at -70^{o}C.

II.B.3 V8 Protease Analysis of Mutant *Src* Proteins

Growing cultures were labelled exactly as described for the phosphoamino acid analysis (section II.B.2.3.1). After lysis as described, 300ul of the clarified RIPA lysate was incubated for 1hr on ice with 50ul normal rabbit serum. The lysate was then cleared by incubation with 250ul heat-killed, formalin fixed *Staphylococcus aureus*. After brief centrifugation, the supernatant was transferred to a fresh tube and mixed with 1ug MAb327[309]. Immunoprecipitates were formed overnight on ice. Complexes were collected by incubation with 50ul *S. aureus* precoated with rabbit anti-mouse IgG (300ug \ ml, 2hrs 4^{o}C) for 2hrs on ice. Immune complexes were washed three times in lysis buffer containing phosphatase and protease inhibitors (10mM sodium pyrophosphate, 10mM B-glycerophosphate, 10uM sodium *ortho*vanadate, 2mM EDTA, 2mM PMSF, ~1ug \ ml aprotinin), and then twice in 20mM PIPES-NaOH, pH 6.8. Final pellets were resuspended in SDS-PAGE sample buffer and separated on a 10% acrylamide gel. pp60 was located by autoradiography of the unfixed dried gel, and limited V8 proteolysis carried out according to Harlow and Lane[312]. Protease was used at 1, 10 and 100ng per well, and digestion products were separated on a 12% acrylamide gel. After drying and fixing, digestion products were visualized by autoradiography at -70^{o}C.

II.B.4 *In vitro* Protein Kinase Assays

II.B.4.1 Immunoprecipitation of pp60

In vitro kinase activity was measured using a conventional assay[41,42]. pp60 was partially purified by immunoprecipitation from cultures at the appropriate temperature, and kinase activity towards self or exogenous substrates measured in the presence of Mn^{2+}, Mg^{2+} and [γ^{32}P] ATP. Typically, subconfluent cells in 60mm dishes were placed on ice and washed once with ice-cold PBS. Cultures were lysed in 1ml of NP40 buffer (20mM tris-HCl, pH 6.8, 150mM NaCl, 1% NP40, 20mM sodium pyrophosphate, 10uM sodium *ortho*vanadate, 2mM PMSF, ~1 ug \ ml aprotinin) for 20min on ice, before scraping to cooled 1.5ml eppendorf tubes. Lysates were clarified by centrifugation at 14,000 rpm for 10min at 4°C in a microcentrifuge, and the protein concentration of the supernatant estimated using the Micro BCA assay supplied by Pierce. 200ug of protein from each lysate was diluted to 1ml in cold lysis buffer and mixed with 25ul normal rabbit serum on ice for 30min. Normal rabbit serum was removed by the addition of 250ul heat-killed, formalin fixed *S. aureus* cells (10% (w\v), washed twice in lysis buffer) for 30min on ice. After removal of bound complexes by brief centrifugation at 4°C, the supernatant was mixed with 0.2ug of MAb327[309] and incubated for 1hr on ice. Specific immune complexes were collected by the addition of 50ul precoated *S. aureus* cells for 30min on ice. Precoating was carried out by incubating a washed 10% (w\v) suspension of *S. aureus* cells with 300ug \ ml rabbit anti-mouse IgG for approximately 2hrs at 4°C. Precoated cells were washed twice with lysis buffer and resuspended in lysis buffer at 10% (w\v) before use. Immune complexes were washed twice in NP40 buffer and once in kinase buffer (100mM PIPES-NaOH, pH 6.8, 20mM $MnCl_2$, 10uM sodium *ortho*vanadate). 25ul of kinase buffer was added to each pellet and immunoprecipitates were frozen at -70°C for later assay.

In some experiments, polyclonal rabbit antiserum was used to prepare immunoprecipitates. Typically, 5ul of antiserum (G950.4, MW2[94] or TBR-RLE[313]) was

used in place of MAb327, and complexes collected on uncoated *S. aureus* cells. The protocol used was otherwise identical.

II.B.4.2 *In vitro* Kinase Assay

Most kinase assays utilized acid denatured enolase as a protein substrate. This substrate was prepared from an ammonium sulphate precipitate of rabbit muscle enolase as follows[42]. An aliquot of the precipitate was centrifuged at 14,000 rpm for 30min at 4°C and the supernatant discarded. The pellet was resuspended in buffer A at 10mg \ ml (buffer A is 50mM HEPES-NaOH, pH 7.0, 1mM DTT, 1mM $MgCl_2$). The suspension was stored on ice for 30min before an equal volume of glycerol was added. Solubilized enolase was stored in 25ul aliquots at -70°C. Immediately prior to use, the substrate was acid denatured by incubation with an equal volume of 50mM acetic acid at 30°C for 10min. Assays employing enolase as an exogenous substrate contained:

4ul acid denatured enolase

4ul distilled water

1.5ul kinase buffer

0.5ul [γ^{32}P] ATP (5uCi, 3000Ci \ mmol)

and were initiated by the addition of 10ul of resuspended immunoprecipitate. Kinase buffer replaced enolase in assays of immunoprecipitates formed using TBR-RLE[313]. Assays were incubated at 30°C for 20min before termination with 500ul of wash buffer (wash buffer is 10mM tris-HCl, pH 8.0, 400mM NaCl, 1mM EDTA, 1% NP40, 0.25% sodium deoxycholate). Pelleted *S. aureus* cells supporting immune complexes were washed twice with wash buffer before resuspension in 20ul of 2x SDS-PAGE sample buffer. Under these wash conditions pp60 and enolase remain with the pellet. Proteins were resolved by SDS-PAGE using 10% acrylamide gels, and fixed, dried gels were typically exposed to X-ray film for 1-4hrs at room temperature or -70°C.

Other assays employed a commercially available gastrin-related peptide substrate, Raytide. Lyophilized peptide was reconstituted to a concentration of 1mg \ ml in kinase buffer and stored at 4°C. Each assay contained the following components :

3ul distilled water

1.5ul kinase buffer

0.5ul [γ^{32}P] ATP (5uCi, 3000Ci \ mmol)

5ul Raytide substrate in kinase buffer

and reactions were initiated by the addition of 10ul of resuspended immunoprecipitate. Reactions were terminated after 20min at 30°C by the addition of 80ul 10% H_3PO_4. *S. aureus* cells were pelleted by microcentrifugation, and 80ul aliquots of the supernatant were spotted on 2x2cm squares of phosphocellulose paper (Whatman P81). Filters were washed extensively with 0.5% H_3PO_4 and once with acetone. After drying at room temperature, filters were counted in 5ml Ecoscint. Radioactivity was normalized to Rat1 controls at the appropriate temperature.

II.B.5 Western Blotting

Extracts of interest were resolved on SDS-PAGE gels together with prestained molecular weight markers. Proteins were blotted onto nitrocellulose membranes using a Sartorius semi-dry blotting apparatus as follows. Twelve pieces of Whatman 3MM paper and one piece of membrane were cut to the size of the gel to be blotted and equilibrated in transfer buffer (60mM tris, 50mM glycine, 1.6mM SDS, 20% (v\v) methanol). The gel was rinsed in transfer buffer and placed upon 6 pieces of 3MM paper. The membrane was placed on the gel and topped with the remaining pieces of 3MM. Air bubbles were carefuly removed and transfer effected at full power for 15-40min. The membrane was removed after transfer of appropriate prestained markers was complete. At this stage, the membrane could be dried and stored at 4°C if not processed immediately. The membrane was blocked for 30min at room temperature in blotto (2.5% non-fat milk powder, 0.1% NP40 in PBS) before incubation with antiserum (typically anti-*src*, MW2[94] or anti-*gag*, 5202, 1:5000 in blotto) overnight at 4°C. The membrane was subsequently washed three times in blotto before incubation with anti rabbit IgG conjugated to alkaline phosphatase (1:5000 in blotto) for 2hr at room temperature. The membrane was washed extensively with blotto and alkaline phosphatase buffer (100mM

tris-HCl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$) before development with chromogenic alkaline phosphatase substrate. This substrate was prepared by dissolving 16mg nitro blue tetrazolium and 8mg of 5-bromo-4-chloro-3-indolyl phosphate in 50ml of alkaline phosphatase buffer. Substrate solution was added to the membrane and developed as necessary before termination in running water.

II.C. RECOMBINANT DNA TECHNIQUES[314]

II.C.1.1 Host Cells

E. coli strains HB101 and TG1 were obtained from V. Fincham. Strain DH1 was obtained as competent cells from D. Crouch. Host cells and derivatives were grown at 37°C with good aeration in L-broth (see materials) supplemented with appropriate antibiotics. Ampicillin and kanamycin were used at final concentrations of 100 and 25ug \ ml respectively.

II.C.1.2 Competent Cells

2ml of an overnight culture of HB101 or TG1 was diluted to 200ml in sterile L-broth and grown at 37°C with good aeration until the absorbance at 550nm reached 0.4-0.6 (1cm path length, L-broth blank). The log phase cells were collected by centrifugation at 10,000 rpm for 5min at 4°C in a Sorvall GSA rotor. The supernatant was decanted and the pellet carefully resuspended in 200ml sterile, ice-cold 80mM $CaCl_2$. The suspension was incubated on ice for 15min before the cells were collected by centrifugation according to conditions described above. The pellet was gently resuspended in 20ml of sterile, ice-cold 80mM $CaCl_2$ \ 15% glycerol and stored on ice for 15min. The suspension of competent cells was then divided into 0.5-1ml aliquots on dry ice before storage at -70°C.

II.C.1.3 Transformation of Bacterial Hosts

II.C.1.3.1 HB101 and DH1

E. coli strains HB101 and DH1 were used for routine plasmid transfections. The appropriate amount of plasmid DNA or ligation mix was pipetted into a precooled

eppendorf tube. An aliquot of competent cells was thawed on ice, and 200ul mixed gently with the DNA of interest. After incubation on ice for 30min, the cells were heat shocked by incubation at 25°C for 15min. 0.5ml of prewarmed L-broth was added and the suspension was placed at 37°C for 30min to allow expression of the antibiotic resistance marker. Cells were then pelleted by brief centrifugation and resuspended in a small volume of supernatant before plating on agar plates (2% agar in L-broth) supplemented with the appropriate antibiotic. Plates were inverted and incubated overnight at 37°C to allow colony formation.

II.C.1.3.2 TG1

E. coli strain TG1 was used for the propagation of bacteriophage M13. Competent cells were thawed on ice and 100-200ul pipetted to a plastic universal. The appropriate amount of phage DNA (single or double stranded) or ligation mix was mixed in gently, and the suspension stored on ice for 40min. During this time, an overnight culture of TG1 was diluted 1:10 in 2YT broth and grown further at 37°C. (2YT broth is 1% bactotryptone, 1% yeast extract, 1% NaCl (w\v) in sterile distilled water). 10min before the transfection was completed, a lawn mix was prepared. This consisted of (for each transfection):

200ul log-phase TG1 cells

25ul IPTG (24mg \ ml in distilled water)

30ul X-gal (2% in dimethylformamide)

This mixture was left at room temperature whilst the transfection was completed. In addition, top agar was prepared for the plates by melting 0.5% agarose in 1YT broth in a microwave. This was allowed to cool in a 42°C water bath whilst the transfection was completed. The DNA \ competent cell mix was heat shocked at 42°C for 2min and then mixed with 200ul lawn mix at room temperature. 3-5ml of top agarose was added and the mixture poured onto a prewarmed YT plate (2% agar in 2YT broth). The top agarose was allowed to set at room temperature before the plates were incubated inverted overnight at 37°C. M13mp18 and M13mp19 encode an IPTG-inducible β-galactosidase

marker which in the presence of the chromogenic substrate X-Gal yields a blue colouration. This enables one to distinguish between plaques containing recombinant bacteriophage and religated parental bacteriophage.

II.C.1.4 Glycerol Stocks

E. coli host strains or hosts bearing useful plasmids were stored as glycerol stocks for future retrieval. Stationary cultures in L-broth were mixed with an equal volume of glycerol in sterile plastic bijous and frozen at -70^{o}C. Cells were retrieved from these frozen stocks using sterile plastic loops.

II.C.2 Restriction Digests and Agarose Gel Electrophoresis

II.C.2.1 Restriction Digests

Restriction digests were carried out in small volumes buffered using buffer concentrates supplied with the enzymes. Small quantities of plasmid DNA (<2ug) were digested in a total volume of 20ul using 1-10 units of enzyme per ug of DNA, depending on the enzyme used and the number of sites present. Digestion was allowed to proceed for 1-2hr at 37^{o}C. Larger, preparative digests were carried out in proportionately larger volumes. For double digests, manufacturers' information was consulted and the appropriate buffer used. Digests of genomic DNA were carried out essentially as described except digestion was normally continued overnight. Ocassionally, a second aliquot of enzyme was required for complete digestion of genomic DNA. Reactions were terminated by the addition of one tenth volume gel loading buffer (see section II.C.2.2).

II.C.2.2 Agarose Gel Electrophoresis

DNA fragments were resolved on non-denaturing agarose gels and visualized by ethidium bromide staining. Agarose gel concentration varied according to the fragments under study, though 0.8% gels were usually sufficient. Gels mixes were prepared by microwaving the agarose in the appropriate volume of 1xTAE buffer (50xTAE buffer is 2M tris base, 50mM EDTA, 57.1ml \ l glacial acetic acid) until all particles had

dissolved. The gel mix was cooled to approximately 50°C, made 1ug \ ml in ethidium bromide and cast in the appropriate gel former. Gels were allowed to set at room temperature before removal of well formers and installation in the tank. Electrophoresis was carried out in 1xTAE buffer. Samples for electrophoresis, and molecular weight standards (bacteriophage λ, HindIII digested, bacteriophage ϕx174, HaeIII digested) were mixed with one tenth volume of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll in water), loaded and resolved by electrophoresis towards the anode. A permanent record of the gel was made using a polaroid camera whilst the gel was illuminated from below using a *uv* source.

II.C.3 Elution, Extraction and Quantitation Techniques

II.C.3.1 Elution of DNA Fragments from Agarose Gels

Fragments of interest were recovered from agarose gels by centrifugation, electroelution or "Genecleaning". Recovery by centrifugation was found to be particularly straightforward and reliable. The gel slice containing the fragment of interest was removed and trimmed of excess agarose over a *uv* source. A small (0.5ml) eppendorf tube was punctured at the base and plugged with glass wool. The gel slice was placed upon the glass wool, and the plugged tube was placed inside a 1.5ml eppendorf. The tubes were then centrifuged at 5000 rpm for 5-10min at room temperature in a bench top minifuge. This resulted in the DNA and gel buffer collecting in the larger eppendorf tube, with the agarose remaining with the glass wool. The fragments could be used directly for ligation, or could be concentrated by ethanol precipitation if required.

Electroelution was used on few occasions. The trimmed gel slice was placed in a dialysis bag with a minimum of TAE buffer and electrophoresis continued for 30min at 170mA. The current was then reversed for 2min to release the DNA from the face of the dialysis membrane. After checking that elution was complete by *uv* transillumination, the TAE buffer containing the fragment of interest was extracted twice with phenol \ chloroform and once with chloroform before ethanol precipitation overnight at -20°C.

"Geneclean" was used according to manufacturers' instructions.

II.C.3.2 Extraction with Organic Solvents and Ethanol Precipitation

DNA samples were extracted with chloroform and \ or phenol as necessary to remove contaminants that might interfere with subsequent manipulations, e.g. residual enzyme activities (restriction, nuclease), detergents. Typically, the sample was first extracted with an equal volume of phenol \ chloroform. Phenol \ chloroform was freshly prepared by mixing equal volumes of NTE-saturated phenol (see section II.B.2.3.1) and chloroform \ isoamyl alcohol (24:1). The aqueous and organic phases were mixed thoroughly by vortexing, and then separated by centrifugation in a bench top microfuge at 14,000 rpm for 5min. This extraction was repeated if necessary. The aqueous phase was carefully removed and extracted with an equal volume of chloroform (chloroform:isoamyl alcohol, 24:1) by vortexing and centrifugation as described above. The aqueous phase was again collected and transferred to a fresh tube. Chloroform extraction of small sample volumes was facilitated by the addition of more aqueous phase (TE buffer, 10mM tris-HCl, pH 8.0, 1mM EDTA). The final supernatant from these extractions was then ethanol precipitated.

Ethanol precipitation was used to collect and concentrate samples of DNA. To the aqueous solution of DNA was added one tenth volume of 3M sodium acetate and 2-2.5 volumes of ethanol. The sample was mixed by inversion, and stored at -20^{o}C overnight, or alternatively placed on dry ice for 15-30min. If the DNA was very dilute (<10ug \ ml), yeast tRNA was added to facilitate precipitation. Precipitated DNA was recovered by centrifugation in a bench top microfuge for 10-15min at 14,000 rpm at room temperature or 4^{o}C. The precipitated material was washed with 70% ethanol, and dried briefly under vacuum before resuspension in TE buffer to the appropriate concentration.

II.C.3.3 Spectrophotometric Quantitation of DNA

The concentration of DNA in aqueous solutions was measured spectrophotometrically. Samples were diluted as appropriate in TE buffer and

absorbance readings taken at 260 and 280nm in a quartz cuvette with a path length of 1cm. The spectrophotometer was calibrated using a TE blank. An A_{260} value of 1 was taken as being equivalent to 40ug \ ml of plasmid or genomic DNA, and 30ug \ ml oligonucleotide. The chemical purity of DNA solutions was assessed using the ratio of absorbance at 260 and 280nm. An $A_{260} \setminus A_{280}$ ratio of 1.8-2.0 indicated that the preparation was essentially pure. Preparations of DNA with a ratio significantly less than 1.8 were purified further by extraction and \ or precipitation.

II.C.4 Enzymatic Modification of DNA

II.C.4.1 Dephosphorylation of 5' Termini

Linearized plasmid DNA was dephosphorylated using calf intestinal alkaline phosphatase essentially according to Maniatis *et al.*[314] except that reactions were carried out in restriction buffer. Dephosphorylated DNA was extracted twice with phenol \ chloroform and precipitated with ethanol.

II.C.4.2 Phosphorylation of 5' Termini

Oligonucleotides used for *in vitro* mutagenesis were phosphorylated according to manufacturers' instructions. Oligonucleotides for hybridization to dot-blots were labelled as follows. 15pmol of oligonucleotide was phosphorylated in a total volume of 30ul kinase buffer (100mM tris-HCl, pH 8.0, 10mM $MgCl_2$, 7mM DTT) using 2 units polynucleotide kinase and 30uCi [γ^{32}P] ATP (3000Ci \ mmol). The reaction was allowed to proceed for 30min at 37^{o}C.

II.C.4.3 Ligation of DNA Fragments

Ligations were effected in a minimum volume, typically 10-20ul. The following components were mixed on ice (for a final reaction volume of 20ul):

10-20ng linear, dephosphorylated vector DNA (in TE)

2-10 fold molar excess of purified insert DNA (in TE)

4ul 5x ligation buffer (250mM tris-HCl, pH 7.8, 50mM

$MgCl_2$, 100mM DTT, 5mM ATP, 250ug \ ml BSA)

distilled water to 19ul

1ul T4 DNA ligase

For the ligation of cohesive termini, reactions were incubated overnight at 12-16^{o}C. Blunt-end ligations were carried out overnight at 4^{o}C. Ligation reactions were used to transform bacterial host cells immediately or were stored at -20^{o}C.

II.C.4.4 Generation of Blunt Ends from Cohesive Termini using Klenow Fragment of DNA polymerase I

Recessed 3' termini were filled using Klenow enzyme. Reactions were carried out in 50mM tris-HCl, pH 7.2, 10mM $MgCl_2$, 0.1mM DTT, 50ug \ ml BSA supplemented with each dNTP at 2mM. Klenow enzyme was added and reactions incubated at room temperature for 30min. The enzyme was then denatured by heating at 70^{o}C for 5min.

II.C.4.5 Bal 31 Nuclease Digestion

Nuclease Bal 31 was used to truncate v-*src* inserts 5' and 3'. This removed viral *env* and LTR sequences, whilst leaving the v-*src* coding sequence and splice acceptor intact. Preliminary experiments to assess the rate of deletion were carried out using HindIII digested bacteriophage λ DNA as substrate. Using 20ug of λ fragments in a 100ul reaction (20mM tris-HCl, pH 8.0, 12mM $MgCl_2$, 12mM $CaCl_2$, 200mM NaCl, 1mM EDTA), 0.1 unit of nuclease Bal 31 was capable of deleting approximately 50 base pairs from each terminus in one minute at 30^{o}C.

Required *src* inserts were excised from pLA29 (proviral 3.1kbp EcoRI *ts*LA29 *src* fragment cloned in pUC13[94,315]), RAV 32 and RAV PrA (obtained from V. Fincham) using EcoRI. The 3.1kb EcoRI fragment containing *src* was gel purified and dissolved in TE. Approximately 10ug of purified fragment was digested with 0.1 unit of Bal 31 in 100ul for 6min at 30^{o}C. 20ul aliquots were withdrawn at 2, 3, 4, 5 and 6min into 5ul ice-cold 100mM EDTA. Tris-HCl, pH 8.0, $MgCl_2$, spermidine and water were added to each tube to create suitable conditions for subsequent digestion with XhoI. The volume of the sample was increased three-fold at this stage to reduce the salt concentration to the

appropriate level. After XhoI digestion, samples were extracted with phenol \ chloroform and ethanol precipitated. Recessed 3' termini were then repaired using Klenow enzyme (see II.C.4.4) and the samples analyzed by agarose gel electrophoresis. Lanes containing fragments of approximately 1.9-2kb were extracted and the isolated DNA ligated to SmaI digested, phosphatase treated pMP323 (plasmid Cla12 in ref. 306). This step generated plasmids pMP 29, pMP 32 and pMP PrA (see chapter III). Appropriate clones were chosen for subcloning to fpGV-1[301] by restriction mapping and single-strand sequencing in bacteriophage M13.

II.C.5 Site Directed Mutagenesis

Oligonucleotide-directed mutagenesis was carried out in bacteriophage M13 using a commercially available kit according to manufacturers' instructions. Mutagenesis was carried out using oligonucleotide A2:

5' C_{7125} ACC ATG GCA AGC AGC AA_{7142} 3'

The nucleotides underlined indicate those which differ from the *ts*LA29 *src* sequence. This mutation results in a glycine to alanine substitution at position 2. The mutant was named *ts*LA29A2 to indicate this change. This mutation prevents myristylation of the *Src* protein[37,111].

Oligonucleotide A2 was 5' phosphorylated according to manufacturers' instructions. The parental template, M13 *ts*LA29 *src*, was generated by ligation of an EcoRI-BamHI fragment from pMP29 (see results) to M13mp19. Single strand template DNA was prepared as described (II.C.6.2.1). After mutagenesis, five putative mutant plaques were picked and expanded for the production of single-stranded and replicative (RF) form DNA. Single-stranded DNA from each putative mutant together with starting template M13 *ts*LA29 *src* and non-recombinant M13mp19 were dot-blotted and screened for the presence of the A2 mutation using 5' ^{32}P-labelled oligonucleotide A2 according to manufacturers' instructions. Under stringent conditions, 3 of the 5 putative mutant clones were found to retain labelled oligonucleotide. The presence of the required

mutation was confirmed by sequencing. RF form mutant M13 *ts*LA29A2 *src* DNA was prepared (II.C.6.2.2) to enable subcloning to pMP323 and fpGV-1.

II.C.6 Preparation of DNA

II.C.6.1 Preparation of Plasmid DNA

II C.6.1.1 Plasmid Minipreparations

Solutions required:

GTE	50mM glucose
	25mM tris-HCl, pH 8.0
	10mM EDTA
5M acetate	60ml 5M potassium acetate
	11.5ml glacial acetic acid
	to 100ml with sterile distilled water

Single bacterial colonies were picked from agar plates and grown overnight in 5ml L-broth supplemented with the appropriate antibiotic. 1.5ml of the overnight culture was pelleted at low speed for 1min in a bench top microfuge. The drained pellet was resuspended by vortexing in 100ul of GTE containing 5mg \ ml lysozyme. After incubation for 5min at room temperature, alkaline lysis was effected by the addition of 200ul ice-cold 0.2M NaOH \ 1% SDS. The samples were mixed gently by inversion, and stored on ice for 5min. 150ul ice-cold 5M acetate was added and mixed gently by inversion before incubation for 5min on ice. The precipitated material was pelleted by brief centrifugation. The supernatant was extracted with an equal volume of phenol \ chloroform (see II.C.3.2), and the aqueous phase precipitated with 0.6 volumes of *iso*-propanol for 15min at room temperature. The precipitate was collected by centrifugation in a bench top microfuge for 1min at 14,000 rpm. The pellet was washed once in 70% ethanol and dried under vacuum. The final pellet was then redissolved in 50ul TE buffer and treated with RNase A (50ug \ ml) for 10min at room temperature. Minipreparation

DNA was stored at -20^{o}C. Typically, 5ul of this minipreparation was digested for mapping purposes.

II.C.6.1.2 Large Scale Plasmid Preparations

Plasmid DNA for transfection purposes was prepared by a modification of the method outlined above in II.C.6.1.1. A large overnight culture (500-1000ml) was harvested in a Sorvall GS3 rotor at 7000 rpm for 10min at 4^{o}C. The pellet was resuspended in a total of 10ml GTE, and solid lysozyme added to 5mg \ ml. The suspension was mixed thoroughly and incubated at room temperature for 5min. 20ml of ice-cold 0.2M NaOH \ 1% SDS was added and mixed gently by inversion before incubation on ice for 5min. Protein and detergent was then precipitated by the addition of 15ml of 5M acetate and incubation on ice for 15min. Precipitated material and cellular debris were collected by centrifugation under the conditions outlined above. The supernatant was filtered through a double thickness of gauze, and precipitated by the addition of 0.6 volumes of *iso*-propanol for 15min at room temperature. Precipitated material was collected by centrifugation in a Sorvall HB4 rotor at 8000 rpm for 10min at 4^{o}C. Pellets were drained thoroughly and excess *iso*-propanol wiped from the walls of the tubes before dissolution in 8.5ml TE. Precisely 8ml of crude plasmid DNA solution was added to 8g of CsCl in a plastic universal. This solution was transferred to screw cap polycarbonate ultracentrifuge tube and balanced to within 0.1g of the other tubes before the addition of 0.5ml of 10mg \ ml ethidium bromide. The balanced tubes were centrifuged at 40,000 rpm for 40-48hrs at 16-20^{o}C in a Sorvall T1270 rotor. Plasmid DNA was carefully removed from the gradient and extracted with 2-butanol to remove ethidium bromide before dialysis overnight against 2l TE buffer. Dialysed plasmid DNA was subsequently precipitated with ethanol and resuspended in TE at 0.5-2.0mg \ ml. Purified plasmid DNA was stored at 4^{o}C or -20^{o}C.

II.C.6.2 Preparation of Bacteriophage M13 DNA

II.C.6.2.1 Single Stranded MI3 Preparation

Distinct white plaques were picked from YT plates (see II.C.1.3.2) using sterile toothpicks. Plaques were expanded in 1.5ml of exponential TG1 in 2YT broth (1:100 dilution of an overnight culture in 2YT) for 4.5hr at 37°C. After transfer to eppendorf tubes, bacteria were pelleted by centrifugation at high speed for 5min. The supernatant was tipped carefully into a clean tube and mixed with 150ul 2.5M NaCl \ 20% PEG. After incubation for 30min at room temperature (or 4°C overnight) precipitated bacteriophage particles were collected by centrifugation for 5min at room temperature. The supernatant was aspirated from the small white pellet and the tube centrifuged briefly to facilitate complete removal of the NaCl \ PEG. The pellet was resuspended in 200ul TE and then mixed thoroughly with 100ul 3:1 phenol:chloroform by vortexing for 10sec. The mixture was stored at room temperature for 10min before a second period of vortexing. Aqueous and organic phases were separated by centrifugation for 5min at high speed, and the aqueous phase removed to a clean tube. Single stranded DNA was collected by ethanol precipitation overnight at -20°C, washed with 70% ethanol, dried and resuspended in 20ul TE. This DNA was suitable for transformation of competent TG1 cells, single-strand sequencing and site directed mutagenesis.

II.C.6.2.2 Replicative Form (RF) M13 Preparation

Mutants generated by oligonucleotide-directed mutagenesis in M13 were subcloned to pMP323. This required the preparation of double stranded insert DNA from mutant M13. Appropriate mutants were selected by dot-blot analysis and sequencing and re-transfected into competent TG1 cells (see II.C.1.3.2). Appropriate plaques were picked and expanded as described in II.C.6.2.1. Infectious bacteriophage was obtained by pelleting the host cells and decanting the supernatant to a fresh tube. 60ul of this supernatant was used to infect 10ml of log-phase TG1 (A_{550} ~0.4, 1cm path length, 2YT blank) in 2YT. After growth for 4.5hr at 37°C, double stranded, replicate form (RF) M13 was prepared from infected host cells as per plasmid minipreparations (see II.C.6.1.1).

The mutant insert could then be recovered by digestion with appropriate restriction enzymes (EcoRI and BamHI).

II.C.6.3 Preparation of Genomic DNA

Cells (10^7-10^8) in T175 flasks were washed twice in PBS at room temperature. The monolayers were then lysed in 6-8ml DNA lysis buffer (10mM tris-HCl, pH 8.0, 100mM NaCl, 2mM EDTA, 2.5% SDS) containing proteinase K at 100ug \ ml. The flasks were incubated at 37°C for 60min before the viscous contents were scraped to 50ml Falcon tubes. Incubation was continued at 37°C for a further 24-72hr. The viscous solution was extracted sequentially with phenol, phenol \ chloroform and chloroform. The tubes were mixed gently at each stage to prevent shearing of the high molecular weight DNA, and after centrifugation at 3000 rpm for 5min, aqueous material was transferred to fresh tubes using wide-bore pipettes. DNA was then precipitated by the addition of one tenth volume 0.1M magnesium acetate, one tenth volume of 3M sodium acetate and 2.5 volumes of ethanol at room temperature. The precipitate which formed at the interface was collected on the tip of a blocked Pasteur pipette and washed in 70% ethanol. After air-drying, the DNA was dissolved in sterile TE buffer to a concentration of ~0.5mg \ ml.

II.C.6.4 Preparation of Oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems model 381A DNA synthesiser. 5' trityl groups were removed on the machine before the oligonucleotide was eluted from the column in concentrated ammonia solution (29%) for 1.5hr at room temperature. The oligonucleotide in concentrated ammonia solution was sealed in a glass vial and incubated overnight at 55°C. Deprotected oligonucleotides were ethanol precipitated and washed extensively in 70% ethanol at room temperature to remove salt. Dried DNA was dissolved in TE to a concentration suitable for sequencing or 5' end-labelling.

II.C.7 Sequencing Methods

II.C.7.1 Single Stranded Sequencing

Single strand sequencing was used to determine the 5' and 3' endpoints of the *src* inserts used, and also to confirm the presence of appropriate mutations in *ts*LA32 *src* and *ts*LA29A2 *src*. Oligonucleotides used for this work are described below (sequence numbered according to Schwartz *et al.*[114]) :

M13 universal primer	5' GTAAAACGACGGCCAGT 3'
Oligonucleotide 107	5' T_{7396}ACGACTACGAGTCCTG$_{7412}$ 3'
Oligonucleotide 300	5' G_{7955}CTGCTTTGGAGAGGTC$_{7971}$ 3'
Oligonucleotide 419	5' C_{8320}TGGTGTGCAAGGT$_{8333}$ 3'
Oligonucleotide 3' *src*	5' A_{8696}GGTCGCTGAATAAGTACGAG$_{8716}$ 3'

Oligonucleotides 107, 300 and 419 are sequences found 5' to the mutations in *ts*LA32 *src*[142]. These reagents enabled sequencing of the mutations in my molecular clones. Oligonucleotide 3' *src* corresponds to the junction between the end of *src* coding sequences and the 3' untranslated region of RSV[114].

Single strand M13 template DNA was prepared according to section II.C.6.2.1, and sequenced using commercially available kits from Pharmacia (Klenow kit) or USB (Sequenase kit) according to manufacturers' instructions. Reactions were resolved on 6% denaturing acrylamide gels at a constant 40W. Gels were fixed for 15-30min in 10% methanol, 10% acetic acid in water and dried before autoradiography at room temperature.

II.C.7.2 Double Stranded Sequencing

Double stranded sequencing was used to confirm the presence of mutations in fpGV-32 (see chapter III) and the A2 mutation in fpGV-29A2 (see chapter VIII). Minipreparation plasmid DNA was prepared and sequenced as follows according to an unpublished method of D. Crouch. 50ul of RNase treated DNA from a plasmid

minipreparation (see II.C.6.1.1) was mixed with 30ul of 2.5M NaCl \ 20% PEG and placed on ice for 60min. The precipitated material was collected by centrifugation and washed with 70% ethanol. The dried DNA was dissolved in 16ul TE and denatured by adding 4ul of 1M NaOH. 2ul of 3M sodium acetate and 2 volumes of ethanol were added and the denatured template precipitated on dry ice for 30min. After collection by centrifugation and washing with 70% ethanol, the pellet was dissolved in 20ul TE. 7ul of this preparation was mixed with 2ul sequencing reaction buffer (USB Sequenase kit) and 1ul primer (30ng \ ul) and annealed for 30min at 37°C. Sequencing reactions were then carried out using the USB Sequenase kit according to manufacturers' instructions. Reactions were resolved as described above in section II.C.7.1.

II.C.8 Southern Blotting

10-20ug of genomic DNA was electrophoresed on 0.8% agarose gels together with ^{32}P-labelled HindIII digested λ DNA (2000cpm) to serve as markers. The gel was depurinated in 0.25M HCl until the dyes had changed colour, and then left for an additional 10min. After a brief rinse in distilled water, the gel was blotted using 0.4M NaOH as the transfer buffer. Transfer to Hybond N+ membrane and hybridization were according to manufacturers' instructions. The probe used was a v-*src* PstI fragment (8050-8662, a gift from V. Fincham) oligo-labelled using a commercially available kit from Pharmacia. Hybridization was carried out in a minimal volume at 65°C overnight in a shaking water bath. After washing to high stringency according to manufacturers' instructions, excess liquid was removed and the membrane subject to autoradiography at -70°C.

II.D. MATERIALS

II.D.1 Antiserum

Goat anti-rabbit IgG, alkaline phosphatase conjugate	Promega, Madison, Wisconsin, USA.
Mouse monoclonal MAb327[309]	Oncogene Scienc Inc., Manhasset, New York, USA.
Normal rabbit serum	Laboratory stocks
Rabbit anti-*gag* 5202	Laboratory stocks
Rabbit anti-mouse IgG	Sigma Chemicals Co. Ltd., Poole, Dorset, England.
Rabbit anti-*src* G950.4	Laboratory stocks
Rabbit anti-*src* MW2[94]	Laboratory stocks
Rabbit anti-*src* TBR-RLE[313]	A gift of Dr J. Neil, Beatson Institute, Glasgow.

II.D.2 Bacterial Hosts

E. coli host strains DH1, HB101 and TG1 were obtained from laboratory stocks held by Dr D. Crouch (DH1) and V. Fincham (HB101 and TG1). HB101 for RCAN work (see chapter III) was obtained from Dr R. Brown, Department of Medical Oncology, University of Glasgow.

II.D.3 Cells

Rat1 cells[302] and Rat1 *ts*LA29[19,94] were obtained from laboratory stocks. CEF were prepared as described[303], from White cross Brown Leghorn chicken embryos supplied by Wickam Laboratories, Hants., England.

II.D.4 Chemicals

Supplier-Amersham International PLC, Amersham, Bucks., England.

Amplify

^{3}H-methylthymidine 25Ci\mmol

[γ^{32}P]ATP 3000Ci\mmol

^{32}P-orthophosphate, carrier-free

Supplier-Bethesda Research Laboratories (UK), Gibco Ltd., Paisley, Scotland.

