STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE

FUJINAMI SARCOMA VIRUS TRANSFORMING PROTEIN

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

FACULTY OF GRADUATE STUDIES

DEPARTMENT OF MICROBIOLOGY

UNIVERSITY OF BRITISH COLUMBIA

We accept this thesis as conforming

to the required standards

University of British Columbia

June, 1985

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Date _June 12, 1985_

ABSTRACT

The phosphorylation of the Fujinami sarcoma virus transform-
ing protein (FSV $P140^{gag-fps}$) is complex, reversible and affects
its tyrosine specific protein kinase activity and transforming
function. The sites of phosphorylation within FSV $P140^{gag-fps}$
have been localized to various regions of the protein using partial
proteolysis. The two major phosphotyrosine residues and a major phos-
phoserine residue are located in the C-terminal portion of the fps
region, which contains the kinase active domain. A comparative tryptic
phosphopeptide analysis of the gag-fps proteins of three FSV variants
shows that the phosphotyrosine containing peptides have similar
mobilities.

To determine whether tyrosine phosphorylation affects protein
function and to evaluate the substrate specificity of the protein
kinase intrinsic to FSV $P130^{gag-fps}$, oligonucleotide-directed
mutagenesis was used to change tyrosine-1073, the major site of
$P130^{gag-fps}$ phosphorylation. Tyrosine-1073 was mutated to a
phenylalanine and a glycine, amino acids that cannot be phosphorylated,
and to the other commonly phosphorylated hydroxyamino acids, serine and
threonine. Neither serine nor threonine were phosphorylated when
substituted for tyrosine-1073 indicating a strict specificity for
tyrosine. All of the FSV tyrosine-1073 mutants had depressed enzymatic

and oncogenic capacities. These data indicate that tyrosine phosphory-lation stimulates the biochemical and biological activities of FSV P130$^{gag-fps}$ and suggest that tyrosine phosphorylation modulates protein function.

Mutations within the putative ATP-binding site of P130$^{gag-fps}$ at lysine-950 destroy both its kinase and transforming activities, supporting the idea that the tyrosine kinase activity intrinsic to P130$^{gag-fps}$ is essential for its transforming function. The mutant protein was also shown to be phosphorylated at a second tyrosine site, which has been previously identified in wild-type P130$^{gag-fps}$ as a site exclusively phosphorylated in vivo. Phosphorylation of secondary tyrosine residues within a mutant protein devoid of intrinsic tyrosine protein kinase activity suggests that the FSV P130$^{gag-fps}$ may be a target for phosphorylation by cellular tyrosine specific protein kinases.

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LIST OF ABBREVIATIONS

A	adenine
ADP	adenosine 5'-diphosphate
AEV	Avian erythroblastosis virus
AK	adenylate kinase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
APE	alanine-proline-glutamic acid
ASV	Avian sarcoma virus
bp	base pair
C	cytosine
cAPK	cyclic-AMP dependent protein kinase
CEFs	chicken embryo fibroblasts
cGPK	cyclic-GMP dependent protein kinase
CHO	Chinese Hamster Ovary
Ci	Curie
cpm	counts per minute
C-terminal	carboxy-terminal portion of a protein
dATP	2'-deoxyadenosine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	disodium ethylene diaminetetraacetic acid
EGF	epidermal growth factor
ESV	Esh sarcoma virus
F	phenylalanine
FAV	Fujinami associated virus
FBS	fetal bovine serum
FSBA	p-fluorosulfonylbenzoyl-5'-adensoine
FSV	Fujinami sarcoma virus
G	guanine
G	glycine
x g	times gravity
$\Delta G°$	free energy

GA-FeSV	Gardner-Arnstein feline sarcoma virus
Gly	glycine
GMP	guanosine 5'-monophosphate
gs^-	group specific antigen negative
HAT	hypoxanthine-aminopterin-thymidine
IGF1	insulin-like growth factor-1 (somatomedin c)
K	lysine
kb	kilobase
kbp	kilobase pair
kcal	kilocalories
kd	kilodaltons
K_m	Michaelis-Menton constant
Lys	lysine
$\underline{M}$	Molar
uCi	micro-Curie
ul	microliter
mCi	milli-Curie
$\underline{mM}$	millimolar
NAD	nicotinamide adenine dinucleotide
NCP98	normal avian cellular fps gene product
N-terminal	amino-terminal portion of a protein
PDGF	platelet derived growth factor
PCS	Phase Combining System scintillation cocktail
PMSF	phenylmethylsulfonylfluoride
pTk1	plasmid containing the Herpes Simplex-1 thymidine kinase gene
PRC II	Poultry Research Centre II sarcoma virus
PRC IV	Poultry Research Centre IV sarcoma virus
R	arginine
Rf	relative mobility
RF	replicative form
RNA	ribonucleic acid

RSV	Rous sarcoma virus
S	serine
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
ST-FeSV	Snyder-Theilen feline sarcoma virus
T	threonine
T	thymine
TBE	Tris-Borate-EDTA buffer
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
Thr	threonine
TK	thymidine kinase
TK⁻	thymidine kinase minus mutant
TLC	thin layer cellulose
TNE	Tris-NaCl-EDTA buffer
TPA	phorbol tetradecanoate acetate
TPCK	L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone
tr	temperature resistant
ts	temperature sensitive
Tyr	tyrosine
UR1	University of Rochester 1 sarcoma virus
UR2	University of Rochester 2 sarcoma virus
vol	volume
wt	wild-type
wt/vol	weight by volume
X	an unspecified amino acid
Y	tyrosine
Y73	Yamaguchi sarcoma virus

ACKNOWLEDGEMENTS

I would like to express my appreciation to my supervisory committee Drs. Julia Levy, George Spiegleman and Ross MacGillivray, and to my research supervisor Dr. Tony Pawson for his support and constant enthusiasm during my research.

In addition, I would like to thank the following people whose interest, support and assistance has contributed greatly to the successful completion of this thesis. All the past and present members of the Pawson group, especially Ehleen (knee-bone) for her friendship, encouragement and expert management of the laboratory, and Alasdair (Lord MacAuley) for taking-off numerous gels and lyophilizations and for alwaying being a good listener. The "Smith lab" for making mutagenesis possible and fun, and more specifically to Drs. Gary Pielak, David Goodin and Mike Smith for many enthusiastic, interesting, entertaining and informative discussions. Dr. Gerry Weeks for his advice, friendship, support and squash games. Gale Dawson who processed the words of this thesis with the expert care that only a sister could.

Finally, I wish to express my deepest appreciation and sincerest thanks to Dennis for his constant encouragement, support, total friendship and love and especially his critical reading of this thesis.

I would like to dedicate this thesis with love to my parents, Doris and David Weinmaster, whose constant love and support has sustained me throughout my student years.

CHAPTER 1

1.0 INTRODUCTION

1.1 Classification of Retroviruses

Retroviruses have been isolated from a wide variety of verte-
brates, as well as invertebrates and have been grouped according to
common morphological, biochemical and physical properties (for a com-
plete review, see Teich, 1982). The Retroviridae virus family includes
all viruses containing an RNA genome and an RNA-dependent DNA poly-
merase (Fenner, 1975). The family is divided into three subfamilies:
(1) Oncovirinae, including all the oncogeneic viruses and many closely
related non-oncogenic viruses; (2) Lentivirinae, the "slow" viruses,
such as visna virus; and (3) Spumavirinae, the "foamy" viruses that
induce persistent infections without any clinical disease.

1.2 Structure of Retroviruses

The genomic RNA of a replication competent virus is a 60-70S
dimer complex composed of two idential subunits, and which resembles
eukaryotic mRNA molecules in that there is a methylated cap structure
at the 5' end and a polyadenylated tract at the 3' end (Teich, 1982).
The genome can be used to direct protein synthesis in an in vitro
translation system and therefore is considered to be of positive-sense.
The genomes of all replication-competent retroviruses contain three

genes encoding the following viral proteins: (1) gag encodes the internal structural proteins found in the viral capsid; (2) pol codes for the RNA-dependant DNA polymerase or reverse transcriptase, and (3) env encodes the envelope proteins which are present in the viral membrane. The order of these three genes is: 5'-gag-pol-env-3'.

1.3 Replication of Retroviruses

Retroviruses encode and package the enzyme reverse transcriptase, which has both RNA-dependent and DNA-dependent DNA polymerase activities. Both of these activities are essential to their mode of viral replication which involves double-stranded DNA intermediates. Such intermediates can exist as unintegrated forms (linear or covalently closed circular molecules) or they may integrate into the cellular DNA of the infected host (proviral forms) (Varmus and Swanstrom, 1982). In the integrated state they can be transcribed like other cellular genes. Thus, a unique feature of retroviruses is that they may occur in nature as infectious elements (exogenous viruses) or as stably integrated proviruses within the cellular DNA of the host (endogenous viruses).

The endogenous viruses are genetically transmitted as inherited genes from one generation to the next. These viruses may remain latent, they may be partially transcribed to produce viral mRNAs which are translated into virus-specified proteins, or they may become

activated to undergo a complete replication cycle with subsequent virus production, viremia and perhaps neoplasia (Teich, et al., 1982).

On the other hand, the exogenous viruses are those which do not occur as integrated viral DNA copies until after infection. If this occurs in cells of the germ line, the exogenous virus may then become an endogenous virus and thus become a stable inheritable trait (Varmus and Swanstrom, 1982).

1.4 Oncogenic Retroviruses

The oncogenic retroviruses, also known as RNA tumor viruses, can be divided into two classes based on their ability to transform cells. One class rapidly causes neoplasias in animals due to the presence of an oncogene (onc$^+$ viruses), while the other class of viruses induces malignancies by a complex mechanism which requires a long latent period (onc$^-$ viruses) (Varmus, 1984).

1.4.1 Onc$^+$ Oncogenic Retroviruses

A number of the oncogenic retroviruses contain sequences which are essential for inducing tumors in animals and for causing morphological transformation of cells in culture. These sequences are termed onc, for oncogene, since they are associated with the oncogenic

properties of the virus. In general, the onc sequences are unrelated to sequences found in the genomes of replication competent viruses that lack transforming genes and they are not involved in viral replication. Both conditional and nonconditional mutations affecting the oncogenic potential of such viruses have been mapped to these sequences. Acquisition of viral transforming genes usually involves the loss of viral genes required for replication. As a result, most of the viruses in this class are replication defective and can only be grown in the presence of a helper virus; however, Rous sarcoma virus (RSV) is the exception to this rule (Bishop, 1983; Varmus, 1984).

1.4.1.1 Transduction of Cellular Oncogenes

The origins of retroviral oncogenes were revealed when both avian (Stehelin, et al., 1976) and mammalian (Spector, et al., 1978) DNAs were shown to contain nucleotide sequences closely related to the oncogene of RSV. These findings suggested that all vertebrates possess a highly conserved gene that is related to a viral oncogene. As other retroviral oncogenes were discovered, each was shown to have a cellular homologue from which the viral oncogene was apparently derived. The homologous cellular genes, known as c-oncogenes (c-onc's) (Coffin, et al., 1981), are proto-oncogenes since they are the normal cellular progenitors of the viral oncogenes (v-onc's) and represent the targets for transduction by retroviruses (Varmus, 1984).

The c-onc's are evolutionarily well conserved; control of their expression appears to be modulated during growth and development which suggests that the c-onc encoded proteins may provide important physiological functions required for cellular growth and development (Bishop, 1983; Muller, et al., 1983; Varmus, 1984).

It is generally considered that preexistent retroviruses incorporate c-oncogenes by recombination (Bishop, 1981). Retroviruses appear to be particularly adept at recombination: their genomes frequently exchange segments of variable size during mixed infections (Coffin, 1979) and their DNA forms enter the host genome as part of their normal replication cycle (Varmus, 1982a). Since proto-oncogenes and retroviral genes are unrelated, the transduction of c-onc sequences must be a complex and infrequent event because at least two illegitimate recombinations would be required to transduce a cellular sequence into a retroviral vector (Bishop, 1983; Duesberg, 1983; Varmus, 1984). Specific mutations are probably also necessary to convert a proto- oncogene (c-onc) into an oncogene (v-onc).

1.4.2 Onc⁻ Oncogenic Retroviruses

The second class of oncogenic viruses includes viruses that do not carry onc sequences transduced from host-cellular genomes and do not appear to contain coding sequences for proteins other than those involved in viral replication. Nonetheless, the oncogenic viruses

lacking onc sequences are capable of inducing a variety of neoplasms, but these tumors generally appear only after a long latent period; these viruses are also unable to transform cells in tissue culture (Teich, et al., 1982). Evidence suggests that tumors which arise following infection with v-onc⁻ retroviruses contain mutant cellular oncogenes that have been activated by proviral insertions (Varmus, 1982b). Integration of a provirus into the host genome is potentially mutagenic if integration disrupts a vital region of the host genome (Bishop, 1983). Indeed, it is generally thought that such insertion mutations are primary events in tumorgenesis and their effect is to stimulate expression of a cellular gene (c-onc) through the strong viral promoter or enhancer element present within the proviral long terminal repeat (LTR) (Hayward, et al., 1981; Neel, et al., 1981; Payne, et al., 1981; Payne, et al., 1982; Fung, et al., 1983; Cuypers, et al., 1984; Nusse, et al., 1984).

1.5 Onc and Proto-onc genes

The retroviral oncogenes are transduced, multiply mutated, and highly tumorigenic forms of cellular proto-oncogenes (Varmus, 1984). Even though v-oncogenes appear to be copies of normal cellular genes, the nucleotide sequences of c-oncogenes and v-oncogenes has revealed many differences (Takeya and Hanafusa, 1983; Duesberg, 1983; Bishop, 1983; Huang, et al., 1985)). The v-oncogenes appear to be truncated versions of c-oncogenes that have acquired multiple point

mutations in addition to deletions of various sizes (Bishop, 1983; Duesberg, 1983). However, despite these differences the v-onc's demonstrate considerable homology with c-onc sequences (Karess, et al., 1979; Wang, et al., 1979; Wang, et al., 1980; Takeya, et al., 1982; Sodroski, et al., 1984).

Although the protein products of the viral and cellular genes have extensive structural and functional homologies (Bishop, 1983; Varmus, 1984), significant differences exist, presumably due to the changes mentioned above. The viral proteins are usually expressed at a higher level and are sometimes truncated versions of the cellular proteins fused to other viral or cellular proteins (Duesberg, 1983). In addition, substitutions of single amino acids or blocks of amino acids may occur, and the sites and degree of phosphorylation may differ (Bishop and Varmus, 1982; Duesberg, 1983). These changes may be responsible for the functional differences which have been detected between viral oncogenic proteins and their normal cellular counterparts.

1.6 Retroviral Oncogene Products

Extensive genetic evidence indicates that the protein product of a single RNA tumor virus gene is generally responsible for the malignant transformation of virus infected cells (Bishop, 1983; Duesberg, 1983; Varmus, 1984). Over the past few years the protein

products of a number of different oncogenes have been identified (Table 1.1). Viral oncogenes may be functionally grouped into two broad classes: (1) those that encode or are likely to encode a tyrosine specific protein kinase activity and (2) those that do not encode this enzymatic activity.

1.6.1 Tyrosine Kinase-Negative Class of Oncogene Proteins

The tyrosine protein kinase-negative class of oncogenes can be subdivided into a number of functional subclasses. They consist of: (1) the v-sis oncogene of Simian sarcoma virus, which encodes the B chain of platelet-derived growth factor (PDGF) (Waterfield, et al., 1983; Doolittle, et al., 1983; Johnsson, et al., 1984); (2) the v-fos oncogene of FBJ sarcoma virus, v-myc of MC29 virus and v-myb of Avian myloblastosis virus which encode proteins that are found in the cell nucleus and the latter two oncogene proteins have been shown to bind DNA (Abrams, et al., 1982; Donner, et al., 1982; Hann, et al., 1983; 1983; Alitalo, et al., 1983; Curran, et al., 1984; Eisenman, et al., 1985) and (3) the H-ras and K-ras oncogenes of murine sarcoma viruses, which encode guanosine nucleotide-binding proteins (Papageorge, et al., 1982).

TABLE 1.1
Retroviral Oncogenes

Oncogene	Viral Origin	Viral Gene Product	Cellular Homologue	Activity
src	Rous sarcoma virus	$p60^{v-src}$	$p60^{c-src}$	Tyrosine protein kinase
yes	Y73 avian sarcoma virus	$P90^{gag-yes}$		Tyrosine protein kinase
fgr	Gardner-Rasheed feline sarcoma virus	$P70^{gag-fgr}$		Tyrosine protein kinase
fps	Fujinami sarcoma virus	$P140^{gag-fps}$	$p98^{c-fps}$	Tyrosine protein kinase
abl	Abelson murine leukemia virus	$P120^{gag-abl}$	$p150^{c-abl}$	Tyrosine protein kinase
ros	UR2 avian sarcoma virus	$P68^{gag-ros}$		Tyrosine protein kinase
fes	Snyder-Theilen feline sarcoma virus	$P85^{gag-fes}$	$p92^{c-fes}$	Tyrosine protein kinase
erb-B	Avian erythroblastosis virus	$gp65^{erb-B}$	Truncated EGF receptor	Potential tyrosine protein kinase
fms	McDonough feline sarcoma virus	$gP180^{gag-fms}$		Potential tyrosine protein kinase
mil	MH2 avian virus	$P100^{gag-mil}$		Potential serine/threonine protein kinase
raf	3611 murine sarcoma virus	$P75^{gag-raf}$		Potential serine/threonine protein kinase
mos	Moloney murine sarcoma virus	$p37^{mos}$		Potential protein kinase
sis	Simian sarcoma virus	$p28^{sis}$	PDGF B-chain	PDGF agonist
Ha-ras	Harvey murine sarcoma virus	$p21^{v-H-ras}$	$p21^{c-H-ras}$	GTP binding
Ki-ras	Kirsten murine sarcoma virus	$p21^{v-K-ras}$	$p21^{c-K-ras}$	GTP binding
fos	FBJ murine osteosarcoma virus	$p55^{fos}$		Possible DNA binding protein
myc	Avian myelocytomatosis virus MC29	$P110^{gag-myc}$	$p58^{c-myc}$	Binds DNA
myb	Avian myeloblastosis virus	$p48^{myb}$		Possible DNA binding protein

The table lists a number of oncogenes, a virus strain which contains the oncogene, the viral gene product encoded by the oncogene and its known or proposed activity and where identified the cellular homologue. The information in the table was obtained from Heldin and Westermark (1984) and various references sited in the text.

1.6.2 Tyrosine Protein Kinase-Positive and Kinase-Related Class of
       Oncogene Proteins

The oncogene products in this class all share structural
and/or functional homology with the transforming protein of RSV and are
collectively known as the src family of kinases. This family is
encoded by a number of independently isolated, acutely transforming
retroviruses (Table 1.1). Each of these viruses carries one of six
transduced cellular genes, all of which apparently originated from loci
different from src. These oncogenes include yes, fgr, fps, fes, abl and
ros; all of which encode proteins that have detectable tyrosine kinase
activity.

These viral oncogenes have been molecularly cloned and
hybridization studies have revealed that the region of strongest
homology corresponds to the kinase domain. These homologous regions
are in the 3' ends of src, yes, fes, fps, and fgr, the 5' end of abl
and the middle of ros (Takeya and Hanafusa, 1983; Kitamura, et al.,
1982; Shibuya and Hanafusa, 1982; Hampe, et al., 1982; Naharro, et al.,
1984; Reddy, et al., 1983; Neckameyer and Wang, 1984). The correspond-
ing amino acid sequences deduced for the proteins encoded by these
genes are all similar to the carboxy-terminal domain of $p60^{src}$
that carries the kinase activity (Levinson, et al., 1981; Brugge and
Darrow, 1984). Using the amino acid sequence of $p60^{src}$ as a
reference point (Schwartz, et al., 1983), the computed homologies are

as follows: yes 90% (Kitamura, et al., 1982); fps 48% (Shibuya and
Hanafusa, 1982); abl 51% (Reddy, et al., 1983); fgr 82% (Naharro,
et al., 1984) and fes 45% (Hampe, et al., 1982). The regions of these
oncogene proteins that contain the major phosphotyrosine acceptor site
show either identical or conservatively altered amino acid sequences
(Figure 1.1), all of which result in predicted secondary structures
that are identical (Mark and Rapp, 1984). This area also contains the
highly conserved ala-pro-glu (APE) sequence which is thought to specify
a functional domain that is essential for the tyrosine protein kinase
and cellular transforming activities of $p60^{src}$ (Byrant and
Parsons, 1984). These comparisons indicate that the genes encoding
tyrosine protein kinase activity comprise a single family of genes
(Figure 1.1).

In fact all protein kinases, irrespective of amino acid
specificity, may be encoded by a single family of genes that have
diverged from a common origin. This was first reported by Barker and
Dayhoff (1982) who used a computer search program to show that the
cyclic-AMP dependent protein kinase (cAPK), the only serine protein
kinase whose complete sequence was known (Shoji, et al., 1981), is
related to $p60^{src}$. The analysis revealed that residues 259 to 485
of $p60^{src}$ have 22% sequence identity with residues 38-258 of the
catalytic subunit of cAPK. Alignment of the two sequences to obtain
maximal amino acid identity aligns lysine-295 in $p60^{src}$ with
lysine-71 in the catalytic subunit. Both of these conserved lysine

Figure 1.1   Amino acid sequences from within the protein-kinase domain of 12 oncogene products and the cyclic-AMP-dependent protein kinase (cAPK) are arranged to show their similarity. A stretch in the middle of each sequence has been omitted; the number of subunits omitted is indicated. Each dot represents a one-amino-acid gap introduced to attain the best alignment. The amino acids are represented by their one-letter codes: A, Alanine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophan; Y, Tyrosine. The sequences blocked are discussed in the text. References for the sequence data are cited in the text.

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src   KL | GQGCFG | EVWMGTWNDTTR.......VAI | K | TLKP -- 83 - VHRDLRAANILVGENL......VCKVADFGLARLIEDNE | Y | TARQGAKF..PIKWT | APE | A
yes   KL | GQGCFG | EVWMGTWNGTTK.......VAI | K | TLKL -- 83 - IHRDLRAANILVGDNL......VCKIADFGLARLIEDNE | Y | TARQGAKF..PIAWT | APE | A
fgr   RL | GTGCFG | DVWLGMWNGSTK.......VAV | K | TLKP -- 83 - IHRDLRAANILVGERL......VCKIADFGLARLIEDNE | Y | NPRQGAKF..PIKWT | APE | A
abl   KL | GGGQYG | EVYEGVWKKYSLT......VAV | K | TLKE -- 84 - IHRDLAARNCLVGENH......LVKVADFGLSRLMTGDT | Y | TAHAGAKF..PIKWT | APE | S
fps   RI | GRGNFG | EVFSGRLRADNTP......VAV | K | SCRE -- 85 - IHRDLAARNCLVTEKN......TLKISDFGMSRQEEDGV | Y | ASTGGMKQI.PVKWT | APE | A
fes   QI | GRGNFG | EVFSGRLRADNTL......VAV | K | SCRE -- 85 - IHRDLAARNCLVTEKN......VLKISDFGMSREEADGV | Y | AASGGLRLV.PVKWT | APE | A
ros   LL | GSGAFG | EVYEGTALDILADGSGESRVAV | K | TLKR -- 91 - IHRDLAARNCLVSEKQYGSCSRVVKIGDFGLARDIYKND | Y | YRKRGEGLL.PVRWM | APE | S

erb-B VL | GSGAFG | TIYKGLWIPEGEK..VTIPVAI | K | ELRE -- 84 - VHRDLAARNVLVKTPQ......HVKITDFGLAKLLGADE | K | EYHAEGGKV.PIKWM | ALE | S
fms   TL | GTGAFG | KVVEATAFGLGKED.AVLKVAV | K | MLKS - 155 - IHRDVAARNVLLTSGR......VAKIGDFGLARDIMNDS | N | YIVKGNARL.PVKWM | APE | S
mil   RI | GSGSFG | TVYKGKWHGD.........VAV | K | ILKV -- 85 - IHRDMKSNNIFLHEGL......TVKIGDFGLATVKSRWS | E | SQQVEQPTG.SILWM | APE | V
raf   RI | GSGSFG | TVYKGKWHGD.........VAV | K | ILKV -- 85 - IHRDMKSNNIFLHEGL......TVKIGDFGLATVKSRWS | G | SQQVEQPTG.SVLWM | APE | V
mos   RL | GSGGFG | SVYKATYHG........VPVAI | K | QVNK - 100 - LHLDLKPANILISEQD......VCKISDFGCSQKLQDLR | G | RQASPPHIGGTYTHQ | APE | I
cAPK  TL | GTGSFG | RVMLVKHMETGNH......YAM | K | ILDK -- 86 - IYRDLKPENLLIDQQG......YIQVTOFGFAKRVKGRT | W | T.LCGT....PE.YL | APE | I
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residues have been shown to react specifically with the ATP analogue, p-fluorosulfonylbenzoyl 5'-adenosine (FSBA) (Zoller, et al., 1981 Kamps, et al., 1984). FSBA also reacts with the homologous lysine residue in the cyclic-GMP dependent protein kinase (cGPK), which has 42% sequence homology with cAPK within the catalytic region (Hashimoto, et al., 1982). Thus, the three-dimensional structure of the ATP-binding regions of both the cyclic nucleotide dependent serine kinases and p60^{src} tyrosine kinase probably orient the homologous lysine residue in a similar conformation. In addition to this highly con-served lysine residue, these proteins all contain a linear array of glycines that have been proposed to function in nucleotide binding (Adams, et al., 1973; Rossmann, et al., 1974; Pai, et al., 1977; Wierenga and Hol, 1983). Together, these data provide convincing evidence that the sequence homology between the tyrosine protein kinases and the serine protein kinases reflect structural and func-tional homology, and indicate that the protein kinases, irrespective of their amino acid substrate specificity, share a common ancestry.

In addition to the oncogenes discussed above, there are five viral transforming genes whose protein sequences show lesser and vary-ing degrees of homology to p60^{src} and are therefore considered to be only related to the src family of tyrosine kinases. These related proteins are encoded by the oncogenes fms (Hampe, et al., 1984), mil (Jansen, et al, 1984), raf (Mark and Rapp, 1984), mos (Van Beveren,

et al, 1981) and erb-B (Privalsky, et al., 1983; and Yamamoto, et al., 1983). The existing homologies include the cluster of glycines and the conserved lysine contained within the putative ATP binding site, as well as the conserved APE sequence discussed above (Figure 1.1). However, the homologous phosphotyrosine acceptor site is not conserved and, except for erb-B (Gilmore, et al., 1985) and fms (Barbacid and Lauver, 1981) there is no evidence to date that these proteins possess tyrosine protein kinase activity. Recently, the protein products of the raf and mil oncogenes have been reported to be associated with a serine and threonine protein kinase activity (Moelling, et al., 1984). In addition, a serine-threonine protein kinase has been reported to be associated with one mos-containing viral protein (Kloetzer, et al., 1983). It is interesting that site-specific mutagenesis of lysine-121 in mos, which is homologous to lysine-295 in $p60^{src}$, destroys the transforming activity of mos (M. Hannick and D.J. Donoghue, personal communication).

The meaning of this kinship is not yet fully understood. It is likely that those transforming proteins related to the src family of oncogenic tyrosine kinases also possess some of the functions of protein kinases. On the other hand, a phylogenetic tree of the src family of oncogenes, constructed on the basis of the relatedness of their conserved sequences (Mark and Rapp, 1984), corroborates the existing data and indicates that the src family of oncogenes has indeed evolved from a common ancestor.

1.7 Cellular Tyrosine Protein Kinases

The diversity of tyrosine kinase encoding genes captured by retroviruses suggests that the vertebrate genome codes for a number of such proteins. In fact the first tyrosine kinase identified in normal cells was the cellular homologue of p60^{src} (Collett, et al., 1978; Oppermann, et al., 1979; Collett, et al., 1979b). Since then a number of normal cellular homologues of the oncogenic tyrosine kinases have been identified. Their differential expression in various cell types suggests that tyrosine phosphorylation plays a role in normal cellular growth and differentiation (Bishop, 1983). Although transformation was previously thought to result from the introduction of a totally foreign activity into the cell, the discovery of normal cellular tyrosine kinases suggested that either over-expression of a normal enzyme or a subtly altered version of that enzyme was responsible for the cellular transformation.

The idea that tyrosine kinases may be involved in the regulation of normal cellular growth is substantiated by the fact that the receptors for epidermal growth factor (EGF) (Cohen, et al., 1980; Ushiro and Cohen, 1980; Buhrow, et al., 1982), platelet-derived growth factor (PDGF) (Ek, et al., 1982; Heldin, et al., 1983), insulin (Petruzzelli, et al., 1982; Roth and Cassell, 1983) and somatomedin C (IGF1) (Jacobs, et al., 1983a; Rubin, et al., 1983) all possess tyro-sine specific protein kinase activity. The binding of the respective

growth factors to their receptors results not only in the phosphoryla-
tion of the receptor, but also in the phosphorylation of cellular
substrates at tyrosine residues.   This suggests that tyrosine phos-
phorylation is involved in the transmission of the mitogenic signal
(Cooper, et al., 1984b).