All DNA modifying enzymes and appropriate buffer concentrates were obtained from BRL unless otherwise stated. The following reagents were also obtained from BRL:

Agarose (ultrapure grade)

Bacteriophage λ (HindIII digested)

Bacteriophage ϕx174 (Hae III digested)

Prestained protein molecular weight markers

Urea (ultrapure grade)

Supplier-BDH Chemicals Ltd., Poole, Dorset, England.

All organic solvents (AnalaR grade) were obtained from BDH unless otherwise stated. The following compounds were also obtained from BDH (AnalaR, Biochemical or Electran grades):

Acylamide	Phosphorus pentoxide
Ammonium chloride	PEG 6000
Ammonium persulphate	Potassium acetate
Aqueous ammonia	Potassium chloride
bis-acrylamide	Potassium hydroxide
β-mercaptoethanol	Sodium acetate
Calcium chloride	Sodium chloride
D-glucose	Sodium citrate

Disodium orthophosphate
EDTA
Glycerol
Glycine
Hydrochloric acid
Magnesium acetate
Magnesium chloride
Manganese II chloride
Orthoboric acid
Orthophosphoric acid
Sodium deoxycholate
SDS
Sodium fluoride
Sodium hydroxide
Sodium *ortho*vanadate
Tris
Xylene cyanol

Supplier-Beta Lab., East Mosely, Surrey, England.

Yeast extract

Supplier-BioRad, Richmond, California, USA.

TEMED

Supplier-Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Caesium chloride
Calf intestinal alkaline phosphatase
Klenow fragment *E. coli* DNA polymerase
Proteinase K
RNase A

Supplier-James Burrough Ltd., Witham, Essex, England.

Ethanol

Supplier-Difco Laboratories, Detroit, Michigan, USA.

BactoAgar
Bactotryptone

Supplier-Gibco Europe, Life technologies Ltd., Paisley, Scotland.

Chicken serum
10x DMEM concentrate
Foetal calf serum
200mM Glutamine
G418
Lipofectin reagent
Methionine-free DMEM
Newborn calf serum
Phosphate-free DMEM
7.5% sodium bicarbonate
100mM sodium pyruvate
S. aureus (killed, fixed)
Trypsin
Tryptose phosphate broth

Supplier-Koch-Light Ltd., Haverhill, Suffolk, England.

Isoamyl alcohol

Supplier-Merck, Darmstadt, Germany.

Ninhydrin

Supplier-Millipore UK Ltd., Watford, Herts., England.

All reagents required for 2D-gel electrophoresis were obtained from Millipore.

Supplier-National Diagnostics, Manville, New Jersey, USA.

Ecoscint A

Supplier-New England Nuclear, DuPont UK Ltd., Stevenage, Herts., England.

^{35}S-methionine

Supplier-Oncogene Science Inc., Manhasset, New York, USA.

Raytide (peptide substrate, tyrosine kinase)

Supplier-Pharmacia Ltd., Milton Keynes, Bucks., England.

Dextran sulphate	Sephadex G100
dNTP	T4 polynucleotide kinase
Restriction endonuclease PvuI	yeast tRNA

Supplier-Rathburn Chemicals Ltd., Walkerburn, Scotland.

Phenol (water-saturated)

Supplier-Safeway supermarket, Glasgow.

Non-fat milk powder ("Marvel")

Supplier-Sigma Chemical Co. Ltd., Poole, Dorset, England.

Ammonium bicarbonate	IPTG
Ampicillin	Lysozyme
Aprotinin	Nitroblue tetrazolium
ATP	NP40
β-glycerophosphate	Phosphoserine
5-bromo-4-chloro-3-indolyl phosphate	Phosphothreonine
Bromophenol blue	Phosphotyrosine
BSA (fraction V)	PIPES
Calf thymus DNA	PMA
Coomassie brilliant blue R	PMSF
DTT	Polyvinylpyrrolidone 360
Enolase (rabbit muscle)	*S. aureus* V8 protease
Ethidium bromide	Sodium pyrophosphate
Ficoll	Spermidine
HEPES	X-gal

Supplier-Unipath Ltd., Basingstoke, Hants., England.

PBS (Dulbecco A, tablet form)

II.D.5 Kits

Supplier-Amersham International PLC, Amersham, Bucks., England.

Oligonucleotide-directed *in vitro* mutagenesis kit, version 2.

Supplier-Bio 101 Inc., Stratech Scientific, Luton, England.

Geneclean kit

Supplier-Pharmacia Ltd., Milton Keynes, Bucks., England.

Oligo-labelling kit

^{35}Sequencing kit

Supplier-Pierce, Life Science Laboratories Ltd., Luton, England.

Micro BCA protein assay kit

Supplier-United States Biochemical, Cleveland, Ohio, USA.

Sequenase version 2.0 kit

II.D.6 Membranes, Paper, TLC plates and X-ray film

Supplier-Amersham International PLC, Amersham, Bucks., England.

Hybond N+

Supplier-Anderman Co. Ltd., Kingston upon Thames, England.

Schleicher and Schuell nitrocellulose membrane BA83 and BA85

Supplier-Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland.

Dialysis tubing

Supplier-Eastman Kodak Co., Rochester, New York, USA.

Duplicating film (DUP-1)

X-ray film (XAR-5)

Supplier-Fuji Photo Film Co. Ltd., Japan.

X-ray film (RX)

Supplier-Phase Separations Ltd., Deeside, Clywd, Wales.

20x20cm cellulose TLC plates (without fluorescent indicator)

Supplier-Sartorius GmbH, Gottingen, Germany.

Dialysis bags

Supplier-Whatman International Ltd., Maidstone, Kent, England.

GF-C filters

3MM chromatography paper

P81 phosphocellulose paper

II.D.7 Plasmids and Bacteriophages

The following plasmid and bacteriophage DNAs were kind gifts of V. Fincham, Beatson Institute, Glasgow:

bacteriophage M13mp18

bacteriophage M13mp19

RAV 32 (Rous-associated virus containing *ts*LA32 *src* sequences)

RAV PrA (Rous-associated virus containing *wt*PrA *src* sequences)

The following plasmid DNAs were kind gifts of Dr D. Crouch, Beatson Institute, Glasgow:

pMP323 (Cla12 in ref. 306) RCAN, subgroup A[306]

The following plasmid DNA was a kind gift of Dr M. J. Welham, Imperial Cancer Research Fund, London EC1:

pLA29 (3.1kbp EcoRI *ts*LA29 *src* fragment cloned in pUC13[94,315])

The following plasmid was a kind gift of Drs E. Liebl and G. S. Martin, University of California, Berkeley, California USA:

fpGV-1[77,301]

II.D.8 Research Supplies, Miscellaneous

Supplier-Beatson Institute Central Services.

Kanamycin

L-broth

Penicillin

Sterile CT buffer	Nacl	6.0g
	trisodium citrate	2.96g
	tricine	1.79g
	phenol red	0.005g
	distilled water	700ml
	pH	7.8

Sterile distilled water

Sterile PBS

Streptomycin

II.D.9 Tissue Culture and Bacteriological Plasticware

Supplier-A/S Nunc, Roskilde, Denmark.

T25, 75 and 175 tissue culture flasks

Supplier-Becton Dickinson Labware, Plymouth, England.

35, 60 and 90mm tissue culture dishes

Supplier-Bibby Sterilin Ltd., Stone, Staffs., England.

60 and 90mm bacteriological dishes

II.D.10 Water

Distilled water for gel buffers and buffer stocks was obtained from a Millipore MilliRO 15 system. Water for tissue culture, protein / enzyme work or recombinant DNA procedures was further purified on a Millipore MilliQ system to 18MΩ cm.

Chapter III

III GENERATION AND BIOLOGICAL CHARACTERISTICS OF CLONAL Rat1 DERIVATIVES

III.A. Introduction

Initial attempts to obtain stable Rat1 cell lines expressing *ts*LA32 RSV by direct infection were disappointing. Information obtained in this laboratory and others indicates that a majority of integrated proviruses are silent in Rat1 cells, and indeed many of those which are expressed are subject to spontaneous silencing[300]. I therefore decided to express *ts*LA32 *Src* under the control of a murine retroviral promoter. A previously characterised temperature sensitive *Src* mutant, *ts*LA29[29,93,94,299], and wild type Prague A (*wt*PrA) *Src* were expressed in parallel with *ts*LA32 *Src* using the same promoter system. The vector chosen, fpGV-1, was a kind gift of E. Liebl and G. S. Martin[77,301], and a map is shown in figure III.1a. Inserts can be placed under the control of a Moloney murine leukaemia virus LTR, and transfected cells selected by virtue of the co-linear *neo* resistance marker[77].

As stated in the introduction to this thesis, v-*src* transformed fibroblasts show a number of striking changes in morphology and growth control which distinguish them from normal fibroblasts in tissue culture. This chapter will describe the phenotypes of clonal Rat1 cell lines obtained after transfection of the murine expression constructs described.

III.B. Results

III.B.1 Plasmid Construction

A series of manipulations was required to subclone the inserts of interest into fpGV-1. A summary of these steps appears below, with a detailed description appearing in Materials and Methods. Nucleotide numbering is after Schwartz *et al.*[114]

a) purification of the 3.1kb EcoRI *src* inserts of interest

b) truncation 3' by nuclease Bal31 digestion to remove 3' LTR sequences, leaving the *src* coding sequence intact

c) truncation 5' by XhoI digestion at 6983, removing 5' *env* sequences whilst retaining the *src* splice acceptor site

Figure III.1

a) Linear representation of fpGV-1 showing relevant sequences and restriction sites. For clarity, the LTR polyadenylation signal is shown to the right-hand side of the diagram. fpGV-1 confers kanamycin resistance in *E. coli* and G418 resistance in appropriate eukaryotic cells. Restriction sites : BamHI 3658, EcoRI 3676, ClaI 4332. *SD, splice donor, ColE1 ori, ColE1 origin of replication, SV40 ori, SV40 origin of replication,* neo^R*, neomycin resistance marker, polyA, polyadenylation signal.*

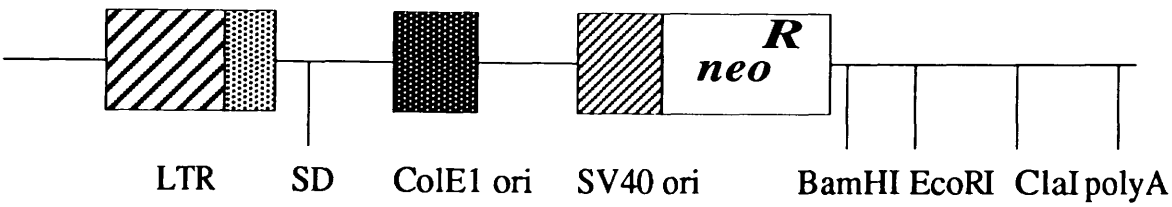
b) The proviral 3.1kbp EcoRI fragment containing *src* was digested with nuclease Bal31 and XhoI as described in the text, and ligated to SmaI digested pMP323. Sequencing in M13 revealed appropriate clones lacking 5' *env* and 3' LTR sequences. The 3' extent of RSV sequence is tabulated in table III.1. The orientation of appropriate clones in pMP323 is indicated, with relevant restriction sites. *SA, splice acceptor.*

i) orientation of *ts*LA29 *src* insert

ii) orientation of *ts*LA32 and *wt*PrA *src* inserts

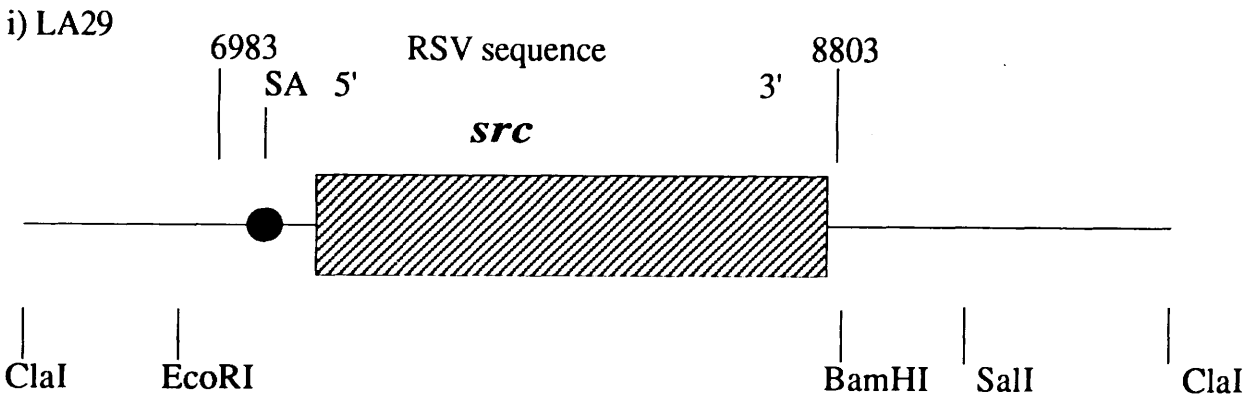
Figure III.1

a) fpGV-1 expression vector (5kbp)

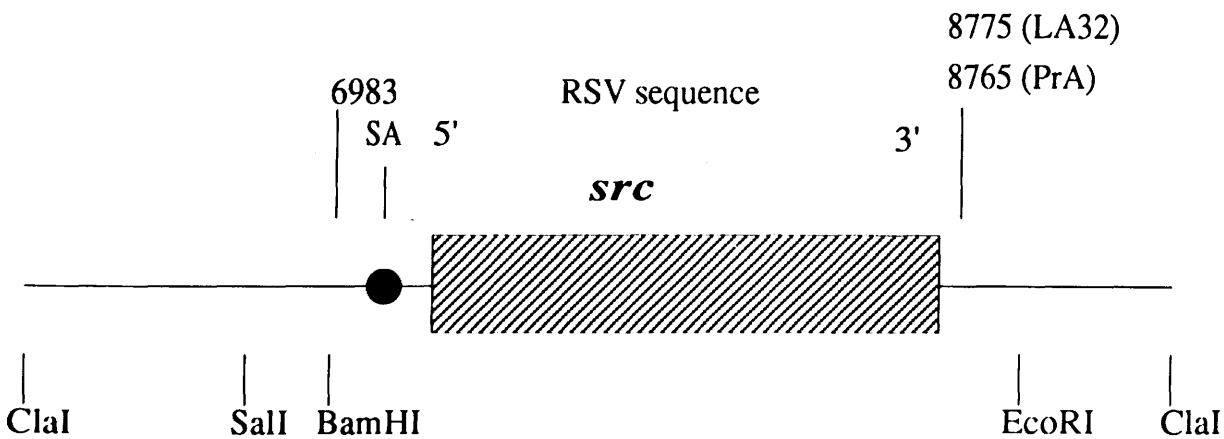


b) orientation of *src* inserts (~1.9kbp) in pMP323 polylinker

i) LA29



ii) LA32 and PrA



d) end filling to generate blunt termini

e) ligation to SmaI cut pMP323[306]. This step generated the ClaI sites necessary for subcloning to fpGV-1.

f) after extensive analysis by restriction digest, appropriate fragments were subcloned into bacteriophage M13. Single strand sequencing was used to confirm the presence of mutations and to assess the extent of 3' and 5' truncation. The 3' endpoints of the *ts*LA29, *ts*LA32, and *wt*PrA *src* inserts are tabulated in table III.1. The orientation of the inserts with respect to the pMP323 polylinker is shown in figure III.1b.

g) appropriate clones were subcloned to ClaI cut fpGV-1, and expression constructs selected after restriction digest analysis (figure III.2a).

TABLE III.1

INSERT	3' ENDPOINT
*ts*LA29	8803
*ts*LA32	8775
*wt*PrA	8765

Appropriate clones of fpGV-29, fpGV-32, fpGV-PrA, or the fpGV-1 vector itself were transfected into Rat1 cells using standard techniques. For some aspects of the work, it was found necessary to express the *src* inserts in primary chick embryo fibroblasts (CEF). The vector employed for this work was the replication-competent avian retroviral vector RCAN[306]. Inserts were subcloned to the ClaI site in RCAN (figure III.2b), and appropriate clones chosen after restriction mapping. (It was found that RCAN constructs were highly susceptible to deletion and rearrangement in some bacterial hosts, even those marketed as lacking known restriction systems. One particular batch of *E. coli* HB101 cells was found to be successful and used for all RCAN work). CEF transfections were carried out according to Materials and Methods.

Figure III.2

a) Restriction analysis of fpGV-1 *src* constructs. *src* inserts were subcloned to the unique ClaI site of fpGV-1 as outlined in the text. Presence of insert was assessed by digestion with ClaI, and orientation derived by digestion with BglI or SmaI. fpGV-29 is used as an example in this figure. Identical results were obtained for fpGV-32 and fpGV-PrA. For additional information see figure III.1b. *B, BglI, C, ClaI, S, SmaI, M, molecular weight markers.*

i) predicted restriction map of fpGV-29

ii) restriction analysis of fpGV-29. Digestion products resolved by 0.8% agarose gel electrophoresis.

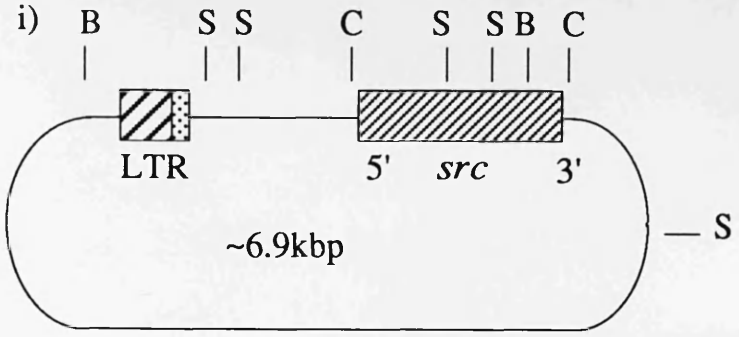
b) Restriction analysis of RCAN *src* constructs. *src* inserts were subcloned to the unique ClaI site of the replication competent avian retroviral vector as described in the text. Orientation of *src* inserts was assessed by digestion with SalI. SalI cuts approximately 900bp upstream of the ClaI cloning site in RCAN. See figure III.1b for position of SalI site in *src* inserts. *SD, splice donor, SA, splice acceptor, S, SalI, C, ClaI, pPH, E. coli replicon, M, molecular weight markers.*

i) restriction map of RCAN *src* constructs

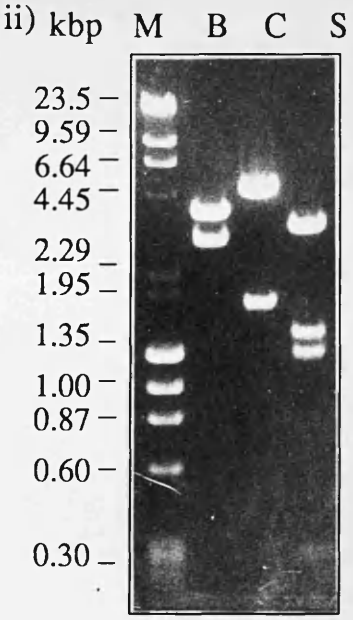
ii) SalI restriction analysis of RCAN *src* constructs. Digestion products resolved by 0.8% agarose gel electrophoresis. Lane 1, RCAN vector, 2, RCAN 29, 3, RCAN 32, 4, RCAN PrA

Figure III.2

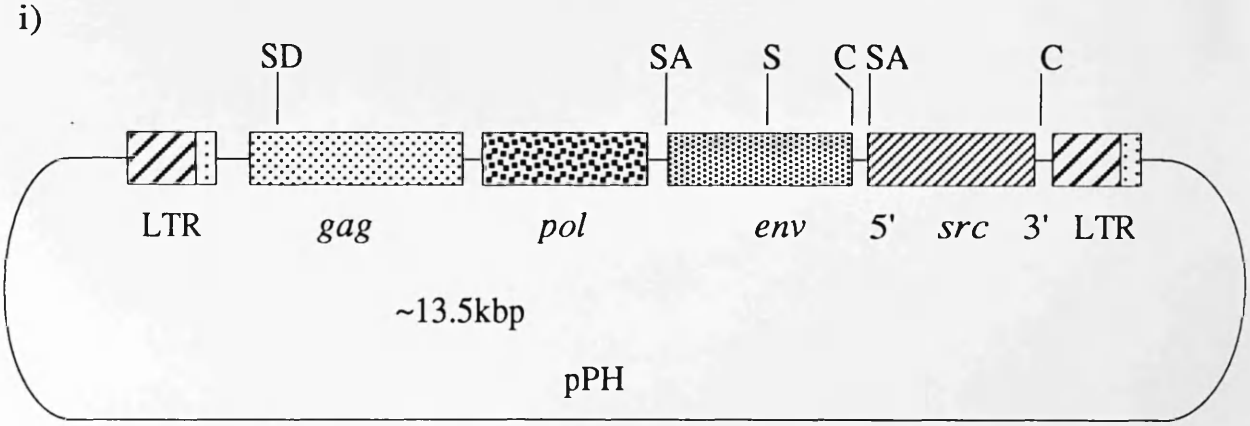
a) restriction mapping of fpGV-1 *src* constructs

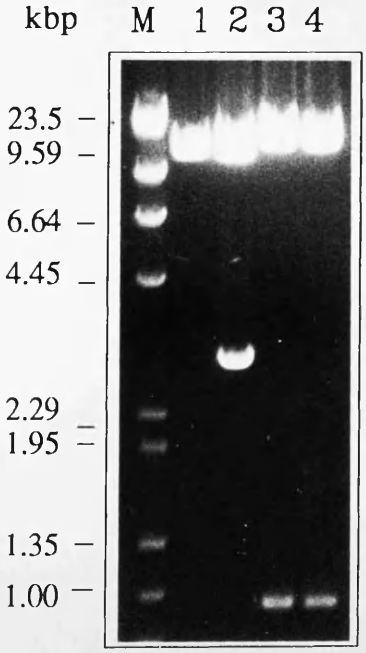


B 2410 (fpGV-1), 8416 (RSV)
C 4332 (fpGV-1)
S 3413, 3665, 4950 (fpGV-1), 7631, 7916 (RSV)



b) restriction mapping of RCAN *src* constructs





III.B.2 Generation of Clonal Rat1 Derivatives

Transfected Rat1 cultures were treated with G418 according to Materials and Methods, and resistant colonies scored after 10-20 days. Typically Lipofection of 1ug of fpGV-1 based constructs yielded 100-300 G418-resistant Rat1 colonies. Approximately the same yield of colonies could be obtained by transfecting 10ug of plasmid using the calcium phosphate coprecipitation method.

Colonies obtained by transfection of fpGV-1 were uniformly flat, and indistinguishable from parental Rat1 cells. Colonies obtained by transfection of fpGV-29, fpGV-32 or fpGV-PrA expression constructs showed a range of morphologies at permissive temperature. In any such transfection approximately 50% of the G418-resistant colonies were indistinguishable from parental Rat1 cells. The remaining colonies showed varying degrees of morphological transformation. This yield of transformed and non-transformed morphologies has been observed before[77] and presumably reflects (in part) the configuration of integrated plasmids. Western blot analysis indicated that pp60 was undetectable in flat colonies, whereas various levels of expression were apparent in lysates of transformed colonies (data not shown). Typical flat (fpGV-1) or transformed colonies (fpGV-29, fpGV-32 or fpGV-PrA) were cloned by limiting dilution and stocks stored in liquid nitrogen. Clonal lines chosen for study are shown in table III.2.

TABLE III.2

TRANSFECTED PLASMID	CELL LINE NAME	CLONE
fpGV-1	Rat1 f	a1
fpGV-29	Rat1 f29	IIC8
fpGV-32	Rat1 f32	aC7
fpGV-PrA	Rat1 fPrA	3C5

III.B.3 Plasmid Copy Number

Genomic DNA was prepared from Rat1 and the clonal lines listed in table III.2. Genomic DNA from an RSV-transformed Rat1 clone, VIT[316], was obtained from V. Fincham for use as a positive control. DNA was digested to completion with EcoRI (VIT, Rat1, Rat1 f, Rat1 f29) or BamHI (Rat1 f32, Rat1 fPrA). VIT contains two copies of the v-*src* gene as a result of proviral rearrangement and duplication, and yields *src* containing sequences of 3.1 and 3.5kbp upon digestion with EcoRI (V. Fincham, personal communication). EcoRI and BamHI cut the fpGV-1 *src* constructs as indicated in figure III.1, generating *src* containing sequences whose size is dependent upon the insertion site within the Rat1 genome. Southern blotting and subsequent hybridization using a *src* probe should estimate the plasmid copy number in the transformed clones. The Southern blot shown in figure III.3a suggests that Rat1 f29 contains three *src* inserts, with Rat1 f32 and Rat1 fPrA containing 3 and approximately 8 copies respectively.

III.B.4 Morphological and Growth Characteristics of Clonal Lines

III.B.4.1 Monolayer Morphology

Clonal lines were maintained in normal growth medium at either permissive (35^{o}C) or restrictive temperature (40^{o}C) for at least 48hrs prior to observation under the light microscope. The morphology of the various Rat1 cell lines is shown in figure III.4. The chosen cell lines exhibit the expected characteristics, with both *ts* mutants being fully thermolabile for the transformed morphology. Rat1 f32 displays a fusiform morphology at permissive temperature, in agreement with the behaviour of this mutant in CEF. Fusiform morphology has been observed in cells transformed with other SH2 \ SH3 mutants of v-*Src*[77,130,141,142,143]. The wild type PrA transformed cells appear somewhat flatter at restrictive temperature, consistent with previous results obtained in virally infected CEF[299]. However, these cells are refractile and fail to arrest at confluence. The cloned wild type PrA gene used contains a point mutation at the C-terminal end (amino acid 502), this possibly conferring a partial *ts* phenotype on the protein (V. Fincham, personal communication).

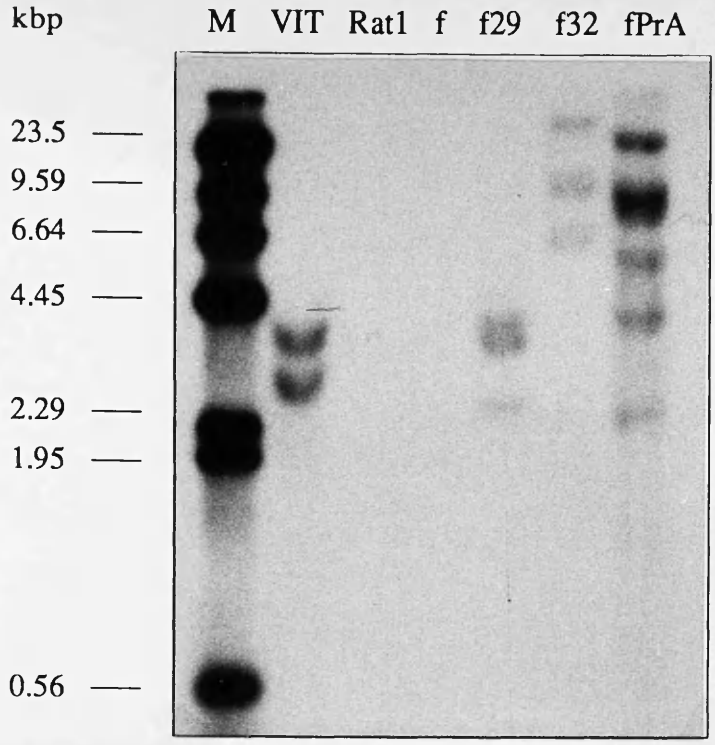
Figure III.3

a) Plasmid copy number of Rat1 *src* clonal lines. Genomic DNA was prepared from clonal lines (Table III.2) and digested to completion with EcoRI (VIT, Rat1, Rat1 f, Rat1 f29) or BamHI (Rat1 f32, Rat1 fPrA). Digested DNA was resolved by 0.8% agarose gel electrophoresis and blotted as described in Materials and Methods. Probe used was a 612bp PstI v-*src* fragment, labelled according to Materials and Methods.

b) $pp60^{v\text{-}src}$ expression in Rat1 *src* clonal lines. Whole cell lysates were prepared from cells at permissive or restrictive temperature. Equivalent amounts of protein from each lysate (~30ug, as judged by Coomassie Blue staining) were resolved by SDS-PAGE and blotted as described in Materials and Methods. Primary antiserum was anti-*Src* MW2 at 1:5000. The arrow head indicates pp60. *A, cells cultured at permissive temperature (35^oC), B, cells cultured at restrictive temperature (40^oC).*

Figure III.3

a) Southern Blot analysis of Rat1 *src* clones



b) Western blot analysis of Rat1 *src* clones

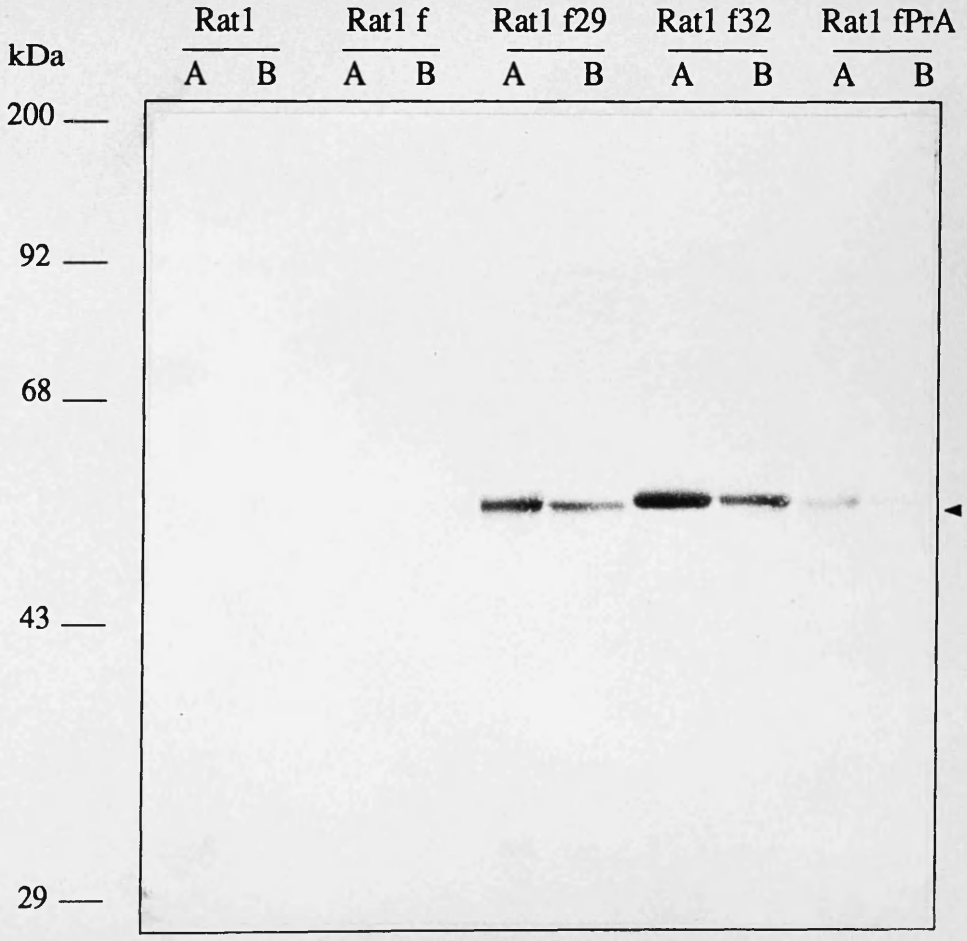
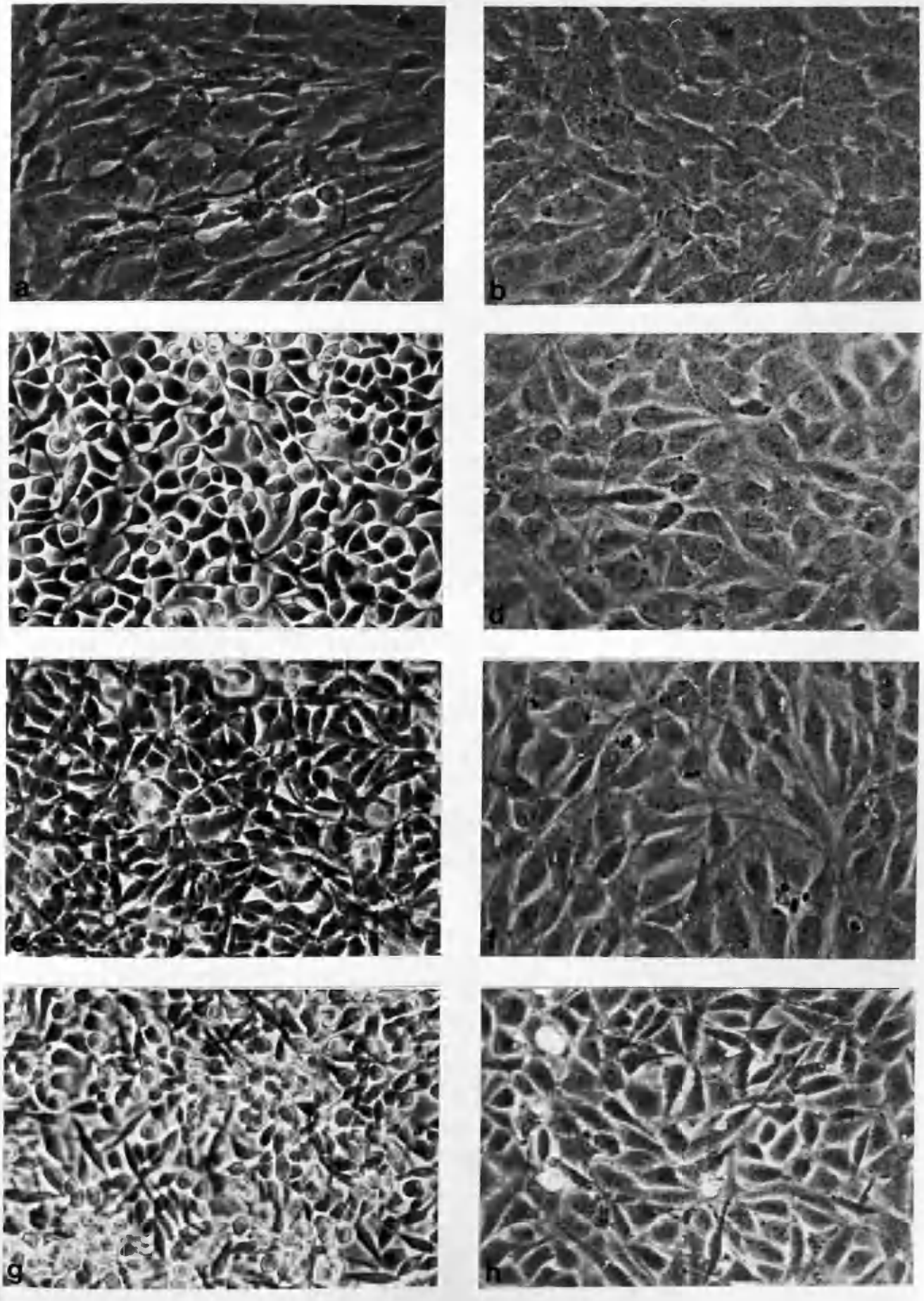


Figure III.4

Monolayer morphology of Rat1 *src* clones at permissive and restrictive temperature. Cultures were maintained in normal growth medium at appropriate temperature for at least 48hr prior to photography. Magnification is x500. *a*, Rat1 35°C, *b*, Rat1 40°C, *c*, Rat1 f29 35°C, *d*, Rat1 f29 40°C, *e*, Rat1 f32 35°C, *f*, Rat1 f32 40°C, *g*, Rat1 fPrA 35°C, *h*, Rat1 fPrA 40°C.

Figure III.4



III.B.4.2 Anchorage Independent Growth

Another characteristic property of v-*src* transformed fibroblasts is their ability to proliferate in an anchorage-independent fashion, this being assayed by growth in soft agar[307]. For these experiments, cells were seeded in soft agar as described in Materials and Methods, and colony formation scored after 14 days. Figures III.5 and III.6 summarise the results of these experiments.

From these data, one concludes that the capacity for anchorage-independent growth correlates well with morphological transformation. Rat1 and Rat1 f cells fail to form colonies at either temperature, even when seeded at 10^5 cells per dish. Rat1 f29 exhibits a fully thermolabile ability for proliferation in soft agar, forming many large colonies at permissive temperature, but no colonies at restrictive temperature. Essentially similar results are obtained with Rat1 f32, although there is residual colony formation at restrictive temperature. Very small colonies appear during the first few days of incubation, these remaining static in size over the course of the next two to three weeks. This is again consistent with data obtained using virally-infected CEF cultures[299]. This behaviour might result from a slight leakiness in the mutant at restrictive temperature, and raises the possibility that given a very high level of expression, the thermolabile properties of *ts*LA32 *Src* may become less obvious. This should be borne in mind when analyzing phosphoprotein patterns since it indicates that important changes may be subtle in nature, i.e. of a quantitative rather than qualitative nature.

In agreement with their morphological characteristics, the Rat1 fPrA cells showed a reduction in their capacity for anchorage independent growth at restrictive temperature, though this decrease did not approach the striking reduction in colony formation seen using the temperature sensitive mutants.

Figure III.5

Anchorage independent growth of Rat1 *src* clonal lines. Single cell suspensions were seeded in triplicate in soft agar as described in Materials and Methods, at a density of 10^5 (Rat1, Rat1 f) or 10^4 (Rat1 f29, Rat1 f32, Rat1 fPrA) cells per 60mm dish. Cultures were placed at either permissive or restrictive temperature for 14 days before colony formation was assessed. Representative areas of agar cultures are shown at a magnification of x4. *a*, Rat1 35°C, *b*, Rat1 40°C, *c*, Rat1 f 35°C, *d*, Rat1 f 40°C, *e*, Rat1 f29 35°C, *f*, Rat1 f29 40°C, *g*, Rat1 f32 35°C, *h*, Rat1 f32 40°C, *i*, Rat1 fPrA 35°C, *j*, Rat1 fPrA 40°C.

Figure III.5

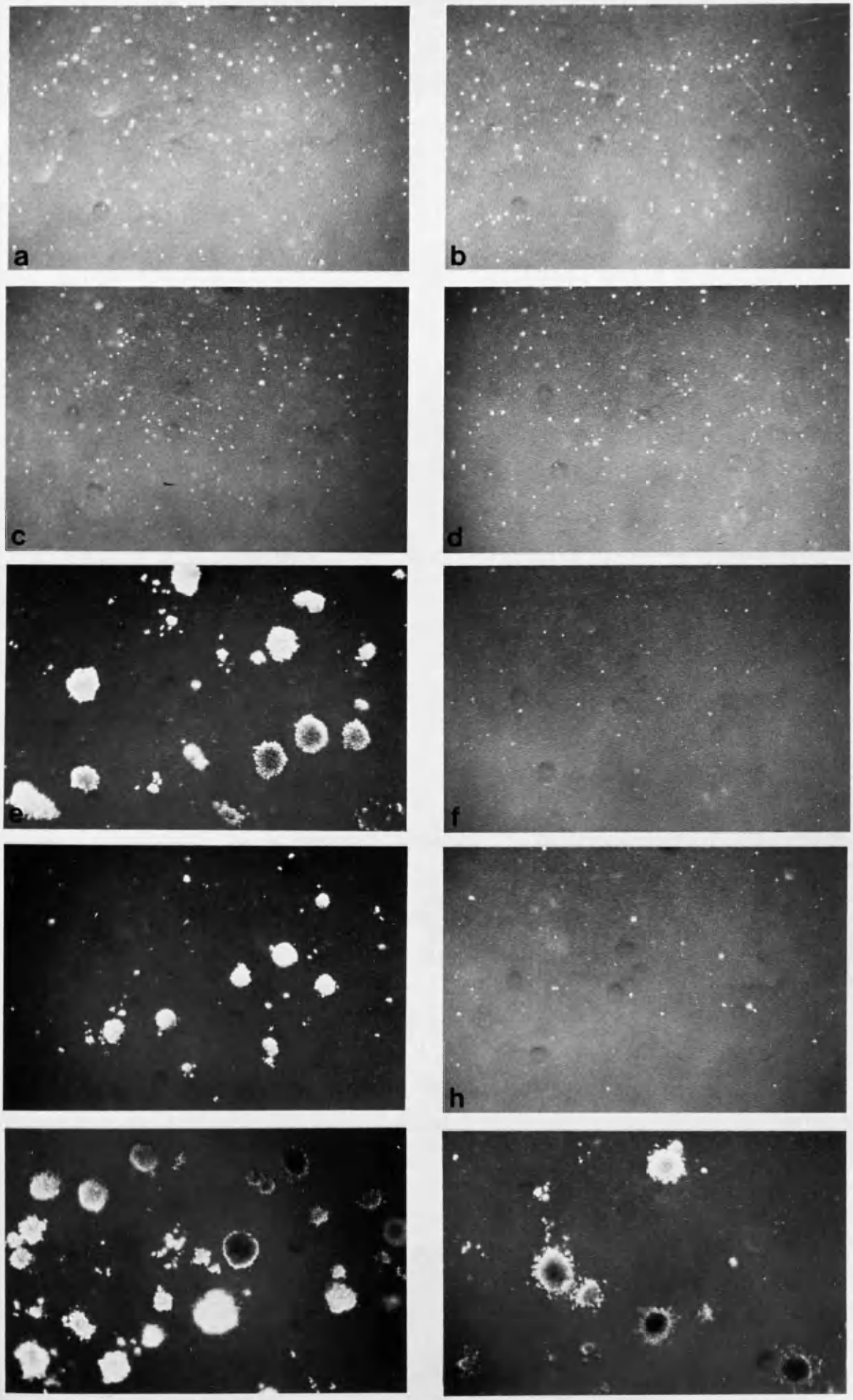
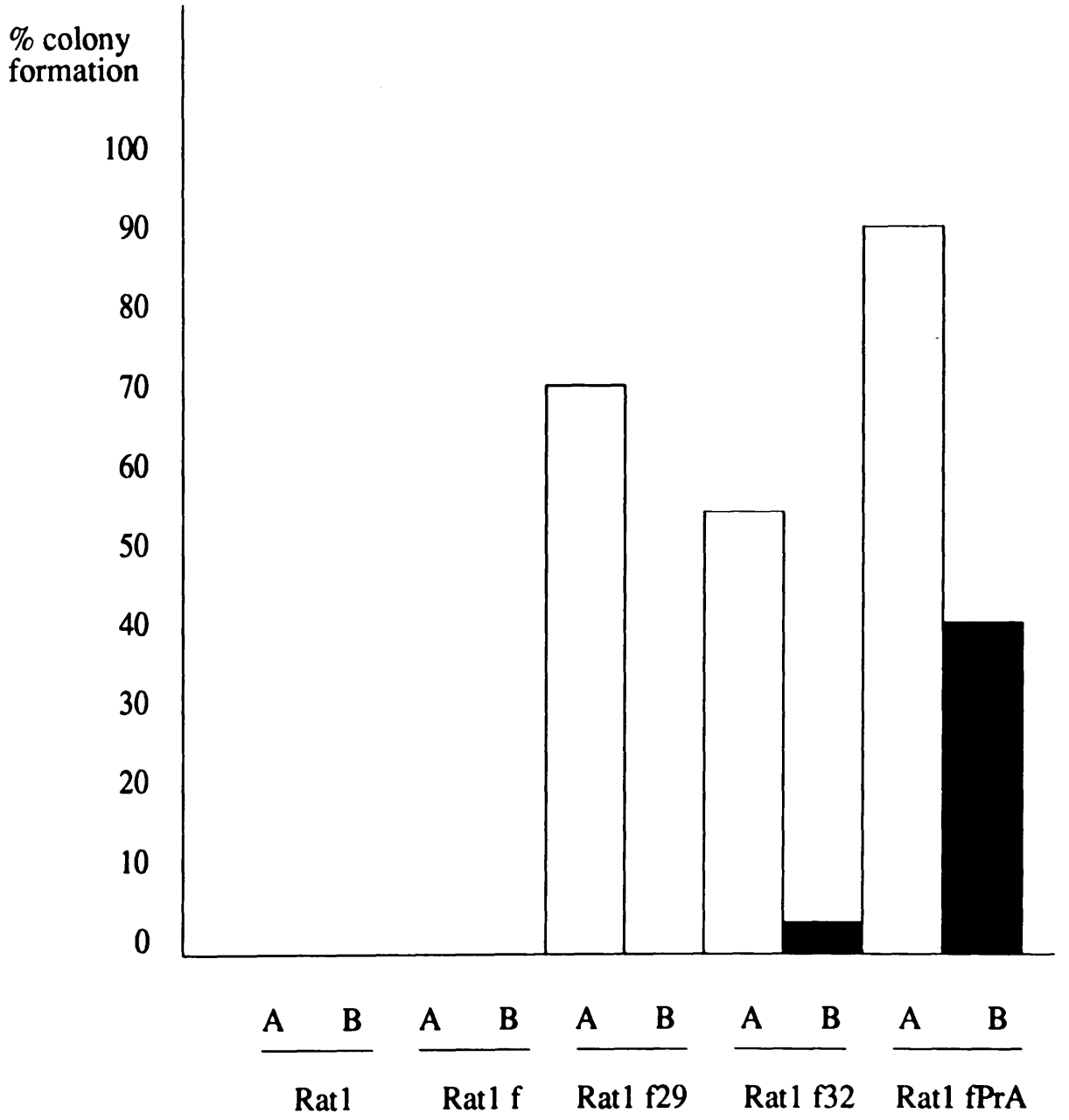


Figure III.6

Anchorage independent growth of Rat1 *src* clonal lines. Colony formation is presented as the percentage of plated cells forming colonies, and is the mean of triplicate determinations. In no case did colony formation deviate more than 6% from the mean value. The data shown are representative of two independent experiments. Note that <5% plated Rat1 f32 cells formed "colonies" at restrictive temperature. These "colonies" consisted of 2-4 cells. See text. *A, cultures at permissive temperature, B, cultures at restrictive temperature.*

Figure III.6

Anchorage-independent growth of Rat1 *src* clones



III.B.5 pp60 Expression

Since the chosen cell lines appeared to have the expected phenotypes, I examined pp60 expression in each of the cell lines at both permissive and restrictive temperatures by Western blot analysis. Cells were grown at the required temperature for 48hrs prior to harvest, and equivalent amounts of protein from each line were blotted according to Materials and Methods. A typical result is shown in figure III.3b, indicating that the cell lines containing *src* plasmids all express detectable levels of pp60. Rat1 f29 and Rat1 f32 contained approximately 5-10 fold more pp60 than Rat1 fPrA, and in each case these levels were not significantly thermolabile. Note that *ts*LA32 pp60 displays a higher apparent molecular weight than either *ts*LA29 or *wt*PrA *Src* proteins on SDS-PAGE gels. This appears to be a secondary structure effect (see section V.B.2). If one assumes that *ts*LA29, *ts*LA32 and *wt*PrA *Src* proteins have equivalent specific activities against relevant substrates in the cell at permissive temperature, then the expression levels of the mutant proteins is well above the threshold required for transformation. This implies that there is negligable leakiness at restrictive temperature.

III.C Discussion

From this summary of the biological and expression characteristics of the clonal lines selected it is apparent that these lines have the expected phenotypes. Both temperature sensitive mutants are fully thermolabile for the parameters of transformation tested, namely morphology in monolayer culture and anchorage independent growth. These results are consistent with data obtained in this laboratory using virally infected CEF[299]. Since the level of *Src* protein appears to be independent of growth temperature one concludes that transformation in this system is not simply the result of exceeding a threshold amount of transforming protein. It follows that transformation probably results from an increase in the functional activity of constant amounts of pp60.

In conclusion, the data presented above suggest that the Rat1 f32 line has the appropriate biological phenotype. The proposed approach to study early events following temperature shift (outlined in chapter I) depends on the cell line and mutant pp60

satisfying a number of other criteria. The biochemical characterization of the *ts*LA32 *Src* protein and further analysis of the Rat1 f32 cell line follow in subsequent chapters.

Chapter IV

IV *IN VITRO* KINASE ACTIVITY

IV.A. Introduction

Immunoprecipitation of pp60 from RSV transformed cells yields immune complexes which, in the presence of Mg^{2+} (or Mn^{2+}) and ATP catalyze both the phosphorylation of pp60 itself (autophosphorylation)[41], and / or exogenous substrates[10,42]. This forms a convenient assay for the kinase activity, and in general this *in vitro* activity reflects the tyrosine kinase activity *in vivo*. As a first step in characterising the kinase activity of the *ts*LA32 *Src* protein, *in vitro* kinase assays were performed on immunoprecipitates prepared from the chosen Rat1 lines. Despite numerous attempts using a variety of antisera, substrates and assay conditions, the results from these assays were not entirely consistent with data obtained by Stoker *et al.* studying RSV in CEF[299]. In order to compare directly the behaviour in Rat1 cells and avian cells, I undertook to express the *src* inserts of interest in CEF using a replication-competent avian retroviral vector, RCAN[306]. The construction of these plasmids is described in Chapter III, and infected cultures were obtained as outlined in Materials and Methods.

IV.B. Results

The *in vitro* kinase activity of pp60 was measured using a conventional assay[10,41,42]. Four substrates were used in these experiments : pp60[41], enolase[42], Raytide (a gastrin-like peptide substrate) and immunoglobulin heavy chain[10]. Briefly, equal amounts of total lysate protein were immunoprecipitated with monoclonal or polyclonal sera to pp60. A portion of this precipitate was incubated in a buffer containing Mn^{2+}, Mg^{2+} ions and gamma-labelled ATP, either with or without exogenous substrate. Assay temperature was standardised to 30°C after preliminary experiments indicated that assay temperature had no significant effect on the kinase activity within any given immunoprecipitate.

IV.B.1 *In vitro* Kinase Activity of Rat1 Cell Lines

Immune complexes were prepared from exponential cultures of Rat1, Rat1 f, Rat1 f29, Rat1 f32 and Rat1 fPrA cells growing at permissive or restrictive temperature. A typical result obtained using monoclonal antibody MAb327[309] is shown in figure IV.1i. Acid-denatured enolase[42] was used as an exogenous substrate in this assay. Immune complexes prepared from Rat1 cells or Rat1 f cells display a very low level of kinase activity towards enolase, either as a result of precipitating pp60$^{c\text{-}src}$ or non-specific precipitation of another endogenous kinase. Precipitates formed from Rat1 f29 cells show the expected temperature sensitivity in pp60 autophosphorylation and kinase activity towards enolase[29,315]. Immune complexes prepared from the Rat1 fPrA line also behave as predicted, displaying activity towards pp60 and enolase when prepared from cells grown at either permissive or restrictive temperature. There is slightly less activity in precipitates formed from Rat1 fPrA cells at restrictive temperature, this reduction correlating with the biological properties of this protein (see chapter III). Recovery of labelled enolase from the Rat1 fPrA lanes of the gel and subsequent hydrolysis demonstrated that all detectable labelled phosphate was incorporated in phosphotyrosine (see figure IV.1ii). Surprisingly, immunoprecipitates prepared from Rat1 f32 cells grown at either permissive or restrictive temperature failed to show any autophosphorylation, or kinase activity towards enolase under these conditions. Increasing the ATP concentration from 0.1uM to 1 or 10uM yielded negative results, and similarly, reducing the assay temperature to 0^{o}C or preparing lysates and immuneprecipitates in the presence of 1mM DTT had no effect (data not shown). In view of this difficulty, I assayed identical MAb327 immunoprecipitates using another substrate, the gastrin-related peptide Raytide, using a filter binding assay. Figure IV.2a shows the results of this experiment. Precipitates prepared from Rat1 f29 and Rat1 fPrA cells behave as expected, with precipitates from Rat1 f32 cells showing negligible activity. (This particular result underestimates the total activity in the active immune complexes since subsequent experiments demonstrated that the peptide substrate was limiting in these assays).

Figure IV.1

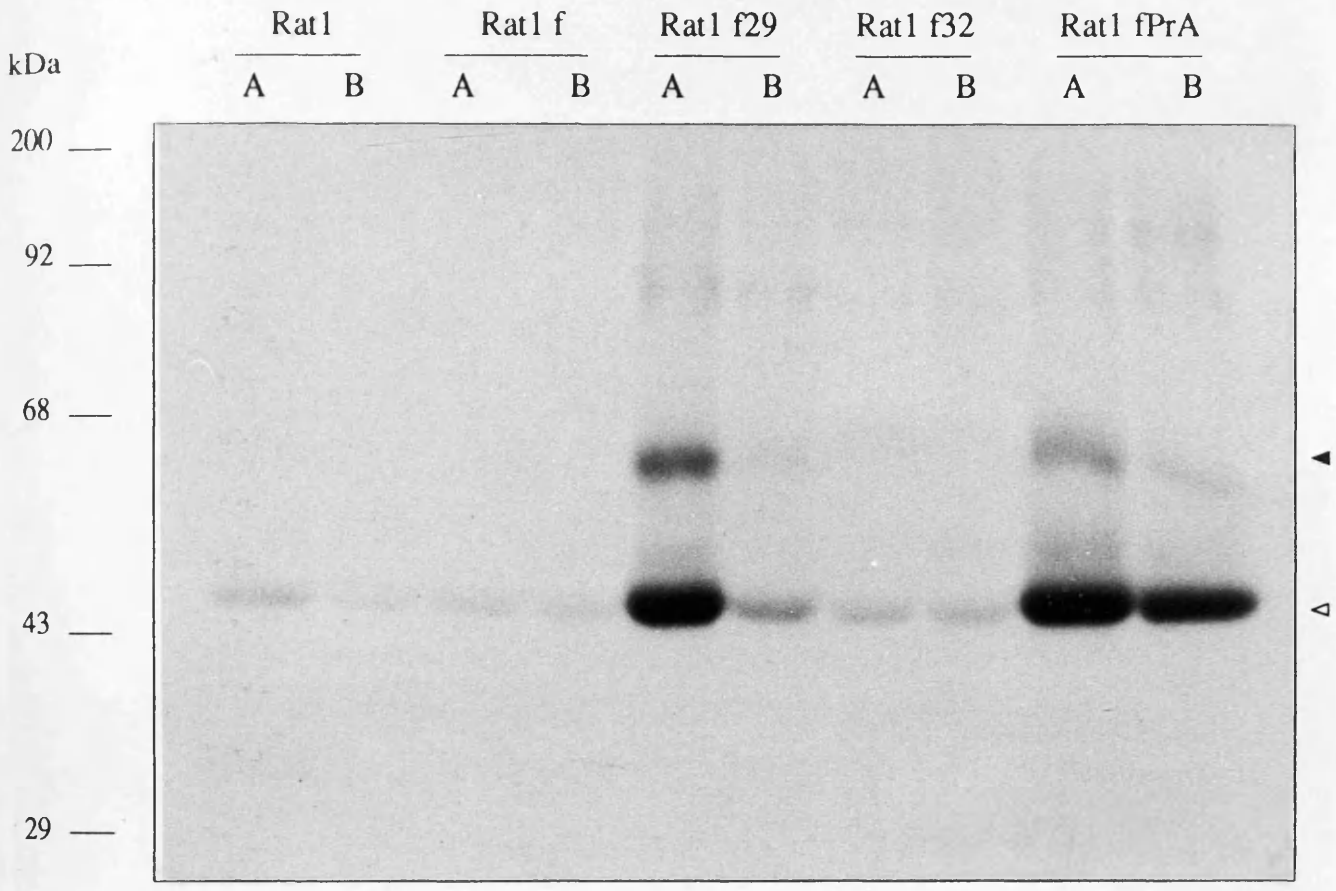
i) MAb327-immune complex kinase assay of Rat1 *src* clones. pp60 was immunoprecipitated from growing cultures as described in Materials and Methods using monoclonal antibody MAb327. Kinase activity in the immune complexes was measured at 30^{o}C using acid denatured enolase as exogenous substrate, as described in Materials and Methods. Exposure time was 4hr at -70^{o}C. The closed arrow indicates labelled pp60 and the open arrow enolase (as judged by Coomassie Blue staining). *A, cells cultured at permissive temperature, B, cells cultured at restrictive temperature.*

ii) Two-dimensional phosphoamino acid analysis of enolase phosphorylated *in vitro.* Labelled enolase was extracted from the Rat1 fPrA lanes of the gel shown in i) and hydrolysed as described in Materials and Methods. Hydrolysed enolase was mixed with unlabelled standards (phosphoserine, phosphothreonine and phosphotyrosine) and resolved by two-dimensional thin layer electrophoresis as described in Materials and Methods. Unlabelled standards were visualized with ninhydrin. Exposure time was 18hr at -70^{o}C. *PS, phosphoserine, PT, phosphothreonine, PY, phosphotyrosine.*

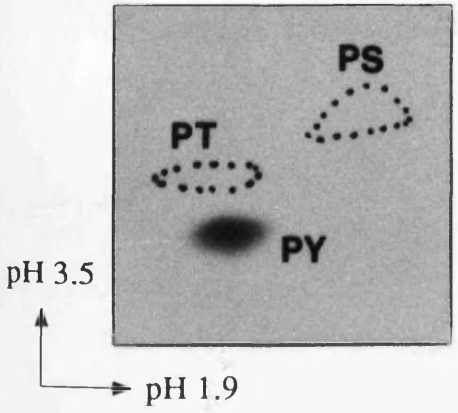
iii) MAb327-immunoprecipitation of ^{35}S-methionine labelled pp60. Immunoprecipitates were formed precisely as described in i) except cultures were labelled for 3hr prior to lysis with 150uCi \ ml ^{35}S-methionine. All cultures were at permissive temperature. Immunoprecipitates were resolved by SDS-PAGE and fluorographed for 30hr at -70^{o}C. The closed arrow indicates pp60. *Lane 1, Rat1, 2, Rat1 f29, 3, Rat1 f32, 4, Rat1 fPrA.*

Figure IV.1

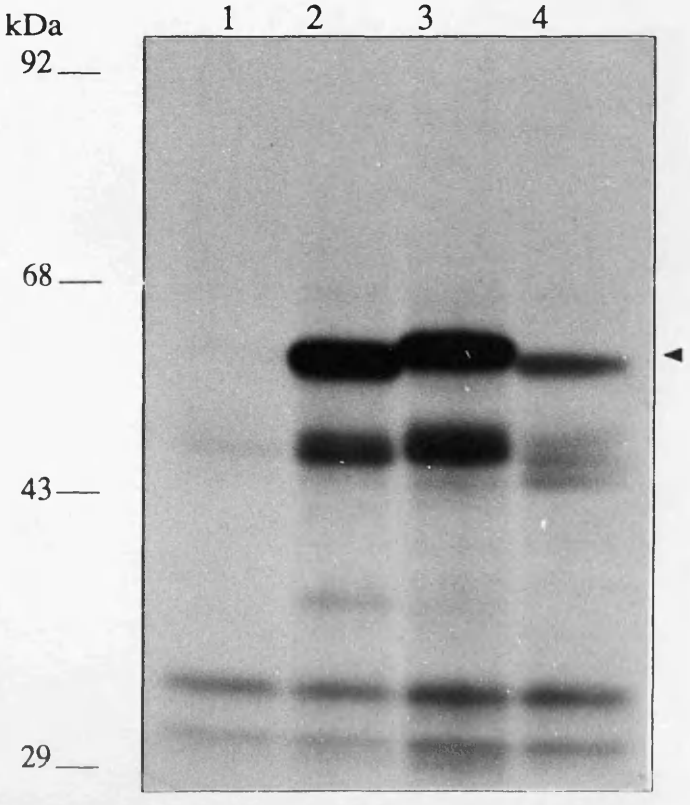
i) MAb327-immune complex kinase assay of Rat1 *src* clones



ii) 2D-PAA



iii) MAb327-immuneprecipitation of pp60



Since MAb327 reacts with an epitope residing within residues 100-110 of the primary sequence of pp60[140] (J. Brugge, personal communication), one possible explanation for these negative results is that the mutation at position 107 in *ts*LA32 *Src* prevents recognition by the antibody. To address this possibility, immune complexes were prepared exactly as described for kinase assays with the exception that the cells were labelled with ^{35}S-methionine prior to lysis. Precipitated proteins were fractionated by SDS-PAGE and detected by fluorography (figure IV.1iii). This demonstrates that pp60 can be immunoprecipitated from Rat1 f32 cells under conditions identical to those used for kinase assays. Additional species of approximately 50kDa are seen in precipitates from *src*-transformed cells; these probably represent degradation products of pp60 and \ or the 50kDa member of the trimeric complex described in chapter I. This result does not preclude the possibility that the interaction between the antibody and the mutant pp60 abrogates kinase activity as a direct or indirect result of the mutation at position 107. To test this possibility, immune complexes were prepared using a number of other polyclonal antisera, and kinase activity towards enolase measured as before. The result of one such experiment utilizing anti-*Src* serum G950.4 is shown in figure IV.2b. As with MAb327, no kinase activity is detectable in precipitates prepared from Rat1 f32 cells. Identical results were obtained using another polyclonal anti-*Src* serum, MW2[94] (data not shown). The preferred substrate in these assays varied with the antiserum used to generate the immune complexes. Hence, the preferred substrate in G950.4 immunoprecipitates was pp60 itself, whereas the majority of incorporated label was found in enolase when using MAb327 precipitates. Immunoprecipitates formed using a tumour-bearing rabbit serum[313], where kinase activity is directed towards pp60 itself and the immunoglobulin heavy chain, yielded identical results (data not shown).

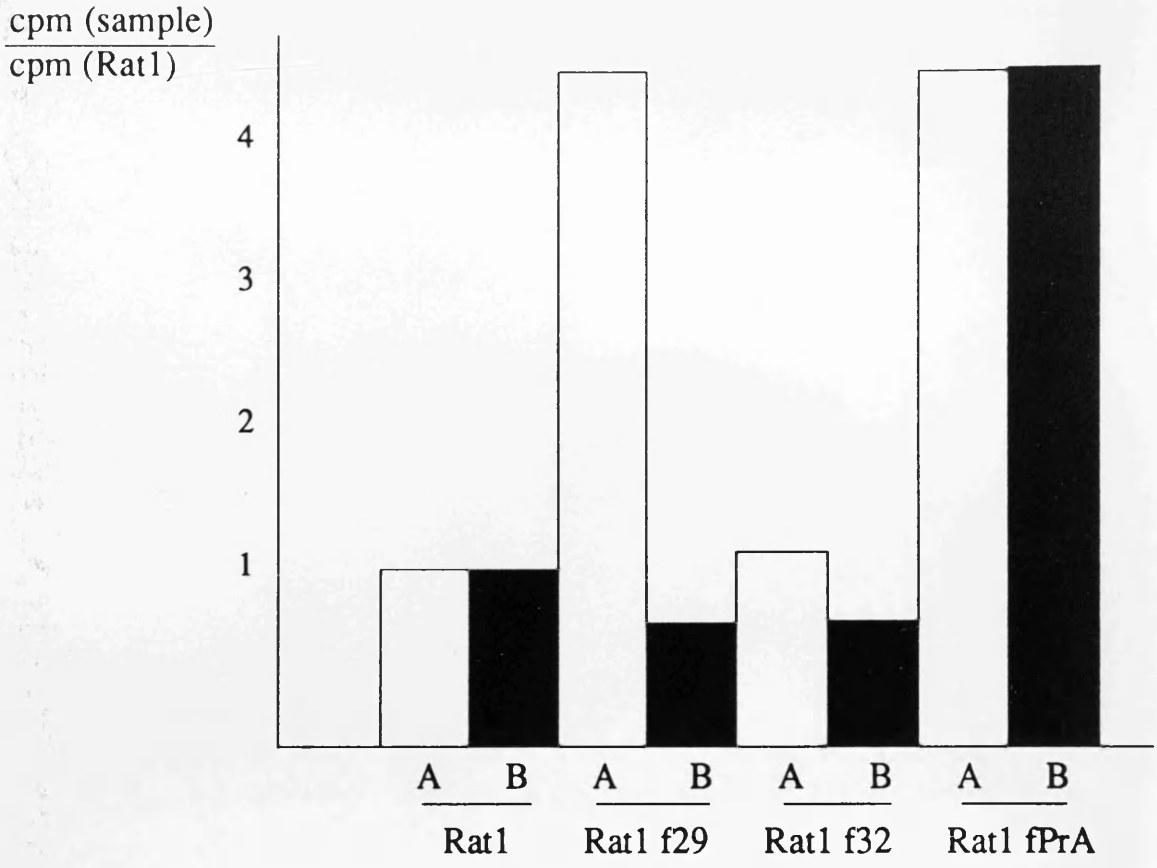
Figure IV.2

a) MAb327-immune complex kinase assay, Raytide substrate. pp60 was immunoprecipitated from growing cultures using MAb327 as described in Materials and Methods. Kinase activity against the gastrin-like peptide Raytide was measured using a filter binding assay as described, and counts were normalised to the Rat1 precipitate at the appropriate temperature. Assay temperature was 30°C. *A, cultures at permissive temperature, B, cultures at restrictive temperature.*

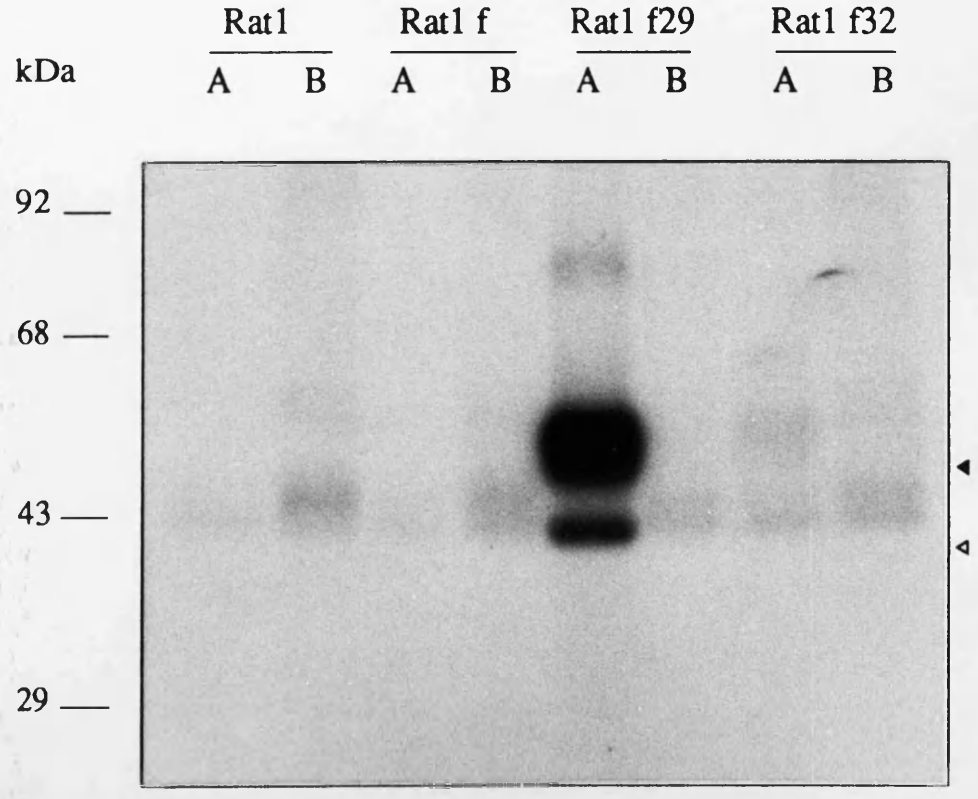
b) G950.4-immune complex kinase assay. pp60 was immunoprecipitated from growing cultures using polyclonal antiserum G950.4 as described in Materials and Methods. Immune complex kinase activity was measured as described using acid denatured enolase as exogenous substrate. Exposure time was 4hr at -70°C. The closed arrow indicates pp60, the open arrow enolase (as judged by Coomassie Blue staining). *A, cultures at permissive temperature, B, cultures at restrictive temperature.*

Figure IV.2

a) MAb327-immune complex kinase assay, Raytide substrate



b) G950.4-immune complex kinase assay



IV.B.2 *In vitro* Kinase Activity of Infected CEF

From the preceeding it is apparent that kinase activity is consistently undetectable in immune complexes prepared from Rat1 f32. I therefore expressed the *ts*LA32 *src* insert in chick embryo fibroblasts (CEF), again using *ts*LA29 and *wt*PrA *src* inserts as controls. Control CEF cultures or CEF cultures transfected with RCAN, RCAN 29, RCAN 32 or RCAN PrA were passaged until uniform infection was achieved. Infection was assessed by Western blot analysis using anti-*Gag* antiserum 5202 (to assess RCAN infection) and anti-*Src* antiserum MW2 (see figure IV.3). Uniformly infected cultures were maintained at permissive (35^{o}C) or restrictive (41^{o}C) temperature for two days prior to lysis and immunoprecipitation. Infected cultures were of the expected morphology at these temperatures (data not shown). Immunoprecipitates were prepared using MAb327, and kinase activity measured using enolase as substrate. Figure IV.4a indicates that these *in vitro* kinase results mimic very closely those seen in the Rat1 cell system, implying that a host-range defect is not responsible for the failure to demonstrate kinase activity in Rat1 f32 immunoprecipitates. To repeat more precisely the method used by Stoker *et al.*[299] immunoprecipitates were prepared from infected CEF cultures using a tumour-bearing rabbit serum, TBR-RLE[313]. Immune complexes were assayed for kinase activity in the absence of exogenous substrate, with pp60 and the immunoglobulin heavy chain serving as substrates in these assays (figure IV.4b). Immunoprecipitates from RCAN 32-infected cells cultured at either permissive or restrictive temperature did exhibit a relatively small kinase activity in this assay, although this activity did not approach that seen in immunoprecipitates from RCAN 29 or RCAN PrA infected cultures. The labelled phosphate incorporated into pp60 and the immunoglobulin heavy chain was stable to treatment with 1M KOH at 55^{o}C[310], suggesting that phosphotyrosine was the predominant phosphoamino acid present (data not shown).