In addition to the cellular tyrosine kinases that are stimu-
lated by growth factors, a number of other tyrosine kinases in normal
cells have been described recently.   It has been possible to detect
these kinases by using synthetic peptide substrates containing tyro-
sine, but lacking serine and threonine (Casnellie, et al., 1982; Wong
and Goldberg, 1983a; Dasgupta and Garbers, 1983; Swarup, et al., 1983).
These cellular tyrosine kinases are unrelated to the oncogenic tyrosine
protein kinases and are not known to be stimulated by growth factors.
For example, a 53,000 to 56,000 dalton tyrosine kinase has been
detected in the LSTRA murine lymphoma cell line and in normal T
lymphocytes (Swarup, et al., 1983; Voronova, et al., 1984).   Also, a
tyrosine protein kinase activity of 75,000 daltons (p75) has been
described in the soluble and microsomal fraction of rat liver (Wong and
Goldberg, 1983a).   Tyrosine protein kinases have also been detected
indirectly by the presence of phosphotyrosine in developing embryos
(Eisenman and Kinsey, 1982), the phosphorylation of tyrosine residues
in Band 3 of erythrocyte membranes (Dekowski, et al., 1983) and the

phosphorylation of synthetic peptides by extracts of various rat tissues and developing embryos (Swarup, et al., 1983; Dasgupta and Garbers, 1983). In total, the normal eukaryotic genome may encode as many as twelve different protein kinases that phosphorylate themselves and other substrates at tyrosine residues.

1.8 Cellular Substrates of Tyrosine Kinases

The activity of virally-coded tyrosine protein kinases accompanies and is necessary for the malignant transformation by these viruses. Transformation of cells by retroviruses encoding tyrosine kinases results in a five to ten fold increase in cellular phosphotyrosine levels (Cooper and Hunter, 1981a), which indicates that the phosphorylation of cellular target proteins may be involved in the mechanism of transformation. Inappropriate phosphorylation of key regulatory proteins could disrupt normal cellular growth control mechanisms. The detection of tyrosine kinase activity associated with certain growth factor receptors and the enhancement of cellular phosphotyrosine following treatment of cells with these growth factors (Hunter and Cooper, 1981; Erikson, et al., 1981; Cooper, et al., 1982) suggest a link between tyrosine phosphorylation and cell growth. It may be that some oncogenic proteins have the same activity and overlapping protein specificity as some growth factor receptors, but are no longer subject to the same regulation.

The link between growth factors and transformation has been
further strengthened by the recent findings that the v-sis oncogene of
Simian sarcoma virus is related to the gene encoding PDGF (Doolittle,
et al., 1983; Waterfield, et al., 1983) and that the transforming gene
of Avian erythroblastosis virus, (erb-B) is apparently a truncated
version of the EGF receptor (Downward, et al., 1984a; Ullrich, et al.,
1984). Both of these findings suggest mechanisms whereby oncogene
products could subvert normal growth control mechanisms and lead to the
malignant state.

The identification and functional characterization of sub-
strate proteins for tyrosine kinases is obviously important, since they
may be involved in the control of cellular growth in both normal and
malignant cells. The cellular proteins phosphorylated at tyrosine in
cells transformed by the members of the src family of viruses are
similar to each other (Cooper and Hunter, 1981b). This is not surpris-
ing considering the proposed common ancestry of the src family cata-
lytic domains, as discussed in section 1.6.2 . It also reinforces the
idea that these viruses probably transform cells by a common mechanism
involving tyrosine phosphorylation.

Malignant transformation by these RNA tumor viruses is a
multifaceted process which alters the cells in a number of ways,
including loss of contact inhibition, abnormal glucose metabolism and
alterations in cell shape (Bishop and Varmus, 1982; Cooper and Hunter,

1983b). A number of cellular targets have been identified by virtue of the fact that they may be directly involved in the above mentioned cellular changes. For example, the change in cell shape is, in some cases, correlated with an enhanced phosphorylation of the cytoskeleton protein vinculin, a protein found specifically localized in adhesion plaques (Geiger, 1979; Rohrschneider, 1980; Shriver and Rohrschneider, 1981). It has been proposed that vinculin acts as a linker between the plasma membrane and the termini of actin-containing microfilament bundles (Burridge and Feramisco, 1980) and would therefore be important in maintaining cell shape. Since p60^{src} is localized in the adhesion plaques in RSV transformed cells (Rohrschneider, 1980; Sefton, et al., 1981; Shriver and Rohrschneider, 1981; Nigg, et al., 1982), phosphorylation of vinculin by p60^{src} may interfere with its function and result in disorganization of the actin-containing micro-filaments leading to an alteration in cell shape. However, several findings indicate that vinculin phosphorylation alone is not sufficient to induce the morphological changes seen upon transformation. Most important, alterations in cell shape and phosphorylation of vinculin are not always correlated (Rohrschneider and Rosok, 1983), and only 1.0% of the vinculin in the transformed cell is phosphorylated (Sefton, et al., 1981). In addition, quantitative changes in both fibronectin and vinculin may contribute to the changes in cell shape (Olden and Yamada, 1977; Iwashita, et al., 1983).

A well known characteristic of tumor cells is an increased rate of aerobic glycolysis, known as the Warburg effect (Racker, 1972). The glycolytic enzymes enolase, phosphoglycerol mutase and lactate dehydrogenase have been shown to contain phosphotyrosine (Cooper, et al., 1983a). However, like vinculin, only a small fraction of these enzyme molecules are phosphorylated, which raises questions as to whether the degree of phosphorylation could produce the enhanced rate of glycolysis observed in transformed cells. In fact, of the eleven enzymes involved in the breakdown of glucose, the three glycolytic enzymes phosphorylated at tyrosine do not catalyze rate limiting steps, and the key enzyme phosphofructokinase does not appear to be phosphory- lated at tyrosine (Cooper, et al., 1983b; Cooper, et al, 1984a) Perhaps the enhanced rate of glycolysis seen with transformed cells is the result of an increased rate in glucose transport by such malignant cells (Bissell, et al., 1973).

The level of phosphotyrosine in a normal cell is less than 0.1% of the total acid-stable phosphoamino acids (Sefton, et al., 1980). Therefore, cellular substrates for tyrosine kinases should be detected by virtue of their enhanced phosphorylation at tyrosine resi- dues following infection with viruses encoding tyrosine kinase activity, or treatment of cells with growth factors that activate receptor tyrosine kinases. This approach has revealed a number of tyrosine protein kinase substrates, but the identity and function of many of these proteins is unknown.

A 36,000 dalton protein (p36 or 36K) is the most extensively characterized tyrosine kinase substrate, being the first cellular target identified (Radke and Martin, 1979) and an abundant cellular protein of chick fibroblasts (Radke, et al., 1980; Cooper and Hunter, 1983a). p36 is phosphorylated at tyrosine in cells transformed by oncogenic viruses that encode tyrosine protein kinase activity and only in some cells following exposure to growth factors (Cooper and Hunter, 1983b). The 36K phosphoprotein co-immunoprecipitates with $gp68^{erb-B}$ from AEV transformed cells (Gilmore, et al., 1985) and with $p60^{src}$ from RSV transformed cells (Dehazya and Martin, 1985), suggesting that these complexes may represent stable enzyme-substrate associations. However, p36 is not extensively phosphorylated under the following conditions: resting or growing normal cells; infection with leukosis viruses; treatment with tumor promoters; and transformation by chemicals, DNA viruses or retroviruses that do not encode tyrosine protein kinases (Cooper and Hunter, 1983b). The phosphorylation of p36 is not always correlated with conditions of mitogenic stimulation (Cooper, et al., 1982; Decker, 1982; Nakamura, et al., 1983) and morphological changes seen with transformation do not always accompany p36 phosphorylation (Nakamura and Weber, 1982; Cooper, et al., 1983b). Both the phosphorylated and unphosphorylated forms of p36 are found predominately in the plasma membrane, where it may perform a structural function (Cooper and Hunter, 1982; Courtneidge, et al., 1983; Greenberg and Eldeman, 1983; Radke, et al., 1983). Even though p36 is abundant in fibroblasts, it is not found in all cells (Cooper and Hunter, 1983b).

A 42,000 dalton phosphoprotein is detected in chick cells, but not in mammalian cells transformed by the avian sarcoma viruses (Cooper and Hunter, 1981a). Treatment of density-inhibited chick cells with a wide variety of mitogenic agents, EGF, PDGF, trypsin and the phorbol ester TPA, stimulates the immediate phosphorylation of two 42,000-dalton cellular proteins at tyrosine (Bishop, et al., 1983; Gilmore and Martin, 1983; Nakamura, et al., 1983; Cooper, et al., 1984b), which indicates that p42 may be involved in the regulation of cell division. Unlike that found in RSV transformed chick cells, treatment of chick cells with mitogenic agents results in very few phosphorylated proteins, although p42 is by far the most prominent of these few (Nakamura, et al, 1983). Phosphorylation of p42 may be involved in the delivery of the mitogenic signal, but as yet its identity and function remain an enigma.

Along with the 36K and 42K proteins, an 81,000 dalton protein (p81) is also phosphorylated at tyrosine following treatment of A431 cells with EGF (Hunter and Cooper, 1981c). The same protein is also found phosphorylated in 3T3 cells transformed by ST-FeSV; however, it is not phosphorylated in other types of transformed cells or in normal cells. The physiological significance of this EGF-induced phosphorylation is questionable, since the EGF dose required to obtain phosphorylation of p81 leads to an inhibition of the growth of A431 cells (Gill and Lazar, 1981). In fact, in fibroblasts responsive to mitogenic stimulation by EGF, neither p36 nor p81 is phosphorylated following treatment with EGF (Cooper, et al., 1984b).

In RSV transformed cells, $p60^{src}$ is found in a complex with a 50,000 dalton protein (p50) and a major heat shock protein (p89) (Sefton, et al., 1978; Hunter and Sefton, 1980; Brugge, et al., 1981; Oppermann, et al., 1981). The oncogenic tyrosine kinases $P105^{gag-fps}$ (Adkins, et al., 1982), $P90^{gag-yes}$ (Lipsich, et al., 1982) and $P140^{gag-fps}$ (Pawson, et al., 1981) are also found in a complex with p50 and p89. It is thought that the complex is involved in shuttling $p60^{src}$ from its site of synthesis on soluble polysomes to the plasma membrane. p50 is phosphorylated at a single tyrosine residue (Hunter and Sefton, 1980; Oppermann, et al., 1981) in transformed cells, but not in normal cells (Brugge and Darrow, 1982; Gilmore, et al., 1982). Whether the tyrosine phosphorylation of p50 is fortuitous or functional, it seems likley that p50 is a substrate for the tyrosine protein kinase with which it is associated.

Despite the identification of several cellular proteins containing high levels of phosphotyrosine in virally transformed cells and cells treated with growth factors, there is no direct proof that the phosphorylation of these proteins is necessary or sufficient for transformation or growth stimulation. If tyrosine phosphorylation is involved in these processes, then phosphorylation of specific proteins must change their function in a manner that contributes to the altered cellular phenotype. However, it has never been shown that tyrosine phosphorylation affects the function of a substrate in a meaningful way. It may be that the physiologically significant substrates for

retroviral kinases are low abundance proteins which have not yet been identified, or that the phosphorylation of substrates other than proteins is crucial for transformation.

1.8.1 Indirect Cellular Substrates of Tyrosine Kinases

Although much attention has been focused on tyrosine phosphorylation, analysis of specific proteins, such as the ribosomal protein S6, reveals that protein phosphorylation at serine residues is also quantitatively altered by the expression of transforming proteins (Decker, 1981; Blenis and Erikson, 1984; Blenis, et al., 1984; Maller, et al., 1985) and treatment of cells with mitogenic agents (Nilsen-Hamilton, et al., 1982, Martin-Perez and Thomas, 1983; Chambard, et al., 1983; Trevillyan, et al., 1984; Martin-Perez, et al., 1984; Novak-Hofer and Thomas, 1984; Blenis, et al., 1984). The wide spread correlation between S6 phosphorylation and the growth-promoting actions of a diverse group of agents suggests that S6 serine phosphorylation plays an important role in growth regulation. The fact that oncogenic tyrosine kinases and growth promoting agents associated with tyrosine specific protein kinases enhance S6 phosphorylation on serine residues implies that such kinases are capable of regulating the enzymes that control S6 phosphorylation.

Recently, some oncogenic tyrosine kinases have been reported to show lipid-phosphorylating activity (Sugimoto, et al., 1984; Macara,

et al., 1984), which implicates these kinases in phosphatidylinositol turnover and the role this turnover plays in the activation of protein kinase C (Marx, 1984; Berridge, 1984). Although there is a certain degree of skepticism regarding the purity of the oncogenic tyrosine kinases used in these studies, they remind us of the possibility that the transforming proteins of these viruses may have some other capacity as critical for transformation as protein phosphorylation. The possibility remains that the src family of transforming proteins may activate pathways leading to the malignant phenotype through the generation of second messengers, and not by the covalent modification of various substrate proteins.

1.9 Characteristics of Tyrosine Protein Kinases

Tyrosine phosphorylation is a recently recognized protein modification first identified in a number of viral transforming proteins (Eckhart, et al., 1979; Collett, et al., 1979a; Hunter and Sefton, 1980; and Witte, et al., 1980). Although it has been assumed that the phosphotransferase activity is intrinsic to these transforming proteins, only src (Gilmer and Erikson, 1981; McGrath and Levinson, 1982), abl (Wang, et al., 1982) and fps (J. Stone, personal communication) have been molecularly cloned in E.coli and the expressed proteins shown to possess tyrosine protein kinase activity. In contrast, the kinase activity associated with middle T antigen of polyoma virus (Eckhart, et al., 1979) has since been shown to be the property

of $p60^{c-src}$, which binds specifically to middle T antigen (Courtneidge and Smith, 1983).

Given the close familial relationship between the oncogenic tyrosine protein kinases, it is not surprising that these kinases share biochemical properties as well. Because tyrosine protein kinases form a class functionally distinct from other protein kinases, the Nomenclature Commission of the International Union of Biochemistry has assigned them a separate number E.C.2.7.1.37 (Recommended name: Protein-tyrosine kinase. Reaction: ATP + protein tyrosine = ADP + protein O-phosphotyrosine). All of the tyrosine specific kinases characterized to date autophosphorylate at tyrosine residues and have activity independent of cyclic nucleotides or Ca^{2+} (Bishop and Varmus, 1982). Many but not all of the enzymes show a preference for Mn^{2+} over Mg^{2+} as a cofactor, but this may reflect inhibition of endogenous phosphatases by Mn^{2+}. Although ATP is the preferred phosphate donor, dATP and GTP can be substituted in some cases (Richert, et al., 1982). Interestingly, the enzymatic properties of the UR2 transforming protein, $P68^{gag-ros}$, are distinctive from those of the other avian sarcoma virus protein kinases in cation preference, pH optimum, and phosphate donors (Feldman, et al., 1982).

Studies using synthetic peptides as substrates have shown that the tyrosine kinases have a tight specificity for tyrosine as the acceptor amino acid and they do not phosphorylate serine, threonine or

hydroxyproline (Pike, et al., 1982; Hunter, 1982; Wong and Goldberg, 1983b). The specificities of the cyclic nucleotide dependent serine and threonine protein kinases have also been thoroughly explored by the use of synthetic peptides (Zetterquist, et al, 1976; Kemp, et al., 1976; Kemp, et al., 1977; Kemp, 1979; Glass and Krebs, 1982). These studies and others indicate that the canonical recognition sequence for these kinases contains charged residues near the target amino acid. However, sequencing of tyrosine kinase recongition sites (Smart, et al., 1981; Neil, et al., 1981; Patschinsky, et al., 1982; Cooper, et al., 1984a) and studies with synthetic peptides (Hunter, 1982) demonstrate that while acidic residues are a factor in substrate recognition, the presence of acidic residues in the primary sequence near the target tyrosine residue is not an absolute requirement for phosphorylation by tyrosine protein kinases (Gallis, et al., 1983; Guild, et al., 1983; Cooper, et al., 1984a). It seems probable that secondary structure is also important for the recognition of phosphorylation sites by these enzymes.

The protein bound tyrosine phosphate is a high energy linkage. The energy of hydrolysis ($\Delta G°$) of protein bound tyrosine phosphate has been reported to be -9.48kcal (assuming an approximate $\Delta G°$ of -10 kcal for hydrolysis of ATP) (Fukami and Lipmann, 1983). This is important since hydrolysis or formation of such a high energy tyrosine phosphate bond in proteins could conceivably bring about a conformational change in the protein resulting in an altered functional state

of the molecule. In support of this, enhanced tyrosine phosphorylation of the insulin receptor kinase and the src kinase has been reported to stimulate the tyrosine kinase activity intrinsic to these proteins (Rosen, et al., 1983; Purchio, et al., 1983; Collett et al., 1984, Brown and Gordon, 1984).

1.10   Phosphorylation of Tyrosine Kinases — A Possible Role in Regulating Activity

1.10.1 Tyrosine Phosphorylation and the Regulation of Kinase Activity

Like the phosphorylation of serine and threonine residues (Krebs and Beavo, 1979), the phosphorylation of tyrosine residues is a reversible event (Fukami and Lipmann, 1983). The dephosphorylation of phosphotyrosine residues is carried out by phosphotyrosine-specific protein phosphatases (Foulkes, 1983), which can be distinguished from phosphoserine or phosphothreonine-specific phosphatases by the use of orthovanadate (Swarup, et al., 1982).

Treatment of RSV transformed cells with vanadate has revealed hyperphosphorylated forms of p60^{src} (Collett, et al., 1984; Brown and Gordon, 1984). This enhanced phosphorylation was due to an increase in tyrosine rather than serine phosphorylation of p60^{src}, and could be correlated with a significant increase in its tyrosine kinase activity. The increase in tyrosine phosphorylation of

p60^{src} was due to the appearance of newly characterized sites of tyrosine phosphorylation in the amino-terminal portion of the molecule. Similar structurally and functionally modified forms of the RSV src protein have also been detected by lysis of transformed cells in the presence of ATP-Mg^{2+} (Purchio, et al., 1983; Collett, et al., 1983). Only when lysis was conducted at high ATP concentrations were the new sites phosphorylated. Perhaps this phosphorylation when sufficiently extensive exerts an allosteric effect on p60^{src} resulting in a net increase in the protein kinase activity of the enzyme. These data introduce the possibility that within transformed cells there may be transient, rapidly modified molecules of p60^{src} that are only detected under conditions which inhibit phosphotyrosine specific phosphatases. Alternatively, vanadate may directly or indirectly activate other kinases which results in the phosphorylation of these recently identified phosphotyrosine sites in p60^{src}.

The vanadate experiments appear to suggest that the level of phosphorylation of tyrosine protein kinases and their cellular substrates will depend upon the phosphotyrosine specific phosphatases in the cell. Since reversible phosphorylation is thought to be a major mechanism for regulating protein function, it seems reasonable to conclude that the phosphorylation of tyrosine protein kinases represents a regulatory mechanism. Additional support for this possibility comes from the observation that incubation of the insulin receptor under conditions in which it autophosphorylates at tyrosine residues

stimulates its ability to catalyze the phosphorylation of exogenous substrates at tyrosine residues (Rosen, et al., 1983). The phosphorylated receptor remained active after the removal of insulin; however, dephosphorylation of the phosphorylated receptor rendered it dependent upon insulin for optimal activity. These results suggest that both the dissociation of bound insulin and dephosphorylation of the receptor may be required to terminate the insulin signal. This study with the insulin receptor and those mentioned above for the src kinase suggest a possible role for tyrosine phosphorylation in the regulation of tyrosine specific protein kinases and this thesis will deal with this aspect in greater detail in the following chapters.

1.10.2 Serine and Threonine Phosphorylation and the Regulation of Kinase Activity

When studied in intact cells, the oncogene derived tyrosine kinases (Bishop, 1983; and Cooper and Hunter, 1983b), as well as the receptors for EGF (Downward, et al., 1984b, Cochet, et al., 1984), PDGF (Heldin and Westermark, 1984), insulin (Kasuga, et al., 1982) and IGF1 (Jacobs, et al., 1983b), were all found to contain phosphoserine, and in some cases phosphothreonine, in addition to phosphotyrosine. In most cases the kinases involved in the phosphorylation of serine and threonine are unknown, as are the effects these phosphorylations have on the activity of the various tyrosine kinases.

The serine phosphorylation of p60^{src} may be the product of the cyclic-AMP dependent protein kinase (cAPK) (Collett, et al., 1979a), however other kinases are probably involved. A mutant of RSV which lacks the major site of serine phosphorylation in p60^{src} is unaltered in both its biochemical and biological activities (Cross and Hanafusa, 1983), suggesting that this site of phosphorylation is not essential for the activity of the protein. However, phosphorylation by cAPK increases the ability of p60^{src} to phosphorylate casein at tyrosine residues (Sefton and Hunter, 1984). Treatment of RSV- transformed Chinese hamster ovary cells (CHO) with cholera toxin or 8-BromocAMP also stimulates the phosphorylation of p60^{src} at serine residues, concomitant with an apparent increase in kinase activity (Roth, et al., 1983). Interestingly, p60^{src} expressed and synthesized in E. coli is not phosphorylated, yet it possesses about 10% of the kinase activity assayed for p60^{src} from eukaryotic cells (Gilmer and Erikson, 1981). These data suggest that phosphorylation is not absolutely essential, but that it may regulate the tyrosine kinase activity.

An increase in cAMP levels in adipocytes has been correlated with a decrease in the binding of EGF and insulin by their respective receptors (Pessin, et al., 1983). Exposure of 3T3 cells to tumor promoters also results in a decrease in EGF binding by its receptor (Magun, et al., 1980). These treatments activate the cAMP dependent serine/threonine protein kinase or the Ca^{2+}-diacylgylcerol

activated serine/threonine protein kinase C. Therefore, these effects may be mediated either directly or indirectly by the phosphorylation of these receptors at serine or threonine residues. Tumor promoters have also been shown to enhance the phosphorylation of both the insulin and IGF1 receptors (Jacobs, et al., 1983b), which suggests a possible role for C-kinase in regulating these receptors as well. Recently, protein kinase C has been shown to phosphorylate the EGF receptor at a specific threonine residue (Hunter, et al., 1984), which subsequently reduces its EGF-stimulated tyrosine protein kinase activity (Cochet, et al., 1984). Taken together, these observations suggest that various functional properties of tyrosine kinases may be regulated by phosphorylation on serine and/or threonine residues.

1.11 Fujinami Avian Sarcoma Virus

Fujinami sarcoma virus (FSV) is an acutely oncogenic retrovirus, which was isolated from a naturally occurring chicken fibrosarcoma in 1909 by Fujinami and Inamoto (Fujinami and Inamoto, 1914). This virus primarily induces solid tumors in chickens and transforms fibroblasts in culture (Lee, et al, 1980; Hanafusa, et al., 1980). Originally it was reported that FSV did not cause any type of leukemia (Lee, et al., 1980; Hanafusa, et al., 1980); however, FSV has recently been shown to transform chicken erythroid cells both in vitro and in vivo (Kahn, et al., 1984). The transforming ability of FSV is due to the expression of an onc gene, termed v-fps, that was acquired from

the normal chicken DNA by recombination with a proto-oncogene sequence called c-fps (Bishop and Varmus, 1982; Bishop, 1983; Varmus, 1984). These c-fps sequences were inserted into a FSV associated nontransforming retrovirus (FAV), where they have replaced the three essential replicative genes and all but the 5' end of the gag gene (Lee, et al., 1980; Hanafusa, et al., 1980). The insertion of v-fps into the FAV genome occurred in frame so that the v-fps gene became attached directly to the partially deleted gag gene. As a consequence of this recombination, FSV encodes a single gag-fps fusion protein and requires a helper virus such as FAV to replicate.

1.11.1 The FSV Genome

The 4.5 kilobase (kb) FSV RNA genome contains a contiguous FSV-specific sequence flanked by 5' and 3' termini related to the RNA of the nondefective helper virus FAV (Lee, et al., 1980; Hanafusa, et al., 1980). The FSV genome has been recently cloned (Shibuya, et al., 1982b) and sequenced (Shibuya and Hanafusa, 1982). The structure of the FSV RNA genome inferred from the cloned DNA nucleotide sequence is: $5'-U_5-leader-gag-fps-C-U_3-R-3'$.   The 5' 1.0 kb of the FSV genome contains a 21 base pair (bp) sequence that is repeated at both termini of the viral RNA (R), an 80 bp unique sequence (U_5) and the leader sequence together with part of the FAV gag gene, which encodes the precursor virion core protein $Pr76^{gag}$. The internal 3.0 kb is FSV specific, since it contains the v-fps gene which is essential for

the transforming activity of FSV. The 3' 0.5 kb of FSV RNA corresponds to the C or "common" region of the FAV RNA, and contains no known coding sequence, a 200 bp unique sequence (U_3) and a 21 bp repeat (R).

1.11.2 Variant Strains of FSV

The FSV genome encodes a single protein having a molecular weight (MW) of 140,000 daltons (P140$^{gag-fps}$) or 130,000 daltons (P130$^{gag-fps}$), depending upon the strain used. From the original FSV/FAV stock (Fujinami and Inamoto, 1914) a number of different strains have apparently arisen spontaneously, which encode transforming proteins of different size and temperature stability (Lee, et al., 1980; Hanafusa, et al., 1980; Hanafusa, et al., 1981; Lee, et al., 1981). Since characterization of the FSV transforming protein has involved the use of a number of different FSV strains, I will refer to the transforming protein of FSV as either P140$^{gag-fps}$ or P130$^{gag-fps}$ throughout this thesis.

1.11.3 The Relationship of FSV to Other Oncogenic Viruses

FSV is one of a number of avian sarcoma viruses which have been grouped into four classes (Table 1.2) based on the relatedness of their cell derived sequences (Wang, et al., 1982; Shibuya, et al., 1982a; Shibuya, et al., 1982b; Shibuya and Hanafusa, 1982; Huang, et

TABLE 1.2

Avian Sarcoma Viruses, Their Cell-derived Sequence

Inserts and Gene Products

Virus	Class	Cell-derived Sequences	Transformation-Specific Protein
Rous sarcoma virus (RSV)	I	src	$p60^{src}$
Avian sarcoma virus B77			
Fujinami sarcoma virus (FSV)	II	fps	$P140^{gag-fps}$
Avian sarcoma virus PRCII			$P105^{gag-fps}$
Avian sarcoma virus PRCIV			$P170^{gag-fps}$
Avian sarcoma virus UR1			$P150^{gag-fps}$
Avian sarcoma virus 16L			$P142^{gag-fps}$
Avian sarcoma virus Y73	III	yes	$P90^{gag-yes}$
Esh sarcoma virus (ESV)			$P80^{gag-yes}$
Avian sarcoma virus UR2	IV	ros	$P68^{gag-yes}$

al., 1984; Neckameyer and Wang, 1984) and their encoded transformation specific proteins (Ghysdael, et al, 1981; Patschinsky and Sefton, 1981; Neil, et al., 1982). The transforming protein of FSV is related structurally and functionally to at least six groups of acutely oncogenic retroviruses (discussed in section 1.6.2). A number of studies have suggested that c-fps and c-fes are the avian and feline versions, respectively, of the same genetic locus (Shibuya, et al., 1980; Barbacid, et al., 1981; Beemon, 1981; Shibuya, et al., 1982a; Groffen, et al., 1983). The viral forms of fps and fes share about 70% of their nucleotide sequences and deduced amino acid sequences (Shibuya and Hanafusa, 1982; Hampe, et al., 1982). Thus it appears, that retroviruses from two completely unrelated groups have acquired the equivalent or related sequences from genomes of two distantly related species.

1.11.4   The FSV Encoded Transforming Protein

The 4.5 kb FSV RNA is translated into a single 140,000 dalton protein which accounts for the entire coding capacity of FSV RNA in one reading frame (Lee, et al., 1980; Hanafusa, et al., 1980). Cell-free translation of FSV RNA has also revealed no FSV-specific products other than $P140^{gag-fps}$, suggesting that it is the sole product of the FSV genome (Lee, et al., 1980; Pawson, et al., 1980). $P140^{gag-fps}$ is immunoprecipitated from transformed cells by antiserum raised against the virion gag proteins, but not by antiserum to the other

replicative gene products (pol and env) (Feldman, et al., 1980). These immunoprecipitation studies, along with tryptic peptide mapping studies of P140$^{gag-fps}$ (Pawson, et al., 1980; Pawson, et al., 1981) and, more conclusively, the deduced amino acid sequence of the FSV transforming protein (Shibuya and Hanafusa, 1982) indicate that it contains an N-terminal region synthesized from a defective gag gene, and a C-terminal region encoded by FSV-specific sequences (fps).

Both genetic and biochemical data have suggested that P140$^{gag-fps}$ is the transforming protein of FSV (Pawson, et al., 1980; Pawson, et al., 1981; Lee, et al., 1981; Hanafusa, et al., 1981; Lee, et al., 1982). P140$^{gag-fps}$ isolated from transformed cells is phosphorylated mainly at serine and tyrosine residues, and possesses a protein kinase activity specific for tyrosine residues (Pawson, et al., 1980; Feldman, et al., 1980; Pawson, et al., 1981). In an in vitro kinase reaction P140$^{gag-fps}$ can phosphorylate both itself and exogenous substrates specifically at tyrosine residues. Cells transformed by FSV have enhanced phosphotyrosine levels compared with nontransformed cells, suggesting that P140$^{gag-fps}$ functions as a tyrosine kinase in vivo. Studies with temperature sensitive (ts) mutants of FSV indicate that phosphorylation of P140$^{gag-fps}$ is necessary for initiation and maintenance of the transformed state of the cell (Pawson, et al., 1980; Lee, et al., 1981; Hanafusa, et al., 1981; Lee, et al., 1982). At the restrictive temperature the degree of

morphological transformation, total cell phosphotyrosine levels, as well as phosphorylation of the transforming protein and its intrinsic kinase activity are all coordinately and reversibly decreased.

1.11.5 Cellular Location of the FSV Transforming Protein

As discussed in section 1.8, the inappropriate phosphorylation of key cellular proteins regulating cell morphology, metabolism and growth could bring about the myriad of changes associated with transformation by FSV. One would anticipate that the intracellular location of P140$^{gag-fps}$ would influence the structures, substrates, and regulators with which it must interact to induce the process of cell transformation. A substantial fraction of P140$^{gag-fps}$ is associated with the plasma membrane or cytoskeletal structures in FSV-infected cells; however, this association is salt sensitive (Feldman, et al., 1983; Moss, et al., 1984). The P140$^{gag-fps}$ encoded by a ts mutant of FSV loses its membrane association at the nonpermissive temperature and becomes soluble (Moss, et al., 1984). This property of the ts P140$^{gag-fps}$ suggests that its intracellular location may be critical to its transforming capacity. The identity and intracellular locations of the physiologically significant targets of P140$^{gag-fps}$ are unknown. However, these studies suggest that the association of the FSV transforming protein with cellular components may be necessary for its interaction with

substrates whose phosphorylation is required to induce and maintain cellular transformation.

1.11.6 The Normal Cellular fps Homologue

Recently the normal cellular homologue of $P140^{gag-fps}$, a 98,000 dalton protein (NCP98), was identified in uninfected chicken tissue (Mathey-Prevot, et al., 1982). NCP98 is homologous to the fps region of $P140^{gag-fps}$ and has an associated tyrosine specific kinase activity. Curiously, NCP98 isolated from cells does not appear to be phosphorylated at tyrosine residues, unlike $P140^{gag-fps}$. NCP98 is expressed in high levels in cells of the myeloid lineage (Shibuya, et al., 1982a; Mathey-Prevot, et al., 1982), suggesting a possible role in hematopoiesis. This is consistent with the idea that the normal cellular proteins encoded by the c-oncs are involved in cellular development and differentiation.

Cell fractionation studies have revealed that NCP98 is a predominantly soluble protein in E26-infected chicken myeloblasts, unlike its homologous transforming $P140^{gag-fps}$, which is mostly associated with cellular structural components in FSV transformed cells (Young and Martin, 1984). This difference in cellular location is probably not due to the absence of gag sequences, since the transform-ing protein of the recombinant virus F36 ($P92^{fps}$) lacks gag sequences (Foster and Hanafusa, 1983), and is also recovered in the

particulate fraction like P140$^{gag-fps}$ (Young and Martin, 1984). The results suggest that an alteration in the fps sequences is responsible for the different cellular localization between the FSV transforming protein and its normal cellular homologue, and that association with membrane or cytoskeletal structures is required for transformation.

1.11.7 Structure of the FSV Gene Product

For analysis, the transforming protein of FSV can be divided into three regions (Stone, et al., 1984): (1) the gag component (amino acid residues 1-309), which is encoded by sequences derived from FAV; (2) the C-terminal region (residues 888-1182), which is homologous to the C-terminal domain of p60^{src}; and (3) the middle or N-terminal fps-specific region (residues 310-388). A number of studies have been directed towards determining which regions of the FSV transforming protein are crucial for cellular transformation and tumorigenicity.

The function of gag-protein determinant fused to the onc gene product is not entirely understood. The gag protein does not appear to influence intracellular locations of the gag-onc fusion protein, since p60^{src}, which contains no gag protein sequences, and P90$^{gag-yes}$ which does, are found at very similar intracellular locations. In addition,   p55^{fos},   P110$^{gag-myc}$,   p48^{myb} and   p60^{myc} are all nuclear proteins, regardless of the absence or presence of

gag sequences (Rohrschneider and Gentry, 1984). To examine the role the gag determinants play in cellular transformation, viral DNA was constructed so that the src gene of RSV was replaced by greater than 90% of the fps sequence from FSV (F36 virus) (Foster and Hanafusa, 1983). Studies with the F36 recombinant virus have suggested that the gag determinants are not required for the transforming capacity, tumor-inducing ability or tyrosine kinase activity of the fps gene product of FSV. Although this data indicates that the gag sequences are not required for transformation, the fact that seven independent isolates of fps-containing acutely transforming retroviruses (FSV, PRCII, PRCIV, UR1, 16L, ST-FeSV, and GA-FeSV) express gag-linked fps-fusion transforming proteins suggests that the association is more than fortuitous (Bishop and Varmus, 1982 Neel, et al., 1982). In fact the transformed foci obtained with the gag-deleted F36 recombinant were morphologically more subtle than those obtained with FSV, suggesting that the gag region is not irrelevant to the activity of the FSV transforming protein. Also, deletion of gag sequences from the transforming gene of Abelson MuLV destroys the viruses ability to induce B-cell lymphomas in mice but the virus can still transform fibroblasts in cell culture (Prywes, et al., 1983). In addition, in-phase insertion mutagenesis of the FSV genome has indicated that insertions in the gag region result in mutants with reduced focus forming activity, further supporting a role for gag in FSV transformation (Stone, et al., 1984).

The same linker-insertion mutagenesis study of the FSV genome has suggested that the N-terminal portion of the _fps_ gene is important in fibroblast transformation (Stone, _et al._, 1984). However, mutations in this region of the protein have no effect on the catalytic activity of the mutant proteins, indicating that the sequences in the N-terminal portion of P130$^{gag-fps}$ are not involved in the phosphotransferase activity of the protein. Perhaps mutations in this area of the gene alter the subcellular location of the mutant protein and/or its interaction with specific cellular substrates.

The C-terminal region of P130$^{gag-fps}$ contains a region of shared homology with the other members of the _src_ family of tyrosine kinases, as well as the other related oncogene products which lack detectable tyrosine kinase activity (section 1.6.2). Genetic and biochemical studies have provided evidence which suggest that this highly conserved region possesses the catalytic site for phosphotransferase activity. Several lines of evidence suggest that the structural and functional properties of the catalytic domain may be shared among different tyrosine kinases. First, a C-terminal proteolytic fragment of P140$^{gag-fps}$ possess kinase activity (Weinmaster, _et al._, 1983), as does the corresponding region of p60^{src} (Levinson, _et al._, 1981; Brugge and Darrow, 1984) and the EGF receptor (Basu, _et al._, 1984). Second, mutations which inactivate the protein kinase activity of these proteins map in this domain (Bryant and Parsons, 1984; Stone, _et al._, 1984). These mutagenesis

studies indicate and support the arguments that the catalytic domain is crucial in the transforming process. A number of other mutagenesis studies have suggested that the major tyrosine phosphorylation site in this domain is not required for activity, but that it may modulate the function of these transforming proteins (Snyder, et al., 1983; Cross and Hanafusa, 1983; Weinmaster, et al., 1984; Snyder and Bishop, 1984).

The catalytic domain also includes a putative ATP binding site which the oncogenic tyrosine kinases share with a number of different functional proteins (see section 1.6.2). Site-directed muta-genesis in the src gene at the codon for lysine-295, which reacts specifically with the ATP analogue FSBA (Kamps, et al., 1984), elimi-nates both the kinase and transforming activities of p60^{src}, confirming the importance of the tyrosine kinase domain in transforma-tion (M. Kamps, personal communication; M. Snyder, personal communi-cation).

1.12 Purpose and Experimental Approach

The transforming protein of FSV is phosphorylated at several serine, tyrosine and threonine residues in transformed cells and possesses an intrinsic protein kinase activity specific for tyrosine residues. The phosphorylation of tyrosine residues within the FSV transforming protein probably results from autophosphorylation occurring either in trans (Mathey-Prevot, et al., 1984), cis, or both.

However, the identity of the kinases involved in the serine and threonine phosphorylations is unknown. Several lines of evidence suggest that various functional properties of tyrosine kinases may be regulated by phosphorylation (section 1.10). Understanding the regulation of the P140$^{gag-fps}$ kinase activity is an important step in understanding the mechanism of transformation by FSV.

In this study I have investigated the phosphorylation of FSV P140$^{gag-fps}$ in detail to establish the relationship between in vivo and in vitro phosphorylation of P140$^{gag-fps}$. To map the multiple sites of phosphorylation on P140$^{gag-fps}$, I have utilized different proteolytic enzymes to generate peptide fragments which were then localized relative to the intact protein, using double digestion experiments and tryptic peptide mapping of the various cleavage fragments (Chapter 3).

Reversible phosphorylation of proteins is a major mechanism for the regulation of protein function (Krebs and Beavo, 1979). Therefore it seemed reasonable to anticipate that the phosphorylation of tyrosine protein kinases may also regulate their activity. To determine whether tyrosine phosphorylation has any effect on the kinase and transforming activities of P130$^{gag-fps}$, I have mutated the major site of tyrosine phosphorylaton within this protein, tyrosine-1073 (Chapter 4).

In order to understand more fully the biological role of P130$^{gag-fps}$ and its kinase activity, it is necessary to characterize certain biochemical properties of this protein. One important property of any protein kinase is its substrate specificity. Therefore, I have investigated the specificity of the protein kinase intrinsic to FSV P130$^{gag-fps}$ by using oligonucleotide-directed mutagenesis to change the codon for tyrosine-1073 to those for the other commonly phosphorylated hydroxyamino acids serine and threonine (Chapter 5).

A protein kinase that functions in phosphotransfer must possess a functional ATP-binding site. If the kinase activity intrinsic to P130$^{gag-fps}$ is crucial for its transforming activity, one would predict that alterations in the P130$^{gag-fps}$ ATP-binding site would eliminate not only its kinase activity, but also its transforming activity. To test this hypothesis and to locate the ATP binding site within the catalytic domain of P130$^{gag-fps}$, I have mutated the highly conserved lysine-950, which by homology with the src kinase is proposed to be involved either in the binding, positioning or catalysis of ATP (Kamps, et al., 1984) (Chapter 6).

The data gained from these studies have allowed the identification of certain structural and functional domains within the transforming protein of FSV and have provided information concerning the molecular mechanisms of transformation by FSV.

CHAPTER 2

2.0 Materials and Methods

2.1 Cells and Viruses

The strain L5 of FSV described by Lee, et al. (1981), is a clonal isolate from a stock obtained by P. Duesberg from H. Temin, which has been traced back to the original isolate of A. Fujinami. FSV pseudotyped by FAV, termed FSV(FAV), and FAV of this strain were obtained from P. Duesberg and provided by T. Pawson. A temperature resistant (tr) derivative, trFSV (Lee, et al., 1981) was also pseudotyped with FAV. Both of these FSV strains encode a 140,000 dalton protein ($P140^{gag-fps}$). FSV clone 12 isolated from a different FSV stock by Hanafusa, et al., (1981) encodes a 130,000 dalton protein ($P130^{gag-fps}$). PRCII rescued with ring-neck pheasant virus was obtained from G.S. Martin. gs$^-$ chicken embryo fibroblasts (CEFs) were obtained from H & N Farms. Cells were infected with virus (5 x 10^7 to 1 x 10^8 focus-forming units per ml), passaged after 3 or 4 days, and used on day 4 or 5. Unless otherwise specified, all cells were maintained at 37°C, in 5% CO_2. The rat-2 thymidine kinase minus (TK$^-$) normal fibroblast line, obtained from W. Topp by J. Stone (Topp, 1981), was grown in 100 mm Falcon dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere.

2.2 Radiolabelling of Cells

Cells were seeded at a density of 3 x 10^5 cells in a 35 mm well (2 x 3 Linbro plate) and the following day were incubated with ^{32}P-orthophosphate (1.0-2.0 mCi/ml, carrier free; ICN Pharmaceuticals, Inc.) in 1.0 ml of phosphate-free DMEM containing 1-2% FBS. After the labelling period the plate was transferred to ice, the radioactive medium removed, and the cells were washed two times with ice-cold phosphate-buffered saline (138 mM NaCl, 2.68 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 [pH 7.2]) and then lysed in a total of 500 ul of lysis buffer (1% Nonidet P-40 [NP40], 0.5% sodium deoxycholate, 10 mM Tris-hydrochloride [pH 7.5], 100 mM NaCl, 1 mM EDTA, 2 mM ATP, and 1% [wt/vol] aprotinin [Sigma Chemical Co.]). The lysate was centrifuged at 4°C at 27,000 x g for 30 minutes, and the supernatant was recovered and incubated with the appropriate antiserum. Cells were labelled with [^{35}S]methionine according to a similar protocol, except that the cells were incubated with [^{35}S]methionine (100 uCi/ml, 1,000 Ci/mmol; Amersham Corp.) in 1.0 ml of methionine-free DMEM containing 1-2% FBS. After the labelling period the radioactive medium was removed, the cells were placed on ice, washed twice with Tris-saline and then lysed in 50 ul of lysis buffer as described above except the lysis buffer contained 1.0% sodium dodecyl sulfate (SDS) (cell lysis buffer). The cell lysates were cleared and treated exactly as described above.

2.3 Immunoprecipitation

Prespun cell lysates were incubated on ice with the appropriate antiserum (2 to 4 ul) for 30 minutes and with 15 volumes of a 10% suspension of Staphylococcus aureus strain Cowan I ((IgGsorb; the Enzyme Center) in cell lysis buffer for a further 30 minutes. The immune complex was then pelleted in a microfuge and washed successively with 1 M NaCl - 10 mM Tris-hydrochloride (pH 8.0) - 0.1% NP40; with 100 mM NaCl -1 mM EDTA - 10 mM Tris-hydrochloride (pH 8.0) - 0.1% NP40 - 0.1% SDS, and with 10 mM Tris-hydrochloride (pH 8.0) - 0.1% NP40. Immunoprecipitates of ^{32}P-labelled cells were washed once more with 1.5 M NaCl - 1 mM EDTA -10 mM Tris-hydrochloride (pH 7.5) - 0.1% NP40. All of these steps were performed at 4°C. Immunoprecipitates were prepared for SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

2.4 Immune Complex Kinase Reaction

Samples of 3 x 10^5 cells/ml from normal or transformed cells were lysed in kinase lysis buffer (1.0% NP40 - 20 mM Tris-hydrochloride (pH 7.5) - 150 mM NaCl - 1 mM EDTA - 0.5% sodium deoxycholate) and immunoprecipitated as described above. The immune complex was washed twice in kinase lysis buffer and twice in 10 mM MnCl$_2$ - 20 mM Tris-hydrochloride (pH 7.5) and incubated with 1-30 uCi of [γ -^{32}P]ATP (3,000 Ci/mmol; Amersham) in 35 ul of 10 mM MnCl$_2$ -

20 mM Tris-hydrochloride (pH 7.5) at 20°C. After the incubation 500 ul
of kinase lysis buffer was added to stop the reaction and the
immunoprecipitate was washed three times with kinase lysis buffer. The
pellet was resuspended in 50 ul of SDS sample buffer at 30°C for 10
minutes to disrupt the immune complex. The released proteins were
recovered in the supernatant following centrifugation in a microfuge
for 3 minutes. The samples were then either stored at -20°C or
prepared immediately for gel electrophoresis. Phosphorylation of the
exogenous substrate enolase was detected by the addition of 5 ug of
enolase, that had been previously treated at 30°C for 5 minutes in 25
mM acetic acid (Cooper, et al., 1984a), to the immune complex kinase
reaction which was then incubated at 30°C for 15 minutes. The reaction
was stopped by the addition of 500 ul of kinase lysis buffer, the
immune complexes were recovered by centrifugation in a microfuge for 3
minutes and subsequently prepared for electrophoresis.

2.5 SDS - Polyacrylamide Gel Electrophoresis

       Samples were heated at 100°C for 3 minutes in SDS sample
buffer (2% SDS, 5% 2-mercaptoethanol, 10 mM Tris-hydrochloride (pH
6.8), 10% [vol/vol] glycerol, .001% Bromophenol blue) and then
electrophoresed using a SDS-polyacrylamide discontinuous buffer system
described by Laemmli (1970). A 15 cm vertical slab gel apparatus
(Richter Scientific; Vancouver, B.C.) contained a 4.5% polyacrylamide
stacking gel and usually a 7.5% separating gel. These gels were

prepared from a stock solution of 29.2% wt/vol of acrylamide (BRL) and 0.8% wt/vol of N-N'-bis-methylene acrylamide (BRL). The final concentrations in the separating gel were as follows: 0.375 $\underline{M}$ Tris-hydrochloride (pH 8.8) and 0.1% SDS. The gels were polymerized chemically by the addition of 0.1% by volume of N,N,N',N'-tetamethylethylenediamine (TEMED) and 0.01% ammonium persulfate. The stacking gels contained 0.125 $\underline{M}$ Tris- hydrochloride (pH 6.8) and 0.1% SDS and were polymerized in the same way as for the separating gels. The electrode buffer (pH 8.3) contained 0.025 $\underline{M}$ Tris, 0.192 $\underline{M}$ glycine and 0.1% SDS. The solubilized proteins were subjected to electrophoresis at a constant power of 2 watts per gel through the stacker and 3 watts per gel through the separating gel. Electrophoresis was stopped when the bromophenol blue reached the bottom of the gel. After electrophoresis, gels of ^{32}P-labelled proteins were either covered with Saran Wrap and exposed to film (Kodak XAR-5) while wet at 4°C or fixed and stained by soaking overnight in, 0.04% Coomassie Brilliant Blue, 7.5% acetic acid and 50% methanol, to locate molecular weight markers of known size. Gels were destained in several changes of 7.5% acetic acid and 23% methanol in distilled water. The destained gels were dried onto 3MM filter paper (Whatman) using a Hoefer slab gel drier. The sensitivity of ^{32}P detection was increased by using an intensifying screen (DuPont; Lightning Plus) with XAR-5 film at -80°C (Laskey and Mills, 1977). Gels of [^{35}S]methionine-labelled proteins were impregnated with En^{3}Hance (New England Nuclear Corp.) before drying and exposed at -80°C (Bonner and Laskey, 1974), unless the proteins were to be

analysed further, in which case fluorography was omitted. The films were developed using a Kodak M11-film processor. Molecular weights of proteins were determined from a linear regression best fit plot of the log of molecular weight versus the Rf of markers of known molecular weight electrophoresed on the same gel. To quantitate the radio-activity in specific proteins the appropriate bands were excised from dried gels and assayed by scintillation counting in the presence of PCS scintillant (Amersham).

2.6   Partial Proteolytic Cleavage with p15 and V8 Protease

Cleavage of immunoprecipitated proteins was as described by Vogt, et al. (1979). Briefly, the washed immune complex was resus-pended in 35 ul of cleavage buffer (1.5 $\underline{M}$ NaCl - 1 m$\underline{M}$ EDTA - 10 m$\underline{M}$ Tris-hydrochloride (pH 7.5) - 0.1% NP40) and incubated for 30 minutes at 37°C with 10 ul of 10 mg/ml NP40-disrupted virions (Prague B. Rous sarcoma virus), and then a further 5 ul of NP40-disrupted virus was added. After a total of 60 minutes, 50 ul of 2 x SDS sample buffer was added to stop the reaction. The immune complexes were prepared for SDS polyacrylamide gel electrophoresis as described above.

Limited proteolysis with V8 protease was performed in situ in SDS-polyacrylamide gels as described by Cleveland, et al. (1977). Briefly proteins were labelled with ^{32}P in vivo or in vitro and purified after immunoprecipitation by SDS-polyacrylamide gel electro-phoresis. The pertinent bands were excised from wet gels, equilibrated

with buffer (125 m$\underline{M}$ Tris-hydrochloride (pH 6.8), 1 m$\underline{M}$ EDTA, 0.1% SDS), and applied to the sample well of a fresh gel with a 5-cm-long stacking gel and a 15% polyacrylamide separating gel cross-linked with 0.0867% bisacrylamide. The sample was overlaid, first with this buffer containing 20% glycerol and then with buffer containing 10% glycerol and V8 protease. Electrophoresis was performed at 2 watts until the dye front approached the separating gel, then the current and the cooling system were turned off for 30 minutes. Electrophoresis was then resumed at 3 watts. Gels were fixed, stained, dried and treated as described in section 2.5

2.7 Analysis of Tryptic Peptides

^{32}P or [^{35}S]methionine labelled proteins were separated by SDS-polyacrylamide gel electrophoresis and the pertinant bands were excised from the gel by using suitable alignment markers. Analysis of the tryptic peptides was essentially as described by Beemon and Hunter (1978) with a few modifications. Gel slices cut from wet gels were crushed directly or in the case of dried gels, the backing paper was removed from the dried gel bands which were then allowed to swell in a small volume of elution buffer (0.05 $\underline{M}$ NH$_4$HCO$_3$, 0.1% SDS). The swollen pieces were crushed and homogenized with the flat end of a disposable 5 ml syringe plunger with more buffer being added when necessary. Including washings, the final volume of buffer used was 5.0 ml. The homogenate was made 5% in 2-mercaptoethanol, boiled

for 5 minutes, and then shaken overnite at 37°C in a shaking water bath to elute the labelled protein. The gel fragments were pelleted for 10 minutes at a setting of 7 on an IEC bench top clinical centrifuge at room temperature. The supernatant was carefuly removed, and the gel fragments were washed for 2 hours at 37°C with 2 ml of elution buffer. The gel fragments were pelleted again, and the second supernatant was pooled with the first. A 25-ug amount of bovine gamma globulin was added as carrier to the pooled supernatants and mixed thoroughly. The protein was then precipitated by making the solution 20% in trichloro-acetic acid (TCA) and leaving at 4°C overnite. The precipitated protein was recovered by centrifugation for 30 minutes at 27,000 x g in a Beckman JA-21 rotor at 4°C. The tube was drained thoroughly by inversion, and the pellet was washed twice with ethanol chilled to -20°C and using the same centrifugation conditions. The dried pellet was dissolved in 100 ul of formic acid and then 25 ul of methanol and 40 ul of performic acid (30% H_2O_2 and 98% formic acid [1:9] which had been incubated for 1 hour at room temperature) were added to the solubilized protein and incubated for 2 hours in an ice-slurry. The performic acid oxidization solution was diluted with 3 ml of water, frozen in a dry-ice/ethanol bath, and lyophilized in a Savant SpeedVac. The oxidized protein was digested with 5 ug of L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone(TPCK)-treated trypsin (Worthington) in 0.5 ml of 0.05 M NH_4HCO_3 for 6 hours at 37°C. The digest was diluted with 2.5 ml of water, frozen and lyophilized as described above. This procedure was repeated twice more until all the NH_4HCO_3 had been

removed. The digest was finally dissolved in 0.5 ml of pH 2.1 electro-phoresis buffer (see below) and lyophilized. The trypsin-digested protein sample was resuspended in 5 ul of electrophoresis buffer and carefully spotted in 1-2 ul aliquotes onto a 20-by-20 cm thin-layer cellulose plate (TLC) (0.1 mm; E. Merck Lab). After the sample had been applied, the plate was dampened with electrophoresis buffer, pH 2.1 (water - 88% formic acid - acetic acid, 90:2:8 by volume) and subjected to 1,000 volts for 60 minutes. During electrophoresis, cooling water was circulated beneath the plate to prevent over-heating and a glass plate was placed upon the thin-layer cellulose plate. After electrophoresis, the plate was air dried and further developed by ascending chromatography in N-butanol - acetic acid - water - pyridine (75:15:60:50, by volume) in the second dimension.

^{32}P was detected by exposing the plates to XAR-5 film at −80°C with the aid of an intensifying screen (Laskey and Mills, 1977). Thin-layer cellulose plates containing [^{35}S]methionine labelled tryptic peptides were sprayed with En^{3}Hance (New England Nuclear) and exposed to film at −80°C (Bonner and Laskey, 1974).

The pattern produced by the two-dimensional separation of tryptic peptides from the various proteins analysed were found to be reproducible. However, the mobilities of the individual tryptic pep-tides were not always identical, presumably due to variation inherent in the preparation of the sample and the mapping procedure. Nonethe-less, tryptic peptide analysis was useful in establishing relationships

among the different proteins examined. Comigration studies were performed to further substantiate the apparent similar or dissimilar mobilities of certain tryptic peptides from different proteins. This was done by separating the tryptic digests from the different proteins on the same TLC plate, and where applicable the results are reported in the text. In some cases individual tryptic peptides were further analyzed for their phosphoamino acid content (as described below in section 2.8) and the approximate location of the peptides within the intact protein was determined. Tryptic peptides characterized in this manner have been assigned a number, while tryptic peptides which were variable or only occurred in minor amounts were not characterized further and were not numbered.

2.8 Analysis of Phosphoamino Acids

Normal or transformed cells growing in 35 mm wells were labelled for 12-16 hours with ^{32}P-orthophosphate at a concentration of 1.0 mCi/ml in DMEM lacking phosphate but supplemented with 2% FBS (not dialyzed). In order to determine the total cellular phosphoamino acids the method described by Cooper et al. (1983c) was followed. Briefly, the monolayers were washed twice with cold buffered saline and the plates were drained thoroughly on ice. To the drained monolayers 0.3 ml of lysis buffer (1.0% NP40, 1.0% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 7.0, 1% Aprotinin, 2mM EDTA) was added and the cells were scraped with a plastic policeman and left at 4°C for 10 minutes to solubilize adherent structures com-

pletely. The cells were then scraped again and transferred to a 1.5 ml eppendorf tube. The samples were centrifuged at 20,000 x g at 2°C for 20 minutes in a JA-21 rotor (Beckman). The cleared supernatant was transferred to a 1.5 ml eppendorf tube containing 0.4 ml of NTE (0.1 $\underline{M}$ NaCl, 0.01 $\underline{M}$ Tris-hydrochloride, pH 7.5, 0.001 $\underline{M}$ EDTA) and 0.4 ml buffer-saturated phenol (redistilled), at room temperature. The sample was vortexed (full speed) for 30 seconds and centrifuged for 1 minute in a microfuge at room temperature and the aqueous layer was discarded. The phenol layer was reextracted once with 0.8 ml of NTE and the aqueous layer was removed carefully to recover the interface above the phenol phase which was subsequently transferred to a 30-ml glass tube (Corex) to which 13 ml of water and 2 ml of 100% wt/vol TCA was added. The sample was mixed well and allowed to stand at 0°C for 1 hour to precipitate the proteins which were recovered by centrifugation at 20,000 x g in a JA-21 rotor for 10 minutes at 2°C and the supernatant was decanted from the pellet of protein and detergent. The pellet was extracted with 5 ml of $CHCl_3$/MeOH (2:1) at room temperature with gentle shaking and the protein precipitate was collected by centrifugation as before. The translucent protein pellet was air-dried, dissolved in 200 ul of 5.7 $\underline{M}$ HCl at 100°C for 2 minutes and transferred to a glass hydrolyzing tube with two, 100 ul washes of 5.7 $\underline{M}$ HCl. The proteins were hydrolyzed at 110°C for 90 minutes in a sealed glass tube. The hydrolyzed proteins were then lyophilized, resuspended in pH 1.9 buffer (88% formic acid-acetic acid - water; 50:156:1794) and 5 x 10^5 Cerenkov counts were applied to a thin-layer cellulose plate along with 0.3-0.5 ug of unlabelled phosphoserine, phosphothreonine and

phosphotyrosine. After the sample had been applied and dried, the plate was dampened with pH 1.9 electrophoresis buffer and subjected to 1,000 volts for 180 minutes towards the anode. After electrophoresis the plate was dried and then re-wetted with pH 3.5 buffer (pyridine: acetic acid:water; 10:100:1890) and electrophoresed at pH 3.5 at 1,000 volts for 80 minutes towards the anode. After electrophoresis, the plate was dried and exposed to film with an intensifying screen at -80°C. Marker phosphoamino acids were identified by spraying the plates with a ninhydrin stain (0.1 gm ninhydrin, 70 ml ethanol, 21 ml acetic acid, 2.9 ml, 2,4,6-collidine) and gentle heating on a hot plate. To quantitate the radioactivity in specific spots on cellulose thin-layer plates, the cellulose was scraped off and counted in 10 ml of PCS (Amersham). The radioactivity in each spot was corrected for the background determined by scraping and counting a clear area on each plate.