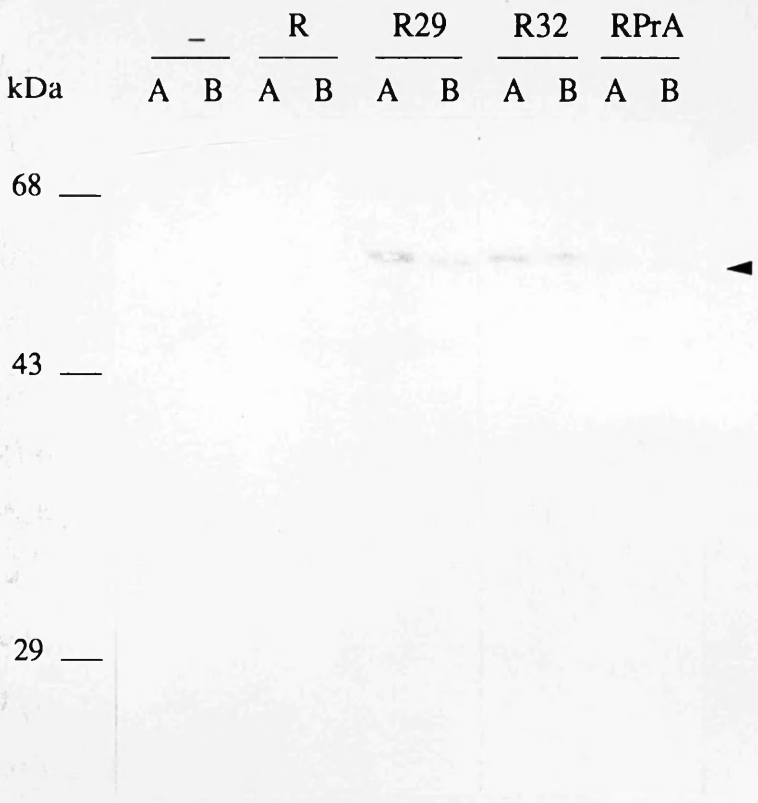
Figure IV.3

a) pp60 expression in infected CEF. Primary chicken embryo fibroblasts were transfected with RCAN expression constructs as described in Materials and Methods. Transfected cultures and mock treated controls were passaged 1-3 times at restrictive temperature to allow virus spread. Cells were grown at either permissive or restrictive temperature for 48hr before lysis. Cultures had aquired the appropriate morphology at this point, indicating uniform infection. Equivalent amounts of lysate protein (as determined by Coomassie Blue staining) were resolved by SDS-PAGE and blotted as described in Materials and Methods. Primary antiserum was anti-*Src* antiserum MW2 at 1:5000. The arrow head indicates pp60. *A, cells at permissive temperature, B, cells at restrictive temperature. Transfected constructs : -, no DNA, R, RCAN, R29, RCAN 29, R32, RCAN 32, RPrA, RCAN PrA.*

b) *Gag* expression in infected CEF. Immunoblotting of *Gag* proteins was used to assess infection by RCAN vector, since CEF expressing this virus appear morphologically normal. Lysates from a) were resolved and blotted as described in a) except anti-*Gag* antiserum 5202 was used (1:5000). All lysates were from cultures growing at permissive temperature. The closed arrow head indicates Pr76 and the open arrow head p27. *Transfected constructs : see a).*

Figure IV.3

a) pp60 expression in infected CEF



b) *Gag* expression in infected CEF

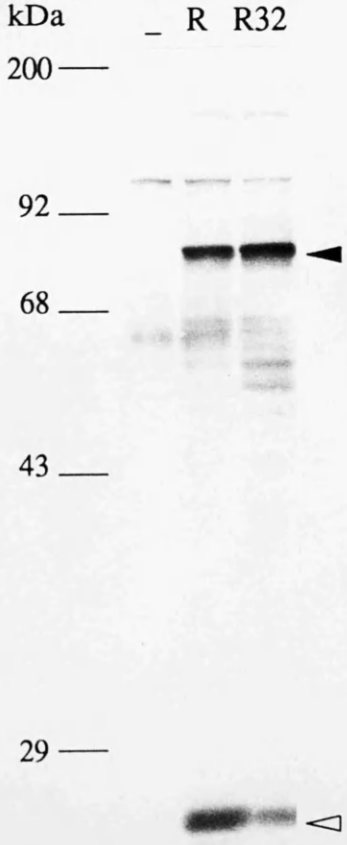


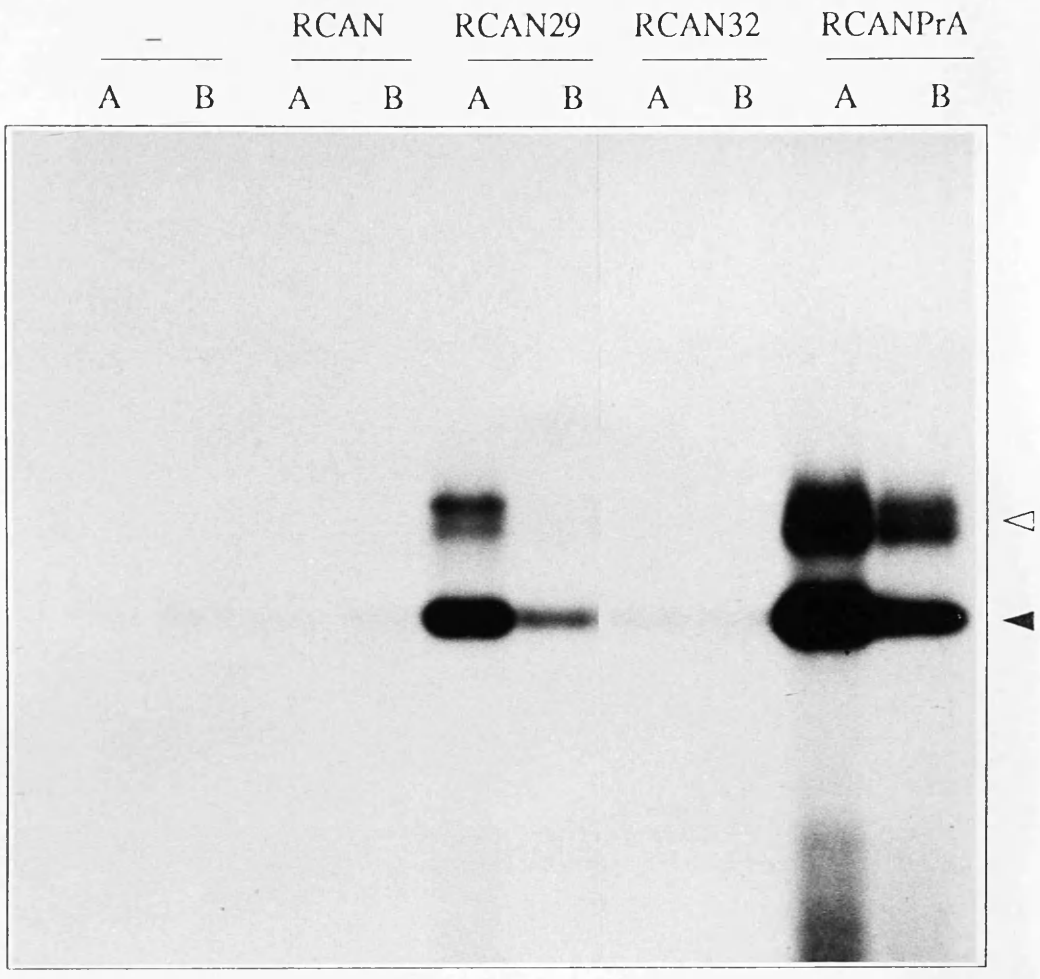
Figure IV.4

a) MAb327-immune complex kinase assay of infected CEF. pp60 was immunoprecipitated from uniformly infected cultures using monoclonal antibody MAb327 as described in Materials and Methods. Kinase activity in the immunoprecipitates was measured using acid denatured enolase as exogenous substrate, as described in Materials and Methods. Assay temperature was 30^{o}C, and exposure time was 4hr at -70^{o}C. The open arrow indicates pp60, and the closed arrow enolase (as determined by Coomassie Blue staining). Transfected constructs are indicated at the top of the figure. *A, cultures at permissive temperature, B, cultures at restrictive temperature.*

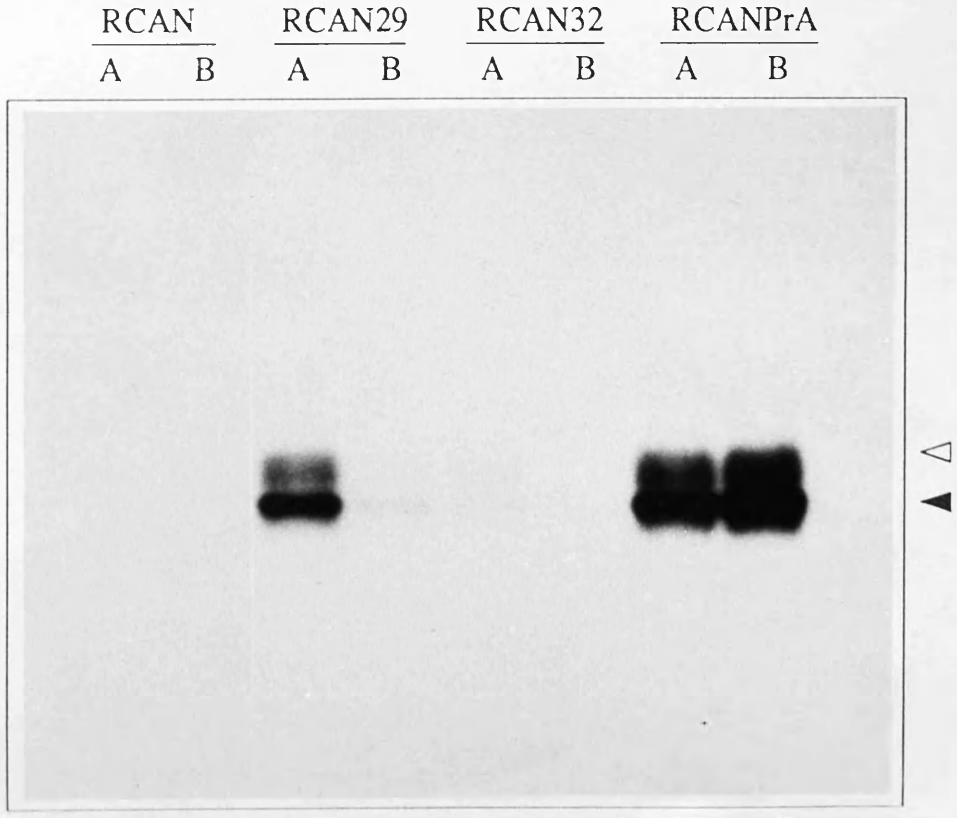
b) TBR-immune complex kinase assay. pp60 was immunoprecipitated from uniformly infected cultures using tumour-bearing rabbit serum TBR-RLE as described in Materials and Methods. Immune complex kinase activity against the immunoglobulin heavy chain and pp60 was measured as described in Materials and Methods. Assay temperature was 30^{o}C and exposure time was 2hr at -70^{o}C. The open arrow indicates pp60 and the closed arrow immunoglobulin heavy chain (as judged by Coomassie Blue staining). Transfected constructs are indicated at the top of the figure. *A, cultures at permissive temperature, B, cultures at restrictive temperature.*

Figure IV.4

a) MAb327-immune complex kinase assay of infected CEF



b) TBR-immune complex kinase assay of infected CEF



IV.C. Discussion

Under conditions where immunoprecipitates of *ts*LA29 pp60 and *wt*PrA pp60 behaved as expected in an *in vitro* assay, immune complexes prepared from Rat1 f32 cells were consistently inactive. This outcome was largely independent of the precipitating antiserum, substrate and host cell background. Identical results were obtained after lysis and immunoprecipitation under reducing conditions. This failure to detect kinase activity in Rat1 f32 precipitates was not the result of poor precipitation of the mutant pp60, as evidenced by precipitation of ^{35}S-methionine labelled protein. The consistent lack of kinase activity appears to result from a defect in the catalytic activity of the protein rather than being the result of poor immunoprecipitation. There are several possible reasons for this observation :

a) *ts*LA32 *Src* transforms cells *in vitro* via a mechanism independent of intrinsic tyrosine kinase activity. This would appear unlikely given the strong correlation between kinase activity and transformation. However, one intriguing idea is that the protein transforms in much the same way as v-*Crk*, by way of its SH2 and SH3 domains[218]. The mutation at position 107 in the SH3 domain is interesting in this respect.

b) the post-translational modification of the protein is defective. This would appear unlikely given that identical results were obtained from both Rat1 and CEF backgrounds. The post-translational phosphorylation of the protein is covered in chapter V.

c) *ts*LA32 pp60 is active against a very limited range of substrates *in vitro*. This possibility could be addressed using biologically relevant substrates.

d) association with a regulatory factor determines kinase activity in *ts*LA32 pp60 immunoprecipitates. This would predict the loss of an activator or the co-precipitation of an inhibitor during sample preparation. To my knowledge, this has not been observed in studies on a large number of v-*Src* mutants, though inhibitors active against specific serine \ threonine kinases are well documented[317]. I have observed the specific precipitation of a 50kDa phosphoprotein in pp60 immunoprecipitates prepared from Rat1 f32 cells growing as transformed cells at permissive temperature. This protein is not seen

in precipitates prepared from Rat1 f32 cells growing at restrictive temperature, nor is it seen in precipitates prepared from Rat1 f29 or Rat1 fPrA. However, upon digestion with V8 protease, this protein yields a phosphopeptide pattern consistent with it being a degradation product of pp60 (data not shown).

e) the presence of a mutation in the SH3 domain could reduce the kinase activity. This could be addressed using appropriate chimaeric molecules.

f) the mutant protein is easily denatured upon lysis and immunoprecipitation.

Experiments to date have not adequately explained the the *in vitro* kinase defect of *ts*LA32 pp60.

Chapter V

V *IN VIVO* KINASE ACTIVITY AND POST-TRANSLATIONAL PHOSPHORYLATION

V.A. Introduction

Since *in vitro* kinase assays were largely uninformative, the *in vivo* consequences of expression of *ts*LA32 pp60 were analyzed in two ways. First, the level of protein phosphotyrosine in the Rat1 cell lines was estimated by steady state metabolic labelling with ^{32}P-orthophosphate. Previous work has indicated that the cellular level of protein phosphotyrosine is independent of growth temperature in *ts*LA32 infected CEF[299]. Second, the *in vivo* phosphorylation state of pp60 was analyzed by limited digestion using V8 protease[318]. This technique provides information on the state of the two major phosphorylation sites, serine 17[58] and tyrosine 416[57]. The phosphorylation state of tyrosine 416 is also an indicator of pp60 kinase activity since tyrosine 416 is believed to be a site of autophosphorylation[57].

V.B. Results

V.B.1 *In vivo* Abundance of Phosphotyrosine

Exponential cultures of Rat1, Rat1 f29, Rat1 f32 and Rat1 fPrA were maintained at permissive or restrictive temperature for at least two days prior to labelling with ^{32}P-orthophosphate (1mCi \ ml) for 18-20hrs as outlined in Materials and Methods. Total cellular proteins were prepared as described, and hydrolyzed in 6M hydrochloric acid. Hydrolysates were resolved by two-dimensional thin-layer electrophoresis at pH 1.9 and pH 3.5 as described by Cooper *et al*[310]. Unlabelled phosphoserine, phosphothreonine and phosphotyrosine were added to hydrolysates to serve as markers following ninhydrin staining. Typical results are shown in figure V.1. Rat1 f29, f32 and fPrA all show elevated levels of phosphotyrosine at both permissive and restrictive temperature when compared to parental Rat1 fibroblasts, where labelled phosphotyrosine is undetectable. Relevant regions of the cellulose plate, together with background areas, were collected and radioactivity quantitated by liquid scintillation counting. The abundance of each phosphohydroxyamino acid is expressed

Figure V.1

Phosphoamino acid analysis of total cellular protein in Rat1 *src* clonal lines. Cultures at permissive or restrictive temperature were labelled to equilibrium with carrier-free ^{32}P-orthophosphate and total cellular protein prepared as described in Materials and Methods. After acid hydrolysis, hydrolysates were supplemented with unlabelled standards (phosphoserine, phosphothreonine, phosphotyrosine) and resolved by two-dimensional thin-layer electrophoresis as described. Standards were visualized using ninhydrin, and labelled phosphoamino acids revealed by autoradiography. Exposure time was ~36hr at -70^{o}C. *PS, phosphoserine, PT, phosphothreonine, PY, phosphotyrosine*. *a*, Rat1 35^{o}C, *b*, Rat1 40^{o}C, *c*, Rat1 f29 35^{o}C, *d*, Rat1 f29 40^{o}C, *e*, Rat1 f32 35^{o}C, *f*, Rat1 f32 40^{o}C, *g*, Rat1 fPrA 35^{o}C, *h*, Rat1 fPrA 40^{o}C.

Figure V.1

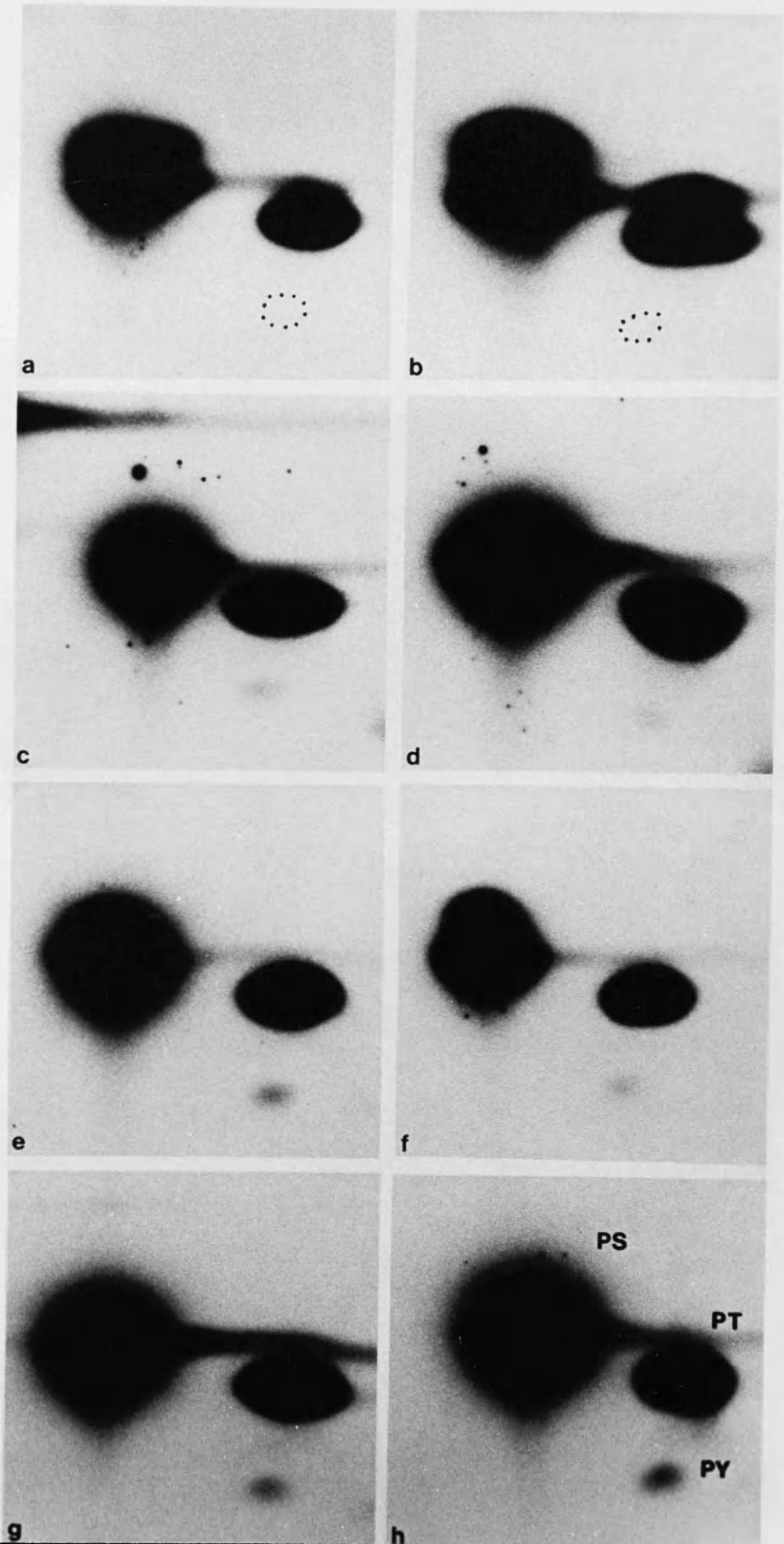


Figure V.2

a) Relative abundance of phosphohydroxyamino acids in Rat1 *src* clonal lines. Phosphoamino acids were scraped from the cellulose plates shown in figure V.1 together with appropriate background areas. After liquid scintillation counting and subtraction of background radioactivity, the percentage abundance of each phosphoamino acid relative to the total radioactivity in phosphoserine, phosphothreonine and phosphotyrosine was calculated. The abundance of phosphotyrosine with respect to growth temperature is shown as a ratio of the percentage in cells at permissive temperature divided by the percentage in cells at restrictive temperature. Very similar results were obtained in three independent experiments. *PY, phosphotyrosine.*

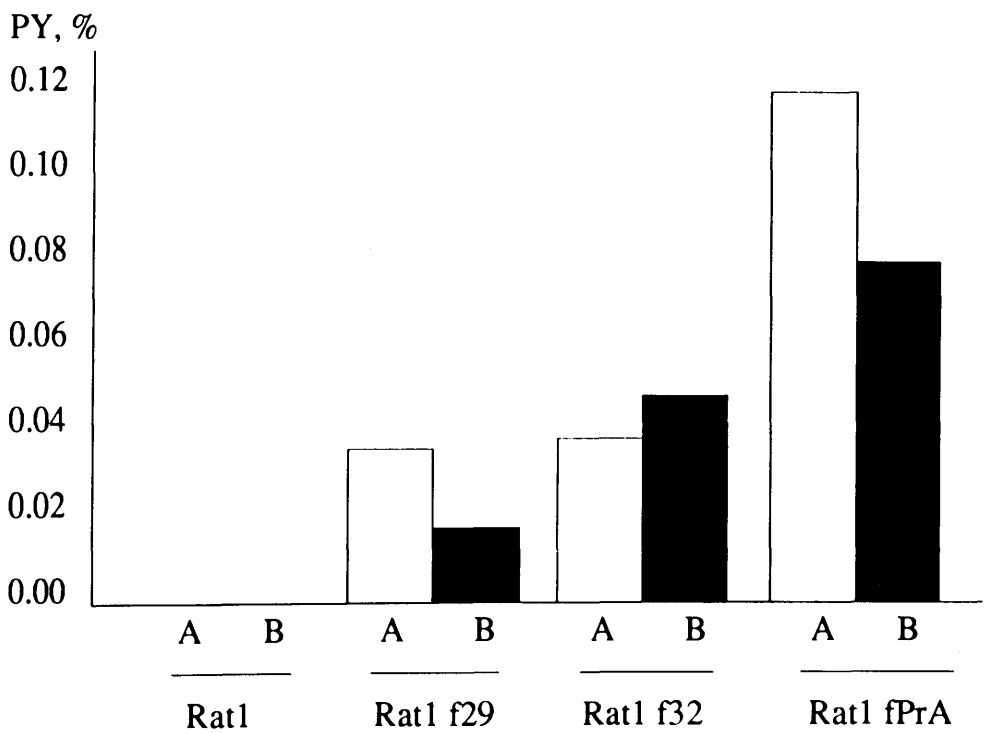
b) Relative abundance of phosphotyrosine with respect to growth temperature in Rat1 *src* clonal lines. Data from a). Abundance of phosphotyrosine is expressed as a percentage of total radioactivity in the three phosphohydroxy amino acids. *PY, phosphotyrosine, A, cells at permissive temperature, B, cells at restrictive temperature.*

Figure V.2

a) Relative abundance of phosphohydroxyamino acids in Rat1 *src* clonal lines

Clone	Phosphoamino acid	35°C	40°C	PY, 35°C/40°C
Rat1	Phosphoserine	87.3%	88.3%	
	Phosphothreonine	12.7%	11.7%	
	Phosphotyrosine	ND	ND	
Rat1 f29	Phosphoserine	89.1%	91.6%	
	Phosphothreonine	10.9%	8.4%	
	Phosphotyrosine	0.036%	0.019%	~2
Rat1 f32	Phosphoserine	91.1%	90.9%	
	Phosphothreonine	8.8%	9.1%	
	Phosphotyrosine	0.037%	0.048%	~0.8
Rat1 fPrA	Phosphoserine	90.3%	88.7%	
	Phosphothreonine	9.7%	11.2%	
	Phosphotyrosine	0.120%	0.080%	~1.5

b) Relative abundance of phosphotyrosine in Rat1 *src* clonal lines



as a percentage of the total radioactivity in all three phosphohydroxyamino acids (figure V.2). As stated above, phosphotyrosine is undetectable in growing Rat1 fibroblasts. In contrast, all cells expressing pp60, whether at permissive or restrictive temperature, exhibit elevated levels of phosphotyrosine. The level of phosphotyrosine in Rat1 fPrA cells is slightly temperature sensitive, correlating well with both the *in vitro* kinase activity and biological behaviour of this "wild type" protein. Somewhat suprisingly, cells expressing the thermolabile kinase mutant *ts*LA29 show only a two-fold reduction in phosphotyrosine level at restrictive temperature. This behaviour was consistent over three separate experiments, and has been observed by other workers[292]. These data contradict the results obtained using *in vitro* assays, where the *ts*LA29 kinase activity is strikingly temperature sensitive. The abundance of phosphotyrosine in Rat1 f32 cells is largely independent of temperature, there being a slight increase at restrictive temperature. These observations suggest that an overall increase in the level of cellular phosphotyrosine is not in itself sufficient for transformation, since both Rat1 f29 and Rat1 f32 cells show elevated levels even when growing non-transformed at restrictive temperature. These observations will be discussed later in this chapter.

V.B.2 Post Translational Phosphorylation of pp60

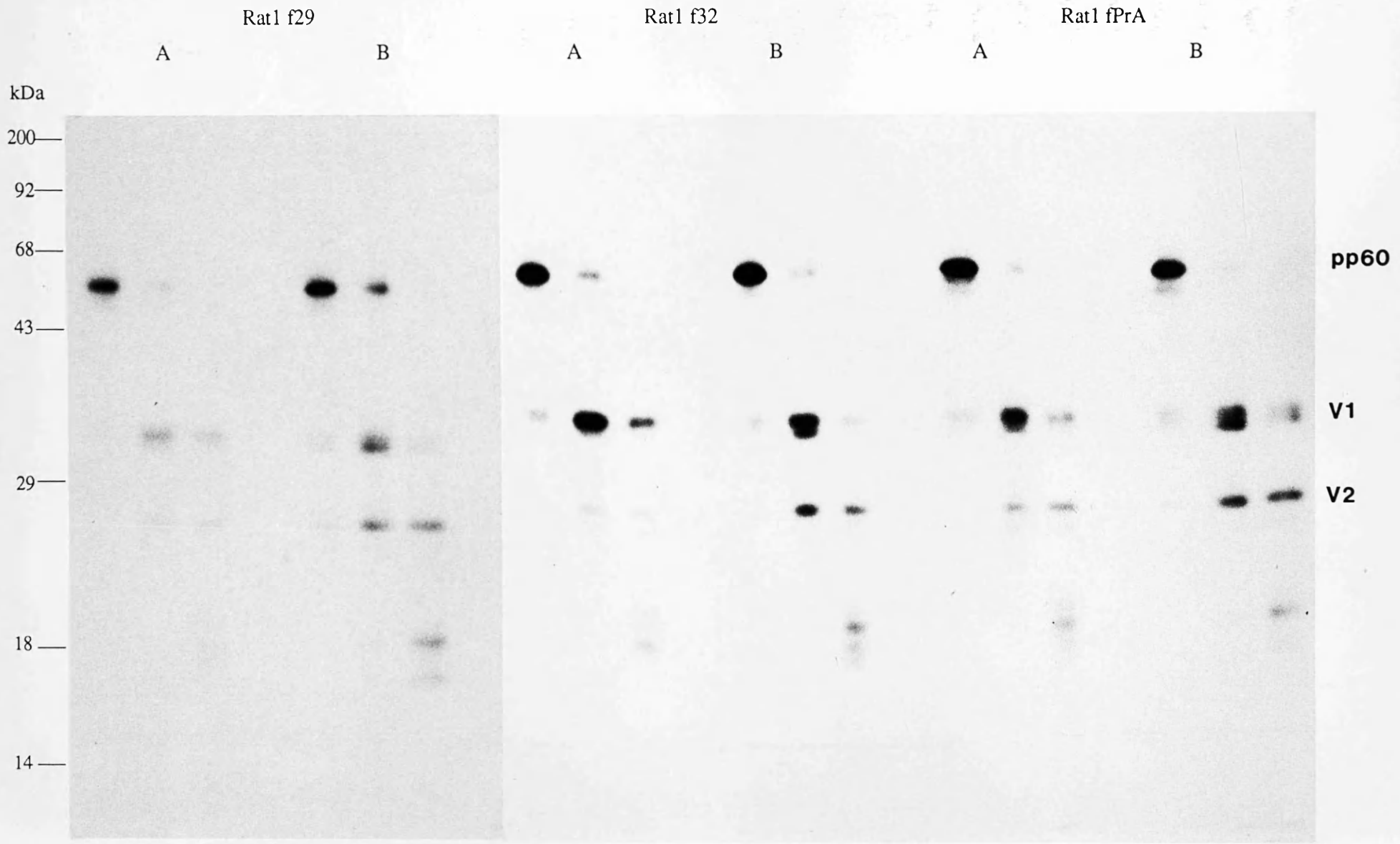
pp60$^{v\text{-}src}$ is post-translationally phosphorylated on at least two residues. The major sites of phosphorylation are serine 17[58] and tyrosine 416[57]. The phosphorylation state of tyrosine 416 correlates well with enzyme activity, and published data suggest that this residue is autophosphorylated *in vitro* and *in vivo* (see chapter I). Serine 17 and tyrosine 416 are separated by limited cleavage with *Staphylococcus aureus* V8 protease, yielding a 36kDa fragment derived from the N-terminus of pp60 (V1), and a 24kDa C-terminal fragment (V2)[319]. V8 cleavage allows assessment of both the kinase activity of pp60 *in vivo*, and the extent of post-translational phosphorylation.

Growing cultures were labelled with ^{32}P-orthophosphate as for the phosphoamino acid analysis. pp60 was immunoprecipitated using MAb327 in the presence of

Figure V.3

Post-translational phosphorylation of mutant and wild-type pp60 in clonal Rat1 *src* lines. Cultures at permissive or restrictive temperature were labelled to equilibrium with carrier-free ^{32}P-orthophosphate and pp60 immunoprecipitates prepared as described in Materials and Methods. Gel purified pp60 was subject to partial digestion with *S. aureus* V8 protease, and the resulting peptides separated by one-dimensional 12% SDS-PAGE as described. pp60 was digested with (from left to right in each set of three lanes) 1, 10 or 100ng V8 protease. The positions of undigested pp60, and primary digestion products V1 and V2 (see text) are indicated. Autoradiography was for 7d at -70^{o}C. *A, cells at permissive temperature, B, cells at restrictive temperature.*

Figure V.3


Rat1 f29
Rat1 f32
Rat1 fPrA
A
B
A
B
A
B
kDa
200
92
68
43
29
18
14
pp60
V1
V2

phosphatase and protease inhibitors and immunoprecipitates fractionated by SDS-PAGE. pp60 bands were excised and digested with V8 protease as described in Materials and Methods. As noted in chapter III, *ts*LA32 pp60 migrates at a slightly higher apparent molecular weight than either *ts*LA29 or *wt*PrA pp60. This apparent size difference is not observed in the resulting phosphopeptides, and is presumably a secondary structure effect. The data presented in figure V.3 indicate that both temperature sensitive mutants appear to be wild type for tyrosine 416 phosphorylation. Similar results was obtained in three experiments. It is somewhat suprising that *ts*LA29 pp60 is tyrosine phosphorylated at restrictive temperature given its striking thermolabile kinase properties *in vitro*. Furthermore, the same mutant appears to be temperature sensitive for *in vivo* tyrosine 416 phosphorylation when these experiments are performed in CEF^{299}. This discrepancy will be discussed more thoroughly below.

Given the absence of a temperature sensitive control in this analysis, the interpretation of these results is somewhat tentative. Assuming that tyrosine 416 phosphorylation is the result of autophosphorylation, the data are consistent with *ts*LA32 pp60 being kinase active at both permissive and restrictive temperatures. This result also excludes the possibility that the lack of *in vitro* kinase activity in Rat1 f32 pp60 immunoprecipitates (chapter IV) is the result of defective post-translational phosphorylation, since the protein is wild type in this respect.

V.C. Discussion

The data presented in this chapter suggest that Rat1 f29, Rat1 f32 and Rat1 fPrA cells contain elevated tyrosine kinase activity at both permissive and restrictive temperatures. Comparing the abundance of phosphotyrosine with the abundance of pp60 in these cells (see figures III.3b and V.2) it is apparent that the wild-type PrA kinase has a greater specific activity than the two temperature sensitive mutants at either permissive or restrictive temperatures. The elevation of cellular phosphotyrosine in Rat1 f29 cells at restrictive temperature was unexpected in view of both the published data generated using *ts*LA29 RSV-infected CEF^{299} and the *in vitro* kinase data presented in chapter IV.

However, it is perhaps pertinent to stress that these mutants were initially selected as being temperature sensitive for transformation in CEF[320]. Restrictive temperature in this system is 41°C rather than 39-40°C in mammalian cell systems. It is formally possible that this slight lowering in restrictive temperature, necessary to allow cell survival, is sufficient to allow the *ts*LA29 kinase to function. This could explain the V8 digestion results and the elevation in cellular phosphotyrosine seen in Rat1 f29 growing at 40°C. A corollary of this argument would be that the major transformation defect in *ts*LA29 pp60 in mammalian cells is its inability to locate to the particulate fraction at restrictive temperature. This would prevent access to important substrates, explaining the conditional biological properties of this mutant. These data also suggest that membrane association and kinase activity are dissociable.

Alternatively, the elevation of cellular phosphotyrosine resulting from kinase activity of *ts*LA29 pp60 at restrictive temperature may be insufficient to elicit transformation, *i.e.* cellular phosphotyrosine must exceed a threshold value before transformation is possible. These alternatives are not necessarily exclusive. One presumes that the lack of *in vitro* kinase activity in pp60 immunoprecipitates from Rat1 f29 cells at restrictive temperature results from instability of the mutant protein.

*ts*LA32 pp60 appears to be somewhat different in that previous work from this laboratory[93] (and my preliminary results, not shown) indicate that this protein retains substantial membrane association at restrictive temperature, likening it to the wild type protein. The results of the total cell protein phosphoamino acid analysis suggest that the transformation defect in *ts*LA32 is rather subtle in nature. One possibility is that this kinase fails to associate with and \ or phosphorylate one or a number of important proteins at restrictive temperature. This model predicts that only a small number of primary tyrosine phosphorylation events would be seen rapidly following shift of Rat1 f32 cells to permissive temperature. This idea has been discussed at length in chapter I.

The tentative conclusion from these studies is that the Rat1 f32 cell line behaves in a manner compatible with the proposed approach to studying early events of v-*src* transformation.

Chapter VI

VI MITOGENIC ACTIVITY OF THE *ts* MUTANTS

VI.A. Introduction

As outlined in the introduction to this thesis, the described approach to studying early events of v-*src* transformation depends on the cell line chosen having a number of characteristics. These are as follows:

a) the cells should be temperature sensitive for transformation

b) the cells should express a mutant pp60 whose membrane localization and kinase activity is not grossly affected by incubation at restrictive temperature

c) one should be able to render these cells quiescent by serum deprivation at restrictive temperature and these cells should re-enter cycle upon shift to permissive temperature in the absence of added factors.

These properties should reduce the number of adventitious phosphorylation events seen following temperature shift, facilitating identification of physiologically relevant changes. The temporal sequence of events can also be followed using this approach, since growth state effects are minimised. Rat1 f29 and Rat1 f32, and parental Rat1 cells were tested for their ability to re-enter cycle upon temperature shift.