^{32}P-labelled proteins eluted from gels, or individual tryptic peptides scraped from TLC plates and eluted from the cellulose with pH 2.1 buffer were acid hydrolyzed and their respective phosphoamino acids were lyophilized, separated by electrophoresis, identified and quantitated exactly as described above.

2.9   Transfection of DNA into Rat-2 Cells

Replicative form (RF) DNAs of phage containing the various FSV genomes described in the text were digested with Sst1, electro-

phoresed on 0.5% agarose gels, and the 4.7 kbp FSV inserts were isolated by electroelution and concentrated by ethanol precipitation. 150 ng (5 pmoles) of each purified 4.7 kbp FSV DNA was incubated with 0.25 units of T4 DNA ligase for 1 hour at 22°C. Ligated FSV DNA and 10 ug of carrier rat-2 DNA was then coprecipitated with calcium phosphate and added to subconfluent rat-2 cells (Topp, 1981) in 100 mm dishes with DMEM containing 10% FBS as originally described by Graham and Van der Eb (1973) and modified by Wigler et al. (1979). Briefly, DNA precipitates were formed by dropwise addition of 150 ng of ligated insert plus 10 ug rat-2 carrier DNA in 0.5 ml of 250 m$\underline{M}$ CaCl$_2$ to an equal volume of a solution containing 50 m$\underline{M}$ Hepes, 1.5 m$\underline{M}$ NaHPO$_4$ and 280 m$\underline{M}$ NaCl (pH 7.05), while bubbling air into the mixture. In the case of cotransfection with the thymidine kinase (TK) gene of Herpes simplex type 1, (pTK$_1$) (Enquist, et al., 1979), 50 ng of pTK$_1$ was added to the DNA solution along with the appropriate FSV insert and rat-2 carrier DNA and TK positive clones were identified by HAT selection (Graham, et al., 1980) (see below).

Twenty-four hours post-transfection the medium was removed and replaced with fresh DMEM containing 5% calf serum and 0.5 u$\underline{M}$ dexamethasone or in the case of HAT selection DMEM was supplemented with 1.0 x 10^{-5} $\underline{M}$ hypoxanthine, 4.0 x 10^{-7} $\underline{M}$ aminopterin, 1.6 x 10^{-5} $\underline{M}$ thymidine plus 10% fetal bovine serum (HAT). The medium was then changed every four to five days until termination of the experiment. Foci of transformed cells and HAT resistant colonies were isolated using cloning cylinders, expanded in mass culture and maintained in

DMEM containing 10% FBS or 5% calf serum with 0.5 uM dexamethasone.
Soft agar colony formation was assayed using 5 x 10^4 cells seeded in
DMEM containing 10% FBS and 0.3% wt/vol Bacto-Agar (Difco) in 60 mm
dishes. Cells were photographed with a Wild photomicroscope.

2.10 Oligonucleotide-directed mutagenesis

Digestion of the λ-FSV-2 vector DNA with Sst1 (BRL), isola-
tion of the 4.7 kbp FSV insert, cloning into the Sst 1 restriction site
of M13mp10 RF DNA, transformation of E. coli JM101 (D lacpro, SupE,
thi1, F', pro AB$^+$, laci$^+$, lac Z D M15 traD36) and identification
and propagation of recombinant phage were carried out as described
elsewhere (Shibuya and Hanafusa, 1982; Zoller and Smith, 1982;
Messing, 1983; Maniatis, et al., 1982; Zoller and Smith, 1983; Zoller
and Smith, 1984). Recombinant phage were screened for FSV inserts in
the correct orientation for mutagenesis by using the mutagenic oligo-
nucleotide as the primer in dideoxynucleotide chain termination
sequencing reactions (Sanger, et al., 1980).

Mutagenic oligonucleotides were synthesized manually using
solid-phase phosphite triester synthesis (Adams, et al., 1983) or on a
Applied-Biosystems Model 380-A oligonucleotide synthesizing machine
using controlled pore glass beads as the solid support and Applied-
Biosystems reagents. Purification of oligonucleotides following
synthesis was carried out using a 20% polyacrylamide-7 M urea sequenc-
ing gel (40 x 20 x 0.05 cm) in TBE (50 mM Tris, 50 mM borate, 1 mM

EDTA, pH 8.3). The desired product was eluted from the gel by a crushsoak method using 0.5 M ammonium acetate, isolated, concentrated by ethanol precipitation and the sequenced confirmed by using the DNA sequencing technique of Maxam and Gilbert (1980).

Phage DNA was mutagenized according to Zoller and Smith (1984), in that a second primer for DNA synthesis, the M13 universal sequencing primer (5'-CCCAGTCACGACGTT-3') was included in the reaction in addition to and at the same molarity as the mutagenic oligonucleotide encoding the desired mutation (see Chapters 4, 5 and 6). This extension reaction was incubated for 8 hours at 15°C in the presence of the four deoxynucleoside triphosphates, E. coli DNA polymerase (large fragment) (BRL) and T4 DNA ligase (BRL) and was then used to transform competent E. coli JM101 cells directly without further purification. Phage from the resulting plaques were grown up in 1.5 ml cultures and their DNAs extracted and resuspended in 30 ul of 10 mM Tris-HCl pH 8.0, 1 mM EDTA (Zoller and Smith, 1984) Two ul was then spotted onto nitrocellulose (Schleicher and Schuell) and screened by hybridization with the mutagenic oligonucleotide which had been 5'-end-labelled with ^{32}P (Zoller and Smith, 1984). In order to sequence the region encompassing the mutation sites of the phage ssDNAs, I obtained from M. Smith an oligonucleotide primer for dideoxynucleotide chain termination sequencing (5'-CCTGAAGATGAAGAAGCT-3') which corresponds to nucleotides 3421-3438 of the FSV genome. Phage containing confirmed mutant or wild type FSV inserts were further plaque-purified and grown to high titer for production of phage RF DNAs, which were isolated by alkaline lysis

of infected bacteria and purified by centrifugation on CsCl gradients (Maniatis, et al., 1982).

2.11 Synthesis of p-Fluorosulfonylbenzoyl-5'-Adenosine

FSBA was prepared according to Pal et al. (1975). The compound had the same UV absorbance pattern described in the literature (Pal, et al., 1975). FSBA was stored dry at room temperature under dessication and was prepared for use by dissolving a measured amount in dimethylsulfoxide (DMSO) (Eastman).

2.12 Reaction of FSBA with P140$^{\text{gag-fps}}$

Immunoprecipitates of FSV-L5 transformed CEFs were prepared as described in section 2.3 and washed once in kinase lysis buffer and once in buffer containing 10 mM Tris-hydrocholoride pH 7.0, 10% glycerol. Reactions with FSBA were carried out at 37°C in the last wash buffer. The reaction was initiated by addition of FSBA and was terminated by addition of 2-mercaptoethanol to a final concentration of 50 mM. Aliquots were removed at the indicated intervals and were assayed for ATP:phosphotransferase activity as described in section 2.4.

CHAPTER 3

3.0 Mapping of Multiple Phosphorylation Sites Within the Structural and Catalytic Domains of the Fujinami Avian Sarcoma Virus Transforming Protein.

3.1 Introduction

The avian sarcoma viruses (ASVs) are a group of acutely oncogenic RNA tumor viruses which contain four distinct transforming genes (Table 1.2). The amino acid sequences of their gene products, predicted from DNA sequence data, show remarkable homology in their C-terminal 300 amino acids, whereas their amino-termini are largely unrelated (Kitamura, et al., 1982; Shibuya and Hanafusa, 1982; Huang, et al., 1984; Neckameyer and Wang, 1985). Reflecting this structural relationship, the ASV transforming proteins are all associated with protein kinases specific for tyrosine residues and are themselves phosphorylated at serine and tyrosine sites. There is much genetic and biochemical data to suggest that the ASV transforming proteins induce cellular transformation by modulating the control of cell growth, structure and gene expression through the pleiotropic effects of protein phosphorylation (see Chapter 1).

The genomic FSV RNA encodes a 140,000 dalton protein ($P140^{gag-fps}$) or a 130,000 dalton protein ($P130^{gag-fps}$), depending upon the variant strain used (section 1.11.2), which is

synthesized from a defective gag gene and the fps gene (Hanafusa, et al., 1980; Lee, et al., 1980). The nondefective gag gene of a replication-competent virus encodes a precursor (Pr76^{gag}) to the five virion core proteins (Vogt, et al., 1975). It has previously been shown that P140$^{gag-fps}$ possesses an N-terminal 40K sequence corresponding to the gag proteins p19, p10 and part of p27, and a C-terminal 100K sequence synthesized from the fps gene (Pawson, et al., 1981). FSV P140$^{gag-fps}$ immunoprecipitated with antiserum directed against antigenic determinants in its gag or fps regions is itself phosphorylated exclusively at tyrosine residues, after incubation in vitro with [γ -^{32}P] ATP and Mn^{2+} (Feldman, et al., 1980; Pawson, et al., 1980; Pawson, et al., 1981). In contrast, P140$^{gag-fps}$ isolated from transformed cells is phosphorylated mainly at serine, tyrosine and possibly threonine residues (Pawson, et al., 1981). However, the tryptic phosphopeptides of P140$^{gag-fps}$ phosphorylated in vitro are similar to those from in vivo-phosphorylated P140$^{gag-fps}$ (Pawson, et al., 1981).

The phosphorylation of the FSV transforming protein is complex and may well affect its activity and function. Since the kinase activity and transforming activity of P140$^{gag-fps}$ are presumably related, I have investigated the phosphorylation of FSV P140$^{gag-fps}$ in detail. The aim of this study was to establish the relationship between in vitro and in vivo phosphorylation of P140$^{gag-fps}$, and to locate the phosphorylation sites within the different structural and functional regions of the protein.

3.2 Results

3.2.1 Tryptic Phosphopeptides of P140$^{gag-fps}$

To identify the sites of phosphorylation in the FSV trans-
forming protein, I have analysed the phosphopeptides produced by
trypsin digestion of P140$^{gag-fps}$ from the temperature sensitive
(ts) L5 strain of FSV. P140$^{gag-fps}$ was isolated by immunoprecipi-
tation from FSV-transformed cells which was either metabolically
labelled with ^{32}P-orthophosphate or phosphorylated in vitro in the
immune complex with [γ -^{32}P] ATP as the phosphate donor. The
sites phosphorylated in vivo were then compared with those labelled
in vitro.

FSV P140$^{gag-fps}$ is phosphorylated exclusively at tyro-
sine residues in the in vitro immune complex kinase reaction (Feldman,
et al., 1980; Pawson, et al., 1980). Tryptic phosphopeptide analysis
of P140$^{gag-fps}$ phosphorylated in vitro revealed five labelled
spots (Figure 3.1C) after electrophoresis at pH2.1 and chromatography
in a butanol-acetic acid-pyridine buffer in a thin-layer cellulose
sheet. Under some conditions of analysis spots 3a and 3b were not
seen, and 3c was the major phosphorylated peptide. Reanalysis of
purified peptide 3c under the experimental conditions used in Figure
3.1 generated peptides 3a and 3b, suggesting that these are derived
from modification of 3c, and that spots 3a through 3c represent

Figure 3.1: Tryptic phosphopeptide analysis of FSV P140$^{gag-fps}$. FSV P140$^{gag-fps}$ was labelled with ^{32}P <u>in vivo</u> by incubation of transformed CEFs with $^{32}P_i$ and isolated by subsequent immunoprecipitation of the labelled protein with anti-p19 serum or phosphorylated <u>in vitro</u> in an immune complex kinase reaction after immunoprecipitation from transformed CEFs with anti-p19 serum. Gel-purified P140$^{gag-fps}$ was then digested with trypsin and separated in two dimensions on thin-layer cellulose plates. An "O" indicates the sample origin. Electrophoresis at pH2.1 was from left to right with an anode on the left, and chromatography was from bottom to top. Tryptic digests were as follows: A, tsFSV P140$^{gag-fps}$, phosphorylated <u>in vivo</u> (18 hour $^{32}P_i$ labelling); B, tsFSV P140$^{gag-fps}$ phosphorylated <u>in vivo</u> (4 hour $^{32}P_i$ labelling); C, tsFSV P140$^{gag-fps}$ phosphorylated <u>in vitro</u>. Phosphoamino acid analysis of tryptic peptide from <u>in vivo</u> labelled P140$^{gag-fps}$ showed that spots 1, 3a through 3c, 4, and 6 contain predominantly phosphotryosine, whereas spot 5 contains predominantly phosphoserine.
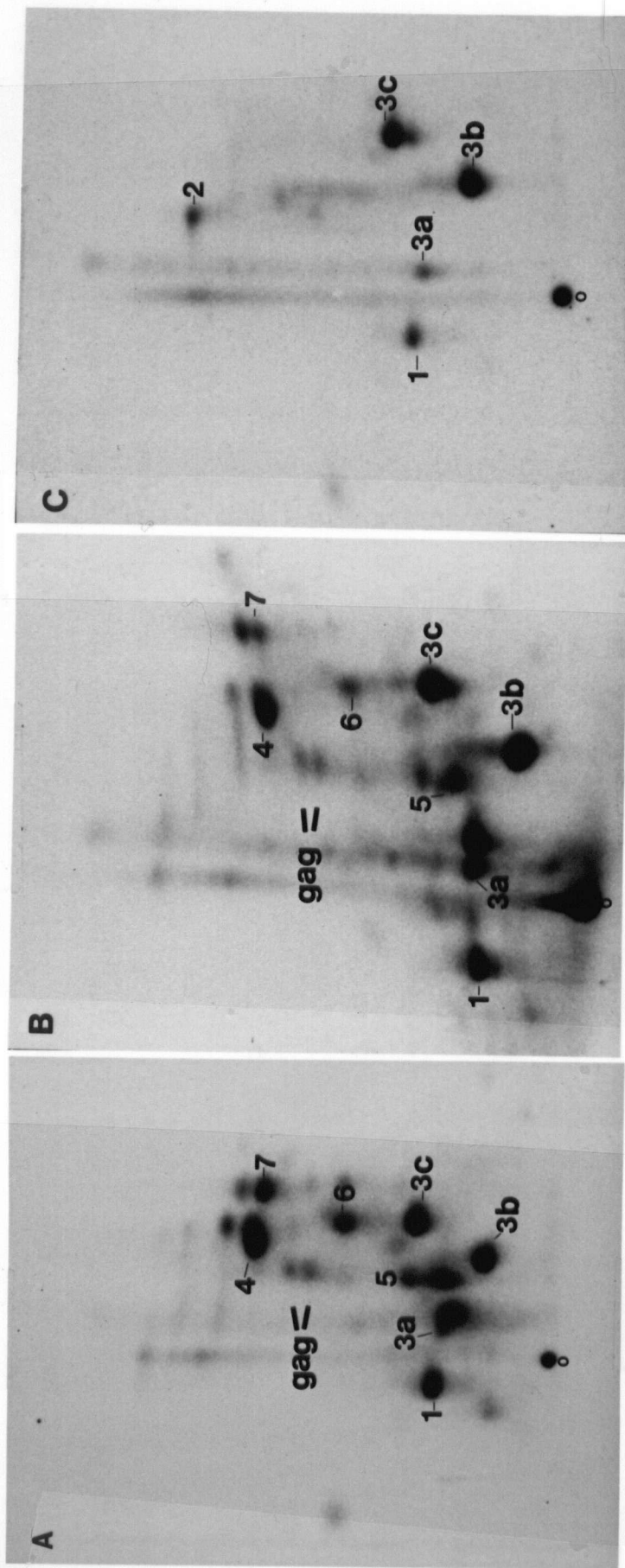
different forms of the same peptide. In addition cleavage of peptides 3b and 3c with Staphylococcus aureus V8 protease, as described by Patschinsky et al. (1982), produced peptides which comigrate in the 2-dimensional system described above suggesting that they are derived from the same tryptic peptide (data not shown). In contrast, the relatively minor tryptic phosphopeptides 1 and 2 apparently represent distinct sites of tyrosine phosphorylation (see below), suggesting that $P140^{gag-fps}$ is phosphorylated in vitro at three different tyrosine residues.

To compare the $P140^{gag-fps}$ sites phosphorylated in vitro by its intrinsic kinase activity with the residues actually phosphorylated in the transformed cell, $P140^{gag-fps}$ was isolated from ^{32}P-labelled ts FSV L5-transformed chicken embryo fibroblasts (CEF) and subjected to tryptic phosphopeptide analysis (Figure 3.1A and B). Secondary analysis of $P140^{gag-fps}$ in vivo labelled tryptic peptides for phosphoamino acid content indicated that there were five major phosphotyrosine-containing spots, of which four (peptides 1 and 3a through 3c) were shown to comigrate with those from in vitro-phosphorylated $P140^{gag-fps}$. A major new tryptic phosphopeptide (spot 4) which contains phosphotyrosine as its sole phosphoamino acid is present in the digest of in vivo-labelled $P140^{gag-fps}$, and peptide 2 of in vitro-phosphorylated $P140^{gag-fps}$ is missing. Mixing experiments on tryptic digests of $P140^{gag-fps}$ phosphorylated in vivo and in vitro show that peptides 2 and 4 migrate differently (data not shown). At least two minor tryptic phosphopeptides of in vivo-labelled

P140$^{gag-fps}$ comigrate with those found in FAV Pr76^{gag} isolated from ^{32}P-labelled, FSV (FAV)-infected cells, indicating that they represent normal sites of gag phosphorylation. In addition, P140$^{gag-fps}$ possesses a strongly labelled tryptic phosphopeptide containing phosphoserine (spot 5) which is not found in FAV Pr76^{gag} and thus represents specific phosphorylation of a serine residue on P140$^{gag-fps}$. These results indicate that ts FSV L5 P140$^{gag-fps}$ is phosphorylated in transformed cells on three tyrosine residues (contained within tryptic peptides 1, 3a through 3c and 4), of which one (peptide 4) is not phosphorylated in vitro, and on several serine residues of which at least one (peptide 5) is specific to P140$^{gag-fps}$. Two other FSV-specific tryptic phosphopeptides from in vivo-phosphorylated P140$^{gag-fps}$ (peptides 6 and 7) have not been analysed in any detail, although peptide 6 is known to contain phosphotyrosine. There is little radioactivity remaining at the origin in these two-dimensional analyses and very little free phosphate, indicating that the majority of tryptic phosphopeptides have been separated. Peptide 1 must have an overall negative charge, as it migrates to the positive electrode at pH2.1, and it therefore is either very small, multiply phosphorylated or contains cysteic acid in addition to a phosphate group since the side chains of glutamic acid and aspartic acid are not ionized at this pH. All of the other peptides migrate toward the negative electrode in the eletrophoretic dimension.

For in vivo-phosphorylated P140$^{gag-fps}$ the relative labelling of the tyrosine residues within tryptic phosphopeptides 1, 4,

and 3a through 3c is approximately 0.2:0.6:1 (where the figure for 3a through 3c is the sum of these spots), although it is difficult to know whether this reflects the actual steady-state levels $\underline{in\ vivo}$. On $\underline{in\ vitro}$-phosphorylated P140$^{gag-fps}$, spots 3a through 3c represent a single major site of tyrosine phosphorylation, with tryptic phospho-peptides 1 and 2 comprising relatively minor phosphorylation sites.

3.2.2 Localization of Phosphorylation Sites on P140$^{gag-fps}$

Proteolytic enzymes were used to cleave FSV P140$^{gag-fps}$ into two or more fragments, and these fragments have been mapped onto the intact protein. By constructing such a proteolytic cleavage map of FSV P140$^{gag-fps}$ I have been able to localize the various phosphorylation sites to different regions of the protein (see Figure 3.8). The cleavage of P140$^{gag-fps}$ by the avian retrovirus virion protease p15 has been previously described (Von der Helm, 1977; Vogt, et al., 1979; Pawson, et al., 1981). p15 cuts P140$^{gag-fps}$ within its N-terminal $\underline{gag}$ region, yielding a 33K, N-terminal, $\underline{gag}$-encoded fragment [N-33K(p15)] and a C-terminal, 120K fragment [C-120K(p15)] that contains a small region of $\underline{gag}$ p27 sequence and the entire C-terminal $\underline{fps}$-encoded portion (Pawson, et al., 1981). To localize phosphorylation sites to these two fragments, P140$^{gag-fps}$ was labelled $\underline{in\ vivo}$ with ^{32}P-orthophosphate or phosphorylated $\underline{in\ vitro}$ in the immune complex reaction and then cleaved with p15 by the

addition of disrupted Rous sarcoma virus virions to the immunoprecipitated protein. Figure 3.2 shows that both the N-33K(p15) and the C-120K(p15) fragments of in vivo- and in vitro-phosphorylated P140$^{gag-fps}$ are labelled with ^{32}P. Phosphoamino acid analysis of the N-terminal 33K fragment of in vitro-phosphorylated P140$^{gag-fps}$ revealed only phosphotyrosine whereas the corresponding 33K fragment from P140$^{gag-fps}$ phosphorylation in transformed cells contained phosphoserine and phosphotyrosine in equivalent amounts and a trace amount of phosphothreonine (data not shown). Tryptic phosphopeptide analysis of the N-terminal 33K gag-fragment of P140$^{gag-fps}$ phosphorylated in vivo yielded the acidic phosphotyrosine-containing tryptic peptide 1 in addition to phosphopeptides which comigrate with those of FAV Pr76^{gag} (Figure 3.3A). The N-33K(p15) fragment of in vitro-phosphorylated P140$^{gag-fps}$ is labelled only at tryptic phosphopeptide 1 (Figure 3.3B). The C-terminal 120K p15 cleavage fragment of in vivo-phosphorylated P140$^{gag-fps}$ gives phosphotyrosine-containing tryptic peptides 3a through 3c and 4 and phosphoserine-containing peptide 5 (Figure 3.3D), whereas, the C-120K(p15) fragment of P140$^{gag-fps}$ labelled in the immune complex reaction contains only peptides 3a through 3c (Figure 3.3E). These results indicate that P140$^{gag-fps}$ is phosphorylated in transformed cells at a tyrosine site (tryptic phosphopeptide 1) within the gag region in addition to sites of phosphorylation shared with FAV Pr76^{gag}. However, the major sites of tyrosine (peptides

Figure 3.2: Cleavage of FSV P140$^{gag-fps}$ with p15. FSV
P140$^{gag-fps}$ was labelled <u>in vivo</u> with $^{32}P_i$
and isolated by immunoprecipitation with anti-p19 serum
or phosphorylated in an immune complex kinase reaction
with [γ-^{32}P]ATP. Both samples were washed exten-
sively, incubated at 37°C for 30 minutes in the presence
or absence of 10 ug of NP40-disrupted RSV(Pr-B), and
then prepared for electrophoresis on a 7.5% SDS-poly-
acrylamide gel. Lanes: A and B, tsFSV P140$^{gag-fps}$
phosphorylated <u>in vivo</u> (18 hour labelling); A,
uncleaved; B, cleaved; C and D, tsFSV P140$^{gag-fps}$
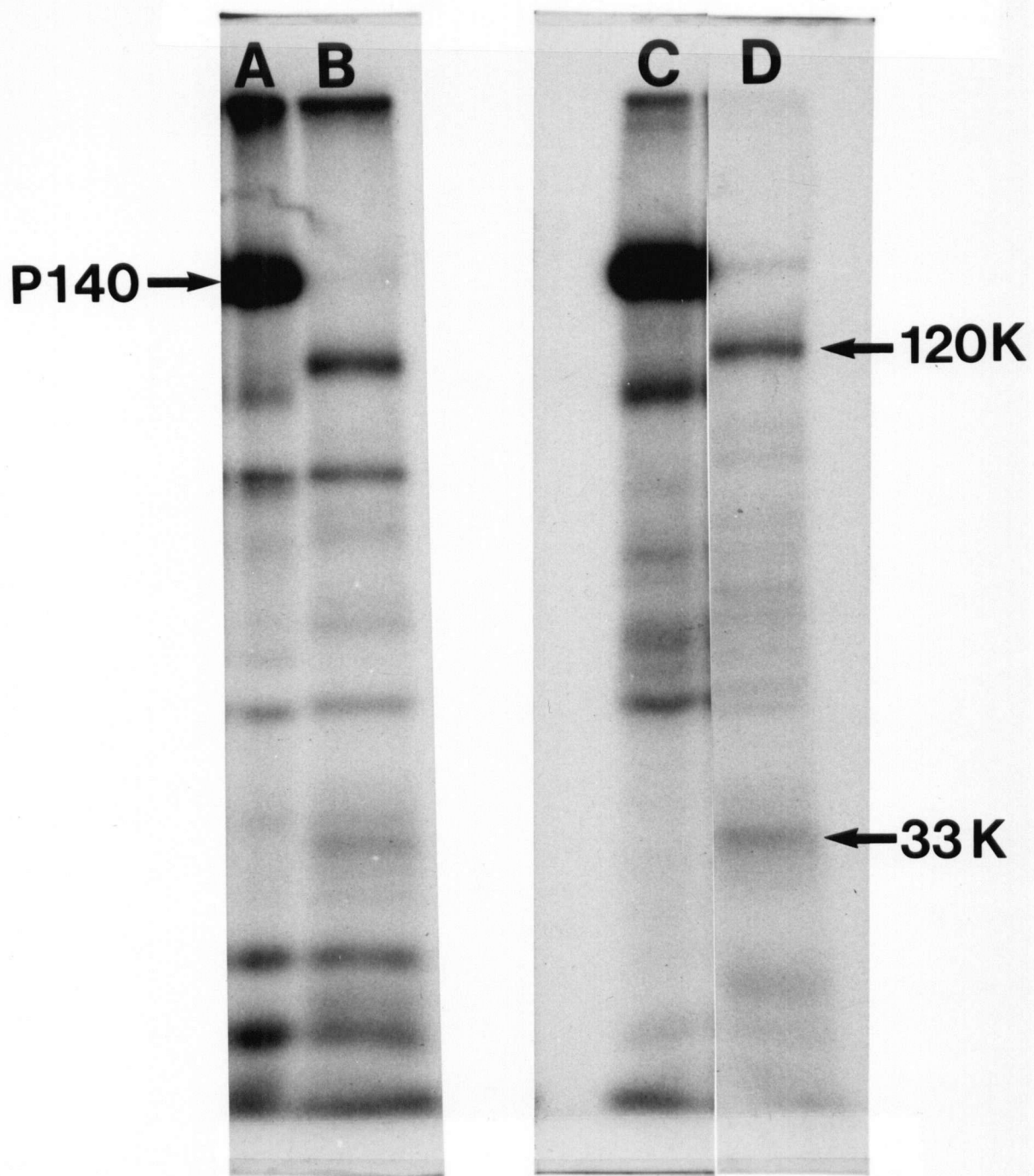phosphorylated <u>in vitro</u>; C, uncleaved; D, cleaved.