VI.B. Results

Quiescent cultures were prepared as described in Materials and Methods, and cells stimulated either by the addition of serum at restrictive temperature, or by temperature shift to 35°C. Rat1 *ts*LA29[19,94], a clonal line demonstrated to re-enter cycle following temperature shift[29] was included as a control for these experiments. Cells were labelled at appropriate time points with tritiated thymidine, and harvested one hour later. Label incorporated into TCA-precipitable material was counted and the data plotted. Figure VI.1 demonstrates that both Rat1 f29 and Rat1 f32 could re-enter cycle following shift to permissive temperature, unlike parental Rat1 fibroblasts which failed to incorporate tritiated thymidine in response to temperature shift. Note that the precise timing of entry

Figure VI.1

Mitogenic activity of the temperature sensitive mutants in Rat1. Rat1, Rat1 *ts*LA29, Rat1 f29 and Rat1 f32 were rendered quiescent at restrictive temperature by serum deprivation as described in Materials and Methods. Cultures were left untreated at 40°C (■), stimulated by addition of newborn calf serum to a final concentration of 5% (v \ v) at 40°C (▲) or shifted to permissive temperature in the absence of added factors (◍). Cultures were pulse-labelled (1hr) with tritiated thymidine at four-hourly intervals, and radioactivity incorporated into TCA-precipitable material was measured by liquid scintillation counting. Incorporation is expressed as ^{3}H cpm x 10^{-4} per 35mm culture dish at each time point. *a*, Rat1, *b*, Rat1 *ts*LA29, *c*, Rat1 f29, *d*, Rat1 f32.

Figure VI.1

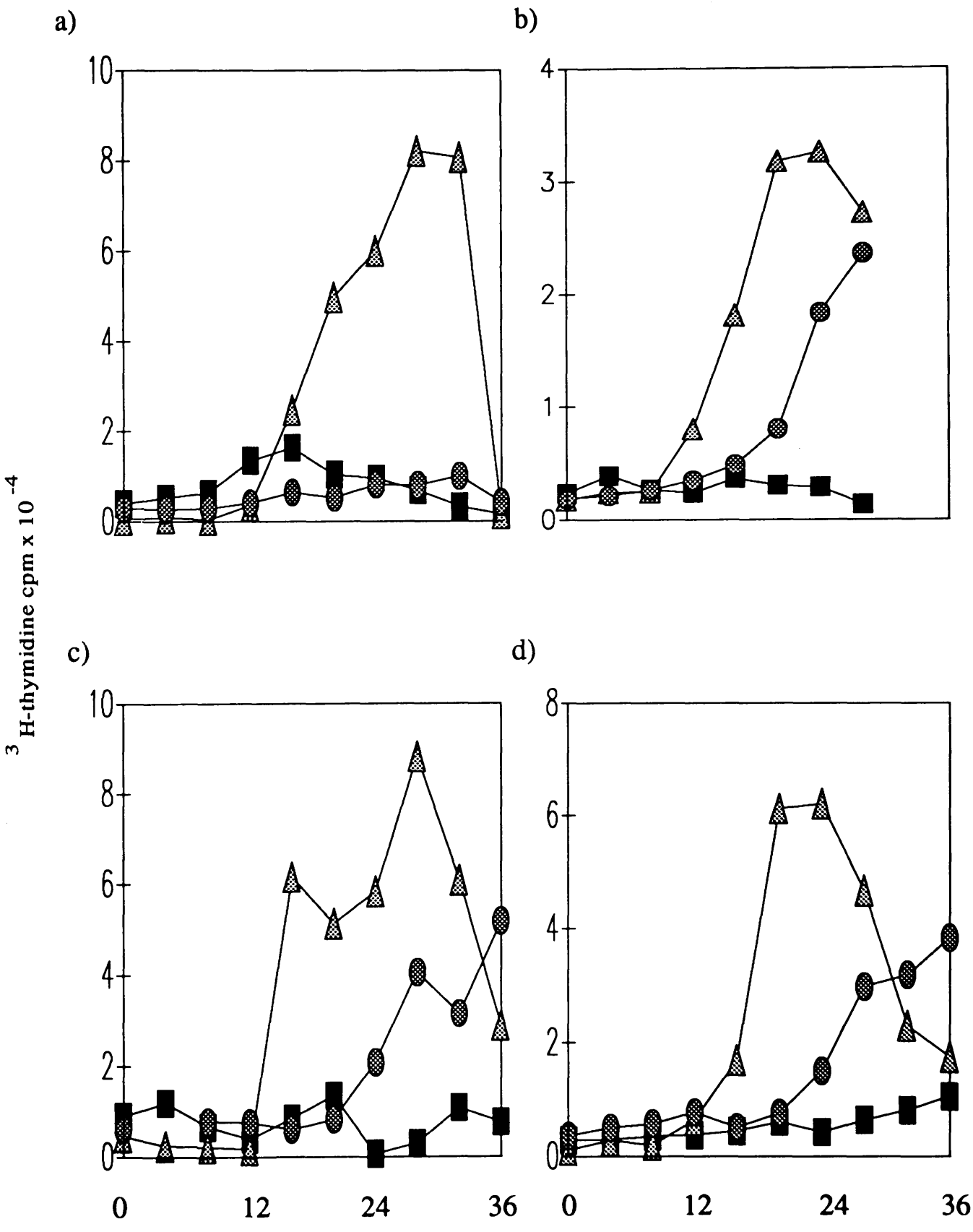


Figure VI.2

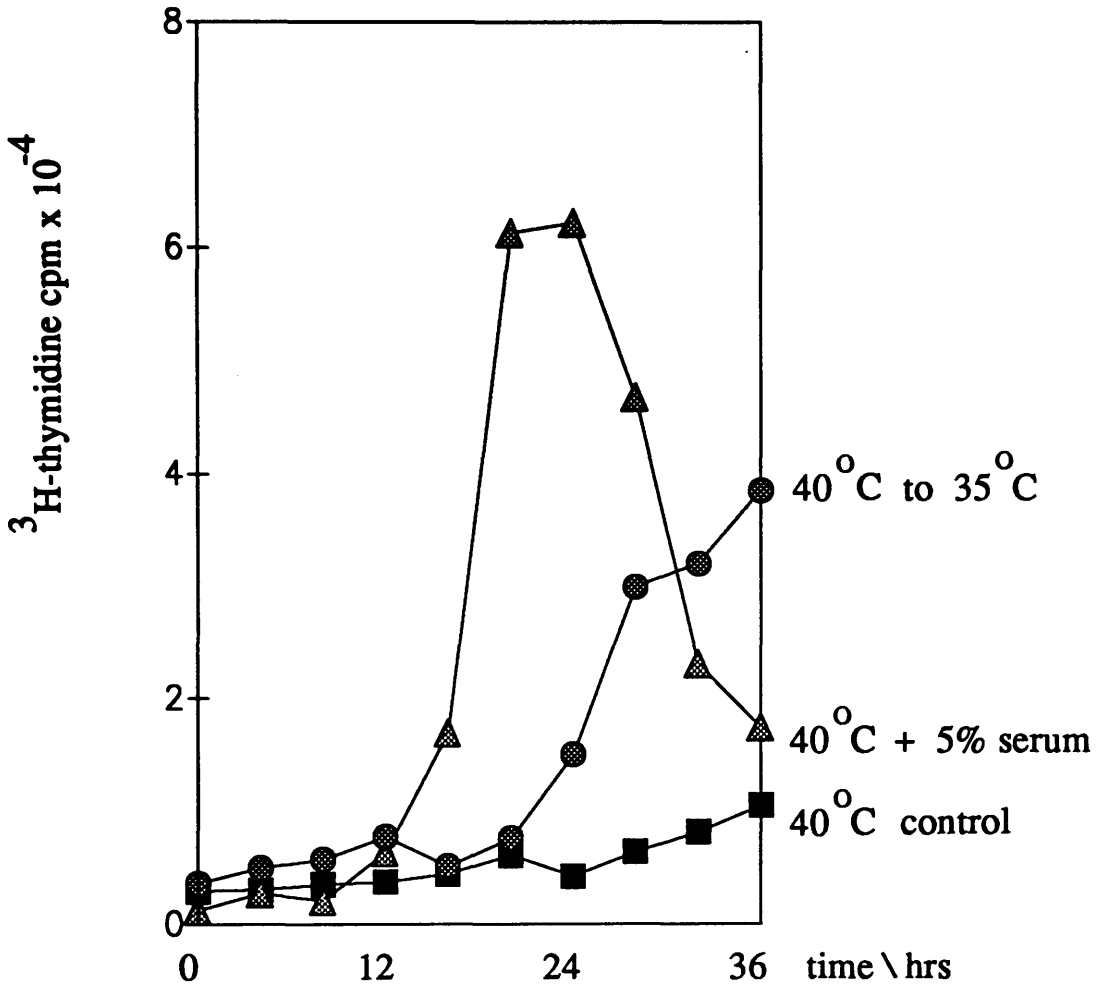
Expression of pp60 in Rat1 f32 following serum stimulation or temperature shift of quiescent cultures.

a) Time course of ^{3}H-thymidine incorporation in Rat1 f32 cells stimulated to enter cycle by addition of serum at restrictve temperature or temperature shift. See figure VI.1 for details.

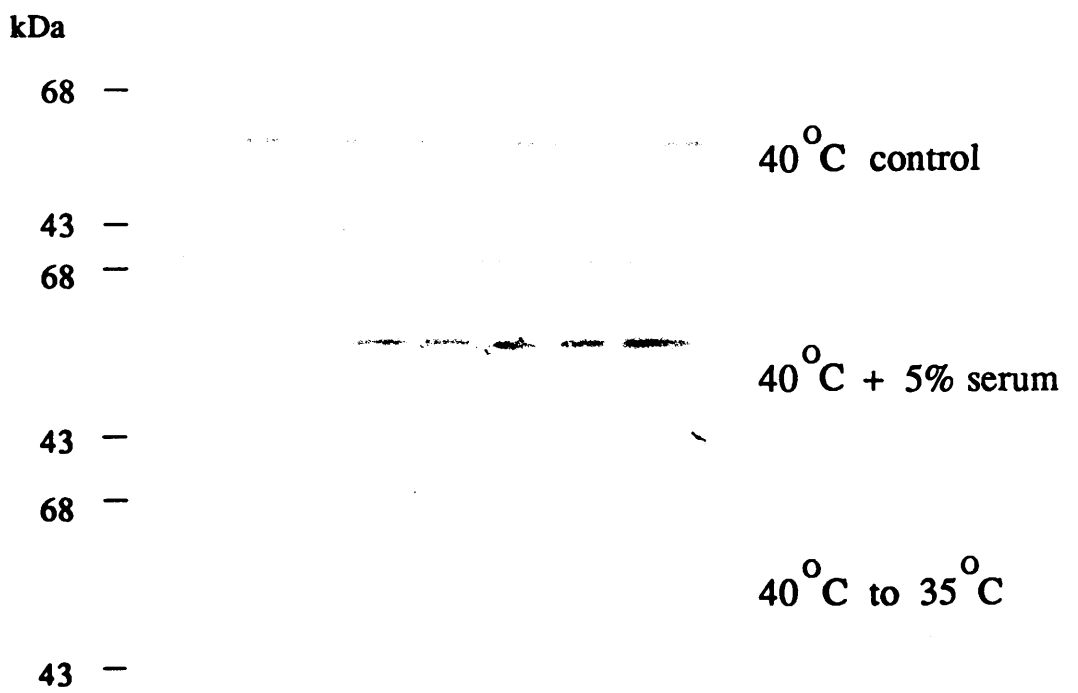
b) Immunoblot analysis of pp60 over time course shown in a). Parallel cultures were treated identically to those in a) except lysates were prepared for immunoblot analysis at 0, 4, 8, 12, 16, 24, 28 and 32hrs. Equivalent volumes of lysate were resolved by SDS-PAGE and blotted according to Materials and Methods. Primary antiserum was MW2 at 1:5000 dilution. Only the relevant areas of the blots are shown, with time increasing from left to right. Culture conditions are shown to the right of each blot.

Figure VI.2

a)



b)



into S phase, and the magnitude of the S phase peak varied somewhat between experiments. However, the onset of S phase was consistently slower and often less synchronous in temperature shifted cultures than in serum stimulated cultures, with DNA synthesis starting at approximately 20-24hrs post temperature shift. Cultures of Rat1 f29 and Rat1 f32 cells were noticably transformed after approximately 16hrs at permissive temperature. Both entry into cycle and transformation appeared not to require gross changes in the level of pp60, since immunoblot analysis throughout this timecourse revealed only modest changes consistent with a doubling in cell number (figure VI.2).

VI.C. Discussion

The mitogenic activity of pp60 has been characterised quite extensively by a number of groups[26,27,29,242,243]. Rat1 cells expressing the thermolabile mutant *ts*LA29 have been characterized extensively in this laboratory, and found to re-enter cycle following shift to permissive temperature[29]. This stimulation of proliferation occurs in the absence of added factors[29]. I have therefore asked whether *ts*LA32 *Src* behaves in a similar fashion, using parental cells and cells expressing *ts*LA29 *Src* as controls. The data shown suggest that Rat1 f32 cells can be rendered quiescent by serum deprivation, in spite of retaining substantial tyrosine kinase activity and, presumably, pp60 membrane localization at restrictive temperature. These cells can then be stimulated to transform and re-enter cycle by shifting to permissive temperature in the absence of added factors. In addition, there is a rapid increase in the ability of nuclear extracts to bind AP-1 consensus oligonucleotides following temperature shift of quiescent Rat1 f32 cells (A. W. Wyke, personal communication). This is in good agreement with the results of Welham *et al.*, using the Rat1 *ts*LA29 system[29]. As with cells expressing *ts*LA29 *Src*[29] or *ts*LA23 *Src*[26], the onset of DNA synthesis is somewhat delayed when compared to the serum response, and in some cases entry into S phase appears to be somewhat less synchronous than for serum stimulated cells at restrictive temperature. This delay does not reflect a requirement for a net increase in pp60 expression as evidenced by the

immunoblot in figure VI.2. The reasons for this delay are not yet clear, though there are several possibilities :

a) the delay is merely a temperature effect.

b) cells stimulated by temperature shift as opposed to serum are limited for factors that allow entry into S phase, *e.g.* nutrients required for an increase in cell mass, nucleotide precursors.

c) cells shifted to permissive temperature secrete a mitogenic factor upon transformation, this functioning in an auto- or paracrine fashion.

d) the mitogenic activity of the *ts* mutants requires turnover of existing pp60.

e) transformation relieves contact inhibition, allowing the mitogenic activity of pp60 to be expressed.

f) events leading to DNA synthesis and mitosis in cells stimulated with pp60 are fundamentally different to those in serum stimulated cells.

At present there is little data to support any of these hypotheses. Note that morphological transformation and mitogenic activity are both temperature sensitive in Rat1 f32. This is interesting in view of the wild type-like *in vivo* behaviour of this mutant protein. It seems likely that these two endpoints are not dissociable, either because they use a common target molecule or because transformation indirectly leads to mitogenesis, perhaps through relief of contact inhibition or via secretion of transformation specific growth factors. Whether morphological transformation and mitogenic activity are separable forms another part of this project and will be described elsewhere in this thesis.

Results detailed in previous chapters demonstrate that Rat1 f32 is conditional for morphological transformation and anchorage independent growth, despite retaining elevated protein phosphotyrosine at restrictive temperature. Previous work from this laboratory using *ts*LA32-infected CEF, and my own preliminary data (not shown) using Rat1 f32 cells indicate that substantial amounts of pp60 are retained in the particulate fraction at restrictive temperature[93]. The results presented in this chapter confirm that these cells can be rendered quiescent at restrictive temperature, despite expressing kinase active, membrane associated pp60. These cells are then stimulated to re-enter cycle

simply by shifting to the permissive temperature. In conclusion, the Rat1 f32 cell line satisfies the criteria outlined at start of this chapter, and would therefore merit use in studying early events of v-*src* stimulated mitogenesis and transformation.

Chapter VII

VII EARLY CHANGES IN PROTEIN SYNTHESIS AND PHOSPHORYLATION FOLLOWING TEMPERATURE SHIFT OF QUIESCENT Rat1 f32

VII.A. Introduction

Many attempts to define substrates important in v-*Src* function have been compromised by the choice of cell line and exact experimental approach. Much emphasis has been placed on the comparison between transformed cells and their normal uninfected counterparts. Results from this type of experiment are difficult to interpret given that observed changes in protein synthesis and modification could be a consequence of, rather than a prerequisite for transformation. Growth state effects are also a potential problem in this type of approach, rendering cell cycle stage-specific events difficult to detect. Indeed, growth-state can be influenced by the techniques employed in detecting changes. For instance, radiolabelling with ^{32}P-orthophosphate or even ^{35}S-methionine can lead to S and G2 arrest[66,321], precluding observation of events in the G1 phase of the cycle. Refined approaches have utilized temperature sensitive mutants of *Src* to study early events following activation of pp60 kinase activity, and combined with appropriate treatment of the cells under study, this has lead to some understanding of the temporal sequence of events following induction of the mitogenic and transforming activities of pp60. However, these approaches have employed pp60 mutants which are typically conditional for kinase activity. Following shift to permissive temperature kinase activity is reactivated against all substrates, leading to phosphorylation of many adventitious substrates, hindering identification of relevant events. I have used a mutant whose biochemical properties are essentially wild type, but whose biological activity is temperature sensitive. The prediction is that cells expressing this mutant should exhibit fewer adventitious events following temperature shift facilitating identification of important changes. Note that this approach should also enable us to identify a group of proteins whose combined tyrosine phosphorylation at restrictive temperature is not sufficient for transformation *in vitro*.

Preceeding characters have dealt with the generation and characterization of the Rat1 f32 cell line, the results obtained suggesting that this line could be of use in the

study of early events of v-*Src* transformation and mitogenesis. Therefore, experiments were performed to address the following questions :

a) can one detect changes in protein synthesis following temperature shift of quiescent Rat1 f32 cultures.

b) can one detect changes in phosphorylation of cellular proteins following temperature shift of quiescent Rat1 f32 cultures.

Preliminary results of these experiments are detailed below.

VII.B. Results

VII.B.1 Protein Synthesis Following Temperature Shift of Rat1 f32

To investigate changes in protein synthesis involved in the G0 \ G1 and G1 \ S transitions, quiescent cultures of Rat1 f32 and Rat1 were shifted to permissive temperature for 0 (i.e. maintained at 40^{o}C), 1, 4, 7 or 24hrs. At one hour before this time period had elapsed, the cultures were incubated in methionine- and serum-free medium for 30 minutes prior to labelling for the remaining 30 minutes with 200uCi \ ml of ^{35}S-methionine in methionine- and serum-free medium. Monolayers were washed in PBS before lysis in 2D-gel sample buffer. 2D-gel electrophoresis was expertly performed by Lynn McGarry, as outlined in Materials and Methods.

Although the 2D-gel system used is able to resolve approximately 2000 methionine-labelled proteins, preliminary results indicate that very few proteins demonstrate detectable changes in rate of synthesis or degradation following temperature shift of quiescent Rat1 f32 cells. The majority of these differences only became apparent following a 24hr shift to permissive temperature, and were not observed at 1, 4 or 7hr timepoints. This correlates with the entry of these cells into S phase (see chapter VI), and also with the onset of morphological transformation. The seven changes observed, designated *a-g*, are indicated by arrows in figure VII.1. These changes (four increases and three decreases in labelling intensity) were not observed in cultures of Rat1 cells treated in parallel, and so probably do not reflect non-specific

Figure VII.1

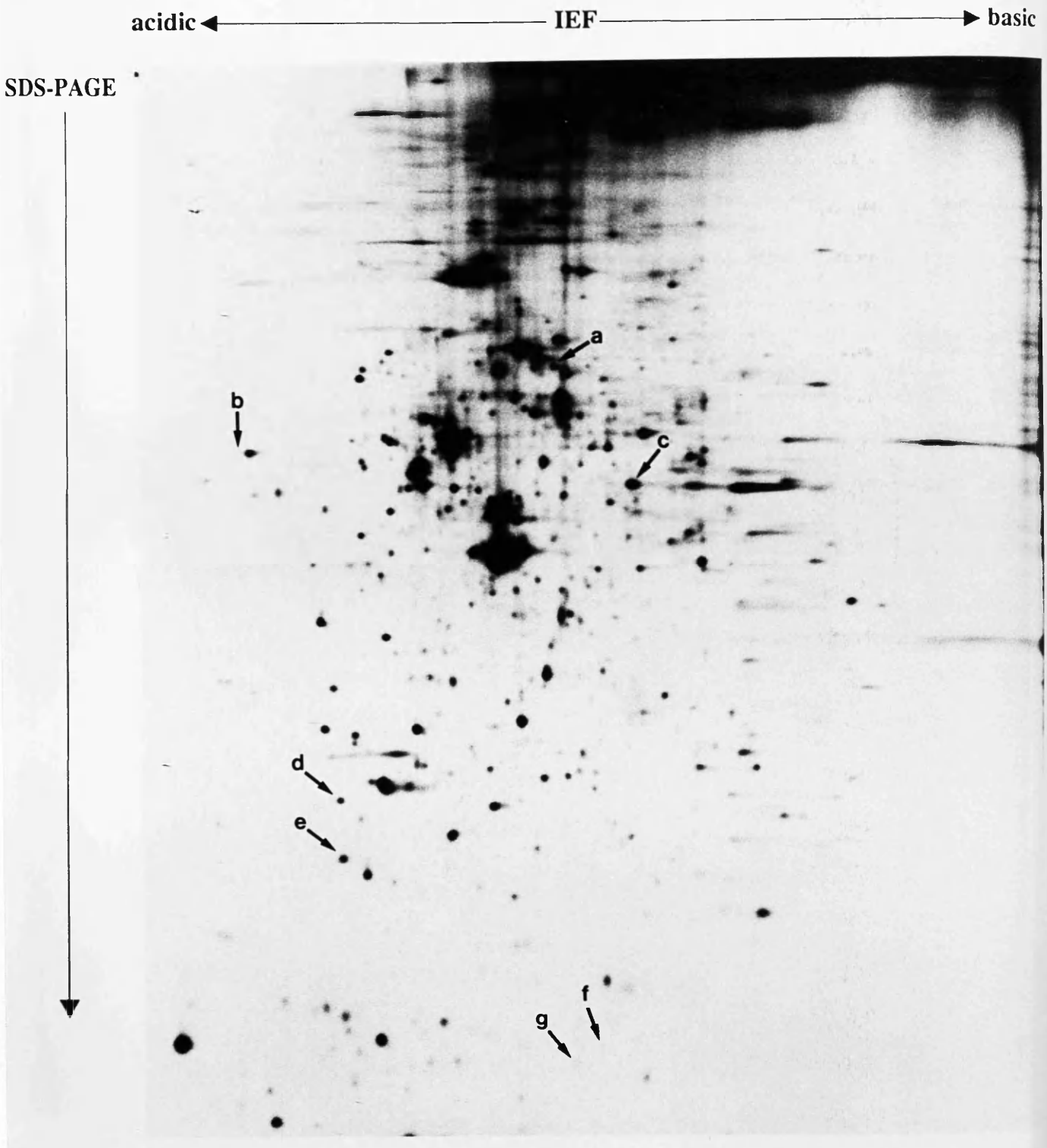
Changes in protein synthesis \ degradation following temperature shift of quiescent Rat1 f32. Quiescent cultures of Rat1 f32 were shifted to permissive temperature for 0 or 24hrs prior to pulse-labelling with ^{35}S-methionine as described in Materials and Methods. Whole cell lysates were prepared and resolved by 2D-electrophoresis as described in Materials and Methods. Indicated (arrows) are proteins whose labelling intensity is modulated by activating the transforming and mitogenic activities of *ts*LA32 pp60 (see text). These changes were not observed in parallel cultures of Rat1. Autoradiography was for 27d at -70^{o}C. *IEF, isoelectric focusing, SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.*

a) quiescent Rat1 f32

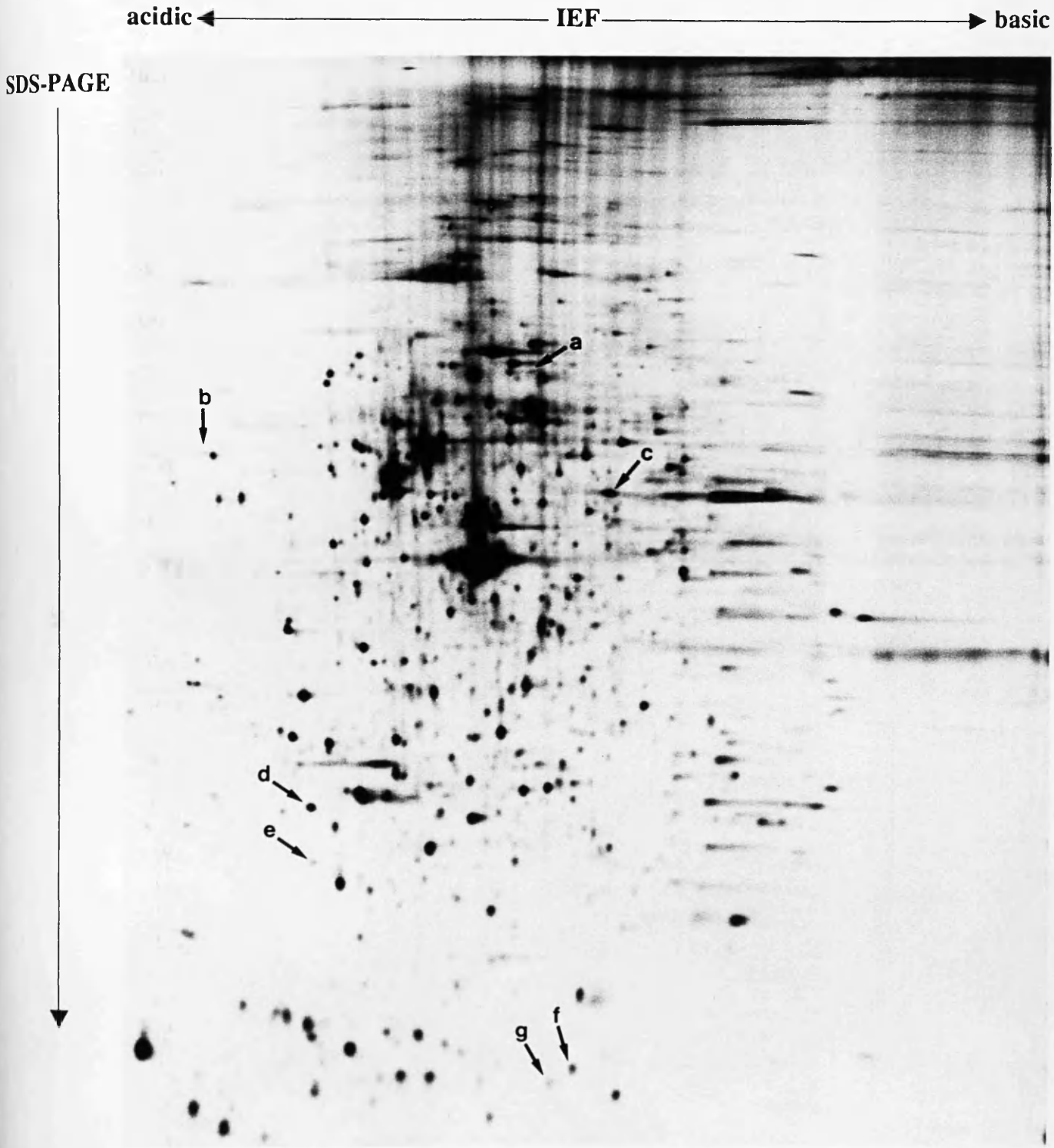
b) Rat1 f32, permissive temperature 24hrs

Figure VII.1

a)



b)



effects of the temperature change. In comparison to quiescent cultures, proteins *a*, *c* and *e* were poorly labelled in shifted cultures whereas proteins *b*, *d*, *f*, and *g* became labelled to higher intensity following a 24hr shift to permissive temperature. Apparent molecular weights for these proteins are listed below in table VII.1.

TABLE VII.1

Protein	Apparent Mol. Weight \ kDa	Labelling ?[a]
a	60	↓
b	45	↑
c	43	↓
d	25	↑
e	20	↓
f	14	↑
g	13	↑

[a] Change in labelling intensity following 24hrs at permissive temperature, ↑ increased labelling, ↓ decreased labelling.

VII.B.2 Protein Phosphorylation Following Temperature Shift of Rat1 f32

One of the main aims of this project is to look at changes in protein phosphorylation early after temperature shift of quiescent Rat1 f32 cultures. There are a number of specific questions that can be addressed using this system:

a) which proteins display changes in phosphate content following temperature shift ?

b) more specifically, which species display increased tyrosine phosphorylation upon temperature shift and hence might constitute primary targets of pp60 kinase activity ?

c) can we identify a group of proteins whose combined tyrosine phosphorylation is insufficient for transformation ?

These questions form a long-term project, and as such results of a very preliminary nature are discussed below.

Quiescent cultures of Rat1 and Rat1 f32 were deprived of phosphate for 30 minutes then labelled with carrier-free ^{32}P-orthophosphate (1mCi \ ml) for a total of 4hrs. Cells were shifted to 35^{o}C for 0, 15, 30, 60 or 120 minutes within this 4hr period before lysis in 2D gel sample buffer. Equivalent TCA-precipitable counts per minute (~500,000 per gel) were resolved by two-dimensional electrophoresis.

Preliminary results of these experiments have been disappointing. Three independent experiments of the type outlined above have failed to yield consistent results. In each experiment rapid changes (maximal by 15 min.) in the intensity of ^{32}P-labelled Rat1 f32 species have been observed, but these appear not to be reproducible between experiments. Indeed, whereas in one experiment label was seen to disappear rapidly from a group of proteins, in the following experiment, these proteins became phosphorylated following temperature shift of quiescent Rat1 f32 cells. These experiments will require careful attention to detail, and some suggestions are offered in the discussion below.

VII.C Discussion

A previous study of protein synthesis in quiescent *ts*LA23-NRK cells stimulated to transform and re-enter cycle has demonstrated that very few proteins are induced upon activation of pp60[27]. Durkin and Whitfield observed seven changes in the incorporation of ^{35}S-methionine into individual proteins of 18.5-44kDa following activation of *ts*LA23 pp60[27]. Three of these pp60-induced proteins also appeared or accumulated during the G1 transit of serum-stimulated cells[27]. My preliminary results support these observations, though the different resolving systems employed preclude a direct comparison with the data of Durkin and Whitfield, and those changes observed in response to PDGF and serum by other workers[322,323]. However, it seems reasonable to assume that the cellular machinery required for the mitogenic response to serum or

serum factors is also required for the mitogenic response to pp60, and hence further studies in this system should yield information on the essential components.

It is somewhat surprising that most of the observed changes were apparent only after an extended period at permissive temperature. This is in contrast to the effect of serum or serum factors upon quiescent cultures of fibroblasts where striking changes are apparent within 40min of stimulation[323]. There are several possible reasons for this difference. First, the magnitude of the mitogenic stimulus might be significantly different. Second, it is plausible that many of the effects elicited by whole serum are not directly related to the mitogenic mechanism. Indeed it is apparent that the overall rate of protein synthesis is increased markedly following serum stimulation of quiescent fibroblasts[27,324], whereas activating pp60 does not result in a significant increase in incorporation of ^{35}S-methionine[27]. pp60, unlike other mitogens might selectively promote the appearance \ disappearance of only a small number of proteins which are essential for transit of G1, DNA replication and cell division. Third, it is formally possible that events leading to DNA synthesis and mitosis in serum stimulated cells are fundamentally different to those in v-*src* transformed cells. The slower, sometimes less synchronous entry of temperature shifted Rat1 f32 or Rat1 f29 cells into S phase suggests that this might be the case (see discussion in chapter VI).

The *Src*-induced changes detected in these studies might represent only a small subset of the total changes in protein synthesis in response to pp60 activation. New proteins synthesized in response to *Src* might be low abundance species or might be expressed only transiently during G1 transit. Proteins which are relatively stable until a particular point in the cycle and are then degraded, for instance some cyclins, might not be detected since the labelling protocol is biased towards detection of newly synthesized proteins. In summary, a range of culture and labelling conditions will be required to define many *src*-induced changes in protein synthesis \ turnover. As a first step it might be useful to label cultures continuously for a number of hours following temperature shift, comparing these cells to parallel cultures maintained at restrictive temperature. Any changes observed could then be analyzed more thoroughly.

Preliminary attempts to investigate changes in phosphoprotein profile following temperature shift of quiescent Rat1 f32 cells have been disappointing. As indicated in VII.B.2, results from three short time-course experiments have not been reproducible for technical problems. Potential sources of error are outlined below, together with measures that might improve reproducibility :

a) growth and labelling media. Variation in the composition and physical properties (pH, osmolarity *etc.*) of medium used might influence the results of these experiments, particularly over short time courses. In particular, any change of pH following shift between incubators might result in spurious results. One could perhaps minimise these problems by labelling and shifting in chemically defined medium, such as a Ringer solution. It might be wise to use a non-bicarbonate buffering system to prevent large pH changes following temperature shift. Stocks of growth and labelling media should perhaps be frozen to reduce variation between experiments.

b) cell density and passage history. There are circumstantial data to suggest that cell density plays a large role in determining the magnitude of a number of *Src*-responsive effects (M. Frame, personal communication). It is important therefore that cells for these experiments are set up at identical densities and are cultured for a defined period before the experiment begins. Control cultures not expressing the *Src* protein should always be included in parallel.

c) labelling protocol. Labelling should be carried out according to a standard protocol. It is important that cells are maintained at the appropriate temperature prior to and during the experiment, and that required temperature changes should be effected as rapidly as possible. This might be achieved by floating culture vessels on water baths. If possible, it would be wise to examine parallel cultures for transformation and re-entry into cycle following temperature shift.

d) detection of protein phosphotyrosine. Since ~99% of protein phosphate in these cells is phosphoserine and phosphothreonine (see chapter V), detection of phosphotyrosine is not straightforward. Two methods are currently available : alkaline hydrolysis of ^{32}P-labelled proteins[310] and Western blotting using antisera specific for

phosphotyrosine[237,238,239,240]. Protein phosphotyrosine is relatively stable to treatment with 1M KOH at 55°C, whereas phosphoserine and, to a lesser extent, phosphothreonine are hydrolyzed[310]. Alkaline enrichment of phosphotyrosine can be carried out in the gel[310], or on Immobilon P membranes[325] (Millipore). The latter method avoids the marked expansion and contraction of the gel seen upon hydrolysis *in situ*. However, there are problems in ensuring reproducible blotting between gels, and indeed consistent blotting within any given gel. These problems are compounded by the large number of gels that are often required in these experiments. Obviously, Western blotting using anti-phosphotyrosine specific antisera suffers from the same blotting problems, though prior labelling of cells is not required. Other problems with this approach include the specificity of the antisera (which can be checked by radiolabelling and phosphoamino acid analysis) and the range of tyrosine phosphorylated proteins recognized by the antisera used. Since different antisera yield significantly different profiles of phosphotyrosine-containing proteins, it is perhaps wise to use mixtures of antisera for this application. In summary, one reasonable approach to detect protein phosphotyrosine might be to blot labelled proteins to Immobilon P membranes for autoradiography before subsequent alkaline hydrolysis. Labelled species resistant to hydrolysis could then be checked by immunostaining with anti-phosphotyrosine antisera.

From the above it is clear that these questions constitute a long-term project, although I believe most of the techniques and materials required are now in place.

Chapter VIII

VIII ARE THE TRANSFORMING AND MITOGENIC ACTIVITIES OF pp60 DISSOCIABLE ?