Figure 3.3: Tryptic peptide analysis of p15 cleavage fragments of FSV P140$^{gag-fps}$. ^{32}P- or ^{35}S-labelled polypeptides were gel purified, digested with trypsin, and analysed by two-dimensional separation on thin-layer cellulose plates. Protein fragments analysed were as follows: A. N-33K(p15) obtained by p15 cleavage of tsFSV P140$^{gag-fps}$ phosphorylated in vivo; B, N-33K (p15) obtained by p15 cleavage of tsFSV P140$^{gag-fps}$ phosphorylated in vitro; C, N-33K(p15) obtained by p15 cleavage of tsFSV P140$^{gag-fps}$ labelled with [^{35}S]methionine in vivo; D, C-120K(p15) obtained by p15 cleavage of tsFSV P140$^{gag-fps}$ phosphorylated in vivo; E, C-120K(p15) obtained by p15 cleavage of tsFSV P140$^{gag-fps}$ phosphorylated in vitro; F, FSV P140$^{gag-fps}$ isolated from tsFSV-transformed CEFs labelled with [^{35}S] methionine; G, Pr76^{gag} isolated from the same [^{35}S]methionine-labelled cells. The numbering of methionine-containing peptides is according to Pawson et al. (1981) and is differentiated by a superscript "S". Tryptic peptides 1^S through 5^S are fps specific.
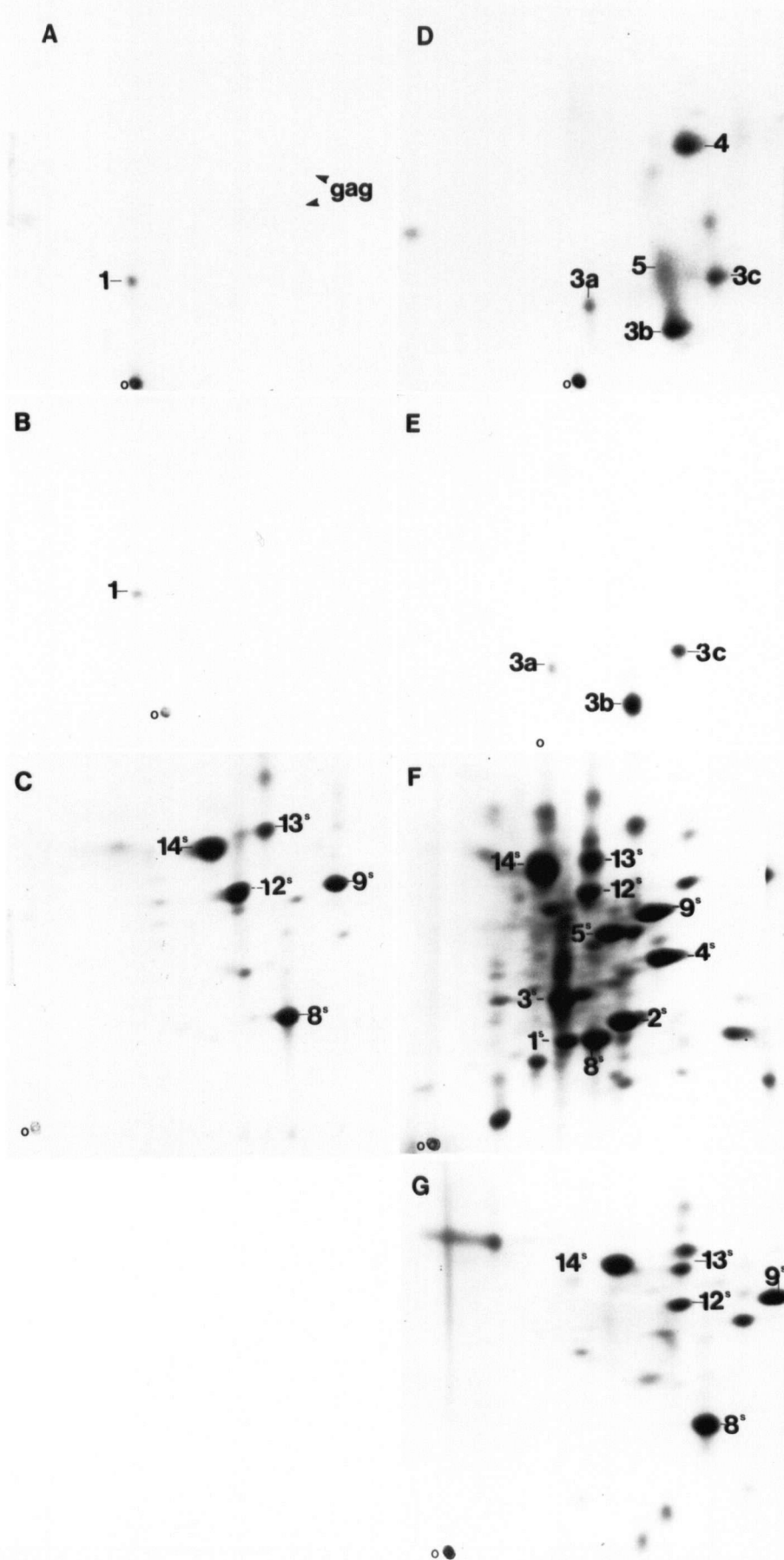
3a through 3c and 4) and serine (peptide 5) phosphorylation are contained within the fps region. To verify the identity of the p15 cleavage fragments used in these experiments, [^{35}S]methionine-labelled P140$^{gag-fps}$ isolated from FSV-infected cells was cleaved with p15 and coelectrophoresed with the ^{32}P-labelled fragments. The [^{35}S]methionine-labelled 33K p15 fragment, intact FSV P140$^{gag-fps}$, and FAV Pr76^{gag} were then subjected to tryptic peptide analysis (Figure 3.3). This confirmed that the 33K fragment contains only gag-encoded sequences.

To localize phosphorylation sites in C-120K(p15) more accurately within the fps-encoded region of P140$^{gag-fps}$ partial proteolytic cleavage fragments were generated with Staphylococcus aureus V8 protease (Houmard and Drapeau, 1972) using the Cleveland gel technique (Cleveland, et al., 1977). Digestion of ^{32}P-labelled P140$^{gag-fps}$ with low concentrations of V8 protease yields two major cleavage products with apparent molecular weights of 78K and 61K [78K(V8) and 61K(V8)] (Figure 3.4). Limited V8 protease digestion of [^{35}S]methionine-labelled P140$^{gag-fps}$ also produces these two major cleavage fragments (Figure 3.5). Tryptic peptide analysis of the V8 protease digestion products of [^{35}S]methionine-labelled P140$^{gag-fps}$ (Figure 3.6) showed that the 61K(V8) fragment contains all of the tryptic peptides previously identified as fps specific (Pawson, et al., 1981) (Figure 3.3). There is no apparent overlap

Figure 3.4: Cleavage of FSV P140$^{gag-fps}$ with V8 protease. Lanes: A through C, tsFSV P140$^{gag-fps}$ labelled with^{32}P$_i$ during an immune complex kinase reaction, purified by SDS-polyacrylamide gel electrophoresis, and then subjected to <u>in situ</u> digestion with V8 protease in a new gel with a 15% polyacrylamide separating gel; A, 500 ng of V8 protease; B, 100 ng of V8 protease; C, 50 ng of V8 protease.
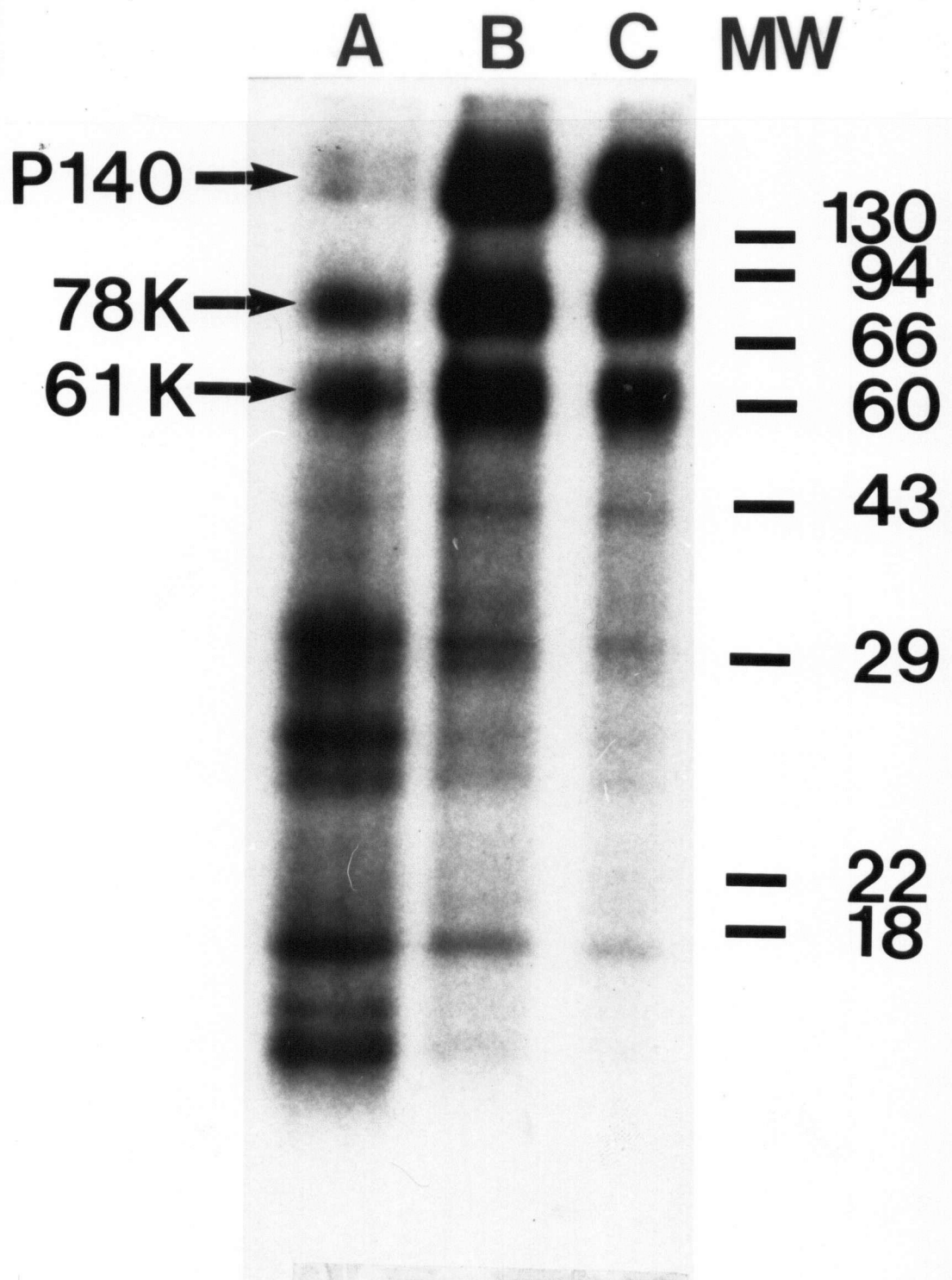
Figure 3.5: Cleavage of [^{35}S]methionine or ^{32}P-labelled FSV P140$^{gag-fps}$ with V8 protease. ^{32}P- or ^{35}S-labelled proteins were isolated from preparative gels and subjected to <u>in situ</u> digestion with V8 protease (50 ng per well) followed by electrophoresis through a 15% SDS-polyacrylamide separating gel. Lane 1, tsFSV P140$^{gag-fps}$ labelled with ^{32}P <u>in vitro</u>; Lane 2, [^{35}S]methionine-labelled tsFSV P140$^{gag-fps}$ obtained by cell-free translation of FSV(FAV) poly(A)-selected heat-denatured 70S virion RNA in a messenger-dependent rabbit reticulocyte lysate.
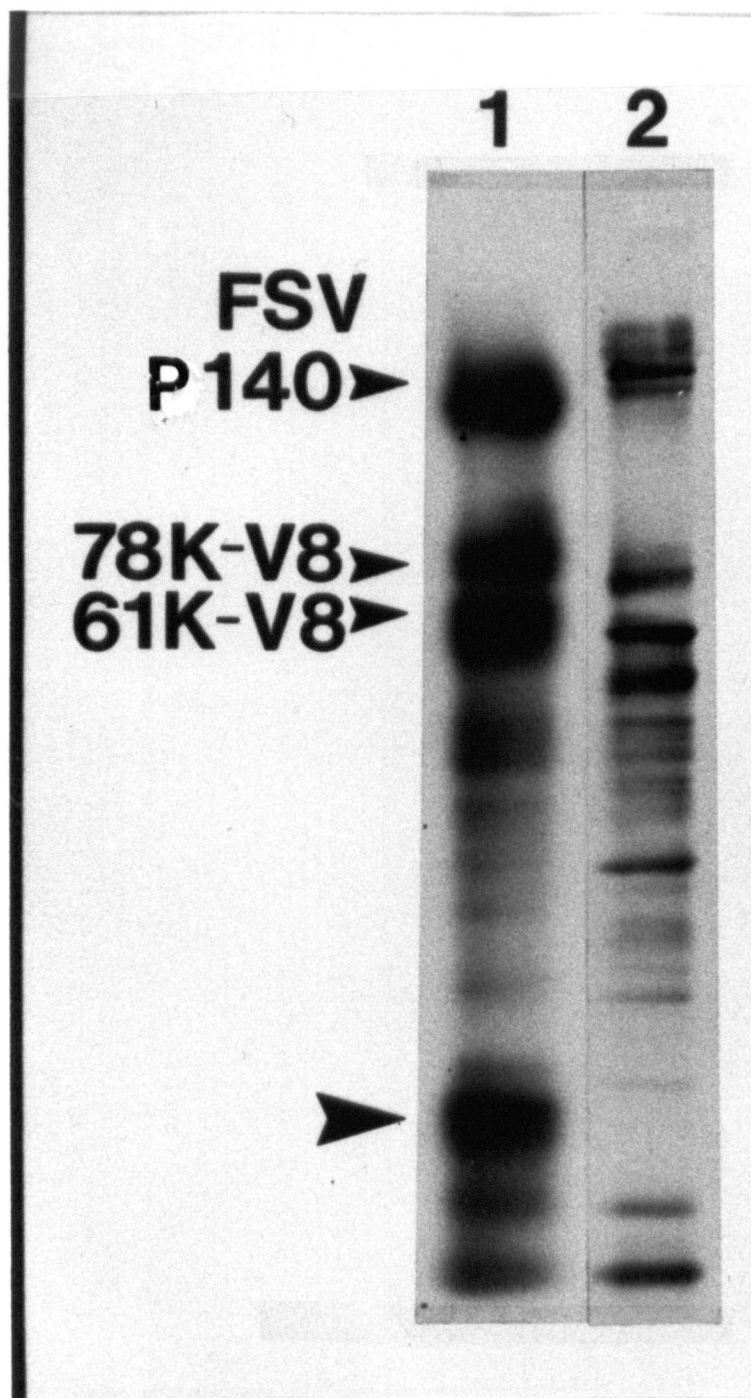
between these two V8 protease cleavage fragments, arguing that they represent a unique N-terminal 78K fragment and a unique C-terminal 61K fragment presumably separated at a single V8 protease cleavage site in the middle of the P140$^{gag-fps}$ region (Figure 3.6 A and D). To test this deduction, I isolated the C-120K(p15) fragment of in vitro-phosphorylated P140$^{gag-fps}$ and digested it with V8 protease (Figure 3.7); this generated the same 61K(V8) fragment contained within the intact P140$^{gag-fps}$, but no 78K(V8) fragment. This would be expected if 78K(V8) corresponded to the N-terminal half of P140$^{gag-fps}$ and 61K(V8) to its C-terminal half since C-120K(p15) has lost most of the gag sequence but retains all of the C-terminal fps sequence. The N-terminal 33K(p15) fragment is resistant to limited V8 protease digestion.

Tryptic phosphopeptide analysis of the N-terminal 78K and C-terminal 61K V8 protease fragment [N-78K(V8) and C-61K(V8)] of P140$^{gag-fps}$ from ^{32}P-labelled ts FSV L5-transformed cells showed that the C-61K(V8) fragment contains peptides 3a through 3c, 4 and 5, whereas, the N-78K(V8) fragment contains peptide 1 (Figure 3.6 B and C). This corroborates the localization of phosphotyrosine- containing tryptic peptide 1 to the N-terminal gag region and indicates that the two major phosphotyrosine sites (peptides 3a through 3c and 4) and the major phosphoserine site (peptide 5) are clustered in the C-terminal portion of P140$^{gag-fps}$. A similar analysis of P140$^{gag-fps}$ phosphorylated in vitro in the immune complex kinase

Figure 3.6:   Tryptic peptide analysis of V8 protease cleavage frag-

ments of FSV P140$^{gag-fps}$. ^{32}P- or ^{35}S-

labelled polypeptides were gel purified, digested with

trypsin, and analysed by two-dimensional separation on

thin-layer cellulose plates. Protein fragments analysed

were as follows: A, N-78K(V8) produced by V8 cleavage

of   [^{35}S]methionine-labelled   tsFSV   P140$^{gag-fps}$

obtained by cell-free translation of FSV(FAV) poly-

adenylic acid-selected, heat-denatured 70S virion RNA in

a messenger-dependent rabbit reticulocyte lysate; B,

N-78K(V8) obtained by V8 protease cleavage of tsFSV

P140$^{gag-fps}$ phosphorylated <u>in vivo</u> (18 hour label-

ling); C, N-78K(V8) obtained by V8 protease cleavage of

tsFSV   P140$^{gag-fps}$ phosphorylated   <u>in vitro</u>;   D,

C-61K(V8)   produced   by   V8   protease   cleavage   of

[^{35}S]methionine-labelled   tsFSV P140$^{gag-fps}$

obtained by cell-free translation of FSV(FAV) poly-

adenylic acid-selected, heat-denatured 70S virion RNA in

a messenger-dependent rabbit reticulocyte lysate; E,

C-61K(V8) obtained by V8 protease cleavage of tsFSV

P140$^{gag-fps}$ phosphorylated <u>in vivo</u> (18-hour label-

ling); F, C-61K(V8) obtained by V8 protease cleavage of

tsFSV   P140$^{gag-fps}$ phosphorylated   <u>in vitro</u>.   The

numbering of methionine-containing peptides is according

to Pawson, <u>et al</u>. (1981) and is differentiated by a

superscript "S". Tryptic pepties 1^S and 5^S are <u>fps</u>

specific.

- 84 -
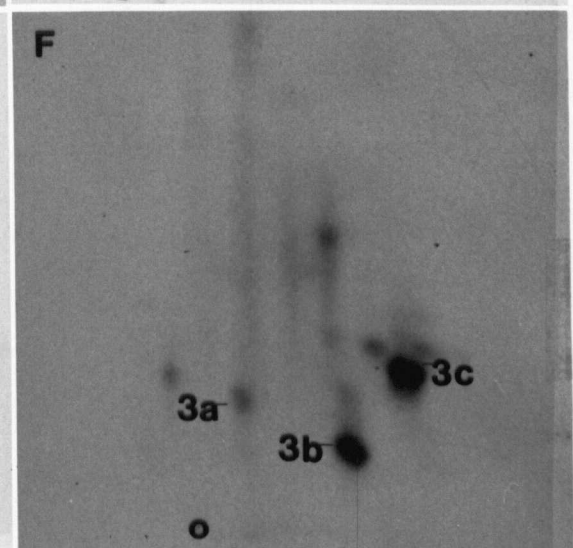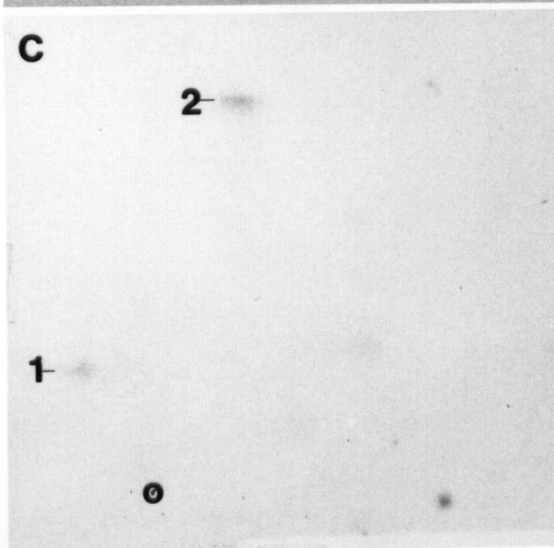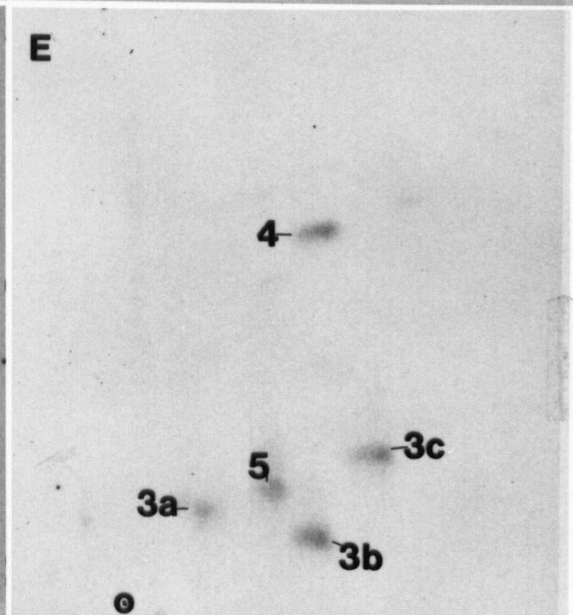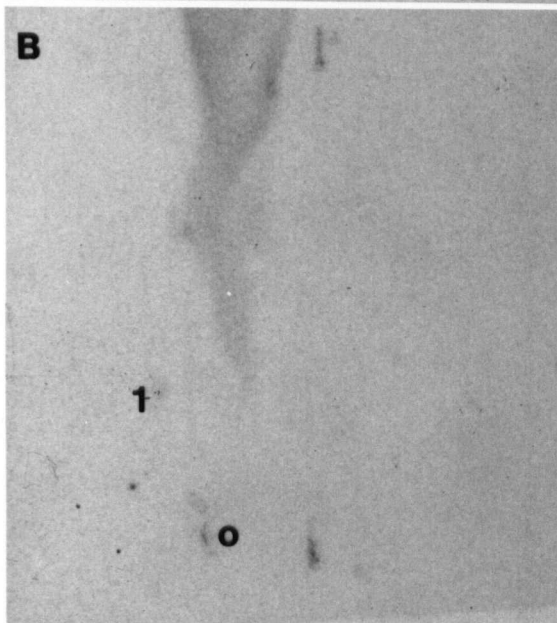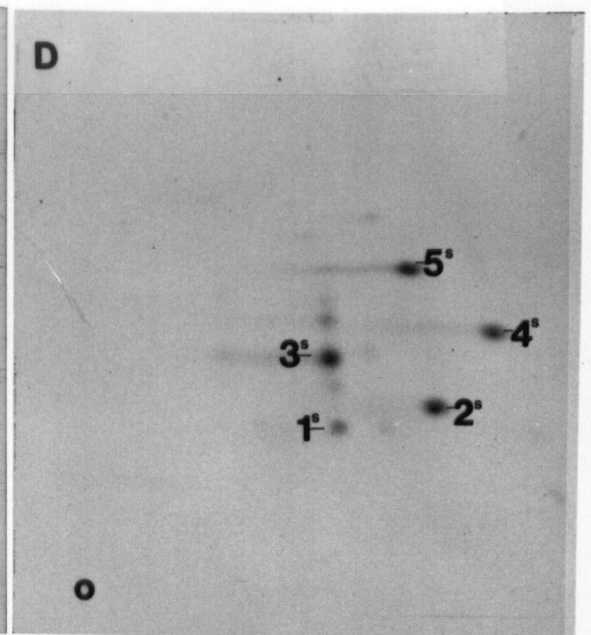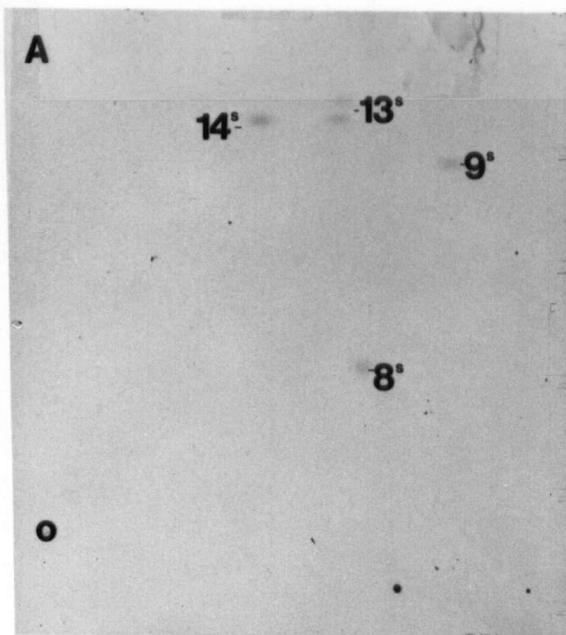
Figure 3.7: Mapping V8 protease cleavage fragments of FSV P140$^{gag-fps}$. The tsFSV P140$^{gag-fps}$ was labelled with ^{32}P$_i$ during an immune complex kinase reaction and cleaved with NP40-disrupted virions. Uncleaved P140$^{gag-fps}$ and the two p15 cleavage fragments 120K(p15) and 33K(p15) were recovered from the same gel and then subjected to in situ V8 protease digestion with 50 ng of V8 protease per sample followed by electrophoretic separation on a 15% SDS-polyacryla-mide gel. Lanes: A, tsFSV P140$^{gag-fps}$; B, 120K(p15) fragment; C, 33K(p15) fragment.
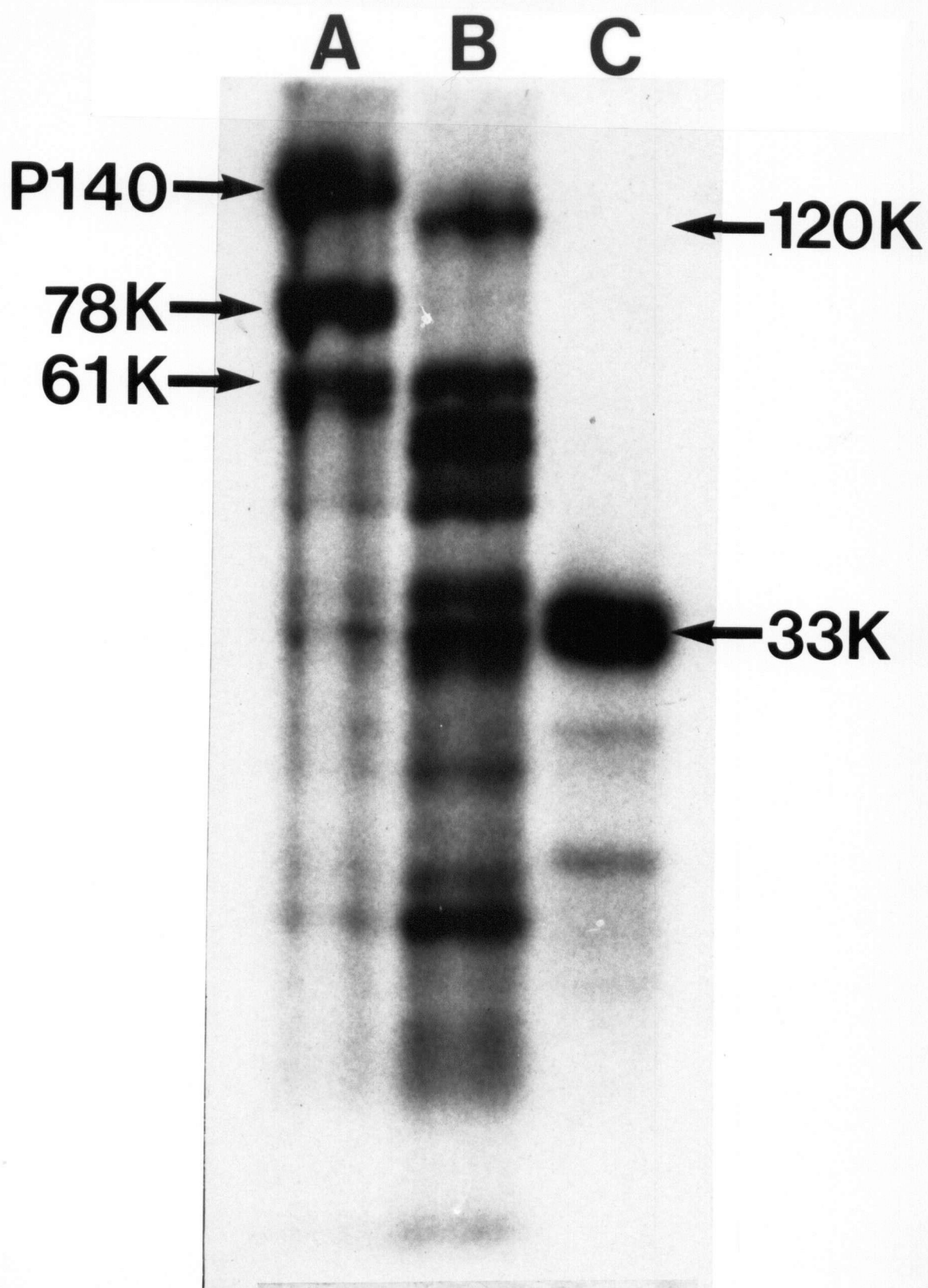
reaction (Figure 3.6 C and D) demonstrates that N-78K(V8) contains tryptic phosphopeptides 1 and 2, whereas C-61K(V8) contains only tryptic phosphopeptides 3a through 3c. This concurs with the observation that the N-78K(V8) from in vitro-phosphorylated P140$^{gag-fps}$ is more heavily labelled with ^{32}P relative to C-61K(V8) than the corresponding fragment from in vivo-phosphorylated P140$^{gag-fps}$ (data not shown). It is possible that the in vitro-phosphorylated peptide 2 is near the site of p15 cleavage, since it is not easily recovered in the p15 cleavage fragments.

The results of these mapping experiments are shown diagramatically in Figure 3.8.