VIII.A. Introduction

The preceeding chapters of this thesis have outlined an approach to the study of early consequences of activating a thermolabile v-*src* mutant. The *ts*LA32 mutant was chosen for this approach on the basis that its behaviour deviates only minimally from the wild type protein. The prediction is that this type of approach should facilitate identification of relevant events. Parallel studies in this laboratory have concentrated on describing the behaviour of the AP-1 transcription factor following temperature shift of *ts*LA29 infected Rat1 fibroblasts. Welham *et al.* have demonstrated that the ability of nuclear extracts to retard oligonucleotides containing consensus AP-1 binding elements increases rapidly upon temperature shift of quiescent cultures of Rat1 *ts*LA29[29]. Since these experiments result in two biological endpoints, namely transformation and re-entry into cell-cycle, equating early events with the correct endpoint is not straightforward. Mutants capable of dissociating these two endpoints would therefore be useful both in characterising early events following temperature shift in the Rat1 f32 system, and in dissecting further the relevance of the AP-1 band-shift assays. Evidence that the various transformation phenotypes can be dissociated is provided by the studies of Becker *et al.*[326], Weber and Friis[327], and also by Hanafusa and Calothy and colleagues[32,33,328,329,330]. In particular, the latter workers have demonstrated that morphological transformation \ anchorage independence and stimulation of proliferation induced by v-*Src* are separable in primary neuroretinal cells of the chick embryo[33], using pp60 defective for membrane localization[32]. Cells infected with these mutants are morphologically normal but exhibit a prolonged proliferative lifespan in culture. Of the mutants studied, those demonstrating the clearest dissociation of mitogenic and transforming activities are proteins defective for myristylation[32]. Although not addressing the mitogenic activity directly, these studies suggest that similar mutants might be useful for dissociating the transforming and mitogenic activities of pp60 in fibroblasts.

This chapter will describe the construction of a mutant non-conditionally defective for myristylation but temperature sensitive for *in vitro* kinase activity, and its properties in Rat1 and CEF.

VIII.B. Results

VIII.B.1 Mutagenesis of *ts*LA29 *src*

Mutagenesis of *ts*LA29 *src* was carried out in bacteriophage M13 using a commercially available kit according to manufacturers' instructions. The oligonucleotide used, oligo. A2, had the following sequence : 5' C ACC ATG GCA AGC AGC AA 3'. Nucleotides which differ from the *ts*LA29 sequence are underlined. This mutation results in a glycine to alanine substitution at position 2 of pp60, a change reported to prevent myristylation[37,111]. The mutant is named "29A2" to indicate this change.

Five plaques generated by the mutagenesis reactions were analyzed by dot-blotting. DNA from these putative mutants and from the starting M13-*ts*LA29 *src* DNA was spotted on Hybond N and hybridized with 5' ^{32}P-labelled oligo. A2 according to instructions supplied with the mutagenesis kit. The membrane was washed three times in 6xSSC at room temperature and exposed to X-ray film overnight. All species retained labelled oligonucleotide A2 after these low stringency washes. To detect phage containing mutant insert, the membrane was washed for five minutes in 6xSSC prewarmed to 51^{o}C. This is five degrees below the calculated T_m of mutant oligo \ non-mutant template hybrids. The membrane was again exposed to X-ray film overnight. This high stringency wash removed all label from the starting template DNA and from two of the five putative mutant DNAs. The remaining putative mutants were sequenced to confirm the presence of the codon 2 mutation, and one of these positive clones, M13 29A2-1 (see figure VIII.1a) was used to prepare double stranded replicative form (RF) DNA. RF DNA from M13 29A2-1 was digested to completion with EcoRI and HindIII and fractionated by agarose gel electrophoresis. A 1.9kb band was recovered from the gel and ligated to EcoRI \ HindIII cut pMP323 to generate the ClaI sites necessary for subsequent subcloning

Figure VIII.1

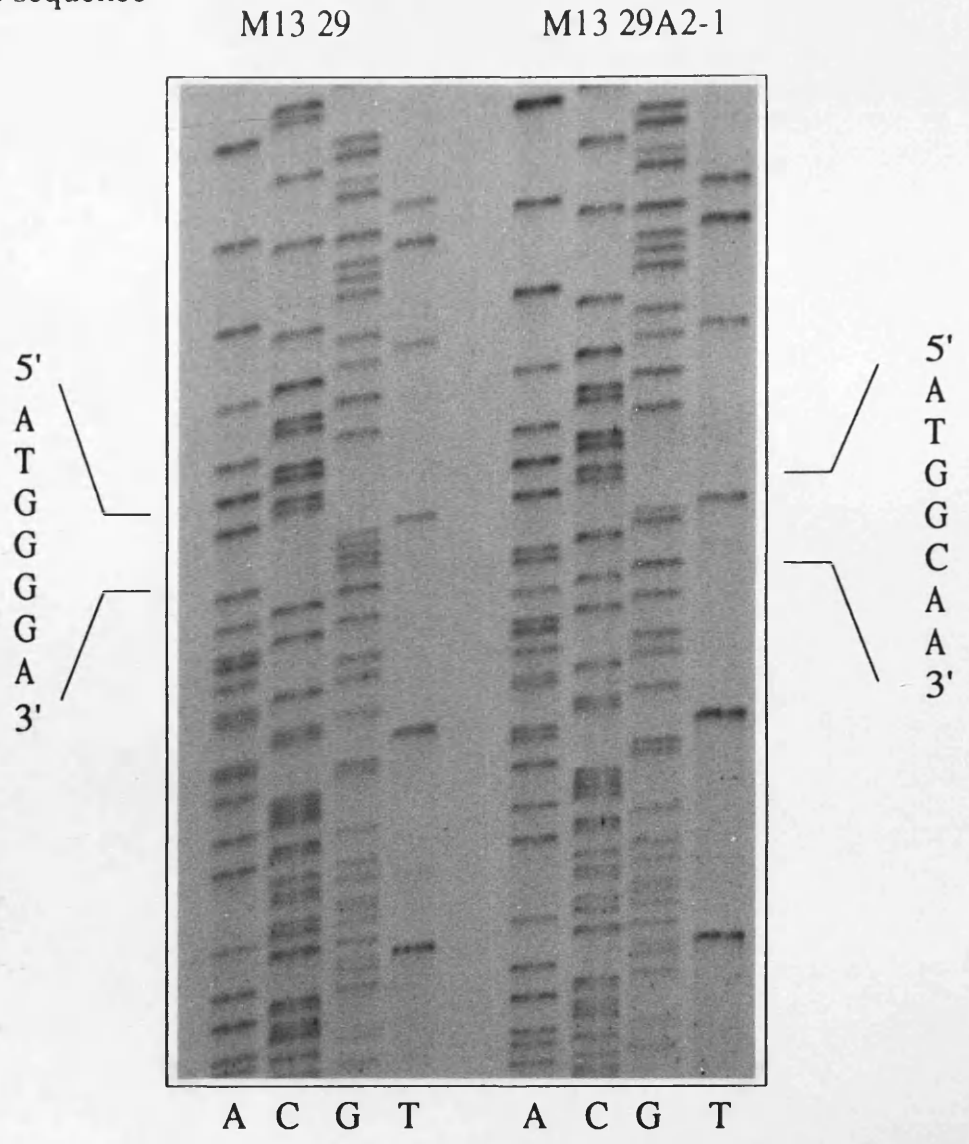
a) Mutagenesis of *ts*LA29. Starting template (M13 29) and putative mutant (M13 29A2-1) were sequenced according to Materials and Methods. Products were resolved by denaturing polyacrylamide gel electrophoresis and revealed by autoradiography (18hrs, room temperature). The starting template and mutant sequences of interest are indicated in the figure. *A, C, G, T-dideoxynucleoside triphosphate in termination reaction.*

b)i) Restriction analysis of fpGV-29A2. 29A2 *src* was subcloned to the unique ClaI site in fpGV-1 as outlined in the text. Presence of insert was assessed by digestion with ClaI and orientation derived by digestion with BglI or SmaI, followed by 0.8% agarose gel electrophoresis. For additional information see figures III.1b and III.2a. *B, BglI, C, ClaI, S, SmaI, M, molecular weight markers*

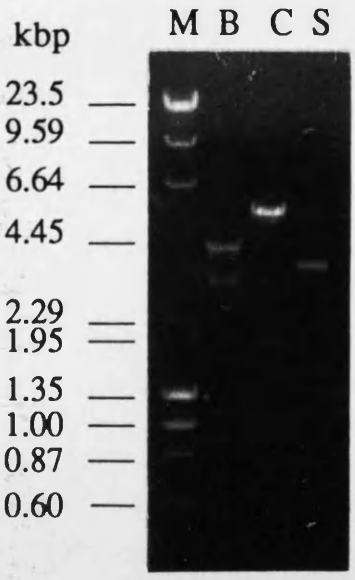
ii) Restriction analysis of RCAN 29A2. 29A2 *src* was subcloned to the unique ClaI site in RCAN as outlined in the text. Presence and orientation of *src* inserts were assessed by digestion with SalI followed by 0.8% agarose gel electrophoresis. For additional information see figures III.1b and III.2b. *M, molecular weight markers, 1, RCAN, 2, RCAN 29, 3, RCAN 29A2.*

Figure VIII.1

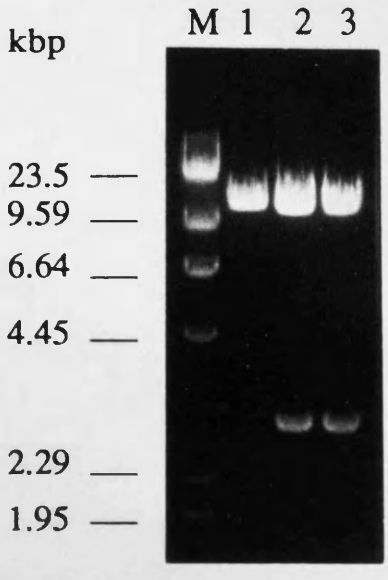
a) 29A2 sequence



b)i) Restriction analysis of fpGV-29A2



ii) Restriction analysis of RCAN 29A2



to the expression vector fpGV-1. Appropriate clones of fpGV-29A2 were identified by restriction mapping (figures VIII.1b and III.2a) and transfected into Rat1 cells according to Materials and Methods. Some aspects of this work required expression of the 29A2 mutant in CEF. The previously described RCAN vector was used for this purpose. Appropriate clones were identified by restriction mapping (see figures VIII.1b and III.2a) and transfected according to Materials and Methods.

VIII.B.2 Derivation of Rat1 f29A2 Clones

Rat1 cultures were transfected with fpGV-29A2 and selected with G418 at permissive temperature according to Materials and Methods. Transfection efficiency was similar to that obtained using the previously described expression constructs (see chapter III). As expected, all G418-resistant colonies obtained from these transfections were morphologically normal. More than thirty independent single cell clones from three independent transfections at permissive temperature were screened for pp60 expression by immunoblot analysis. Results for 23 G418-resistant clones derived from two independent transfections are shown in figure VIII.2. None of these clones express pp60 to a level approaching that seen in the Rat1 f29 clone, although a number do express detectable pp60. The properties of two of these clones (G and H) are described later in this chapter.

Since transfection and selection at permissive temperature yielded only clones expressing low levels of 29A2 pp60, one possibility was that an active cytoplasmic kinase was toxic in these cells. To address this possibility, transfection and selection were carried out at restrictive temperature. Any pp60 expressed at this temperature should have basically the same properties as *ts*LA29 *Src* protein, i.e. it should have a reduced kinase activity and be cytoplasmic in nature. Given that *ts*LA29 *Src* protein appears not to be toxic at restrictive temperature, one would predict that cytotoxicity of the 29A2 protein should be minimized under these conditions. G418-resistant colonies obtained by transfection and selection at restrictive temperature were screened for pp60

Figure VIII.2

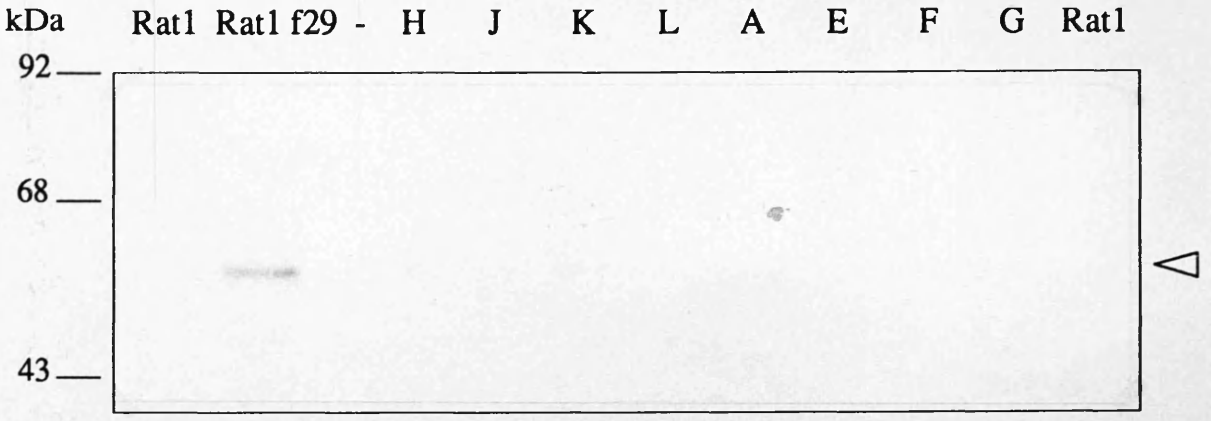
Transfection of fpGV-29A2 into Rat1 cells at permissive temperature. Rat1 cells were transfected with fpGV-29A2 and selected for G418-resistance at permissive temperature as described in Materials and Methods. Colonies or single cell clones derived from these transfections were analysed by Western blotting, using appropriate positive and negative controls. Equivalent amounts of lysate protein (as judged by Coomassie blue staining) were resolved by SDS-PAGE and blotted as described in Materials and Methods. Primary antiserum was MW2 at 1:5000. The arrowhead indicates pp60.

a) single cell clones from transfection #1 at permissive temperature plus positive (Rat1 f29) and negetive (Rat1) controls.

b) colonies obtained from transfection #2 at permissive temperature plus positive (Rat1 f29) and negative (Rat1) controls.

Figure VIII.2

a) fpGV-29A2 transfection #1, permissive temperature



b) fpGV-29A2 transfection #2, permissive temperature

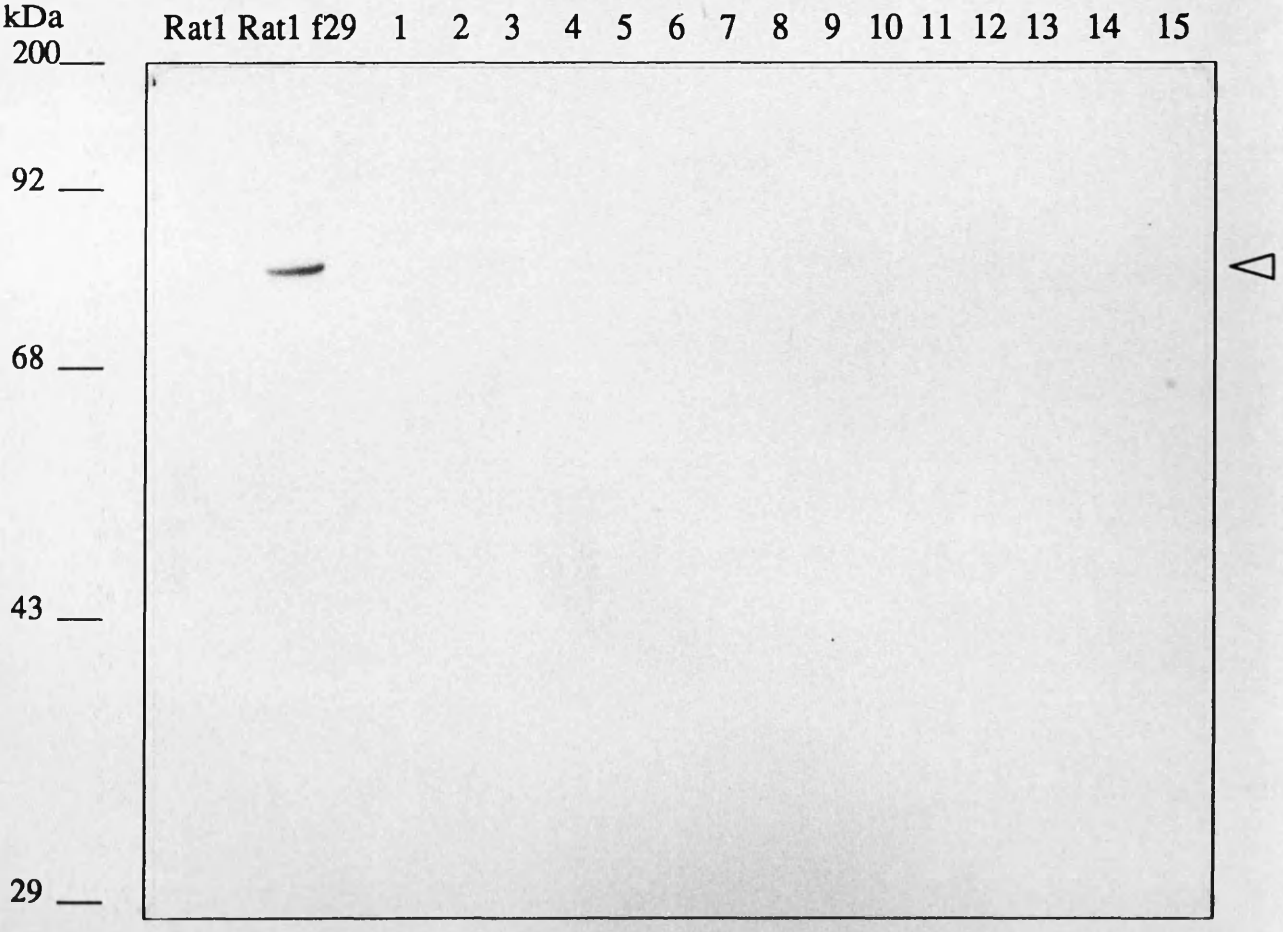
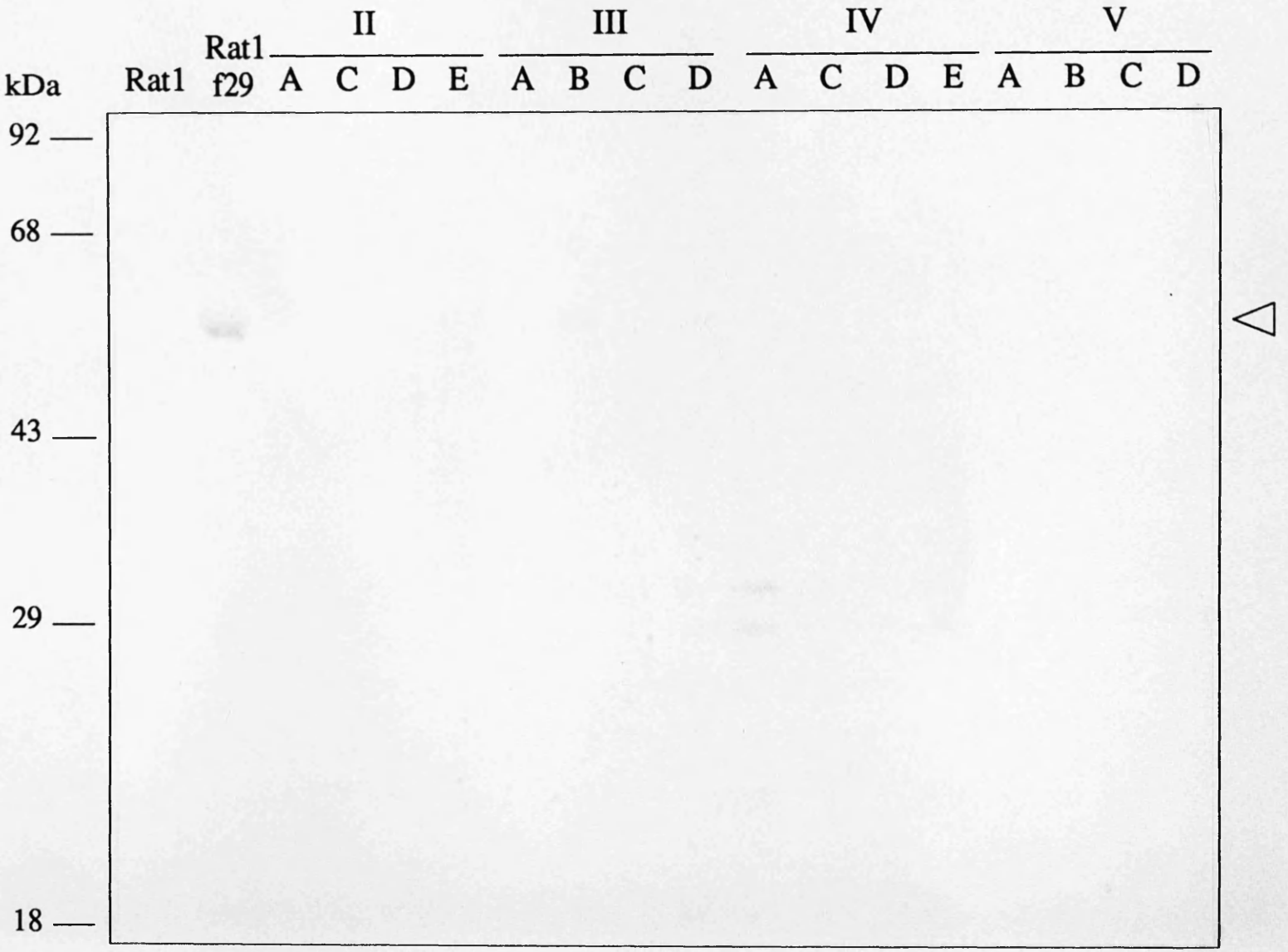


Figure VIII.3

Transfection of fpGV-29A2 into Rat1 cells at restrictve temperature. Four independent Rat1 cultures were transfected at and selected for G418-resistance at restrictive temperature as described in Materials and Methods. Colonies were analysed by Western blotting using appropriate positive and negative controls. Equivalent amounts of lysate protein (as judged by Coomassie blue staining) were resolved by SDS-PAGE and blotted as described in Materials and Methods. Primary antiserum was MW2 at 1:5000. The arrowhead indicated pp60. *II-V represent independent transfections and A-E represent individual colonies from these transfections.*

fpGV-29A2 transfection #3, restrictive temperature



expression by Western blot analysis (figure VIII.3). Although generally similar to the results obtained from transfections at permissive temperature, two colonies did exhibit reasonable expression of pp60. These colonies, 3B and 3D were picked from the same transfection and hence may not result from independent transfection events. Colony 3B was selected for single cell cloning and further study. Although uniformly flat and non-refractile at restrictive temperature, 3B became more refractile at permissive temperature. Upon reaching confluence at 35°C, cells appeared to be lost from the surface of the monolayer. Additionally, colonies formed after plating at low density at permissive temperature demonstrated a range of morphologies, suggesting that 3B was not clonal (not shown). Furthermore, Southern analysis suggested the presence of more than one cell clone in 3B (not shown). Interestingly, the refractile colonies that resulted from plating at low density at permissive temperature grew very poorly. These observations are consistent with the 29A2 mutant protein being somewhat toxic at permissive temperature. Unfortunately, attempts to clone Rat1 f29A2 3B (even at restrictive temperature) yielded uniformly flat lines which failed to express detectable pp60. Hence, with reservations regarding the viability and clonality of Rat1 f29A2 3B in mind, this line was characterised together with the two low expressing clones (G and H) from transfections at permissive temperature. These experiments and the tentative conclusions drawn from them are outlined below.

VIII.B.3 *In vitro* Kinase Activity of Rat1 f29A2

Cultures of Rat1 f29A2 G and H (low expressing clones derived from transfections at permissive temperature, figure VIII.2) and Rat1 f29A2 3B (colony derived from transfection at restrictive temperature, figure VIII.3), together with appropriate control cultures of Rat1 and Rat1 f29, were grown for at least two days at permissive or restrictive temperature. MAb327 immunoprecipitates were prepared according to Materials and Methods, and *in vitro* kinase assays performed. Figure VIII.4 indicates that the

Figure VIII.4

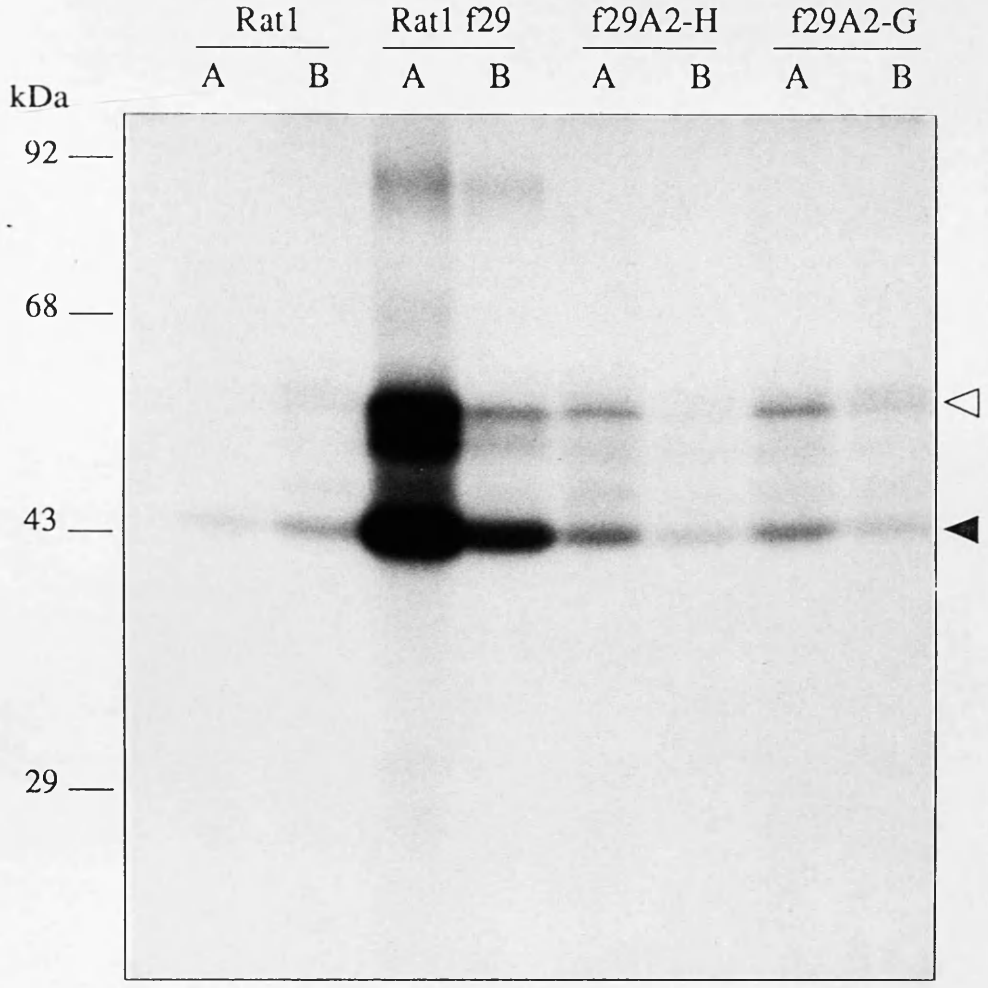
MAb327-immune complex kinase assay of Rat1 f29A2 lines. pp60 was immunoprecipitated from growing cultures of Rat1, Rat1 f29 or the Rat1 f29A2 lines indicated as described in Materials and Methods using monoclonal antibody MAb327. Kinase activity in the immunoprecipitates was measured at 30^{o}C using acid-denatured enolase as exogenous substrate, as described in Materials and Methods. The open arrow indicates pp60 and the closed arrow enolase (as judged by Coomassie blue staining). *A, cells at permissive temperature, B, cells at restrictive temperature.*

i) Rat1 f29A2 clones G and H. Autoradiography was for 3hrs at -70^{o}C.

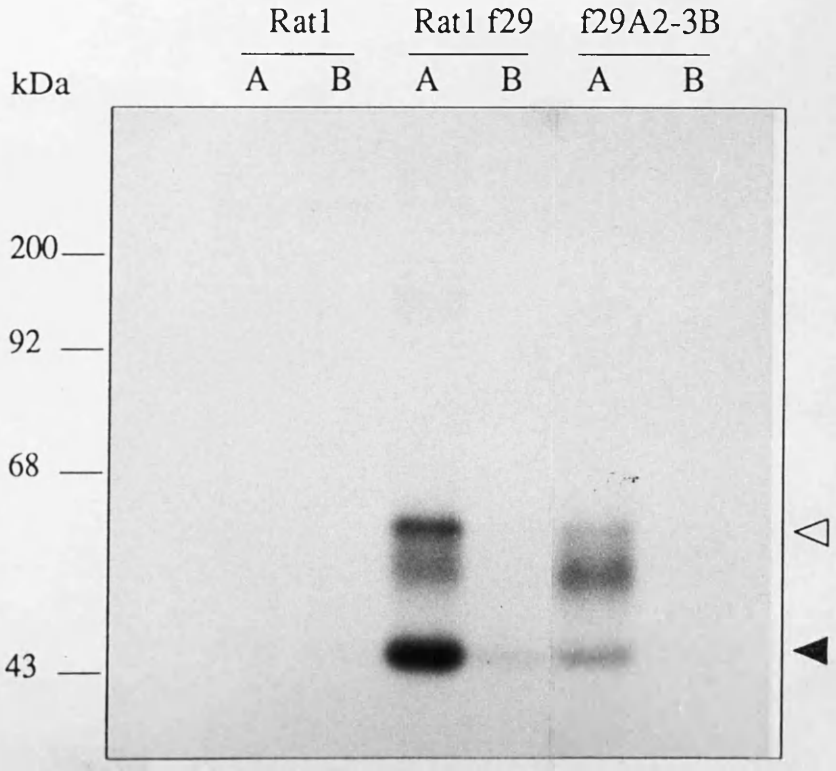
ii) Rat1 f29A2 colony 3B. Autoradiography was for 1hr at -70^{o}C.

Figure VIII.4

i) MAb327-immune complex kinase assay of Rat1 f29A2 clones H and G



ii) MAb327-immune complex kinase assay of Rat1 f29A2-3B



myristylation defective mutant 29A2 derived from *ts*LA29 *src* retains the thermolabile kinase properties of the *ts*LA29 protein in *in vitro* assays, and that the absolute level of kinase activity at permissive temperature mirrors the level of expression of pp60 as assessed by Western blot (see figures VIII.2 and VIII.3).

VIII.B.4 Mitogenic Activity of 29A2 In Rat1 Cells

Quiescent cultures of Rat1 f29 and cells expressing the 29A2 protein were prepared according to Materials and Methods. Cultures were stimulated either by addition of serum to a final concentration of 5% at restrictive temperature, or by shifting the cultures to permissive temperature in the absence of added factors. At appropriate intervals cultures were labelled with tritiated thymidine and incorporation into TCA-precipitable material was estimated by liquid scintillation counting. The results of these experiments are shown in figure VIII.5. Under conditions where Rat1 f29 cells entered S phase in response to temperature shift, cells expressing the myristylation defective mutant 29A2 failed to incorporate labelled thymidine. This was true of clones expressing low levels of the mutant protein (clones G and H) and of the line expressing levels of pp60 approaching that seen in the Rat1 f29 line (Rat1 f29A2 3B). These cells respond normally to serum at restrictive temperature, demonstrating that they are capable of re-entering cycle from a quiescent state. The data suggest that the 29A2 mutant is not itself a complete mitogen in Rat1 fibroblasts when assayed directly. Possible technical reasons for this result will be discussed in more detail later in this chapter.

Since the available Rat1 lines expressing the 29A2 protein did not re-enter cycle following shift to permissive temperature, I examined the properties of this mutant in infected CEF cultures. The 29A2 *src* ClaI fragment was subcloned to the avian retroviral vector RCAN to yield RCAN 29A2 and uniformly infected cultures obtained as described in Materials and Methods. RCAN and RCAN 29 infected cultures were prepared in parallel to serve as controls. The monolayer morphology of these infected cultures is shown in figure VIII.6, demonstrating clearly the normal morphology of RCAN 29A2 infected cultures.

Figure VIII.5

Mitogenic activity of *ts*LA29A2 pp60 in Rat1 cells. Rat1 f29, Rat1 f29A2 G, Rat1 f29A2 H and Rat1 f29A2 3B were rendered quiescent at restrictive temperature by serum deprivation as described in Materials and Methods. Cultures were left untreated at 40°C (■), stimulated by addition of newborn calf serum to a final concentration of 5% (v \ v) at 40°C (△) or shifted to permissive temperature in the absence of added factors (▤). Cultures were pulse-labelled (1hr) with tritiated thymidine at four-hourly intervals, and radioactivity incorporated into TCA-precipitable material was measured by liquid scintillation counting. Incorporation is expressed as ^{3}H cpm x 10^{-4} per 35mm culture dish at each time point. *i*, Rat1 f29, *ii*, Rat1 f29A2 H, *iii*, Rat1 f29A2 3B.