3.2.3 Phosphorylation of the Transforming Proteins of Different fps Viruses

FSV L5 induces a temperature-sensitive, transformed phenotype and encodes a P140$^{gag-fps}$ with a thermolabile protein kinase activity (Pawson, et al., 1980). To determine whether this temperature sensitivity reflects any change in the amino acids sequence immediately surrounding P140$^{gag-fps}$ phosphorylation sites, I analysed the tryptic phosphopeptides of its temperature-resistance derivative, tr FSV (Lee, et al., 1981). Figure 3.9 shows that tr FSV and ts FSV P140$^{gag-fps}$, isolated from FSV-transformed CEFs metabolically labelled with ^{32}P-orthophosphate, have tryptic phosphopeptides

Figure 3.8:   Cleavage sites for p15 and V8 protease on FSV
P140$^{gag-fps}$ yielding the fragments described in the
text.   The numbers indicate the putative location of
tryptic phosphopeptides within FSV P140$^{gag-fps}$ as
discussed in the text.
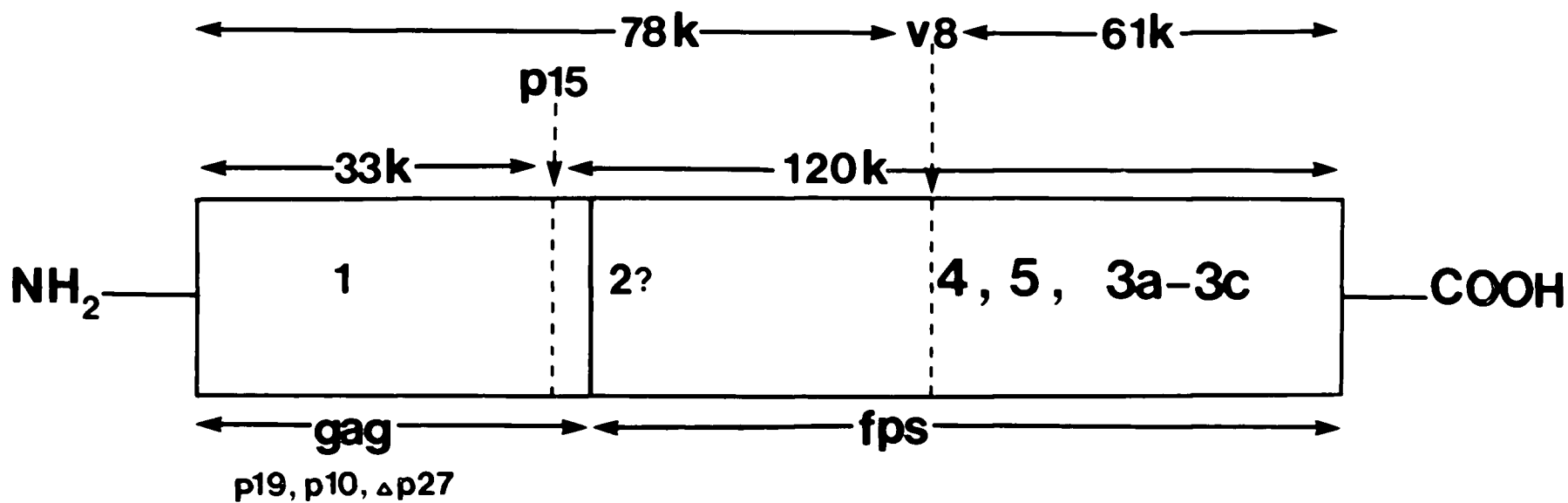
Figure 3.9: Tryptic phosphopeptide analysis of trFSV P140$^{gag-fps}$, FSV P130$^{gag-fps}$ and PRCII P105$^{gag-fps}$. FSV P140$^{gag-fps}$, FSV P130$^{gag-fps}$, or PRCII P105$^{gag-fps}$ was labelled with ^{32}P in vivo by incubation of transformed CEFs with ^{32}Pi and isolated by subsequent immunoprecipitation with anti-p19 serum, or labelled in vitro by phosphorylation in an immune complex kinase reaction with [γ-^{32}P]ATP after immunoprecipitation with anti-p19 serum from transformed CEFs. Gel-purified P140$^{gag-fps}$, P130$^{gag-fps}$, or P105$^{gag-fps}$ was then digested with trypsin and separated in two dimensions on thin- layer cellulose plates. Tryptic digests were as follows: A, trFSV P140$^{gag-fps}$ phosphorylated in vivo; B, FSV P130$^{gag-fps}$ phosphorylated in vivo; C, trFSV P140$^{gag-fps}$ phosphorylated in vitro; D, PRCII P105$^{gag-fps}$ phosphorylated in vitro; E, PRCII P105$^{gag-fps}$ phosphorylated in vivo. The arrows indicate normal migration of spot 4 as determined by a mixing experiment. The identity of spots is based on comigration in mixing experiments with tsFSVL5 P140$^{gag-fps}$. The additional spots in panels B and E have not been analysed further.
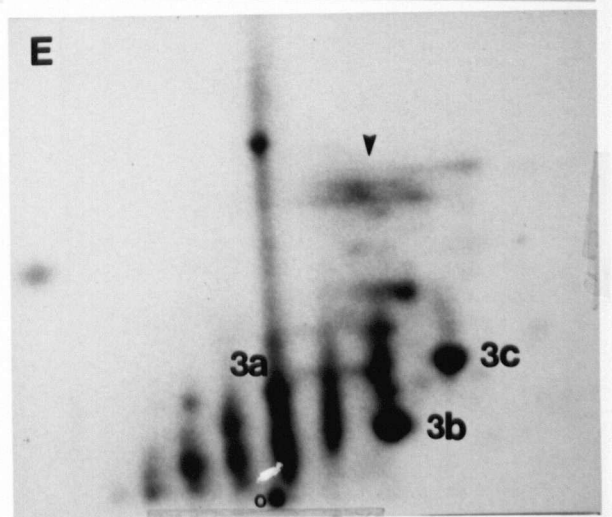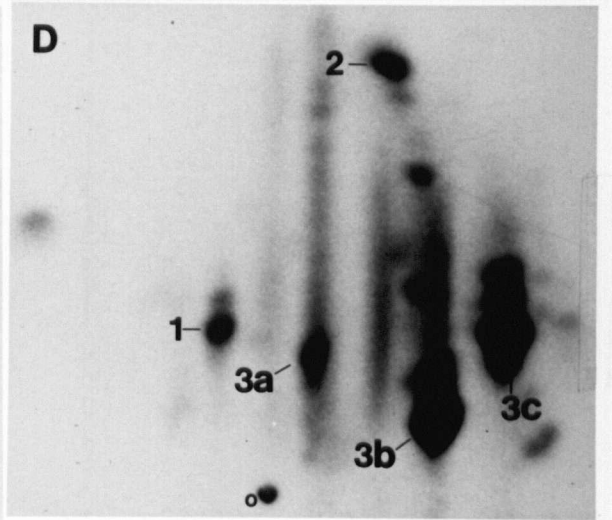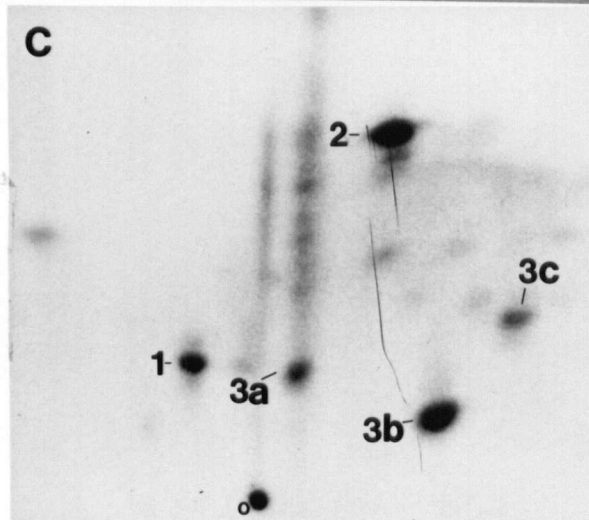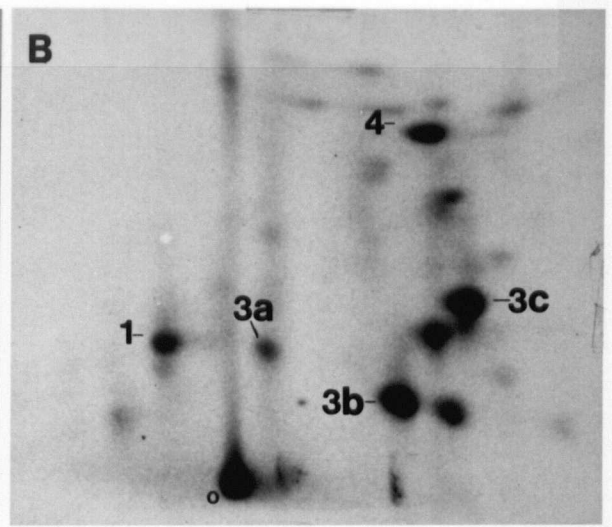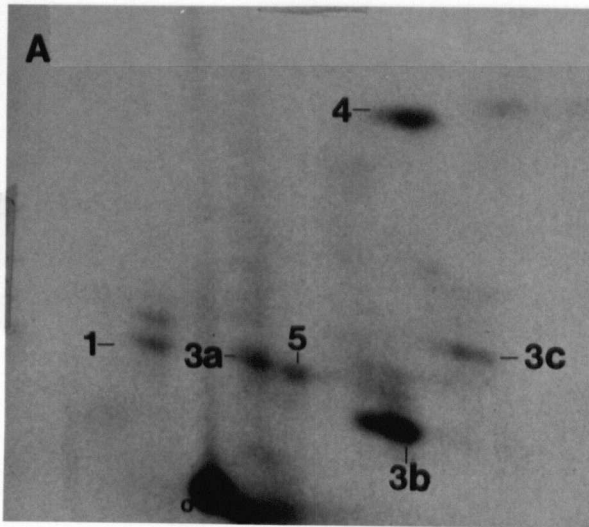
identical to those of their P140$^{gag-fps}$ proteins phosphorylated in the immune complex (peptide 1 and peptides 3a through 3c). Another variant of FSV with a different passage history (Hanafusa, et al, 1980; Hanafusa, et al., 1981) was also analysed. The P130$^{gag-fps}$ from this variant is phosphorylated in vivo with ^{32}P at phosphotyrosine containing tryptic peptides which have similar mobilities with those of ts FSV L5 and tr FSV (Figure 3.9B). However, new phosphoserine-containing peptides are seen, suggesting that there has been either sequence divergence in the tryptic peptide encompassing the major ser-ine phosphorylation site, or that the sites are completely different.

PRCII is an independent isolate of a transforming virus con-taining fps sequences whose encoded protein lacks a sequence of 340 amino acids that is found in the N-terminal half of the fps domain of FSV P130$^{gag-fps}$ (Huang, et al., 1984). PRCII P105$^{gag-fps}$ phosphorylated in the immune complex kinase reaction has a tryptic phosphopeptide map similar to that of in vitro-phosphorylated tsFSV L5 and trFSV P140$^{gag-fps}$, suggesting that it has retained the sequences encoding the conserved tyrosine phosphorylation sites 1 and 3a through 3c (Figure 3.9D). However, PRCII P105$^{gag-fps}$ phosphor-ylated in vivo is labelled at tryptic phosphopeptides 1 and 3a through 3c, but not or very weakly at the phosphotyrosine site represented by tryptic phosphopeptide 4 found in the FSV transforming proteins examined (Figure 3.9E). It is therefore possible that this site is contained within a region of FSV P140$^{gag-fps}$ that is deleted in PRCII P105$^{gag-fps}$.

3.3 Discussion

Phosphoamino acid analysis of FSV P140$^{gag-fps}$ indicates that it is phosphorylated in transformed cells at multiple tyrosine residues. Comparative tryptic phosphopeptide analysis suggests that these residues are contained within a region which is highly conserved between the transforming proteins of different FSV variants. The N-terminal gag region of P140$^{gag-fps}$ contains minor phosphorylated sites shared with Pr76^{gag} (Pawson, et al., 1981 and this study), but surprisingly it is also phosphorylated at a tyrosine residue contained within an acidic tryptic phosphopeptide. The importance of this gag phosphotyrosine site to the functional activity of P140$^{gag-fps}$ is unknown. Clearly, the tyrosine phosphorylation within the gag is specfic, but whether this represents fortuitous phosphorylation owing to the proximity of this sequence to the kinase active site or an important functional modification is yet to be determined (see section 1.11.7). The major fps-specific phosphorylation sites of FSV P140$^{gag-fps}$, including two phosphotyrosine residues and a phosphoserine residue, are clustered in the C-terminal region of fps, as represented by the 61K C-terminal V8 protease fragment. Limited trypsin cleavage experiments indicate that this region of the protein contains the kinase active domain (Levinson, et al., 1981; Weinmaster, et al., 1983; Brugge and Darrow, 1984). The C-terminal localization of the major fps phosphorylation sites and the detection of trypsin fragments with kinase activity support the suggestion that

the C-terminal sequences of the ASV transforming proteins are highly conserved because they encode the kinase domain. The conservation of sequence surrounding the P140$^{gag-fps}$ phosphotyrosine residues argues that these sites are important for activity of the protein and in the following two chapters I will present data to support a relationship between the phosphorylation of P140$^{gag-fps}$ itself and its kinase activity.

Several groups have compared the phosphorylation sites from a number of different viral transforming proteins by using micro-sequencing (Neil, et al., 1981; Neil, et al., 1982; Patschinsky, et al., 1982) and deduced amino acid sequences (section 1.6.1). There is substantial homology between the characteristically acidic amino acid sequences N-terminal to the tyrosine phosphorylation sites (see figure 1.1). For PRCII P105$^{gag-fps}$ and FSV P140$^{gag-fps}$ phosphorylated in vitro, the major tyrosine phosphorylation site has a glutamic acid four residues N-terminal to the phosphotyrosine and a basic amino acid seven residues N-terminal (Patschinsky, et al., 1982). Examination of the amino acid sequence of FSV P130$^{gag-fps}$ deduced from the DNA squence of cloned FSV shows a tyrosine at residue 1073 which fulfills these characteristics (Shibuya and Hanafusa, 1982). The N-terminal amino acid of the tryptic peptide containing this tyrosine residue is glutamine, as initially suggested to account for poor yields in microsequencing (Neil, et al., 1982). Cyclization of the glutamine residue following hydrolysis of its side chain amide group during the

tryptic mapping procedure may yield a tryptic peptide having pyro-glutamic acid at its N-terminal end (3b) which is a less positively charged species at pH2.1 compared to the unmodified peptide containing glutamine at its N-terminus (3c). In addition, the pyro-glutamate ring may open to produce glutamic acid at the N-terminal end, which would be more negatively charged (3a). These observations and those discussed previously suggest that the tryptic peptides 3a through 3c, which represent the major site of tyrosine phosphorylation in vitro, correspond to the tryptic peptide containing tyrosine-1073.

P140$^{gag-fps}$ is also phosphorylated in vivo at a C-termi-nal tyrosine residue contained within peptide 4. It is intriguing that this residue is not phosphorylated in the immune complex kinase reac-tion, perhaps this site of tyrosine phosphorylation is not the product of P140$^{gag-fps}$ autophosphorylating activity but rather is phos-phorylated by a cellular tyrosine kinase. Alternatively, the lack of phosphorylation within peptide 4 could reflect the artificial nature of the in vitro immune complex kinase assay which would also account for the phosphorylation of peptide 2 which is only seen with P140$^{gag-fps}$ phosphorylated in vitro. This lack of phosphorylation within peptide 4 does not affect the apparent kinase activity of P140$^{gag-fps}$ as measured in vitro. It appears doubtful whether peptide 4 is present in PRCII P105$^{gag-fps}$. However, if it is absent from this transforming protein it clearly does not affect its in vitro tyrosine protein kinase activity, but whether it has anything

to do with the decreased oncogenicity reported for PRCII (Breitman, et al., 1981) remains to be seen.

These data raise the possibility that more than one kinase is involved in the phosphorylation of P140$^{gag-fps}$ tyrosine residues in FSV transformed cells. Experiments described in the following chapters were designed to investigate the relationship between the phosphorylation of the FSV transforming protein and its enzymatic and biological activities.

CHAPTER 4

4.0 Oligonucleotide-directed Mutagenesis of Fujinami Sarcoma Virus: Evidence that Tyrosine Phosphorylation of P130$^{gag-fps}$ Modulates its Enzymatic and Biological Activities.

4.1 Introduction

A variety of genetic and biochemical data indicate that the integrity of the P130$^{gag-fps}$ kinase function is essential for FSV to induce neoplastic transformation of infected cells (Pawson, et al., 1980; Hanafusa, et al., 1981; Lee, et al., 1981; Stone, et al., 1984). The aberrant phosphorylation at tyrosine of cellular proteins normally involved in regulating cell growth and metabolism might explain many phenotypic changes associated with expression of the viral transforming protein. A number of cellular proteins do become newly or increasingly phosphorylated at tyrosine upon transformation by FSV, of which three have been positively identified as glycolytic enzymes (enolase, phosphoglycerate mutase and lactate dehydrogenase) (Cooper and Hunter, 1981b; Cooper, et al., 1983a). FSV P140$^{gag-fps}$ and its protease-resistant C-terminal 45 kd fragment phosphorylate enolase and LDH in vitro at the same tyrosine residues as become phosphorylated in FSV-transformed cells (Cooper, et al., 1984a), and these substrates display apparent Kms for P140$^{gag-fps}$ that are within physiological concentrations. In addition, the association of tyrosine-specific

protein kinase activity with the cellular receptors for mitogenic hormones such as epidermal growth factor, platelet-derived growth factor and insulin has suggested a normal role for tyrosine phosphorylation in the induction of cell division (Ushiro and Cohen, 1980; Ek, et al., 1982; Kasuga, et al., 1983).

If these notions are valid then tyrosine phosphorylation would be expected to exert its effect by modulating the enzymatic and biological activities of at least some substrate proteins, as has been fully estabilshed in several instances of phosphorylation by serine-specific protein kinases (Cohen, 1982). However, such a function has yet to be demonstrated for any case of reversible tyrosine phosphorylation; indeed substitution of RSV $p60^{src}$ tyrosine-416 with phenylalanine destroys the major site of $p60^{src}$ tyrosine phosphorylation but has no obvious effect on src transforming ability or kinase activity of $p60^{src}$ (Snyder, et al., 1983). In the previous chapter I showed that $P130^{gag-fps}$ is phosphorylated in FSV-transformed cells at three tyrosine residues and at several serine sites. Therefore I have used FSV $P130^{gag-fps}$ as a model substrate to investigate whether tyrosine phosphorylation can in fact modify protein function. The major site of $P130^{gag-fps}$ phosphorylation in vivo, as in vitro, is tyrosine-1073 which is located in the C-terminal kinase domain and is homologous to the major site of tyrosine phosphorylation in RSV $p60^{src}$ and Y73 $P90^{gag-yes}$ (Kitamura, et al., 1982; Shibuya and Hanafusa, 1982). In this study I have determined the

effects of changing tyrosine-1073 of FSV P130$^{gag-fps}$ to a phenylalanine residue on the enzymatic activity and biological function of this transforming protein.

4.2 Results

4.2.1 Oligonucleotide-directed Mutagenesis of FSV

Oligonucleotide-directed mutagenesis of the FSV genome, cloned into an M13 single-stranded DNA bacteriophage vector has been used to convert tyrosine-1073 within FSV P130$^{gag-fps}$ to phenylalanine. Since phenylalanine is the closest amino acid in structure to tyrosine, which cannot be phosphorylated, this amino acid substitution should render residue 1073 unavailable for phosphorylation, without perturbing the native conformation of the unphosphorylated protein. The amino acid sequence surrounding tyrosine-1073 and the corresponding nucleotide sequence of the FSV genome are shown in Figure 4.1. A mutagenic oligonucleotide 16 nucleotides long was synthesized such that the TAT codon for tyrosine-1073 is replaced by a TTT codon for phenylalanine. However, apart from this single substitution of T for A the sequence of the mutagenic primer is identical to nucleotides 3589-3604 of the FSV genome as defined by Shibuya and Hanafusa (1982) (see Figure 4.1). To provide a template for mutagenesis a 4.7 kbp Sst1 restriction endonuclease fragment corresponding to the entire FSV genome in a circularly permuted form was purified from a λgtWES·λB vector (Shibuya,

Figure 4.1   Synthetic oligonucleotides used to mutate the codon for tyrosine-1073 of FSV P130$^{gag-fps}$. The figure shows (a) the amino acid sequence surrounding tyrosine-1073 and the corresponding wild-type FSV nucleotide sequence (Shibuya and Hanafusa, 1982); (b) the oligonucleotide used to mutate the TAT codon for tyrosine-1073 to a TTT codon for phenylalanine; and (c) the oligonucleotide used to direct the synthesis of a revertant in which the TAT codon is restored. Arrows indicate potential sites of trypsin cleavage in the peptide sequence.

- 100 -

```
                      ▼                    1073                           ▼
        N . . . . ArgGlnGluGluAspGlyVal[Tyr]AlaSerThrGlyGlyMetLys . . . . . C
(a)     5'. . . . CGGCAGGAGGAGGATGGTGTCTATGCCTCCACGGGGGGCATGAAG . . . . 3'    WILD TYPE

                                         Phe
(b)                                 TGGTGTCTTTGCCTCC                          MUTANT

                                         Tyr
(c)                                 TGGTGTCTATGCCTCC                          REVERTANT
```

et al., 1982b) and was cloned into the SstI site of M13mp10 RF DNA (Messing, 1983) (Figure 4.2). Phage containing viral inserts were propagated in E. coli JM101, and those phage ssDNAs with the FSV strand complementary to the oligonucleotide were identified by their ability to act as templates for dideoxynucleotide chain termination sequencing reactions in which the mutagenic oligonucleotide served as a sequencing primer. These reactions also showed that the mutagenic oligonucleotide only hybridized stably to the expected site within the FSV insert. A representative phage (M13mp10FSV-8) was mutagenized by a two-primer method (Zoller and Smith, 1984) in which the mutagenic oligonucleotide and a second oligonucleotide complementary to an M13 sequence (the universal sequencing primer) were both annealed to M13mp10FSV-8 phage ssDNA. E. coli DNA polymerase I (large fragment) was used to extend the primers in the presence of the four deoxynucleoside triphosphates and T4 DNA ligase. This DNA was introduced into competent bacteria, and ssDNAs isolated from the resulting plaque purified phage were screened using the mutagenic oligonucleotide as a hybridization probe under conditions of increasing stringency to identify phage with the desired mutation in their FSV inserts. Of 24 different phage DNAs tested, 22 bound the probe at 40°C but not at 50°C and were presumed to still contain a wild-type FSV sequence with a single base mismatch to the mutagenic oligonucleotide. Two phage DNAs remained hybridized to the probe at 50°C (Figure 4.2), and were presumed to be mutants. DNAs of a putative wtFSV phage (M13mp10wtFSV) and a mutant phage (M13mp10FSV-F(1073)) were sequenced in the region encompassing the

Figure 4.2   Strategy for the oligonucleotide-directed mutagenesis of

FSV. The diagram illustrates the method by which the

4.7 kbp FSV genome was subcloned from λ-FSV-2 into the

Sst1 site of M13mp10 and the TAT codon for tyrosine-1073

of P130$^{gag-fps}$ mutated to TTT using the oligonu-

cleotide displayed in Figure 4.1(b). Putative mutant

phage ssDNAs were dotted onto nitrocellulose and

screened for their abilities to hybridize with the

^{32}P-labelled mutagenic oligonucleotide under condi-

tions of increasing temperature.

ISOLATE 4.7 kbp FSV INSERT OF λ-FSV-2 DNA

CLONE INTO Sst1 SITE OF M13mp10 RF DNA

M13mp10

Sst1

gag   fps   LTR
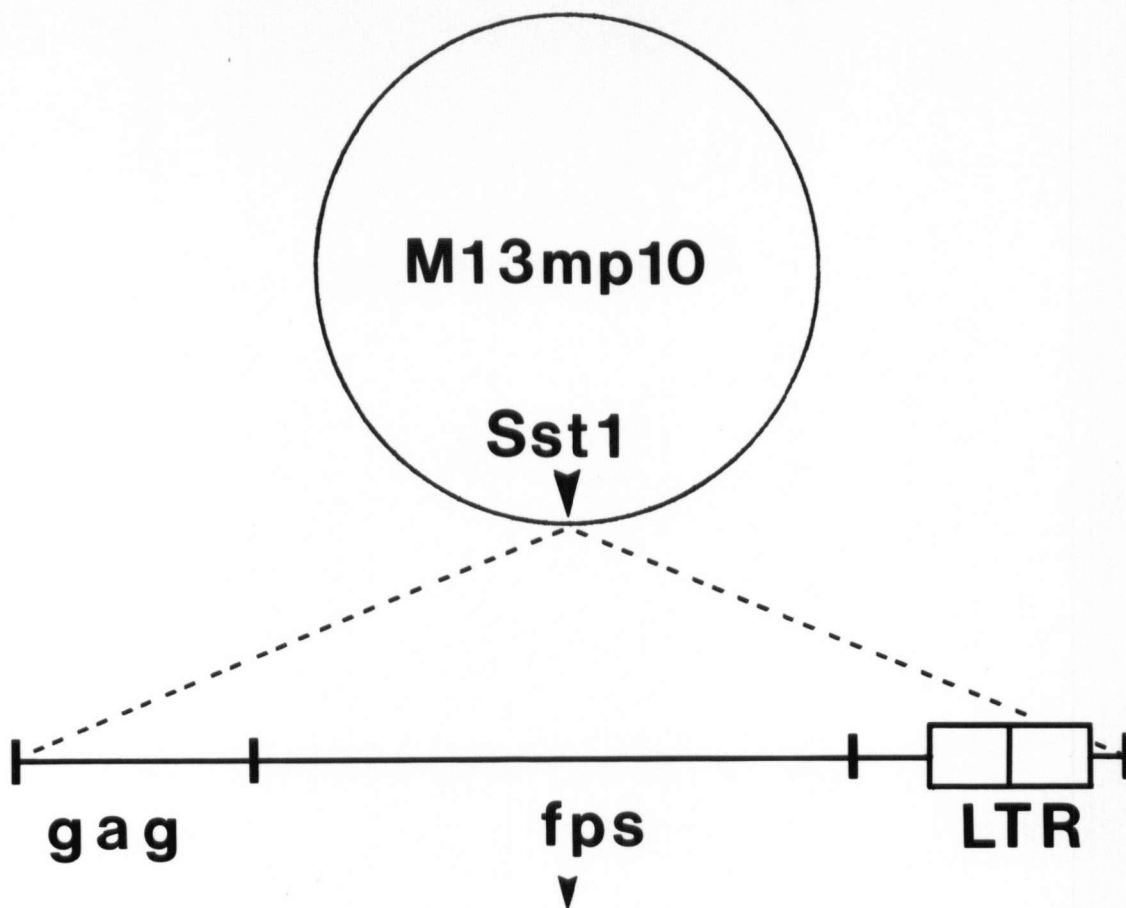
ISOLATE PHAGE WITH FSV INSERT IN DESIRED ORIENTATION

OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

SCREEN RESULTING PHAGE DNAs BY DOT-BLOT HYBRIDIZATION WITH
THE MUTAGENIC OLIGONUCLEOTIDE AS A LABELED PROBE
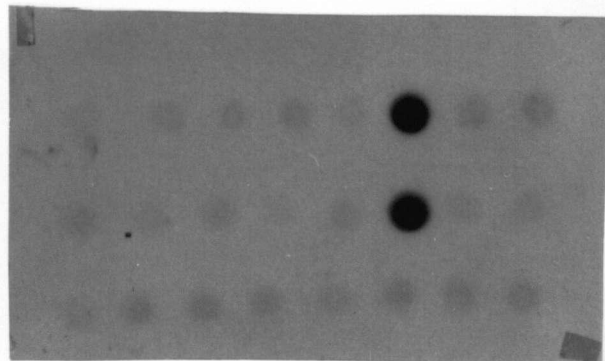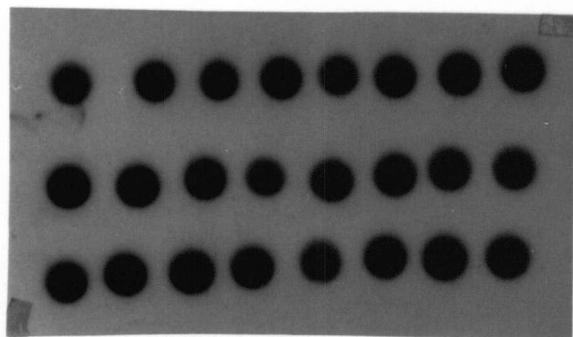
40°C   50°C

mutation site (Figure 4.3). The two sequences are identical except that adenine contained within the TAT codon for tyrosine-1073 of wtFSV DNA is mutated to a thymine in FSV-F(1073) DNA yielding a phenylalanine codon.

4.2.2 Synthesis of a Revertant FSV

In order to prove that any biological or biochemical differences between the P130$^{gag-fps}$ proteins encoded by wtFSV and FSV-F(1073) resulted from the conversion of tyrosine-1073 to phenylalanine and not from a spontaneous mutation at another unrelated site I reverted the mutant DNA back to the wild-type. To do this I synthesized an oligonucleotide 16 long identical to the original wtFSV sequence between nucleotides 3589-3604 (see Figure 4.1) and used this as a mutagenic oligonucleotide with M13mp10FSV-F(1073) phage ssDNA as the template for mutagenesis. The same protocol described above was used to isolate an M13 phage in which the TTT codon for phenylalanine-1073 in the mutant FSV-F(1073) insert has been mutated back to a TAT codon for tyrosine. The nucleotide sequence of this phage M13mp10FSV-Y(1073) is identical to wtFSV in the region of interest (Figure 4.3). Any biological activity of wtFSV that is altered in the mutant FSV-Y(1073) but restored to wild-type function in the revertant FSV-Y(1073) must vary solely in response to the amino acid changes at residue 1073 in P130$^{gag-fps}$.

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Figure 4.3   Partial DNA sequencing of wtFSV, mutant FSV-F(1073) and
revertant FSV-Y(1073) M13mp10 inserts.   The nucleotide
sequences encompassing the codon for tyrosine-1073 of
wtFSV and the mutation sites of FSV-F(1073) and
FSV-Y(1073) DNAs were determined by the dideoxynucleo-
tide chain termination method.   The codons for amino
acid 1073 are arrowed in each case.

C T A G   C T A G   C T A G

$^{T}_{T}$A▶ $^{T}_{T}$T▶ $^{T}_{T}$A▶

wt FSV   FSV-F(1073)   FSV-Y(1073)

WILD TYPE   MUTANT   REVERTANT
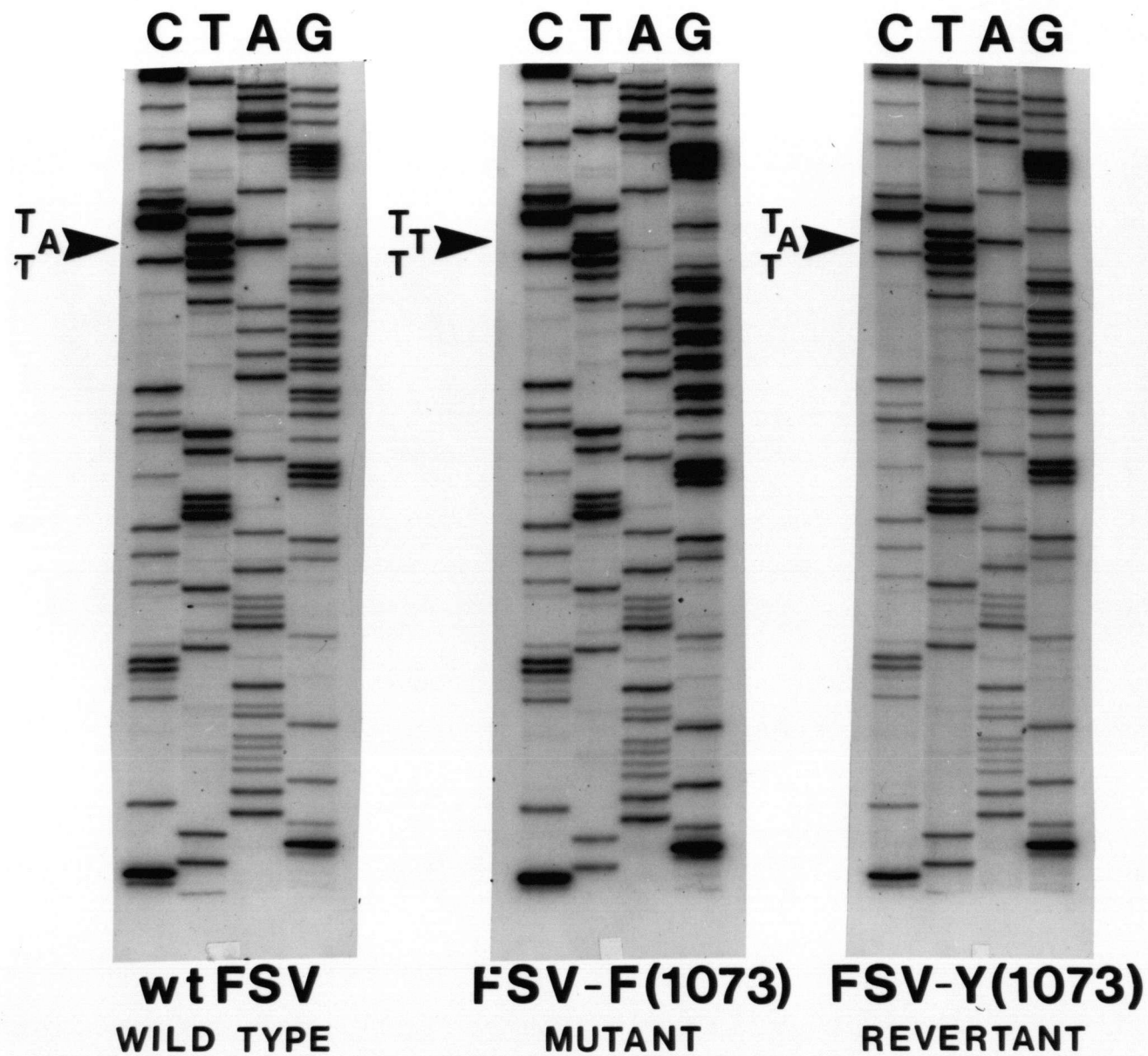
4.2.3 Transforming Activity of Wild-type, Mutant and Revertant FSV

DNAs

The wtFSV, FSV-F(1073) and FSV-Y(1073) sequences from the appropriate M13 vectors were assayed for their abilities to transform rat-2 cells. The 4.7 kbp FSV inserts were purified by agarose gel electrophoresis following Sst1 digestion of RF DNAs isolated from phage-infected cells. FSV inserts were electroeluted from the gels, ligated to reconstruct intact FSV genomes (Shibuya, et al., 1982b) and introduced into rat-2 cells by the calcium phosphate co-precipitation transfection technique (Graham and Van der Eb, 1973). Within 8 days foci of transformed cells with a round, refractile morphology were apparent in dishes of rat-2 cells transfected with wtFSV DNA or with revertant FSV-Y(1073) DNA (Figure 4.4). At two weeks post-transfection wtFSV DNA gave 50-100 foci/ug DNA/5 x 10^6 cells, and by three weeks approximately five times more foci were visible, though many of these may have arisen by secondary spread of transformed cells. In contrast, rat-2 cells transfected with FSV-F(1073) mutant DNA showed no evident morphological change until at least 30 days post-transfection, when some foci of transformed cells with a fusiform morphology became visible (Figure 4.4). If the cells tranfected with FSV-F(1073) mutant DNA were maintained for 2-3 months the initial foci became prominent and acquired a more pronounced, round morphology. Thus, the FSV-F(1073) DNA encoding phenylalanine in place of tyrosine-1073 at position 1073 is still able to induce transformation of rat-2 cells,

Figure 4.4   Transformation of rat-2 cells following transfection
with FSV DNAs. Rat-2 cells were transfected with wild-
type, mutant or revertant FSV DNAs and examined for the
appearance of foci of transformed cells: Normal rat-2
cells (A); a typical focus 15 days after transfection
with wtFSV DNA (B); a focus 44 days after tranfection
with mutant FSV-F(1073) DNA (C); a focus 15 days after
transfection with revertant FSV-Y(1073) DNA (D). Cell
lines of wtFSV-transformed rat-2 cells (E) and
FSV-F(1073)-transformed cells (F) derived from such foci
are shown. The appearance of colonies 3 weeks after
seeding in soft agar are shown for normal rat-2 cells
(G); wtFSV-transformed cells (H); FSV-F(1073)-
transformed cells (I); and FSV-Y(1073)-transformed cells
(J).

RAT-2

wt FSV

FSV-F(1073)

FSV-Y(1073)

FOCI   CELL LINES   COLONIES
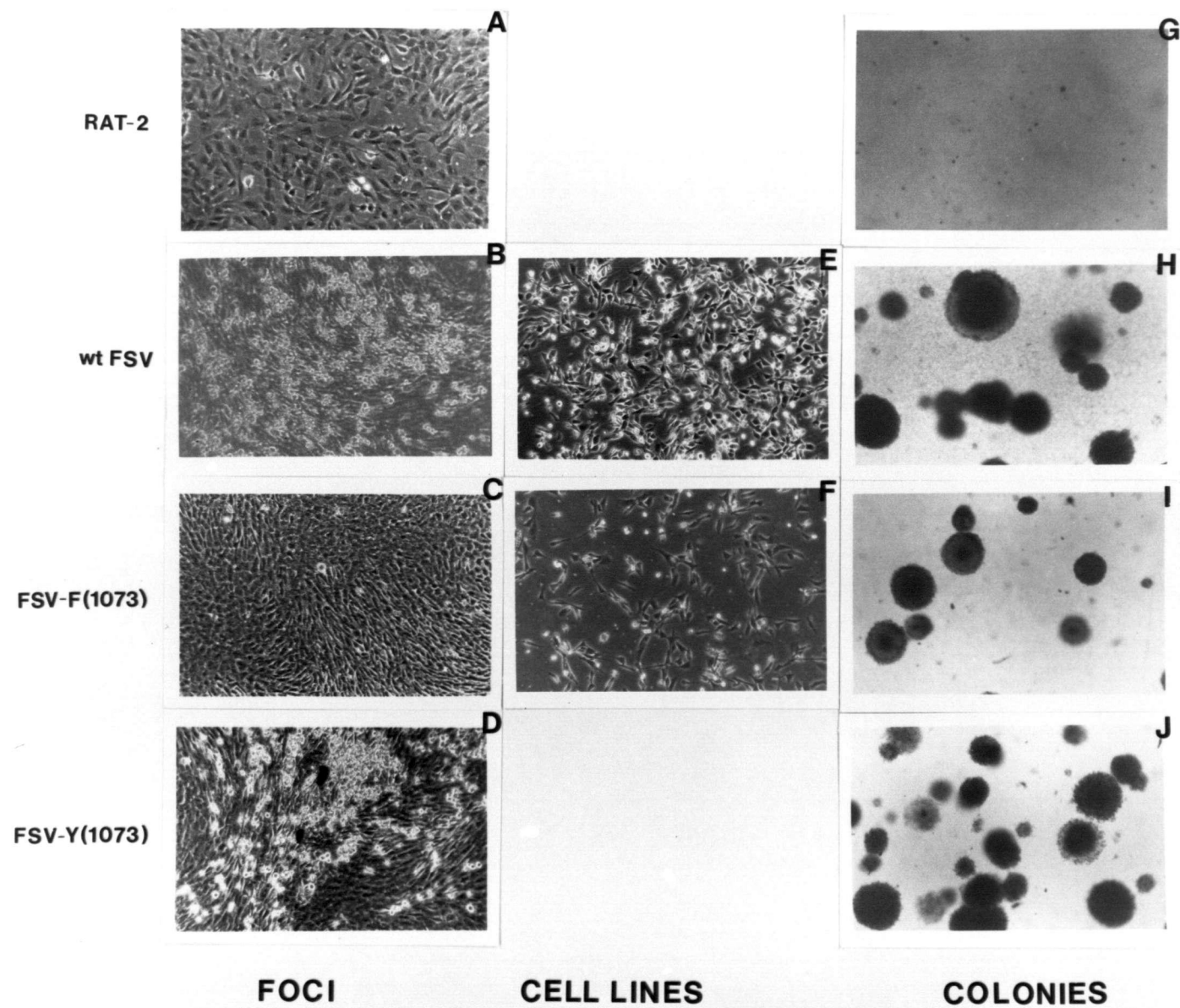
but does so with a much longer latent period than wtFSV or revertant FSV DNAs.

Foci of transformed rat-2 cells which appeared following transfection of wtFSV, mutant FSV-F(1073) or revertant FSV-Y(1073) DNAs were picked, subcloned and expanded in mass culture (Figure 4.4). All such morphologically transformed cell lines, including those induced by FSV-F(1073), formed large colonies in soft agar (Figure 4.4) unlike the normal rat-2 cells (Figure 4.4G).

To determine whether the mutant FSV-F(1073) transformed rat cells could induce tumors in vivo, wtFSV, mutant FSV-F(1073), revertant FSV-Y(1073) or rat-2 cells were injected subcutaneously, at a cell concentration of 1.0 x 10^6 cells per .1 ml into the back of the neck of 4-5 week old female Fischer rats, in triplicate. Approximately three weeks post-injection tumors appeared at the site of injection in rats that had been injected with wtFSV or revertant transformed cells. However, tumors were not obvious until 6-8 weeks post-injection with rats injected with mutant FSV-F(1073) transformed cells. In addition, tumors appeared at the site of injection in some animals that had been injected with normal rat-2 cells, but this occurred only after 5 months following injection and probably reflects the properties of this immortalized, continuous cell line. These results are an average of three independent experiments and they support the enhanced latent period of transformation seen with the mutant FSV-F(1073) in vitro.

Tumors from these animals were excised, grown in tissue culture and subsequently assayed for the expression and activity of P130$^{gag-fps}$ as described below. Tumors induced by mutant FSV-F(1073) transformed cells contained cells which expressed P130$^{gag-fps}$ characteristic of the mutant protein, such as enhanced mobility in SDS gels and reduced in vitro tyrosine specific kinase activity compared to of the wild-type or revertant transforming proteins (see below).

4.2.4 Expression, Structure and In Vitro Kinase Activities of Wild-type and Mutant P130$^{gag-fps}$ Proteins

Equal numbers of cells transformed with wtFSV, FSV-F(1073) or with FSV-Y(1073) were labelled with [^{35}S]methionine for 16 hours, lysed and immunoprecipitated with anti-p19^{gag} serum. Equivalent amounts of labelled P130$^{gag-fps}$ were immunoprecipitated from each line of transformed cells (Figure 4.5). Pulse-chase experiments have shown that the wtFSV and FSV-F(1073) P130$^{gag-fps}$ proteins have approximately the same turnover rates (data not shown) and [^{35}S]methionine-labelling can therefore be used as a relative measure of P130$^{gag-fps}$ levels. The mobilities of the wtFSV and revertant proteins were identical, demonstrating that the mutagenesis procedures had not grossly affected the FSV coding sequence. Mutant P130$^{gag-fps}$ encoded by FSV-F(1073) migrated slightly more rapidly than the wild-type or revertant proteins, possibly as a result of decreased phosphorylation. In a parallel experiment wild-type, mutant or revertant P130$^{gag-fps}$ proteins were immunoprecipitated from

Figure 4.5   Analysis of wild-type, mutant and revertant FSV-trans-
formed rat-2 cells for FSV P130$^{gag-fps}$ synthesis
and tyrosine-specific protein kinase activity. Normal
and transformed rat-2 cells were labelled for 16 hours
with 100 uCi [^{35}S]methionine, immunoprecipitated
with anti-p19^{gag} serum and examined by gel electro-
phoresis to identify P130$^{gag-fps}$ (lanes A-E).
Cells from duplicate unlabelled cultures were immunopre-
cipitated in identical fashion and then incubated with 5
ug of acid-denatured enolase in the presence of 10 mM
MnCl$_2$ and 2.5 uCi [χ-^{32}P]ATP for 15 minutes at
30°C (lanes F-J). Samples were anlaysed by electro-
phoresis through a 7.5% SDS-polyacrylamide gel and auto-
radiography. Immunoprecipitates were from normal rat-2
cells (lanes C and H) or from rat-2 cells transformed
with wtFSV (A and F); with FSV-F(1073) (B, D, G, and I);
or with FSV-Y(1073) (E and J).

A B   C D E   F G   H I J

◄ P130 ►

Enolase►

^{35}S ^{32}P

– 113 –
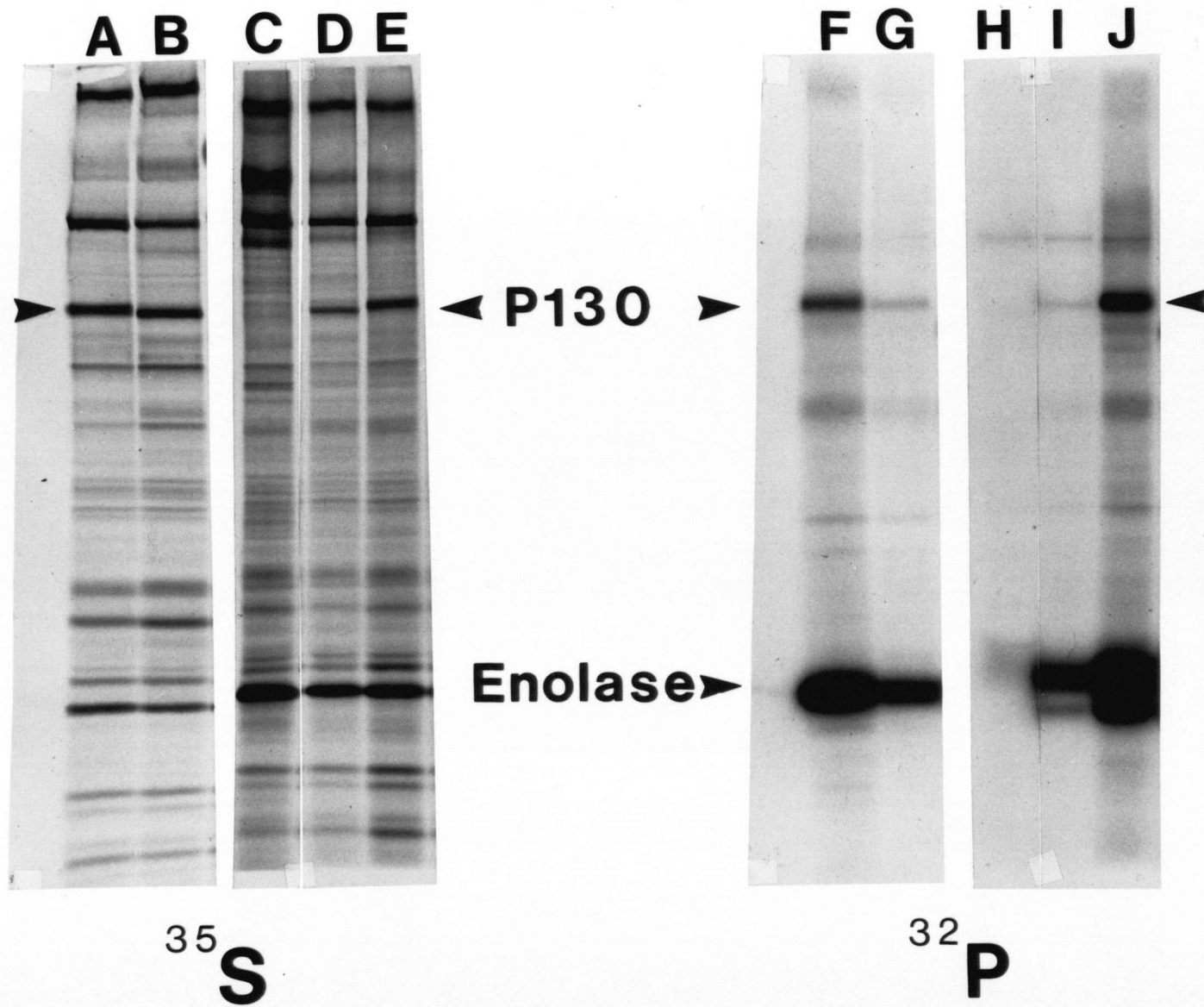
equal numbers of unlabelled cells and the immune complexes incubated with $MnCl_2$, $[\gamma-^{32}P]ATP$ and soluble, denatured rabbit muscle enolase (Figure 4.5). In this reaction wtFSV P130$^{gag-fps}$ is autophosphorylated and also phosphorylates enolase at a single tyrosine identical to that phosphorylated in FSV-transformed chicken embryo fibroblasts (Cooper, et al., 1984a). Mutant FSV-F(1073) P130$^{gag-fps}$ clearly functions in vitro as a protein kinase but is apparently less active in phosphorylating enolase than wtFSV or FSV-Y(1073) P130$^{gag-fps}$. The extent of enolase phosphorylation in each case was normalized to the amount of immunoprecipated P130$^{gag-fps}$ by determining the ^{32}P or ^{35}S cpm in the relevant bands (Table 4.1). The mutant protein is approximately five-fold less active in enolase phosphorylation than the wild-type or revertant proteins when assayed in this way.

The considerable decrease in radiolabelling of FSV-F(1073) P130$^{gag-fps}$ itself in the immune complex kinase reaction (Figure 4.5, Table 4.1) would be expected if the major site of in vitro tyrosine autophosphorylation had been destroyed by substituting tyrosine-1073 with phenylalanine. C-terminal fragments of in vitro-phosphorylated mutant P130$^{gag-fps}$ generated by cleavage with V8 protease were poorly phosphorylated compared with corresponding fragments of the wtFSV protein (data not shown). To confirm that the expected amino acid substitutions had been introduced at residue 1073 and to define the effect of these substitutions on phosphorylation of the mutant and

TABLE 4.1

Quantitation of the Kinase Activities of P130$^{gag-fps}$

Proteins Encoded by Wild-Type, Mutant and Revertant FSV DNAs

Source of P130$^{gag-fps}$	Relative Phosphorylation[a] in vitro by P130$^{gag-fps}$		
	Enolase		P130$^{gag-fps}$ (autophosphorylation)
wtFSV	7.5[b]	9.7[c]	0.6[d]
FSV-F(1073)	1.4	2.2	0.1
FSV-Y(1073)	6.0	ND	0.6

a   Immune complex kinase reactions were performed as shown in Figure 4.5 following immunoprecipitation of P130$^{gag-fps}$ from rat-2 cells transformed by wtFSV, FSV-F(1073) or FSY-Y(1073).   The extent of enolase phosphorylation or P130$^{gag-fps}$ autophosphorylation was measured by counting appropriate dried gel slices for ^{32}P.   The amount of P130$^{gag-fps}$ in each immunoprecipitate was estimated by labelling duplicate dishes of cells with [^{35}S]methionine,   immunoprecipitating   P130$^{gag-fps}$ and counting the gel-purified protein.   The values shown are the ratios of ^{32}P cpm incorporated into the substrate to ^{35}S cpm in P130$^{gag-fps}$.

b,c Values are from two separate experiments.   ND, not done.

d   Mean of two separate experiments.

revertant proteins tryptic digests ofwtFSV, FSV-F(1073) and FSV-Y(1073) P130$^{gag-fps}$, which had been radiolabelled in vivo with $^{32}P_i$ or [^{35}S]methionine or autophosphorylated in vitro using [γ-^{32}P]ATP were analysed. Analysis of the tryptic peptide containing tyrosine-1073 is complicated by the location of a glutamine residue at its N-terminus which, as described in Chapter 3, can be modified during the mapping procedure yielding three separately migrating peptide species. A tryptic phosphopeptide map of P130$^{gag-fps}$ autophosphorylated in an immune complex kinase reaction (Figure 4.6, map C) reveals five spots of which three (previously designated 3a, 3b and 3c) are all derived from the same tryptic peptide thought to contain tyrosine-1073 whereas the remaining two correspond to less prominent sites of phosphorylation (Chapter 3). The tryptic peptide encompassing tyrosine-1073 also possesses a methionine residue, and a tryptic peptide map of [^{35}S]methionine-labelled wtFSV P130$^{gag-fps}$ should therefore contain radiolabelled peptides corresponding both to the phosphorylated species 3a-3c and their non-phosphorylated counterparts in a ratio dependent on the stoichiometry of phosphorylation.

A map of mutant FSV-F(1073) P130$^{gag-fps}$ labelled by in vitro phosphorylation (Figure 4.6, map F) is entirely lacking spots 3a-3c but retains the two minor tryptic phosphopeptides, confirming that 3a-3c represents phosphorylation of tyrosine-1073. In contrast, the tryptic phosphopeptide map of revertant P130$^{gag-fps}$ phosphorylated in an immune complex is identical to that of wtFSV

Figure 4.6   Tryptic peptide analysis of P130$^{gag-fps}$ encoded by
wild-type, mutant and revertant FSVs. P130$^{gag-fps}$
isolated from transformed rat-2 cells labelled <u>in vivo</u>
with [^{35}S]methionine (A, D, G) or with ^{32}P-
orthophosphate (B, E, H) or labelled with ^{32}P
<u>in vitro</u> by autophosphorylation in immune complex kinase
reactions (C, F, I) was subjected to 2-dimensional
tryptic peptide mapping. P130$^{gag-fps}$ was isolated
from rat-2 cells transformed with wtFSV (A, B, C) or
with FSV-F(1073) (D, E, F) or with FSV-Y(1073)
(G, H, I).   tsFSV P140$^{gag-fps}$ was isolated from
tsFSV(FAV)-infected CEFs which were labelled with
[^{35}S]methionine (J, K). CEFs were maintained at
37°C (J) or at 41.5°C (K).   [^{35}S]methionine-
labelled proteins and ^{32}P-labelled proteins were
oxidized, digested with trypsin and separated in two
dimensions in identical fashion. Origins are marked
with an "O". Electrophoresis at pH 2.1 is displayed
from left to right; anodes are to the left and cathodes
are to the right. Chromatography in the second dimen-
sion was from bottom to top. The identities of unmarked
tryptic peptides can be found in Pawson, <u>et al.</u> (1981)
or Chapter 3. The double spot to the right of phospho-
peptide 4 in maps E and F and the spot above 3c in map F
are variable and have also been observed in wtFSV
P130$^{gag-fps}$ (see Chapter 3).
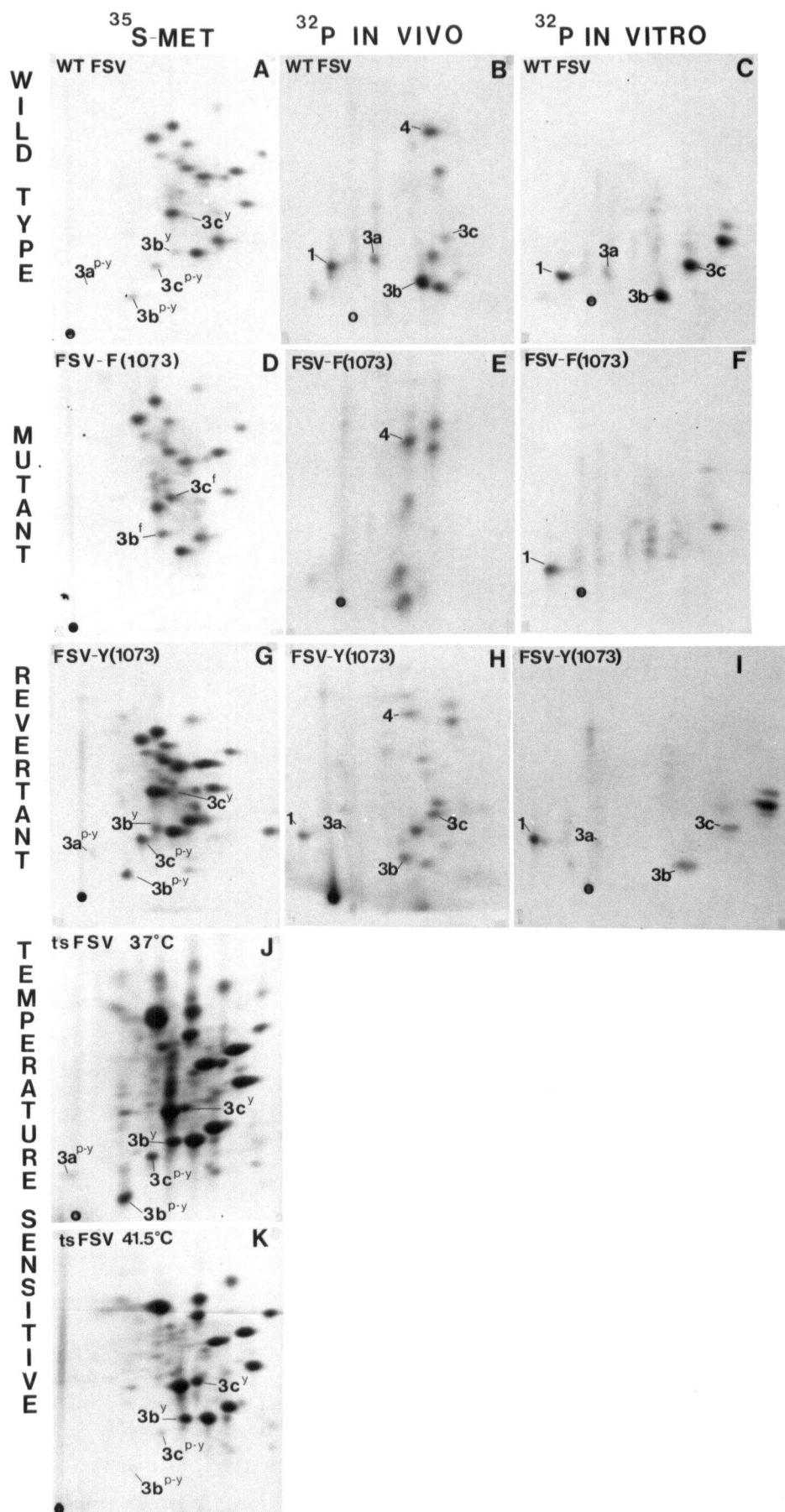
P130$^{gag-fps}$ (Figure 4.6, map I). These findings show that tyrosine-1073 is indeed the major site of wtFSV P130$^{gag-fps}$ auto-phosphorylation, and that this phosphorylation site is lost in the mutant protein but restored in revertant P130$^{gag-fps}$. To undertake a more detailed structural analysis of the P130$^{gag-fps}$ proteins, and to identify the mutant phenylalanine-1073-containing tryptic peptide I used in vivo labelling with [^{35}S]methionine. wtFSV P130$^{gag-fps}$ contains [^{35}S]methionine-labelled tryptic peptide species which co-migrate with tryptic phosphopeptides 3a, 3b and 3c of ^{32}P-labelled P130$^{gag-fps}$ (Figure 4.6, Map A). Presumably they correspond to the phosphorylated form of the tyrosine-1073-containing tryptic peptide with an unmodified (3c) or modified (3a, 3b) N-terminal glutamine, and are designated 3a^{p-y}, 3b^{p-y} and 3c^{p-y} to indicate the presence of a phosphotyrosine. To confirm the identity of these methionine-containing tryptic peptides I have used a variant of FSV which is temperature-sensitive for transformation (tsFSV L-5) and which encodes a P140$^{gag-fps}$ protein which is highly phosphorylated at tyrosine in cells maintained at the permissive temperature for transformation (37°C) but only poorly phosphorylated at tyrosine at the nonpermissive temperature (41.5°C) (Pawson, et al., 1980). tsFSV P140$^{gag-fps}$ from infected chicken embryo fibroblasts grown at 37°C contains [^{35}S]methionine-labelled tryptic peptides 3a^{p-y}-3c^{p-y} in similar yield to wtFSV P130$^{gag-fps}$ (Figure 4.6, map J), but these peptides are barely detectable in P140$^{gag-fps}$ from infected cells maintained at 41.5°C (Figure 4.6,

map K), as would be expected if they contained a reversibly phosphorylated tyrosine residue. I then analysed a tryptic digest of methionine-labelled mutant FSV-F(1073) P130$^{gag-fps}$ (Figure 4.6, map D) and could detect no spots corresponding to phosphorylated 3a^{p-y}-3c^{p-y}, consistent with predicted substitution of phenylalanine for tyrosine-1073. In addition to the absence of 3a^{p-y}-3c^{p-y}, mutant P130$^{gag-fps}$ lacks two further wild type peptides (designated 3b^y, 3c^y on maps of wild-type and revertant P130$^{gag-fps}$) but has acquired two novel peptides (designated 3b^f and 3c^f). The 3b^y and 3c^y wild-type peptides missing from the digest of mutant P130$^{gag-fps}$ migrate as if they were less negatively charged and more hydrophobic than the phosphorylated 3b^{p-y} and 3c^{p-y}. My interpretation of these data is that wild-type peptides 3b^y and 3c^y correspond to the non-phosphorylated forms of the tyrosine-1073-containing tryptic peptide and that the mutant peptides 3b^f and 3b^f represent the same tryptic peptide but with phenylalanine at residue 1073. Mutant 3b^f and 3c^f peptides migrate very similarly to 3b^y and 3c^y, but move more rapidly in the chromatographic dimension of the 2D map consistent with the increased hydrophobicity expected from substituting phenylalanine for tyrosine. With these exceptions, the methionine containing tryptic peptides of mutant P130$^{gag-fps}$ are identical to those of wtFSV P130$^{gag-fps}$. The tryptic peptide map of [^{35}S]methionine-labelled revertant P130$^{gag-fps}$ (Figure 4.6, map G) has a similar pattern to that of the wild-type protein, indicating that the differences between wild-type and mutant tryptic peptides result solely from the substitution at residue 1073.