Figure VIII.5

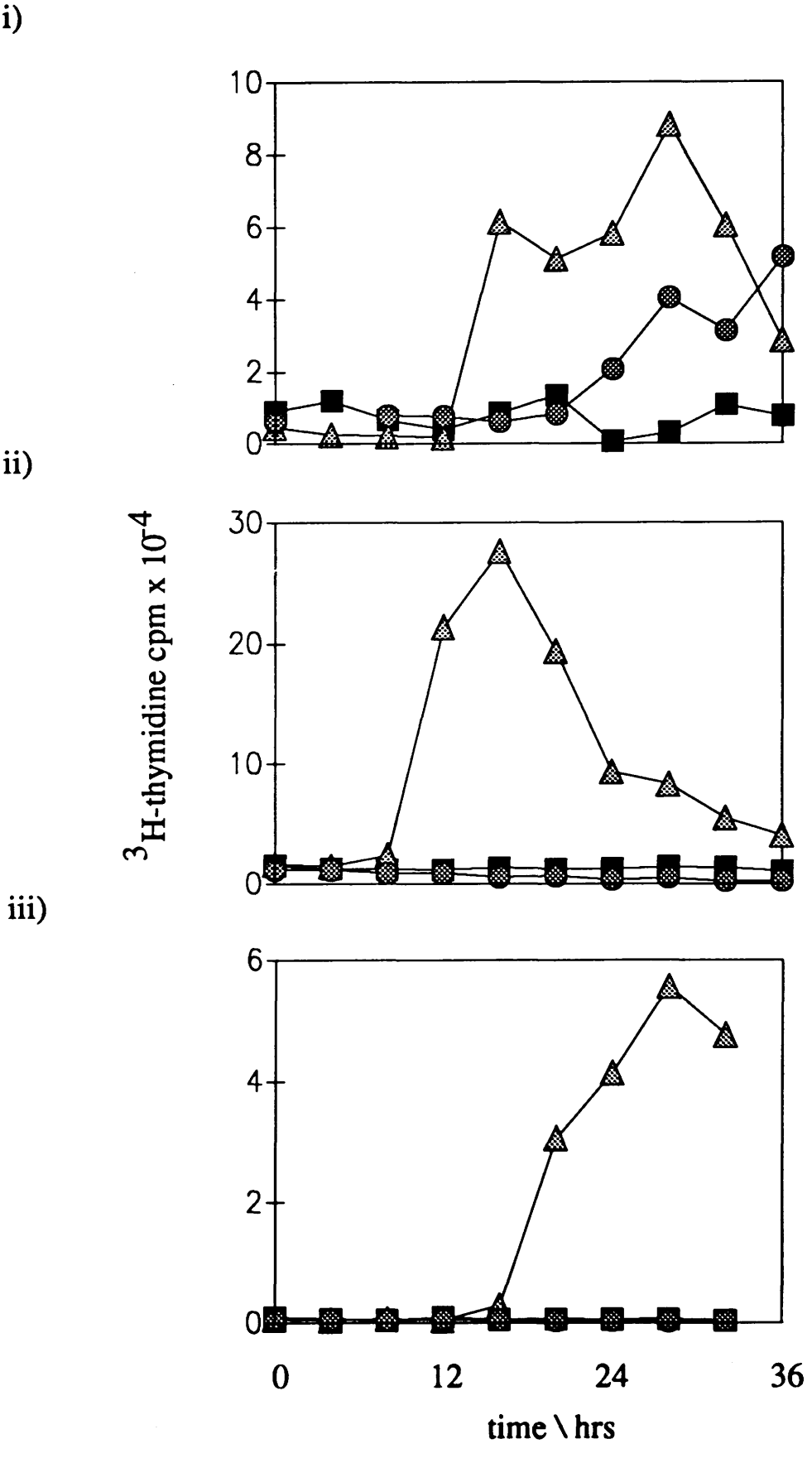
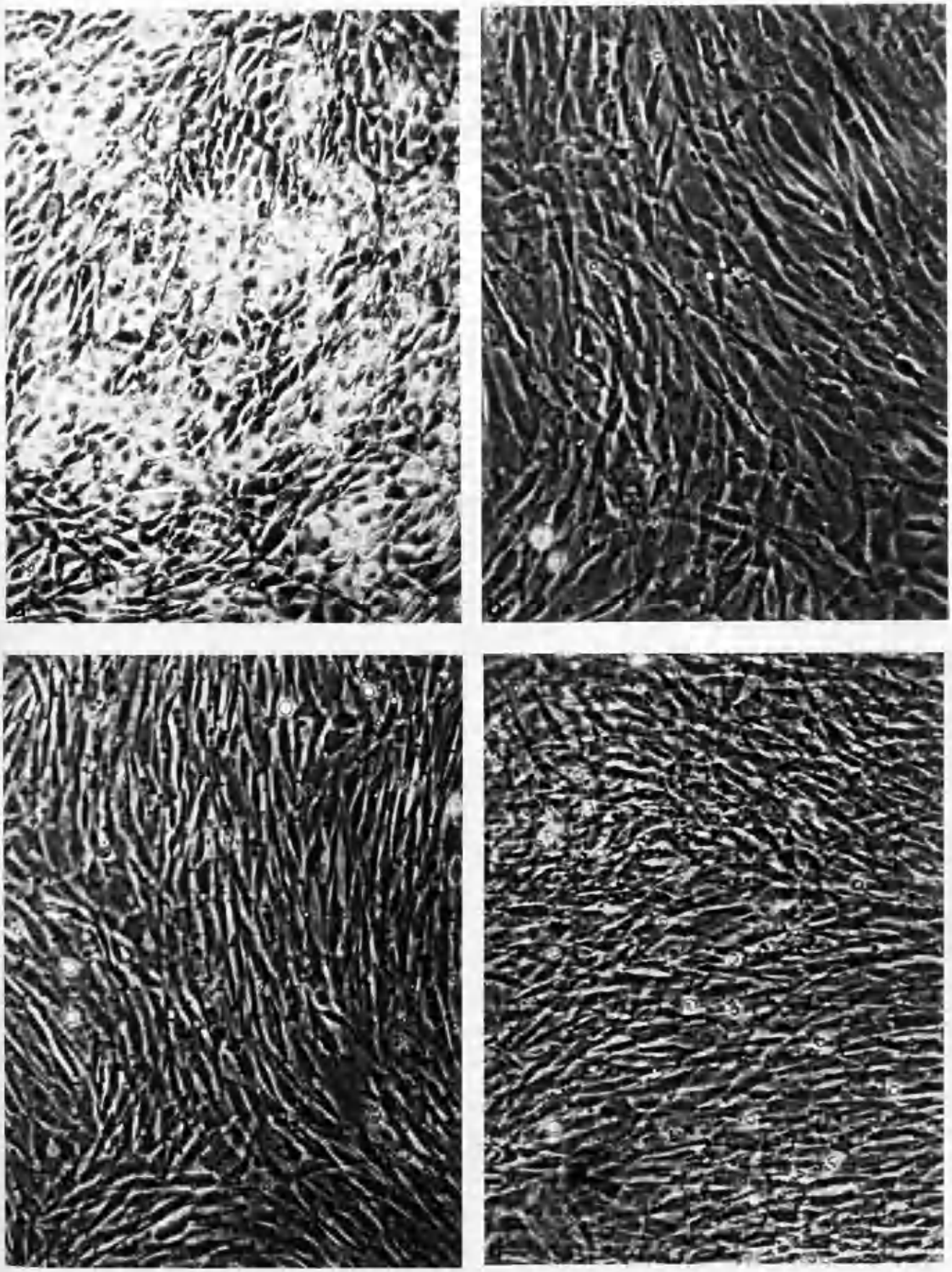


Figure VIII.6

Monolayer morphology of RCAN 29 and RCAN 29A2-infected CEF. Cultures were maintained in normal growth medium at appropriate temperature for at least 24hrs prior to photography. Magnification is ~x750. *a*, RCAN 29 35^{o}C, *b*, RCAN 29 41^{o}C, *c*, RCAN 29A2 35^{o}C, *d*, RCAN 29A2 41^{o}C.

Figure VIII.6



VIII.B.5 *In vitro* Kinase Activity of RCAN 29A2 Infected CEF

Extent of CEF infection was gauged by Western blot analysis using anti-*Gag* serum 5202 (not shown) and anti-*Src* serum MW2 (figure VIII.7a). The 29A2 protein was expressed to the same level as the prototype *ts*LA29 protein in chick embryo fibroblasts, with no obvious toxic effects. The reason for this differential behaviour in Rat1 and chick embryo fibroblasts is not clear. Immunoprecipitates were prepared from infected cultures as described using MAb327, and *in vitro* kinase assays performed using acid-denatured enolase as substrate. Figure VIII.7b demonstrates that the *in vitro* kinase activity of 29A2 pp60 mimics closely the behaviour of the prototype *ts*LA29 protein. When prepared from CEF, both the absolute levels and the temperature sensitivity of these kinases are closely matched when assayed *in vitro*.

The data presented above suggest that growing cultures infected with RCAN 29 and RCAN 29A2 were well matched in terms of pp60 expression and *in vitro* kinase activity, although clearly different morphologically. It was therefore important to determine if both kinases reactivated with the same kinetics following temperature shift. Quiescent cultures of CEF infected with RCAN, RCAN 29 and RCAN 29A2 were prepared as described in Materials and Methods. Appropriate dishes were shifted to permissive temperature for 0, 0.5, 1 or 4hrs before lysis and immunoprecipitation with TBR-RLE. Kinase assays were carried out according to Materials and Methods, and labelled immunoglobulin and pp60 detected by autoradiography. Figure VIII.8 demonstrates that both *ts*LA29 and *ts*LA29A2 kinases reactivate over this timecourse, whereas no activity is detected in immunoprecipitates from RCAN infected cultures. Coupled with the apparent lack of toxicity, these results enabled a more direct comparison of the mitogenic activity of the *ts*LA29 and 29A2 *Src* proteins than had been possible in the Rat1 cell system.

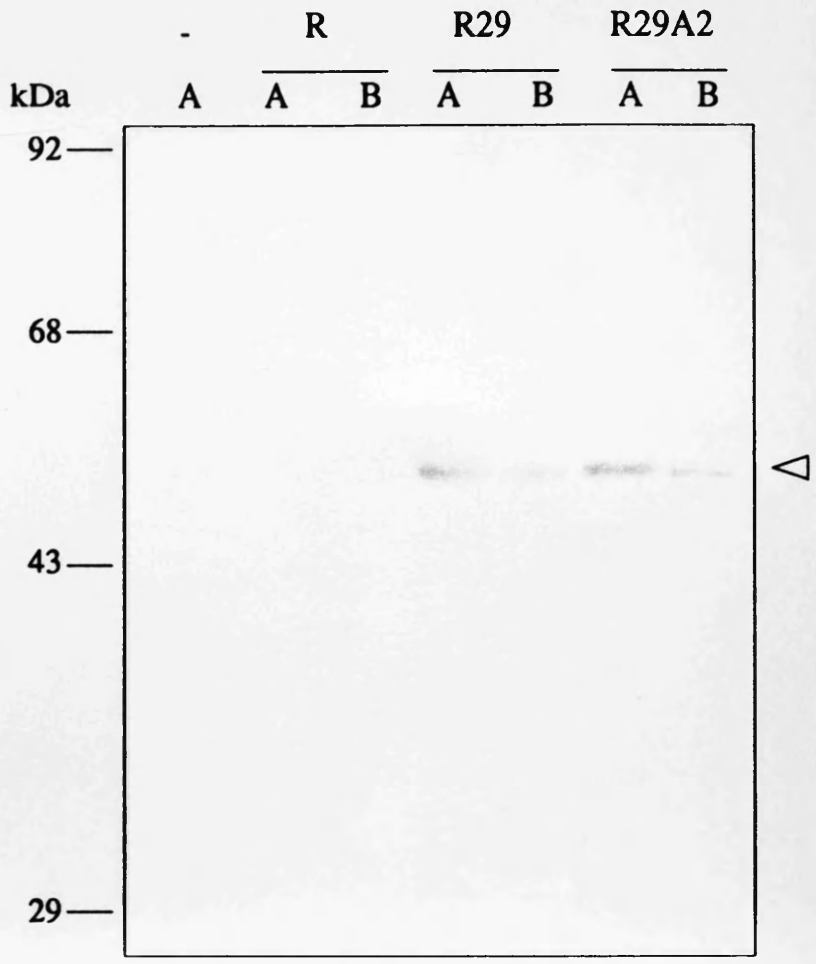
Figure VIII.7

a) pp60 expression in infected CEF. Uniformly infected cultures were maintained in normal growth medium at the appropriate temperature for at least two days prior to lysis. Equivalent amounts of lysate protein (as judged by Coomassie blue staining) were resolved by SDS-PAGE and blotted according to Materials and Methods. Primary antiserum was MW2 at 1:5000. The arrowhead indicates pp60. *A, cells at permissive temperature, B, cells at restrictive temperature, -, mock-transfected CEF, R, RCAN transfected CEF, R29, RCAN 29 transfected CEF, R29A2, RCAN 29A2 transfected CEF.*

b) MAb327-immune complex kinase assay of infected CEF. Uniformly infected cultures were maintained in normal growth medium at the appropriate temperature for two days prior to lysis. pp60 was immunoprecipitated from equivalent amounts of lysate protein using monoclonal antibody MAb327. Kinase activity in the immunoprecipitates was measured at 30^{o}C as described in Materials and Methods using acid-denatured enolase as exogenous substrate. The open arrow indicates pp60 and the closed arrow enolase (as judged by Coomassie blue staining). Autoradiography was for 4hrs at -70^{o}C. *A, cells at permissive temperature, B, cells at restrictive temperature, -, mock-transfected CEF, R, RCAN transfected CEF, R29, RCAN 29 transfected CEF, R29A2, RCAN 29A2 transfected CEF.*

Figure VIII.7

a) pp60 expression in infected CEF



b) MAb327-immune complex kinase assay of infected CEF

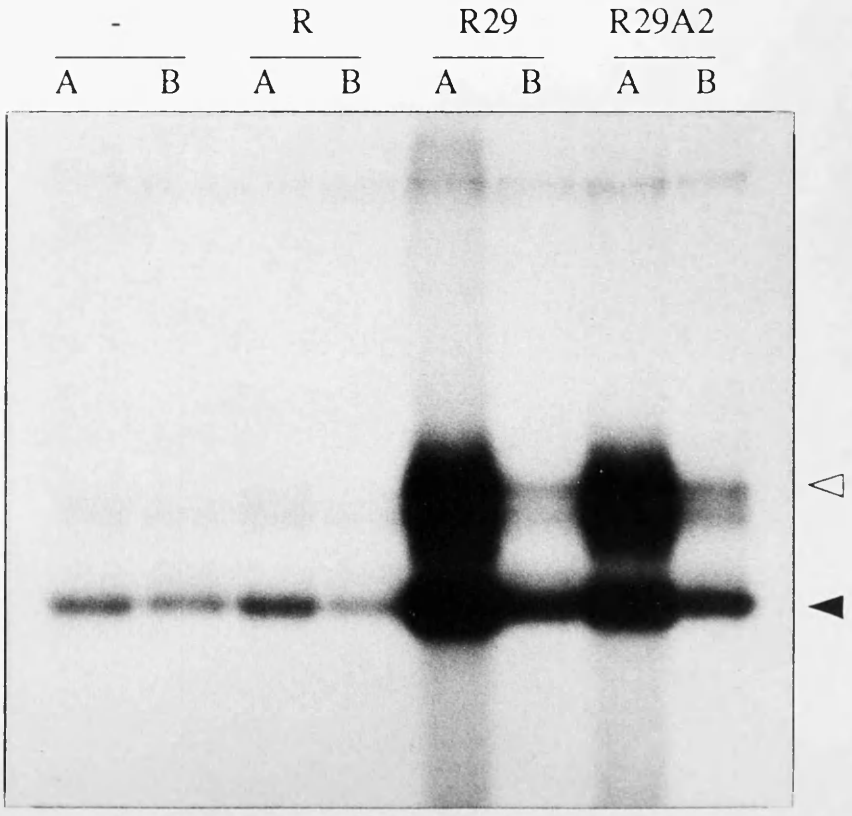
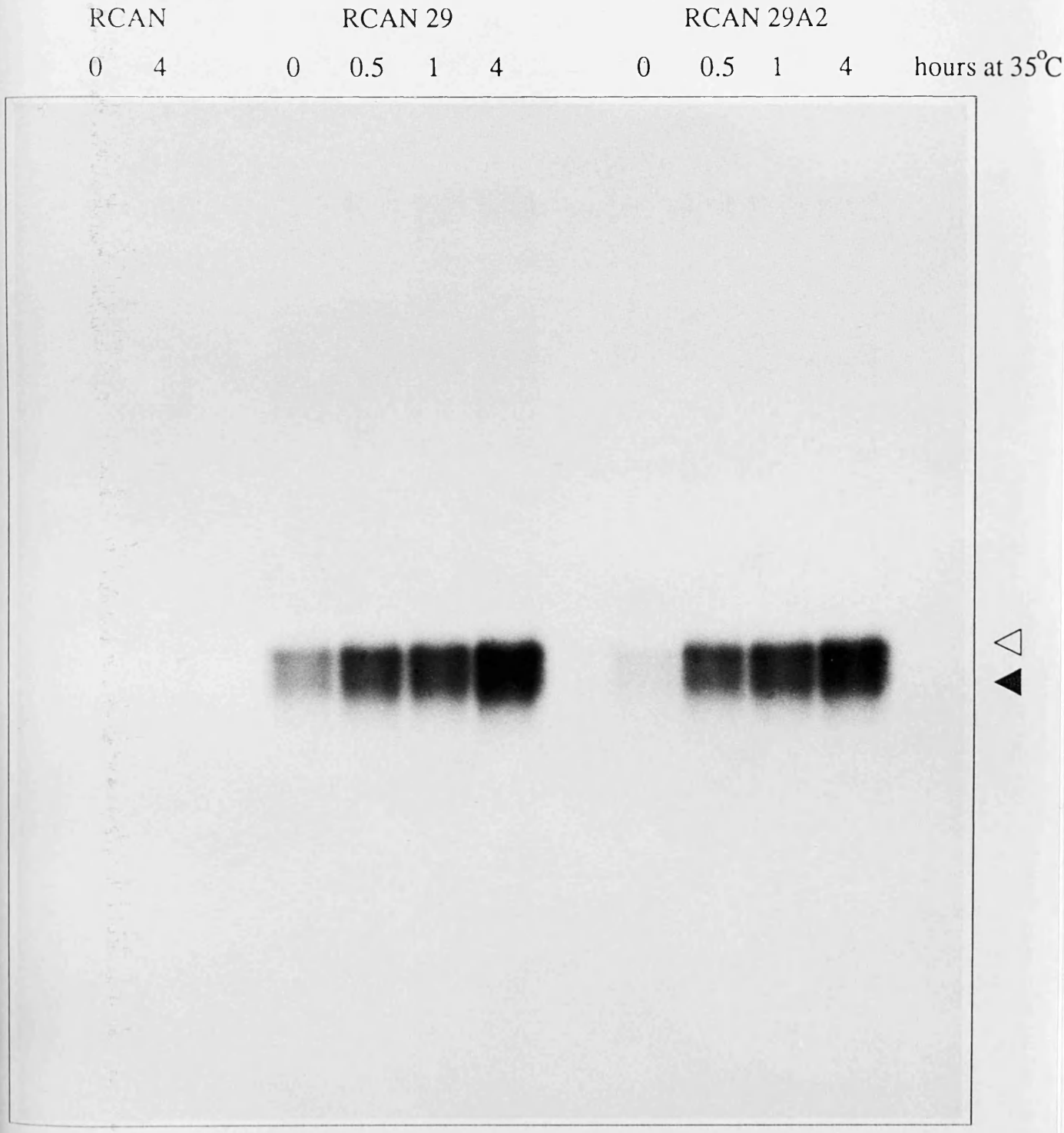


Figure VIII.8

Kinase reactivation in quiescent CEF expressing RCAN 29 and RCAN 29A2. Uniformly infected cultures of CEF were rendered quiescent at restrictive temperature as described in Materials and Methods. Cultures were shifted to permissive temperature for the indicated times and pp60 immunoprecipitates prepared as described using antiserum TBR-RLE. Kinase activity against immunoglobulin heavy chain and pp60 was measured as described. The open arrow indicates pp60, and the closed arrow immunoglobulin heavy chain (as judged by Coomassie blue staining). Transfected constructs are indicated at the top of the figure. Autoradiography was for 1.5hrs at -70^{o}C.

Figure VIII.8

Kinase reactivation in CEF expressing RCAN 29 and RCAN 29A2



VIII.B.6 Mitogenic Activity of 29A2 In CEF

Primary CEF were transfected with RCAN 29 or RCAN 29A2 and passaged until uniform infection was achieved. Infected cultures were rendered quiescent by serum deprivation at restrictive temperature (41^{o}C) as outlined in Materials and Methods. Cultures were then stimulated by addition of serum at restrictive temperature or by shift to permissive temperature in the absence of added factors. DNA synthesis was assessed by pulse-labelling at appropriate intervals with tritiated thymidine. The results from two of these experiments are presented in figure VIII.9. Cultures infected with RCAN 29A2 remained morphologically normal and incorporated little tritiated thymidine above control levels in response to temperature shift, whereas shift of RCAN 29 infected cultures to permissive temperature resulted in entry into S phase and morphological transformation. RCAN 29 and RCAN 29A2 infected cultures responded similarly to stimulation with serum at restrictive temperature, demonstrating that differential viability was probably not responsible for the failure of RCAN 29A2 infected cells to re-enter cycle. In summary, results obtained in chick embryo fibroblasts closely resemble those obtained in the Rat1 cells. These data suggest that a myristylation defective, temperature sensitive kinase is non-mitogenic in fibroblasts when assayed directly.

One well characterised response to activation of the transforming and mitogenic activities of *ts*LA29 *Src* is the early increase in AP-1 oligonucleotide binding activity present in nuclear extracts[29]. The induction of binding activity correlates with re-entry into cycle and transformation, indicating that modulation of the activity of this transcription factor family might be required for either one or both of these biological endpoints. Preliminary data indicate that AP-1 binding activity is not elevated similarly in response to reactivating *ts*LA29A2 pp60 in CEF (M. Frame, A. Lang and A. Catling, figure VIII.10a), correlating with the failure of these cells to re-enter cycle and transform. I was therefore interested to investigate the effects of artificially elevating AP-1 binding activity in CEF infected with the non-transforming, non-mitogenic mutant 29A2. The protein kinase C agonist

Figure VIII.9

Mitogenic activity of *ts*LA29A2 *Src* in CEF. Cultures uniformly infected with RCAN 29 (■,△,◍) or RCAN 29A2 (◇,•,+) were rendered quiescent at restrictive temperature as described in Materials and Methods. Cultures were left untreated at restrictive temperature (■,◇), stimulated with 5% (v\v) newborn calf serum at restrictive temperature (△,•) or shifted to permissive temperature in the absence of added factors (◍,+). Cultures were pulse labelled (1hr) with tritiated thymidine at four-hourly intervals, and radioactivity incorporated into TCA-precipitable material measured by liquid scintillation counting. Incorporation is expressed as ^{3}H cpm x 10^{-4} per 35mm culture dish at each time-point. The results from two independent experiments are shown.

Figure VIII.9

i)

ii)

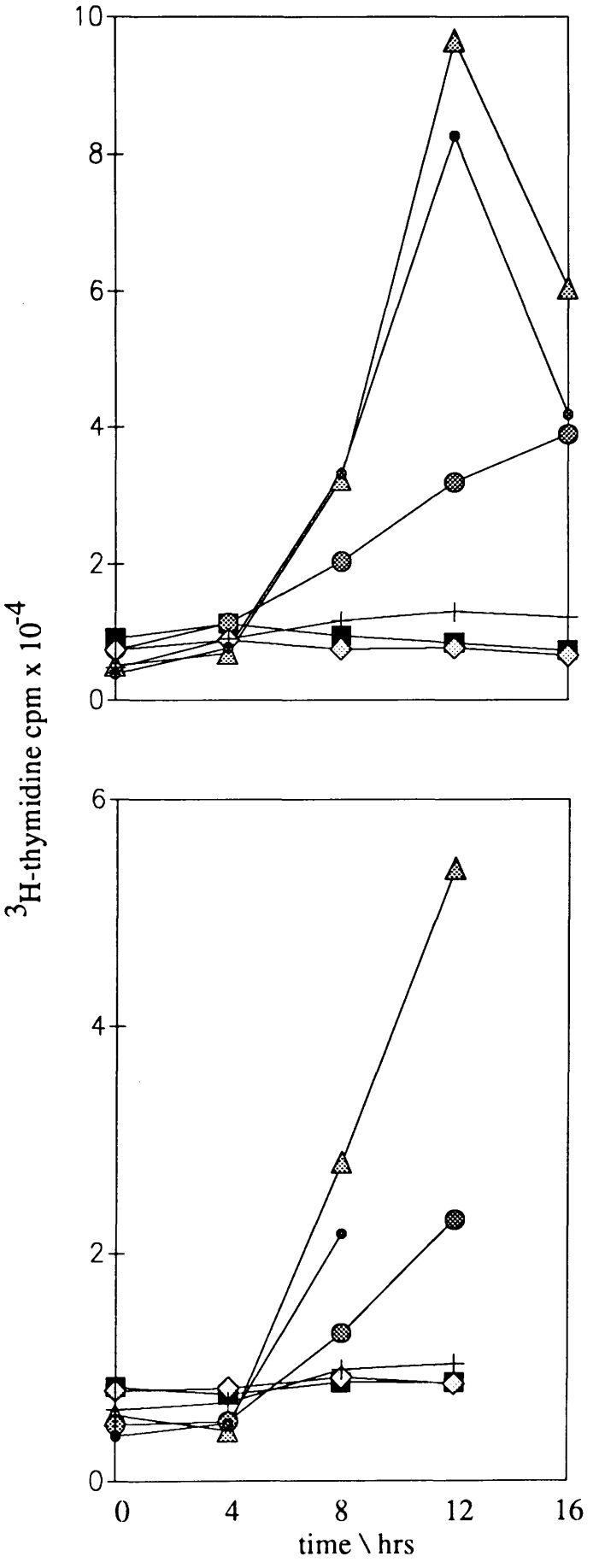


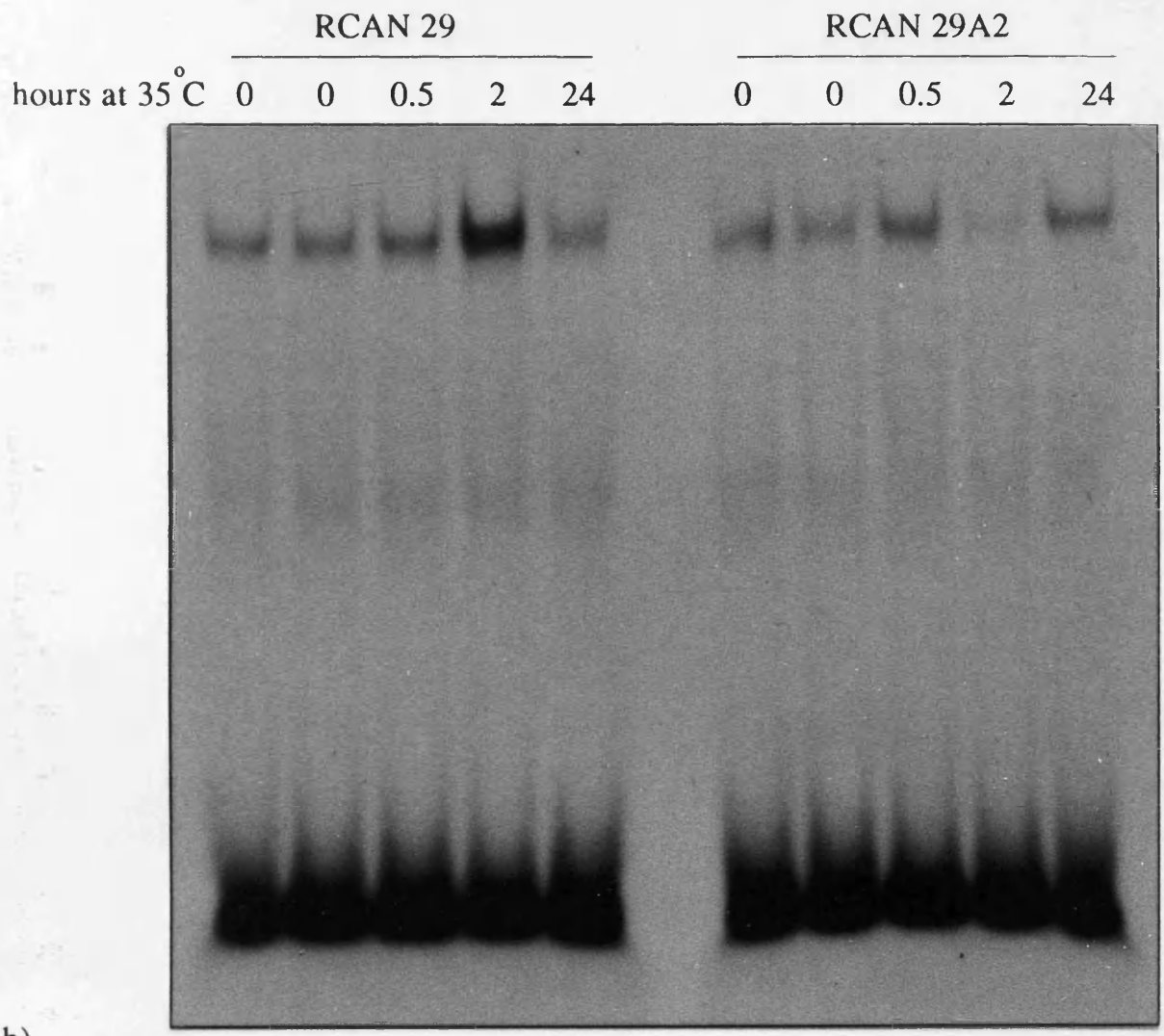
Figure VIII.10

a) AP-1 binding activity after temperature shift of infected CEF. Cultures uniformly infected with RCAN 29 or RCAN 29A2 were rendered quiescent at restrictive temperature as described in Materials and Methods. Cultures were shifted to permissive temperature for the indicated times, and nuclear extracts were kindly prepared by M. Frame and A. Lang. Equivalent amounts of extract protein were incubated with excess ^{32}P-labelled adenovirus E3 AP-1 oligonucleotide, and complexes resolved by non-denaturing polyacrylamide gel electrophoresis. Transfected constructs are indicated above appropriate lanes. Autoradiography was for 18hrs at-70^{o}C.

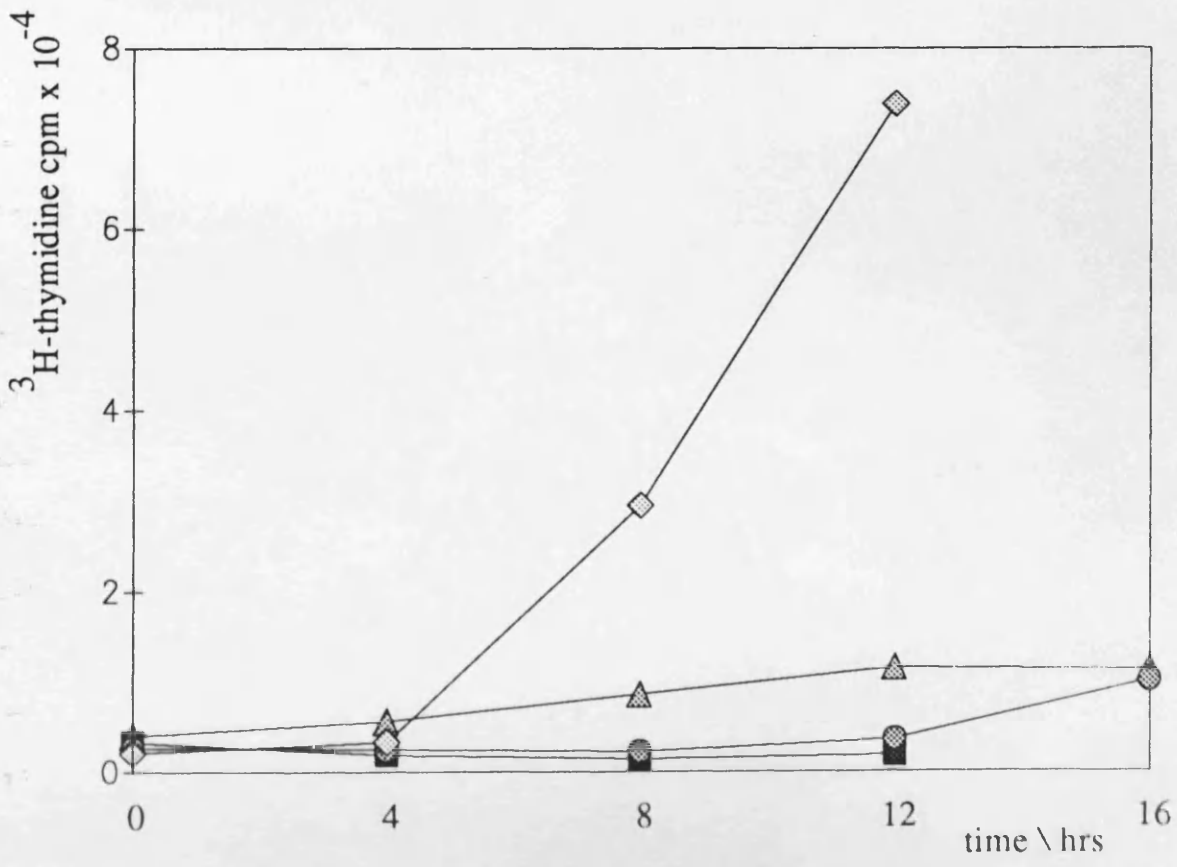
b) PMA is insufficient to stimulate mitogenesis in RCAN 29A2-infected CEF. RCAN 29A2-infected CEF were rendered quiescent at restrictive temperature by serum deprivation as described in Materials and Methods. Cultures were left untreated at restrictive temperature (■), stimulated with 5% (v \ v) newborn calf serum at restrictive temperature (◇), shifted to permissive temperature in the absence of added factors (△) or shifted to permissive temperature in the presence of 100ng \ ml PMA (●). This concentration of PMA elevates AP-1 DNA binding activity under these conditions (M. Frame and A. Catling, not shown). Cultures were pulse labelled (1hr) with tritiated thymidine at four-hourly intervals, and radioactivity incorporated into TCA-precipitable material measured by liquid scintillation counting. Incorporation is expressed as ^{3}H cpm x 10^{-4} per 35mm culture dish at each time point.

Figure VIII.10

a)



b)



phorbol 12-myristate 13-acetate (PMA) is reported to elevate AP-1 activity in quiescent fibroblasts[331]. PMA was used to stimulate the activity of AP-1 in RCAN 29A2 infected CEF. Quiescent cultures of CEF infected with RCAN 29A2 were prepared as described in Materials and Methods. Cultures were treated with 100ng \ ml PMA at restrictive temperature or were shifted to permissive temperature in the presence of 100ng \ ml PMA. Control cultures were shifted to permissive temperature in the absence of PMA or were stimulated at restrictive temperature by the addition of serum to a final concentration of 5%. This concentration of PMA is demonstrated to elevate AP-1 DNA binding activity under these conditions (M. Frame and A. Catling, not shown). Figure VIII.10b suggests that quiescent RCAN 29A2 infected CEF do not incorporate tritiated thymidine in response to this concentration of PMA either at restrictive or permissive temperature. In addition, cell transformation also fails to occur in temperature-shifted cells stimulated with this concentration of PMA. The implication is that transformation and mitogenesis require cell membrane events, in addition to an elevation of nuclear AP-1 DNA binding activity.

VIII.C. Discussion

The data presented above suggest that the myristylation mutant 29A2 is not capable of acting as a complete mitogen in Rat1 and CEF using a direct assay. However, there are a number of potential technical problems associated with the Rat1 cell system that render this conclusion tentative. First, the protein is poorly expressed in Rat1 cells. The failure to detect a mitogenic activity in the low expressing Rat1 clones could therefore result from sub-threshold expression of the mutant protein. Although one colony expressing reasonable amounts of 29A2 pp60 was assayed for mitogenic activity, this too yielded negative results. The interpretation of this result is however not straightforward since the Rat1 f29A2 3B cells appear not to be clonal; non-expressing cells in this population could exert a suppressive effect on those cells expressing 29A2 pp60. Attempts to generate single cell clones from the Rat1 f29A2 3B population yielded only lines in which pp60 was undetectable by Western blot. The reason(s) for the poor

expression of this protein in Rat1 cells is unclear. The glycine to alanine mutation used in this study has been used by numerous groups, none of whom have reported problems in expressing non-myristylated proteins in a number of cell types. The mutation used preserves a consensus sequence for translational initiation, and is more than 100bp downstream of the *src* splice acceptor. This would suggest that the expression problems are unlikely to result from RNA splicing defects or poor translational initiation. However, it is possible that the mutant protein is turned-over rapidly in Rat1 cells, leading to low steady state expression. This possibility has not been addressed.

The second problem encountered in expressing 29A2 in Rat1 cells is the apparent toxicity of the protein. This might in part explain the low levels of expression of this protein in the majority of clones and colonies screened following transfection of fpGV-29A2, and also the failure to generate clonal lines from Rat1 f29A2 3B. It is possible that those cells in the Rat1 f29A2 3B population expressing reasonable amounts of the mutant protein do respond to temperature shift by transiting G1, but fail to incorporate labelled thymidine as the non-specific toxic effects of a cytoplasmic kinase manifest themselves at permissive temperature. Given that other myristylation mutants are not reported to be toxic in mammalian cells, this non-specific toxicity would presumably result from the combination of mutations in the 29A2 protein. Alternatively, the toxicity observed might be rather more specific. One possibility is that the cytoplasmic kinase is capable of initiating some but not all events leading to mitosis, leading to mitotic catastrophe. If this is the case, it might be possible to rescue such cells by adding specific agonists to compensate for these deficiences.

Many of the problems associated with expression of the 29A2 protein in Rat1 cells were overcome in CEF. CEF cultures uniformly infected with RCAN 29 or RCAN 29A2 express similar amounts of pp60 and exhibit equivalent *in vitro* kinase activities. Coupled with the apparent lack of toxicity in the CEF background, this enables a more direct comparison of the mitogenic and transforming activities of the two proteins than has been possible in Rat1 cells. Examination of the mitogenic activity of the myristylation mutant in this CEF background also yielded negative results, as had been

the case in a Rat1 cell background. This is not due to a defect in the reactivation of the myristylation mutant kinase, since under these conditions the *in vitro* kinase activity of *ts*LA29A2 reactivates with kinetics similar to the prototype *ts*LA29 protein. RCAN 29 and RCAN 29A2 infected cultures respond similarly to serum at restrictive temperature, indicating that both cultures are able to re-enter cycle.

In summary, it appears that a temperature sensitive kinase defective for myristylation is not mitogenic in the fibroblast cells studied. This contradicts the results reported by Calothy *et al*. using infected cultures of primary chick neuroretinal cells[32]. These workers found that mutants defective for membrane association, by virtue of mutations in the myristylation domain[32] or through increased pp50 \ pp90 complex formation[328,329], prolonged the lifespan of neuroretinal cells in the absence of transformation. This discrepancy might result from the use of different cell types, or from differences in experimental approach. The stringent assay for mitogenic activity used in this study will reveal complete mitogens whose function is largely independent of exogenous factors. The studies of Hanafusa Calothy and colleagues utilise neuroretinal cells which do not normally proliferate *in vitro*, even when maintained in serum-containing medium. Under these conditions, introduction of a mutant pp60 defective for myristylation is sufficient to stimulate these cells into cycle. It is possible that this mitogenic activity results from synergy between the mutant pp60 and serum factors. This idea could be tested by shifting quiescent cultures infected with RCAN 29A2 to the permissive temperature in the presence of specific agonists which are themselves not complete mitogens. Such studies might include an examination of the properties of conditioned medium from *src*-transformed cells. Preliminary data from an experiment employing the protein kinase C agonist PMA as a potential co-mitogen are described above. Other potentially interesting agonists include insulin and in particular insulin-like growth factor I, since tyrosine phosphorylation of the receptor for this ligand correlates well with transformation *in vitro*[292]. Alternatively, it might be possible to express a myristylation defective *wt*PrA protein in cells already expressing *ts*LA29 *Src*: temperature shift experiments would then enable a thorough examination of the

mitogenic properties of the non-myristylated protein. It is possible that non-myristylated proteins are capable of stimulating early events required for G1 transit, but are insufficient to stimulate entry into S phase. Reactivating a thermolabile transforming mutant in this situation might result in more rapid entry of cultures into S phase, early events having already been completed. One could also design experiments to address any later requirement for *Src* using cells co-expressing suitable non-myristylated and myristylated *Src* proteins.

The studies of Hanafusa and colleagues have indicated that the mitogenic and transforming activities of pp60 show poor correlation with *in vitro* or *in vivo* kinase activity, although both biological properties did correlate with the presence of a v-*Src* kinase domain[328,329]. Furthermore, the thermolability of different parameters of transformation becomes apparent at different temperatures: thus anchorage independent growth of PA104-infected chick embryo fibroblasts is essentially abolished at 37°C, whereas the protein still retains a mitogenic activity in chick neuroretinal cells at this temperature[328]. This differential temperature sensitivity is not reflected in thermolabile complex formation with pp50 and pp90, since the mutant is predominantly complexed at 34, 37 and 41°C[329]. One possible explanation for the observed dissociation of anchorage independent growth and mitogenic activity is that substrate recognition becomes more stringent as temperature is increased, such that substrates required for anchorage independent growth are not phosphorylated at 37°C[329]. Alternatively, it is possible that the absolute level of phosphorylation of a particular substrate involved in eliciting a number of transformation parameters varies with temperature. Since the transformation parameters were measured in different cell types, albeit from the same host, a trivial explanation for these observations would be that the restrictive temperature for this mutant varies with cell type. A more direct test of mitogenic activity of PA104 in CEF would be useful to exclude this possibility.

The experiments reported in this chapter indicate that factors sited in or in proximity to the plasma membranes of fibroblasts are important for both the transforming and mitogenic activities of pp60. Preliminary data indicate that membrane

association of pp60 is required for induction of AP-1 binding activity, since temperature shift of quiescent RCAN 29A2-infected CEF does not result in stimulation of AP-1 binding activity. Furthermore, it is apparent that other events required for mitogenesis and transformation are dependent upon membrane association of pp60, since elevating AP-1 activity (with PMA) in concert with reactivating *ts*LA29A2 pp60 does not result in transformation or re-entry into cycle. If one assumes that both endpoints are mediated through the tyrosine kinase activity of pp60 (but see refs. 328,329), this implies that phosphorylation of common target(s) results in both biological endpoints, or that multiple signal transduction pathways, initiating at the cell membrane, are activated in response to *Src*. The former possibility allows little scope for dissecting the transforming and mitogenic activities. The latter situation might allow dissociation of these two activities in one of two ways :

a) use of mutants defective for substrate recognition and \ or association with other signalling proteins. These might be mutated around the autophosphorylation site at tyrosine 416 or in the N-terminal homology domains of pp60, or mutants such as PA104 described above.

b) use of pp60 derivatives that are membrane associated through interactions other than the pp60-32kDa complex. This might favour interaction with initial targets specific to one or other of the pathways leading to transformation or mitogenesis.

Alternatively, one could select appropriate cell types on the basis of their behaviour. For instance neuroretinal cells might be useful in examining a mitogenic or co-mitogenic activity of *Src* protein using the 2D-gel approach. However, these cells do not normally proliferate in culture, and hence there are practical difficulties associated with this approach. It might also be possible to select appropriate cell lines defective for certain signal transduction pathways. This approach could yield information on the pathway(s) leading to *Src* transformation and mitogenesis.

Chapter IX

IX GENERAL DISCUSSION

One aim of this project was to investigate early consequences of activating the transforming and mitogenic activities of the v-Src protein pp60. In particular, I was interested in defining changes in phosphoprotein profile and protein synthesis that resulted from the induction of these activities. This conceptual approach demanded a system where phenotypically normal cells could be stimulated to transform rapidly and reversibly under experimental conditions. In addition, I needed a system where phenotypically normal cells could be rendered quiescent and yet retain the ability to enter cycle upon activating the mitogenic activity of pp60. Both requirements could be fulfilled by employing a temperature sensitive mutant of pp60. Temperature sensitive-transformation mutants are, by definition, temperature sensitive for transformation and have also been used successfully to investigate the mitogenic activity of pp60[26,27,29,242,243]. Many of these mutants induce wild-type characteristics of transformation at permissive temperature, whilst being essentially biologically inactive at restrictive temperature. The biochemical lesions in a number of these mutants have been described[55,88,234] (see I.7), the most frequent being defects in membrane localization and \ or kinase activity. As outlined in section I.13, this conceptual approach to defining early consequences of the transforming and mitogenic activities of pp60 depends on minimizing the impact of adventitious activities of pp60. For this reason I decided to use a temperature sensitive mutant whose biochemical behaviour deviates only minimally from the wild-type protein. One would predict that such a mutant would express most if not all adventitious activities at restrictive temperature whilst the host cells are still phenotypically normal. These non-transforming activities might manifest themselves in altered protein and phosphoprotein profiles in comparison with parental cells under the same conditions, and therefore one might be able to identify a group of *src*-responsve proteins \ phosphoproteins whose perturbation is not sufficient to elicit transformation. By inducing phenotypic transformation and mitogenesis in this system, one predicts that important early changes would be visible against a preset background of adventitious events.

One mutant generated in this laboratory, *ts*LA32, appeared from previous work to have properties compatible with some aspects of this approach. In chick embryo fibroblasts (CEF) the protein retains both membrane association[93] and kinase activity at restrictive temperature[299], whilst being temperature sensitive for a number of characteristics of transformation. Information on the mitogenic properties of this mutant was not available. This mutant was chosen for further study together with a well characterised temperature sensitive mutant, *ts*LA29 and the wild-type parent *wt*Prague A. In view of the practical difficulties associated with generating equivalent infected CEF cultures for analysis by 2D-gel electrophoresis, I decided to generate clonal immortal fibroblast lines expressing these *Src* proteins. Much of this thesis is dedicated to describing the generation and characterisation of these clonal lines.

Previous attempts to introduce *ts*LA32 RSV into established Rat1 fibroblasts had been largely unsatisfactory (my own data, not shown, M. Welham and J. Wyke, personal communication), so expression of the mutant *Src* was achieved under the control of a murine retroviral promoter. Additional experiments were undertaken in CEF, using replication-competent retroviral constructs. Chapter III documents the construction and transfection of the expression constructs used in these studies, and the biological properties of the clonal lines generated. The results indicate that it was possible to express the *ts*LA32 protein in Rat1 cells, and that this mutant was temperature sensitive for transformation in Rat1 cells.

Subsequent chapters have described the biochemical properties of *ts*LA32 pp60 in Rat1 cells, and in general these match the published data closely. Rat1 cells expressing this mutant exhibit elevated levels of protein phosphotyrosine at both permissive and restrictive temperatures. In addition I have demonstrated that the mutant protein is non-conditionally phosphorylated on tyrosine *in vivo*, presumably as a result of autophosphorylation (see chapter V). Both results indicate that the kinase is functional *in vivo* at both permissive and restrictive temperatures. The significance of these results is however unclear, since the well characterised temperature sensitive mutant *ts*LA29 *Src* also demonstrated significant levels of protein phosphotyrosine at restrictive

temperature, and displayed wild-type *in vivo* autophosphorylation characteristics. Hence, the characteristics of *ts*LA29 *Src* in this clonal Rat1 line differ from those reported for infected CEF[299]. It is noteworthy that Weber and colleagues have reported similar results in other cell types[208,292]. The lower restrictive temperature used might result in significant leakiness in the kinase activity of the *ts*LA29 in rodent cells. This implies that the major transformation defect of *ts*LA29 *Src* in Rat1 cells is its failure to associate with the particulate fraction of the cell at restrictive temperature. Assuming that this is the case, one can make two predictions. Firstly, it should be possible to dissociate membrane association and kinase activity in *ts*LA29 *Src*, and secondly one should be able to use *ts*LA29 *Src* to look for membrane substrates in Rat1 cells. This latter property of *ts*LA29 *Src* might be useful in further defining changes observed using the *ts*LA32 system. Dissociation of the membrane binding and kinase activities of pp60 will be discussed later in this chapter.

In summary, these data suggest that *ts*LA29 *Src* is itself a minimal deviation mutant in Rat1 cells, and that its properties complement those of *ts*LA32 *Src* with regard to the proposed 2D-gel approach to identifying early events in *Src*-induced transformation and mitogenesis.

In vitro kinase activity has been routinely used as an indicator of the *in vivo* activity of numerous mutants of pp60, and in general, the apparent *in vitro* and *in vivo* activities correlate well. Published data indicate that CEF infected with *ts*LA32 RSV contain *in vitro* kinase activity when cultured at either permissive or restrictive temperature, in contrast to *ts*LA29 infected cells where the *in vitro* kinase activity is thermolabile[299]. I have attempted to repeat these results in the clonal Rat1 lines. As documented in chapter IV, I have been unable to detect significant kinase activity in immunoprecipitates prepared from clonal Rat1 f32 or in immunoprecipitates prepared from CEF infected with RCAN 32, under a variety of conditions. In contrast, kinase assay of immunoprecipitates prepared from cells expressing *ts*LA29 *Src* demonstrated clear temperature sensitivity, as had been observed in infected cultures of Rat1 and CEF[94,299]. This raises some interesting questions. Firstly, why is it not possible to detect

in vitro activity of the *ts*LA32 protein when cells expressing this mutant exhibit elevated levels of protein phosphotyrosine ? Secondly, why does the *in vitro* activity of the *ts*LA29 protein not mimic its *in vivo* activity ? Possible reasons for the lack of *in vitro* kinase activity in immunoprecipitates prepared from cells expressing *ts*LA32 *Src* are given in chapter IV. It seems likely that this mutant protein might be particularly susceptible to conformational change upon lysis or interaction with precipitating antibodies. Similar problems were encountered by Sefton *et al.* studying *ts*LA90, *ts*NY68 and *ts*BK5[332]. In addition, this protein might be somewhat susceptible to proteolysis, since a degradation product of pp60 was found in immunoprecipitates prepared from phosphate labelled Rat1 f32 cells grown at permissive temperature (see chapter IV). I am not aware of other transforming mutants that exhibit undetectable *in vitro* activity under the mild conditions of lysis employed.

The behaviour of *ts*LA29 pp60 in *in vitro* assays is also somewhat puzzling. In view of the elevated level of protein phosphotyrosine in Rat1 f29 cells at restrictive temperature, the lack of activity in immunoprecipitates prepared from cells under these conditions suggests one of three things : the mutant protein is labile when prepared from cells at restrictive temperature[332], or the precipitating antibody selectively complexes inactive kinase at restrictive temperature, or an inactive kinase is capable of elevating cellular phosphotyrosine by a mechanism independent of its own enzymatic activity. The latter suggestion would liken *ts*LA29 pp60 at restrictive temperature to the product of the v-*crk* oncogene[105]. However, Snyder *et al.* were unable to detect elevated levels of cellular phosphotyrosine in Rat1 cells expressing SF2, a point mutant (lysine 295 to methionine) of v-*src* non-conditionally defective for *in vitro* kinase activity, suggesting that this mutant is incapable of stimulating endogenous tyrosine kinases[46]. Although this result does not support the hypothesis that *ts*LA29 pp60 elevates cellular phosphotyrosine at restrictive temperature independently of its intrinsic kinase activity, it is noteworthy that SF2 is not detectably phosphorylated either on tyrosine or serine *in vivo*, and indeed, is not a substrate for protein kinase A *in vitro*[46]. Similar results were obtained by Parsons and colleagues using defective kinases generated by point mutations

at amino acids 430-433[44,233]. Cells expressing these CH*pm* mutants display lowered levels of tyrosine phosphorylated p36 protein[44], and both the serine and tyrosine phosphate content of the mutant *Src* proteins is reduced[44,233]. The lack of N-terminal serine phosphorylation might suggest that the native conformation of pp60 is dirupted by the SF2 and CH*pm* mutations, leading to a loss of both kinase and perhaps, other activities. It is formally possible that other kinase inactive mutants whose overall structure is conserved might behave differently. However, studies have demonstrated that there are functional interactions between the catalytic and N-terminal domains of the *src*-family proteins (see chapter I), and hence the mutants required might be difficult to obtain.

One alternative explanation for the lack of kinase activity in immunoprecipitates prepared from Rat1 f29 cells at restrictive temperature might be that the protein is complexed to pp50 and pp90[50], a form which has been shown to exhibit much reduced protein kinase activty *in vitro*[56]. However, Welham and Wyke have demonstrated that most of the *Src* protein at restrictive temperature in Rat1 *ts*LA29 cells is monomeric[94]. It should also be stressed that the lack of *in vitro* catalysis may be due only to the conditions used to measure catalysis and not a true absence of kinase activity[332].

Previous work from this laboratory has demonstrated that *ts*LA29 pp60 is located predominantly in the cytoplasm in Rat1 cells grown at restrictive temperature[93,94]. Assuming that some of this protein retains tyrosine kinase activity, the *in vitro* kinase assay data suggest that the majority of this active protein is not accessible to the precipitating antibody, as a result of its environment or conformation. Cytoplasmic localization *per se* appears not to preclude immunoprecipitation of *in vitro* active kinase, as evidenced by my results obtained using the myristylation mutant *ts*LA29A2 *Src* (see chapter VIII, discussed later in this chapter). Again, it may be that *ts*LA29 pp60 is susceptible to conformational change when precipitated from cells grown at restrictive temperature, such that its inherent kinase activity is reduced. Alternatively, it is conceivable that the catalytic activity of this protein is reduced at restrictive temperature

and that elevation of cellular phosphotyrosine in Rat1 cells results from the thermolabile nature of endogenous protein phosphotyrosine phosphatases.

From the preceeeding it is apparent that the biological and biochemical characteristics of *ts*LA32, and indeed *ts*LA29 *Src* were consistent with these mutants being employed in the aforementioned approach to describing early events of v-*src*-induced transformation and mitogenesis. It remained important to establish whether the Rat1 f32 line could be rendered quiescent by serum deprivation at restrictive temperature, as had been demonstrated for Rat1 cells expressing *ts*LA29 RSV[29]. The results presented in chapter VI indicate that this mutant is temperature sensitive for both the transforming and mitogenic activities of pp60. This is perhaps somewhat surprising in view of the characteristics of this protein. As discussed in chapter VI, it is plausible that the transforming and growth-regulatory properties of pp60 are effected through common mediators, and as such are not dissociable in fibroblasts. Experiments undertaken in an attempt to dissociate the mitogenic and tranforming activities of pp60 are described in chapter VIII, and will be discussed later in this chapter.

Preliminary experiments to define changes in protein profile in temperature-shifted quiescent cultures of Rat1 f32 cells have demonstrated a small number of proteins whose synthesis \ degradation is modulated by activating the transforming and mitogenic activities of *ts*LA32 *Src*. The small number of changes I have observed is in good agreement with data published by Durkin and Whitfield[27], who have defined six proteins which appear or disappear as quiescent *ts*LA23-infected NRK cells transform and re-enter cycle following shift to permissive temperature. However, due to the differences in separation techniques employed in these studies, I am unable to comment on whether the changes I have observed correspond to those observed in other systems. Indeed, it is important that these changes are investigated in cells expressing other temperature sensitive mutants, for instance *ts*LA29 and *ts*LA29A2 to ascertain correlation with transformation and mitogenesis.

Preliminary attempts to define early changes in the phosphoprotein profile of quiescent Rat1 f32 cells following temperature shift have been disappointing. Possible

technical reasons for the failure of these experiments are given in the discussion to chapter VIII. In addition to these technical problems, parallel analysis of candidate substrates might facilitate interpretation of data from these experiments. These candidates might include the *Ras* GTPase activator protein, GAP[138], members of the MAP kinase family[255], c-*Raf-1*[333] and the insulin-like growth factor I receptor[292]. With a knowledge of the labelling and electrophoretic behaviour of these candidate substrates \ effectors, it would be possible to use them as internal reference points in the 2D-gel analysis.

Data obtained from the 2D-gel approach outlined above will correlate changes in protein synthesis and phosphorylation with both exit from the quiescent state and transformation. To further define the role (if any) of these biochemical events it would be useful to test mutants that are thermolabile for one biological endpoint whilst non-conditionally defective for the other. Since all temperature sensitive transformation mutants tested to date appear to be thermolabile for both the morphological and growth regulatory aspects of transformation in fibroblasts, I have attempted to generate a mutant defective for morphological transformation which nonetheless should retain a thermolabile kinase activity. Studies by Hanafusa and Calothy and colleagues indicate that pp60 proteins defective for membrane localization by virtue of mutations in the myristylation domain are still capable of stimulating proliferation of primary chick neuroretinal cells in the absence of morphological transformation[32]. I therefore mutated the myristylated glycine of *ts*LA29 pp60 to alanine, a residue that does not serve as a substrate for the myristyl-transferase[37,111]. The generation and properties of this mutant have been described in detail in chapter VIII. From the data presented, it would appear that this mutant is defective in activities leading to both morphological tranformation and mitogenesis, despite a retention of *in vitro* kinase activity. Together with the previously discussed properties of *ts*LA29 pp60 in Rat1 cells, these data suggest that a cytoplasmic *Src* protein with associated tyrosine kinase activity is not sufficient to stimulate mitogenesis in quiescent fibroblasts. A number of technical explanations are offered for the failure of this protein to stimulate mitogenesis in Rat1 cells, although many of these

problems were overcome in a CEF background. As discussed in chapter VIII, this result implies interaction with or phosphorylation of common targets results in both biological endpoints, or that multiple signal transduction pathways, initiating at the cell membrane are activated in response to *Src*. Alternatively, it is plausible that changes in cell structure or cell-cell communication resulting from morphological transformation are required to enable expression of the mitogenic activity of pp60. This is not to say that morphological transformation of fibroblasts invariably leads to deregulated growth control, since the growth of rat fibroblasts transformed with v-*fos* is at least partially serum dependent (R. Hennigan, personal communication). It would be interesting to express *ts*LA29A2 *Src* in quiescent v-*fos*-transformed fibroblasts such that reactivation of pp60 could be effected in a transformed cell background. The apparent toxicity of the protein in Rat1 cells might be a problem, though it should be possible to co-express both v-*fos* and *ts*LA29A2 *src* in CEF. If this approach allowed the temporal dissociation of mitogenesis and transformation, changes in protein synthesis and phosphorylation could be investigated using the 2D-gel approach.

An alternative approach to the problem of dissociating mitogenesis and morphological transformation in *Src*-transformed fibroblasts employs cells defective for aspects of certain signal transduction pathways involved in mitogenesis. Cells for these studies can be obtained in one of two ways. Firstly, a number of cell lines have been selected on the basis of their non-responsiveness to specific agonists, and in at least two cases, the molecular basis of the defect is partially understood[247,289]. Secondly, it is possible to obtain cells defective for signalling pathways using specific agonists or antagonists. For instance, protein kinase C can be downregulated in Swiss 3T3 fibroblasts in response to chronic TPA treatment[334], this enabling an examination of the requirement for this enzyme in a given situation. At this point it is worth noting that downregulation of protein kinase C abolishes the mitogenic activity of activated *Ras*, without affecting morphological transformation[202,204]. Indeed, microinjection studies have suggested that *Ras* functions downstream of tyrosine kinase oncogene products in the induction of transformation and stimulation of cell proliferation[182]. Additional studies

have indicated a requirement for two parallel *Ras*-dependent pathways, one dependent on protein kinase C, in mitogenesis[335], and it is tempting to speculate that GAP and associated proteins play a role in one or both of these pathways. However, the role of protein kinase C in *Src*-induced mitogenesis and transformation is at present unclear. A number of studies in NRK cells[336], NIH 3T3[337] and CEF[338] have indicated that protein kinase C activity is increased in response to activating thermolabile pp60 or *Src*-induced transformation. However, other studies suggest that prior downregulation of the enzyme does not prevent *Src*-induced mitogenesis or transformation, and therefore may be irrelevant to these endpoints[150]. Preliminary data from this laboratory indicate that chronic treatment of Rat1 *ts*LA29 cells with phorbol dibutyrate has no effect on either the mitogenic or transforming activity of pp60 upon temperature shift of quiescent cultures (A. W. Wyke, personal communication). Experiments to test this result in Swiss 3T3 cells, where the response to chronic phorbol ester treatment is well characterised, are underway (M. Unlu, personal communication). These data are relevant in consideration of the published *Src*-induced changes in AP-1 activity[28,29], since phorbol ester agonists of protein kinase C are potent stimulators of AP-1 activity[331]. It is conceivable that the observed transient increases in AP-1 activity following temperature shift of Rat1 *ts*LA29 are merely a consequence of the increase in protein kinase C activity following activation of pp60. Although there is some evidence to suggest that transcripts from an AP-1 responsive gene, stromelysin, accumulate following temperature shift of quiescent Rat1 *ts*LA29 cells (A. W. Wyke, personal communication), the mechanism (*i.e.* transcriptional or post-transcriptional) and factors required are not defined. Indeed it has been reported that transcriptional activity of the stromelysin gene is mediated through sequences in addition to AP-1 elements[339], perhaps by the c-*ets* family proteins[340]. Further experiments are required to confirm or otherwise the functional involvement of AP-1 in mediating early events following activation of thermolabile pp60. In particular, a study of *Src* mutants defective for transformation and \ or mitogenic activity might allow correlation of increased AP-1 activity with one or other of these biological endpoints, or with a particular property of pp60, such as

membrane localization or kinase activity. Complementary approaches designed to abrogate AP-1 activity, by expression of either dominant inhibitors of functional complex formation or expression of anti-sense RNA might also yield information on the requirement for AP-1 following temperature shift. However, one should appreciate that the number of structurally distinct AP-1 complexes *in vivo* is potentially large, and that each member of this family is likely to respond differently to inhibition.

CONCLUSIONS

Protein serine \ threonine phosphorylation and dephosphorylation is a well documented mechanism whereby cells regulate the activity of critical enzymes. Protein tyrosine phosphorylation is presumed to offer the cell another means of covalently regulating specific enzymes. Many studies have been undertaken on the assumption that $pp60^{v\text{-}src}$ transforms fibroblasts *in vitro* by modulating the activity of critical membrane proteins in an allosteric fashion. This model predicts that key regulators of *Src* transformation might be identified by virtue of their tyrosine phosphorylation in transformed cells. Despite a great deal of effort, non-candidate approaches to identifying targets for the tyrosine kinase activity of pp60 have been largely unsuccessful. A number of tyrosine phosphorylated proteins have been identified, although in no case is there inviolate correlation between transformation and phosphorylation of these substrates (see I.9-12). In view of these results and the technical problems involved in this approach, many groups have taken to examining candidate substrates for differential phosphorylation and activity following *Src* transformation. This approach has identified a number of potential substrates for the tyrosine kinase activity of the *Src* protein, and tyrosine phosphorylation of a number of these candidates has been demonstrated to correlate with transformation using a battery of partially, conditionally and non-conditionally defective mutants (see I.9-12). In addition, tyrosine phosphorylation of a number of these proteins has been demonstrated to occur in mitogenic stimulation of normal cells, indicating that these proteins may play a role in normal growth control (see I.6). These studies have not however examined the temporal sequence of events

following activation of *Src*, and indeed many of the described changes may in themselves be consequences of transformation.

Recent data from diverse systems have indicated that the primary role of protein tyrosine phosphorylation may not be in the allosteric control of enzyme activity. Much data (see I.6) indicates that an important function of tyrosine phosphorylation is to promote the formation of very specific, stable protein-protein interactions. Tyrosine phosphorylation and specific complex formation occur in response to both growth factor stimulation and oncogenic agents such as retroviral tyrosine kinases. Available data strongly suggest that formation of these specific complexes is dependent both on tyrosine phosphorylation of certain components and structural features, SH2 domains, of other components. One model accounting for these features has been described in chapter I of this thesis. Briefly, this model suggests that tyrosine phosphorylation in response to mitogenic or transforming agents functions to recruit specific signalling molecules to appropriate sites in the cell, often at the plasma membrane. These signalling proteins (*e.g.* GAP, PI 3-kinase, PLCγ-1) are then able to utilize localised concentrations of substrates to generate specific intracellular signals. This allows a highly co-ordinated and rapid response to stimulation, and suggests that allosteric modulation might not be essential to this mechanism of control; relocation of enzymes to their substrates may be the overriding regulatory mechanism. It follows that important early effectors for growth factor receptors and oncogenic kinases might not be substrates for tyrosine phosphorylation, and hence might not be identifiable by conventional labelling approaches; recruitment to appropriate membrane locations might be dependent only on pp60 autophosphorylation. I speculate that the v-*Src* product pp60 may transform fibroblasts *in vitro* by recruiting specific proteins to appropriate plasma membrane and cytoskeletal environments through formation of specific phosphotyrosine-dependent complexes. This could account for two main transformation requirements of pp60: tyrosine kinase \ autophosphorylation activity and membrane localization. Furthermore, one can envisage a number of similar but distinct routes to transformation based on

subtle variations of this model. These models and their implications with respect to the search for crucial *Src* effectors are outlined below.

Chapter I presented evidence supporting the contention that autophosphorylated growth factor receptors and oncogenic tyrosine kinases, which are themselves tyrosine autophosphorylated, form specific complexes with SH2 domain containing proteins (see I.6). Two identified complex proteins, PI 3-kinase and PLC-γ1 act upon lipid substrates and hence are dependent on relocation to membrane environments for functional activity. A third SH2-containing complex member, GAP, associates with and stimulates the GTPase activity of membrane bound *Ras* proteins. Signals generated by these complexes would be dependent on the integrity of the membrane complex, and hence on the tyrosine phosphorylation of specific components. Signal termination could occur by internalisation and proteolysis of the the complexes, or by dephosphorylation of specific autophosphorylation sites. Interesting in this respect is the identification of an SH2-containing protein tyrosine phosphatase which might plausibly promote signal termination[224]. pp60$^{v\text{-}src}$ might transform cells by promoting constitutive complex formation and signalling in a number of ways. Since pp60$^{v\text{-}src}$ autophosphorylation is apparently ligand-independent, signalling complexes could form around the *Src* protein; the constitutive tyrosine kinase activity of pp60$^{v\text{-}src}$ would preclude dephosphorylation and signal termination. Furthermore, this could account for the behaviour of SH2-domain mutants of pp60 that are host-range restricted for transformation; the prediction is that these proteins fail to associate with appropriate signalling molecules in the non-permissive host. As outlined in chapter I, a number of biochemical activities have been identified in association with pp60$^{v\text{-}src}$.

Alternatively, pp60$^{v\text{-}src}$ kinase activity might promote signal complex formation upon other membrane targets. The most likely candidates for such a model would be tyrosine kinase-type receptor molecules. I speculate that the reported phosphorylation of growth factor receptors in *Src* transformed cells might result in inappropriate formation of otherwise ligand-dependent signalling complexes. The constitutive tyrosine kinase activity of pp60$^{v\text{-}src}$ might preclude dephosphorylation and inactivation of these

complexes. This model could account for the host-range specific transforming activity of some pp60$^{v\text{-}src}$ mutants defective for autophosphorylation[77] : signal complex formation is not dependent on the structural integrity of the pp60$^{v\text{-}src}$ autophosphorylation site, and full transformation would require the presence of appropriate host receptors. Note that this model suggests that parameters of transformation might be dissociable using appropriate autophosphorylation mutants in the correct cellular background. However, this model has practical implications for attempts to define important targets for *Src*. To illustrate this point I will draw parallels with polypeptide growth factor function. Complete polypeptide mitogens elicit a discrete number of early biochemical events that appear necessary for ligand-induced DNA synthesis. One current hypothesis is that DNA synthesis will result in these cells if this spectrum of early events can be achieved by treatment with a combination of factors that individually are non-mitogenic (see I.6 and ref. 163). At the molecular level, this implies that DNA synthesis will result from a complete mitogen recruiting all required activities to its cognate receptor or alternatively DNA synthesis will result from activities recruited to two or more distinct receptor types in response to stimulation with co-mitogens. Assuming that transformation occurs in a similar fashion, it follows that the initial (receptor) targets for *Src* activity might vary with the particular mutant or cell type under study *i.e* transformation can result from functional redundancy at the level of the initial target. Correlating early events with the biological endpoint is therefore difficult unless one has a knowledge of the intermediate steps linking the intitial target with the end result. As noted above, these intermediate steps need not involve tyrosine phosphorylation nor changes in the intrinsic activity of particular enzymes, and are therefore potentially very difficult to identify by classical methods of *Src* research. Candidate approaches designed with the action of polypeptide mitogens in mind might be the best way of addressing these problems, and I believe that these studies should not be biased towards identification of tyrosine phosphorylated intermediates for the reasons alluded to above.

A third model based on the functional properties of isolated SH2-domains and the behaviour of p47$^{gag\text{-}crk}$ might also be invoked in explaining the transforming activity of

pp60$^{v\text{-}src}$. A recent report has indicated that recombinant SH2 domains from PLC-γ can inhibit dephosphorylation of the EGF receptor *in vitro*, leading Rotin *et al.*[341] to speculate that the resulting increase in tyrosine phosphorylation and signal molecule recruitment might result in tranformation[341]. The behaviour of p47$^{gag\text{-}crk}$ is particularly interesting since this oncoprotein elevates cellular phosphotyrosine in the absence of an identifiable kinase domain[105]. As discussed in chapter I, v-*Crk* is believed to transform via a modulation of endogenous enzyme activity, and SH2 domains have been demonstrated to be important determinants in v-*Crk* oncogenesis[218]. v-*Src* might transform in a similar fashion by locking receptor molecules or other tyrosine phosphorylated proteins in an active form through interaction with the kinase SH2 domain. The apparent requirement for v-*Src* kinase activity is more difficult to accommodate in this model. Tyrosine kinase activity might be required for initiating the transformed state by phosphorylating appropriate targets which are then protected by the adjacent SH2 domain. This would predict that maintenance of the transformed state might not require continuing *Src* kinase activity. To my knowledge, this has not been tested, and indeed mutants of pp60$^{v\text{-}src}$ conditional for kinase activity but wild-type for transformation would not be identified by conventional biological screening. An alternative idea is that exposure of the pp60 SH2 domain is important in conferring oncogenicity, and that activated kinase activity is merely a consequence of the structural requirements for exposure of SH2. Some published data support the idea[217] that tyrosine 527 phosphorylation promotes interaction between the catalytic and SH2 domains of pp60$^{c\text{-}src}$[176,342], and indicate that the protein has little kinase activity in this form[83]. Overexpression of this protein is non-transforming in fibroblasts[54]. If tyrosine 527 phosphorylation is prevented by point mutation[82,83] or C-terminal truncation[84], the biochemical and transforming activities of the protein increase dramatically. It is plausible that these mutations prevent *cis* interaction between the kinase and SH2 domains, exposure of SH2 allowing binding of tyrosine phosphorylated ligands. Binding of v-*Src* SH2 domains to these ligands might protect them from dephosphorylation and inactivation. Polypeptide growth factors (promoting autophosphorylation of their

cognate receptors) or pp60 kinase activity might be required for initiating transformation.

In summary, the described models make five general points :

a) transformation may occur through one or more of a number of related but distinct pathways based upon formation of signalling complexes

b) there may be redundancy at the level of the primary tyrosine phosphorylation event(s)

c) relocalization of specific proteins to their substrates may be more important than overt changes in their intrinsic catalytic activity

d) tyrosine kinase activity might be dispensable for the maintenance of transformation, or might be a consequence of structural changes activating another transforming activity of pp60, *e.g.* exposure of SH2. The latter suggestion does not rule out the involvement of kinase activity in transformation; it merely indicates that tyrosine kinase activity alone is insufficient

e) most and maybe all protein tyrosine phosphorylation events seen upon v-*Src* transformation are adventitious, resulting from association with pp60 or indiscriminate activity against other proximal proteins.

The conceptual approach to identifying pp60 substrates outlined in this thesis might therefore be inadequate to detect physiological effectors of *Src* function. The models presented indicate that primary events in v-*Src* function need not be tyrosine phosphorylation events, or that if these initiating events are marked by tyrosine phosphorylation, there is likely to be functional redundancy at this level. This suggests that even discriminatory mutants such as *ts*LA32 and *ts*LA29 *Src* are perhaps unlikely to be useful in the optimised 2D gel approach I have outlined in this thesis, since observed changes in tyrosine phosphorylation might simply reflect subtle changes in pp60 localization. However, many of the speculative suggestions noted above are at present untestable, since it appears likely that appropriate kinase activity is important in the context of SH2 domain interactions. Abrogation of kinase activity, even by conservative

point mutation tends to have long-range effects on pp60 conformation[45,46] and hence, it is difficult to analyse the effect of pp60 kinase activity and pp60 SH2 domains in isolation.

The properties of *ts*LA29 and *ts*LA32 pp60 are consistent with some features of the models presented above. First, both proteins increase protein phosphotyrosine *in vivo*, and both are tyrosine phosphorylated *in vivo* at restrictive temperature, suggesting that tyrosine phosphorylation alone is insufficient for transformation. The models presented above would predict that *ts*LA29 pp60 might participate in the formation of appropriate signalling complexes at restrictive temperature, though these would be functionally inactive through cytosolic localization until shift to permissive temperature. The observed *in vivo* kinase and autophosphorylation activity of *ts*LA29 pp60 are consistent with this possibility. Second, the reported membrane localization of *ts*LA32 pp60 at restrictive temperature indicates that correct localization together with kinase activity is also insufficient to elicit transformation. One possibility therefore is that other pp60 sequences determine the transforming potential in this background, either by directing subtle changes in localisation or complex formation. The *ts*LA32 SH3 domain mutation is interesting in this regard. This particular mutant would be useful in defining changes in complex formation following temperature shift given its wild-type biochemical properties. Experiments to assess the effect of the SH3 domain mutation upon binding of tyrosine phosphorylated ligands to the adjacent SH2 domain might be particularly informative. Furthermore, one might suggest that the composition of the proposed signalling complex formed around *ts*LA29 pp60 at restrictive temperature is unlikely to change following temperature shift; relocation of associated enzymes to substrates would elicit transformation. This again predicts that any observed tyrosine phosphorylation events might be adventitious. Although the speculative models outlined above suggest that the described optimised approach to identifying early tyrosine kinase targets for *Src* is perhaps naive, it is clear that the same mutants and cell lines might be useful in investigating the role of signal complex formation in *Src* transformation. Studies on the complex forming activity of *Src* mutants would also be complemented by

parallel studies on growth factor receptor complex formation in these same cells in view of the proposed involvement of these molecules in *Src* transformation.

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