These data all support the assertions that tyrosine-1073 is the major site of P130$^{gag-fps}$ tyrosine phosphorylation, that this residue has been substituted with phenylalanine and is no longer phosphorylated in FSV-F(1073), and has been restored to tyrosine and is once more phosphorylated in FSV-Y(1073).

4.2.5 Tyrosine Phosphorylation in wtFSV and FSV-F(1073)-transformed Cells

wtFSV P130$^{gag-fps}$ is phosphorylated at three tyrosine residues in FSV-transformed cells. A minor and variable site of tyrosine phosphorylation lies within the N-terminal gag region (peptide 1) and two phosphotyrosine sites are located in the fps-encoded region: the major site at tyrosine-1073 (peptides 3a-3c) and an additional fps site contained within peptide 4 (Figure 4.6, map B). Several serine residues are also phosphorylated. wtFSV and FSV-F(1073) P130$^{gag-fps}$ proteins were isolated from ^{32}P-labelled transformed rat-2 cells and analysed for phosphoamino acid content and by tryptic phosphopeptide mapping. The mutant protein contained approximately two-fold less phosphotyrosine, relative to its total phosphoamino acids, than the wild-type protein (Figure 4.7, Table 4.2) and this is explained by the absence of tryptic phosphopeptides 3a-3c from digests of in vivo-labelled FSV-F(1073) P130$^{gag-fps}$ (Figure 4.6, map E). The mutant protein is still phosphorylated in vivo at the second fps tyrosine site (peptide 4) and at the minor gag tyrosine site

Figure 4.7   Phosphoamino acid analysis of P130$^{gag-fps}$.   Rat-2
cells transformed with wtFSV (A) or mutant FSV-F(1073)
(B) were labelled with ^{32}P-orthophosphate for 4
hours, lysed, immunoprecipitated with anti-p19^{gag}
serum and the immunoprecipitates analysed by gel elec-
trophoresis. ^{32}P-labelled   P130$^{gag-fps}$ as
recovered from the gels, acid-hydrolyzed and analysed
for phosphoamino acids by two-dimensional electro-
phoresis at pH 1.9 and pH 3.5 followed by autoradio-
graphy.   The positions of marker phosphoserine (S),
phosphothreonine (T) and phosphotyrosine (Y) as revealed
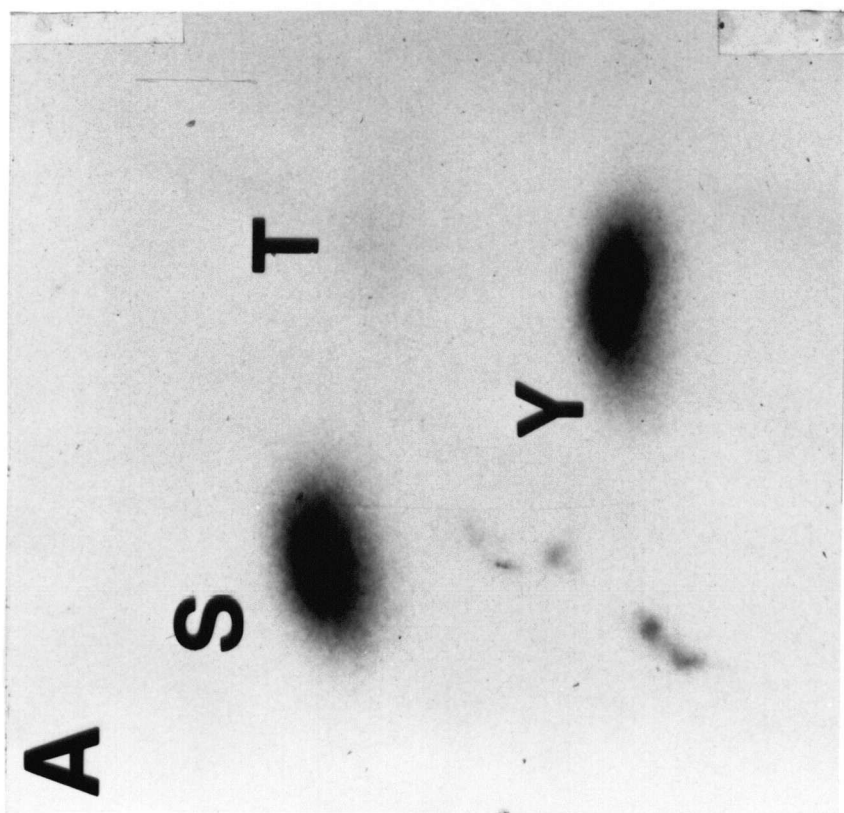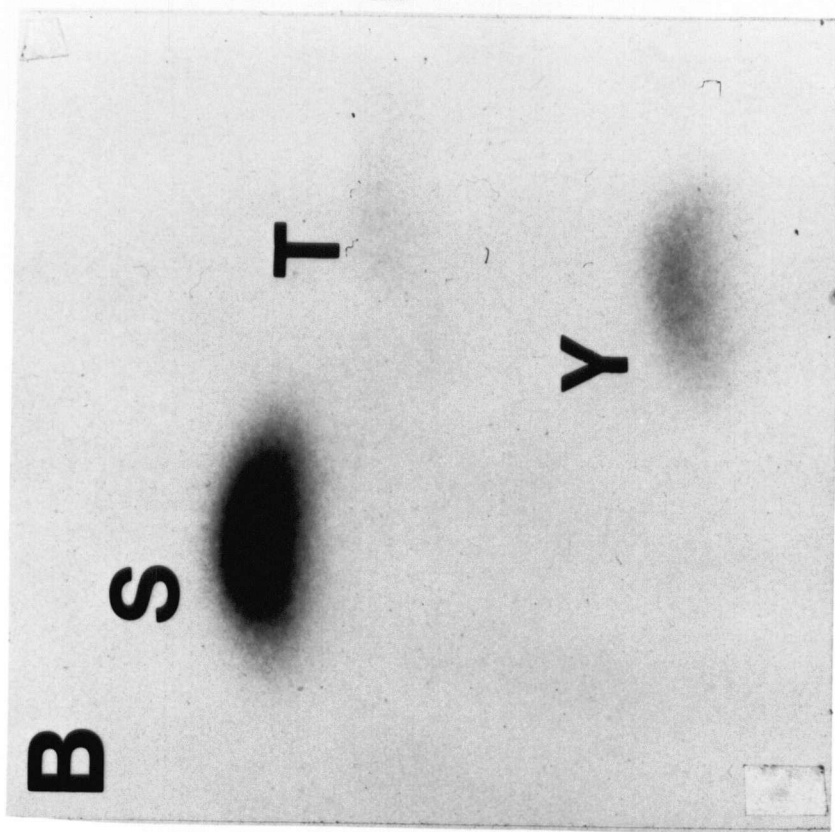by ninhydrin staining are indicated.

TABLE 4.2

Phosphoamino Acid Analysis of P130$^{gag-fps}$

and Total Cellular Protein

Phosphoamino acid	P130$^{gag-fps}$		Total Cell Protein		
	wtFSV	FSV-F(1073)	rat-2	wtFSV^a	FSV-F(1073)^b
p-serine	65^c	82	95	90	91
p-threonine	13	8	5	9.7	8
p-tyrosine	22	10	.07	.39	.27

Radioactivity in individual phosphoamino acids of P130$^{gag-fps}$ isolated from ^{32}P-labelled cells or of total cellular ^{32}P-labelled protein was determined by scintillation counting of aspirated thin-layer cellulose spots following electrophoretic separation of acid hydrolysates (Figures 4.7 and 4.8).

a   wtFSV-transformed rat-2 cells.

b   FSV-F(1073)-transformed rat-2 cells.

c   Values for each phosphoamino acid are expressed as a percentage of total phosphoamino acids for that sample.

(peptide 1), though this latter phosphopeptide is not apparent in the map shown in Figure 4.6. In addition FSV-F(1073) $P130^{gag-fps}$ is phosphorylated at the same serine sites <u>in vivo</u> as wtFSV $P130^{gag-fps}$, as judged by the migration of phosphoserine-containing tryptic peptides. Thus the loss of tyrosine-1073 does not appear to qualitatively affect the phosphorylation of other tyrosine or serine residues within $P130^{gag-fps}$. A tryptic phosphopeptide map of revertant FSV-Y(1073) $P130^{gag-fps}$ labelled <u>in vivo</u> with $^{32}P_i$ (Figure 4.6, map H) once more contained peptides 3a-3c. In a series of independent tryptic digests the only consistent difference between the tryptic phosphopeptides of wtFSV, FSV-F(1073) and FSV-Y(1073) $P130^{gag-fps}$ was the absence of 3a-3c from the mutant protein.

Transformation by FSV induces an increase in total cell phosphotyrosine resulting from phosphorylation of a variety of cellular proteins at tyrosine (Pawson, <u>et al</u>., 1980; Cooper and Hunter, 1983b). Normal rat-2 cells and the rat-2 lines transformed by wtFSV or the mutant FSV-F(1073) were labelled with ^{32}P-orthophosphate and analysed for whole cell phosphoamino acid content (Figure 4.8; Table 4.2). In one experiment wtFSV-transformed cells had 5.6-fold more phosphotyrosine than normal rat-2 cells, and rat-2 cells transformed by the mutant FSV-F(1073) showed a 3.8-fold elevation of phosphotyrosine. FSV-F(1073) can therefore still induce the phosphorylation of cellular proteins at tyrosine, indicating that the mutant protein is active as a kinase <u>in vivo</u>.

Figure 4.8   Whole cell phosphoamino acid analysis. Normal rat-2 cells (A) or rat-2 cells transformed by wtFSV (B) or mutant FSV-F(1073) (C) were labelled for 5 hours with $^{32}P_i$ and total cellular protein was extracted, acid-hydrolyzed and separated in two dimensions by electrophoresis at pH 1.9 and pH 3.5. The mobilities of marker phosphoamino acids are phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) as identifed by ninhydrin staining.
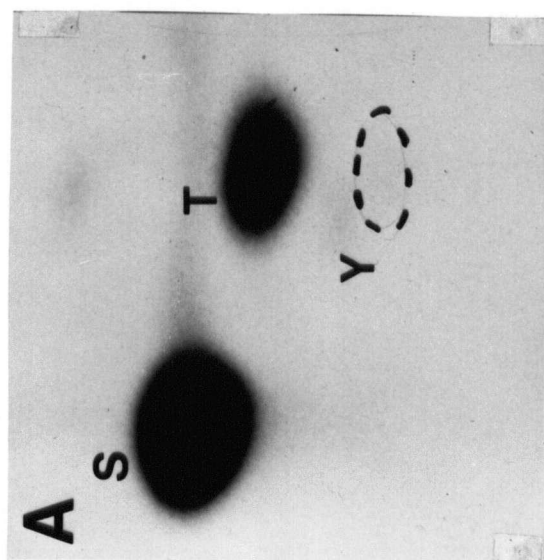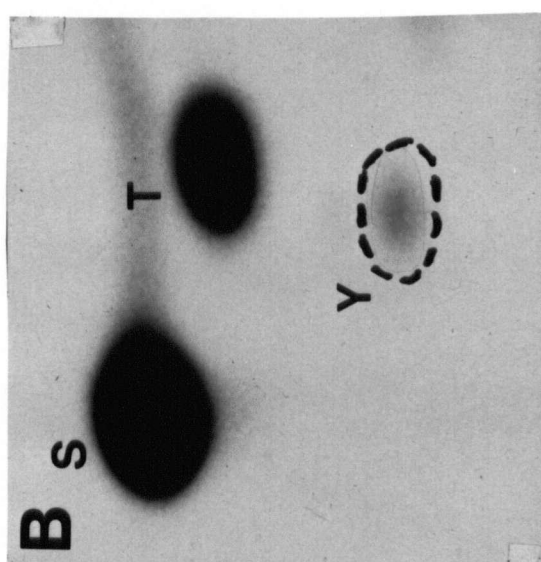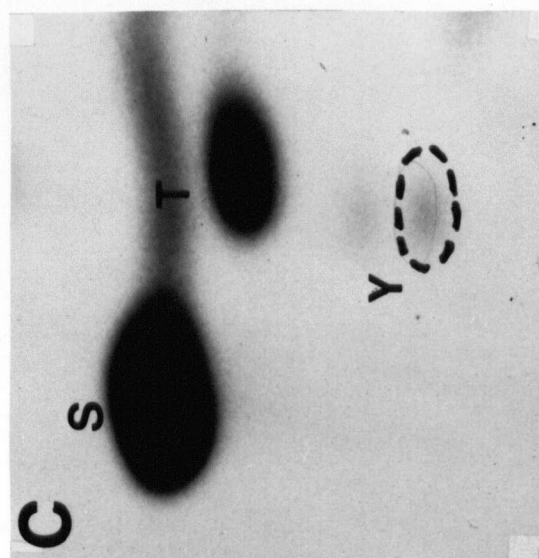
4.3 Discussion

The substitution of phenylalanine for tyrosine at residue 1073 of P130$^{gag-fps}$ does not abolish the ability of FSV to transform rat-2 cells. However there is a marked delay in the appearance of foci in rat-2 cells transfected with the mutant compared with those transfected with wtFSV. The long latent period of focus formation of FSV-F(1073) must reflect the loss of tyrosine-1073 since the revertant FSV-Y(1073) is rapidly transforming, and this suggests that the mutant protein is functionally altered as a result of the conversion of tyrosine-1073 to phenylalanine. The tyrosine-specific kinase activity of the mutant protein, as measured by in vitro phosphorylation of rabbit muscle enolase, is reduced five-fold compared with wtFSV or revertant P130$^{gag-fps}$. It is reasonable to propose that the reduced kinase activity of the mutant FSV-F(1073) P130$^{gag-fps}$ results from its inability to become phosphorylated at residue 1073 and that this reduced enzymatic activity is responsible for the ineffi-ciency of the mutant FSV-F(1073) in inducing transformation of rat-2 cells. This conclusion rests on the assumption that phenylalanine at position 1073 does not disturb the conformation and activity of P130$^{gag-fps}$.

The functional differences between the mutant and wild-type P130$^{gag-fps}$ can be attributed to the substitution at residue 1073, since the wtFSV and revertant FSV-Y(1073) DNAs and P130$^{gag-fps}$ proteins are identical in all aspects of structure and biological

activity tested. These findings also demonstrate the stability of the 4.7 kbp FSV genome in the M13mp10 vector through two cycles of oligonucleotide-directed mutagenesis.

There are several examples of serine-specific protein kinases whose activities are enhanced by their own phosphorylation at serine; the stimulation of phosphorylase kinase activity by phosphorylation of its B-subunit by cAMP-dependent protein kinase is well-documented (Cohen, 1982). There is some circumstantial evidence that the activity of tyrosine-specific protein kinases may be stimulated by their own phosphorylation at tyrosine (see section 1.10.1). Preincubation of purified insulin receptor or RSV p60^{src} under conditions promoting their autophosphorylation enhances their subsequent ability to phosphorylate exogenous substrates (Rosen, et al, 1983; Purchio, et al., 1983). The 98 kd product of the normal avian cellular fps gene (NCP98) is expressed in bone marrow cells (Shibuya, et al., 1982a). NCP98 possesses in vitro autophosphorylating tyrosine-specific kinase activity but is not detectably phosphorylated at tyrosine in cultured cells (Mathey-Prevot, et al., 1982). It is possible that the phosphorylation of P130$^{gag-fps}$ in vivo represents a unique feature of the viral transforming protein which fortuitously leads to increased kinase activity. Perhaps, NCP98 becomes transiently phosphorylated at tyrosine in the animal in response to a specific environmental signal (which is lost in tissue culture) resulting in turn in a short-lived stimulation of NCP98 activity and function at a defined stage of hematophoiesis.

The decreased kinase activity and inefficient transforming function of the FSV-F(1073) mutant protein indicate that the high level of wtFSV P130$^{gag-fps}$ phosphorylation at tyrosine-1073 results in constitutive enzymatic activation which in turn is important in the induction of unregulated cellular proliferation.

The basis for the long latency of transformation by FSV-F(1073) is unknown. Recombination with c-fps resulting in restoration of the codon for tyrosine-1073 does not seem likely since all of the P130$^{gag-fps}$ detectable in FSV-F(1073)-transformed cells retains phenylalanine at residue 1073 as judged by peptide mapping and migrates more rapidly than wtFSV P130$^{gag-fps}$ in SDS-polyacrylamide gel electrophoresis. It is possible that the latent period reflects the requirement for activation of a cellular gene that co-operates with mutant P130$^{gag-fps}$ in inducing the transformed phenotype, as suggested for the transformation of B-lymphocytes by Abelson murine leukemia virus (Ab-MuLV) whose transforming protein is also a tyrosinespecific protein kinase (Whitlock, et al., 1983). Even though the molecular basis of the enhanced latent period of transformation associated with the mutant FSV-F(1073) is unknown, it must reflect the oncogenic status of the mutant transformed cells since these cells also induce tumors in animals only after a longer period of time than that required by the wtFSV or revertant FSV-Y(1073) transformed cells.

The phenotypic consequences of changing tyrosine-1073 of FSV P130$^{gag-fps}$ are much more dramatic than those observed for corresponding mutants of RSV src. The major site of RSV p60^{src} tyrosine phosphorylation, tyrosine-416, which is homologous with P130$^{gag-fps}$ tyrosine-1073 has recently been changed to phenylalanine (Snyder, et al., 1983) or deleted (Cross and Hanafusa, 1983). Cross and Hanafusa reported a slightly longer latent period for transformation, decreased tumorigenicity and possible reduction in kinase activity of p60^{src} for the pSR-XDT10-1 deletion mutant of RSV src. However, since this construction contained a small deletion and substitution these biological effects could not be certainly ascribed to the loss of tyrosine-416. Synder, et al., (1983) have observed that the substitution of p60^{src} tyrosine-416 with phenylalanine has no apparent effect on transformation of mouse fibroblasts in culture or p60^{src} kinase activity in vitro, in contrast to my results with FSV. Although further investigations reveal that this src mutant is very poorly tumorigenic suggesting that it has indeed sustained a functional lesion (Snyder and Bishop, 1984).

These results do not exclude the possibility that some activity of FSV P130$^{gag-fps}$ other than tyrosine phosphorylation is involved in its transforming ability. However, these data strongly suggest that tyrosine phosphorylation can modulate enzymatic activity, and therefore strengthen the case for involvement of tyrosine phosphorylation in transformation by FSV and similar viruses. The

apparent effect of tyrosine-1073 phosphorylation on FSV P130$^{gag-fps}$ activity is consistent with the general concept that reversible protein phosphorylation at a single site can enhance or depress activity by converting an enzyme from one structural form to another, but does not usually act as an absolute functional activator or inhibitor. Apparently phosphorylation of the FSV transforming protein at tyrosine is an important factor in regulating its oncogenic action.

CHAPTER 5

5.0 The Protein Kinase Activity of FSV P130$^{gag-fps}$ Shows a Strict Specificity for Tyrosine Residues.

5.1 Introduction

A number of oncogenic viruses encode transforming proteins with protein kinase activities specific for tyrosine residues (Bishop and Varmus, 1982; Cooper and Hunter, 1983b). Tyrosine phosphorylation is a relatively rare event and appears to be correlated with cellular transformation. As a result, the basis on which oncogenic tyrosine protein kinases select their substrates is of interest as it likely relates to the mechanism of viral transformation.

Other kinases, such as the cAMP-dependent protein kinases appear to recognize their substrates by the primary sequence around the phosphorylated residue. Studies using synthetic peptide substrates show that the serines phosphorylated by the cAMP-dependent protein kinases usually have one or two basic residues on the N-terminal side of the phosphorylated amino acid (see section 1.9). However, a number of other studies have indicated that these enzymes must also recognize a specific secondary structure in their substrates (Shenolikar and Cohen, 1978; Zetterquist and Ragnarsson, 1982).

Synthetic peptides have also been used to determine the substrate specificity of tyrosine protein kinases. The presence of acidic residues on the N-terminal side of the phosphorylatable tyrosine appears to be a factor in substrate recognition by several tyrosine protein kinases (Patschinsky, et al., 1982; Pike, et al., 1982; Hunter, 1982). However, some of the sites phosphorylated in proteins do not have acidic residues on the N-terminal side of the phosphorylated tyrosine residue (Gallis, et al., 1983; Guild, et al., 1983; Cooper, et al., 1984a). In addition, the angiotensin peptides are reasonably good substrates for a number of tyrosine protein kinases, even though none of them have a large number of acidic residues in their sequences (Wong and Goldberg, 1983b). Therefore it appears that factors other than the presence of acidic residues contribute to the recognition of phosphorylation by tyrosine kinases. As in the case of the cyclic nucleotide dependent protein kinases, secondary structure is probably also an important recognition factor. In any case, the studies using synthetic peptides as substrates suggest that tyrosine kinases have a strict specificity for phosphorylating tyrosine residues (Pike, et al., 1982; Hunter, 1982; Wong and Goldberg, 1983b).

In contrast, there is evidence to suggest that some of the tyrosine kinases may phosphorylate non-protein substrates such as glycerol (Richert, 1983), diacylglycerol and phosphatidylinositol (Sugimoto, et al., 1984; Macara, et al., 1984), which raises questions as to their substrate specificity in general, and the physiological

relevance of tyrosine phosphorylation in particular. However, the high concentrations of glycerol required to detect the phosphorylated product makes it unlikely that the enzyme functions as a glycerol kinase in vivo. In addition, the purity of the enzyme preparations used to demonstrate lipid phosphorylation are somewhat questionable.

I have investigated the specificity of the protein kinase activity intrinsic to FSV $P130^{gag-fps}$ by using site-directed mutagenesis to change the codon for tyrosine-1073 to those for the other commonly phosphorylated hydroxyamino acids serine and threonine. This approach has several advantages over the use of synthetic peptides to define the protein kinase recognition site. Most important, the protein containing the altered target site may be expressed in intact cells, which allows the specificity and dynamics of phosphorylaton to be examined in vivo. As a consequence, such studies will not only allow the primary structure of the enzyme recognition site to be considered, but may also allow the contributions of secondary and tertiary structure to be evaluated.

5.2 Results

5.2.1 Oligonucleotide-directed Mutagenesis of FSV

The 4.7 kilobase FSV genome was subcloned from λ-FSV-2 (Shibuya, et al., 1982b) into the M13mp10 bacteriophage vector to

provide a template for oligonucleotide-directed mutagenesis as previously described (section 4.2.1). The oligonucleotides used to change the TAT codon for tyrosine-1073 within P130$^{gag-fps}$ to those for serine, threonine or glycine residues are shown in Figure 5.1. The procedures for mutagenesis, bacterial transformation, isolation of phage and the hybridization technique used to screen for mutant phage were exactly as described in section 4.2.1 of the preceding chapter. DNAs isolated from the putative mutant phage were sequenced in the region encompassing the mutation site to confirm the desired mutations (Figure 5.2). The sequences are all identical except for the TAT codon for tyrosine-1073 which has been changed to a TCT codon for serine in [FSV-S(1073)], a ACT codon for threonine in [FSV-T(1073)] and a GGT codon for glycine in [FSV-G(1073)].

To ensure that any functional changes associated with the mutants were due to the specifically induced mutations and not the product of random second-site mutations that could have occurred during the mutagenesis procedure, the mutated genomes were each reverted using an oligonucleotide encoding the wild-type sequence (see Figure 5.1). The same protocol described above was used to revert the mutant genomes. The nucleotide sequences of these revertant phage were shown to be identical to the wtFSV in the region of interest (data not shown). Therefore any biological activity of wtFSV that is altered in any of the mutants but restored to wild-type function in the revertant FSV-Y(1073) genomes must be due solely to the amino acid change at residue 1073 in P130$^{gag-fps}$.

Figure 5.1:   The synthetic oligonucleotides used to mutate the codon for tyrosine–1073 of FSV P130$^{gag-fps}$.   Oligonucleotides 16 long were designed to alter the codon for residue 1073 by one or two nucleotides to code for a serine, threonine or glycine as indicated.   The mutagenic oligonucleotide encoding the wild type codon TAT was used to revert each of the mutants to FSV–Y(1073).   The figure displays amino acids 1066–1080 of wtFSV P130$^{gag-fps}$ and their encoding nucleotide sequence.

N .... ArgGlnGluGluAspGlyVal<u>Tyr</u>AlaSerThrGlyGlyMetLys C

5' .... CGGCAGGAGGAGGATGGTGTC<u>TAT</u>GCCTCCACGGGGGGCATGAAG .... 3'   WILD TYPE FSV

        5' – TGGTGTC<u>TCT</u>GCCTCC – 3'   Ser FSV–S(1073)

                        ACT   Thr FSV–T(1073)

                        GGT   Gly FSV–G(1073)

                        TAT   Tyr FSV–Y(1073)

Figure 5.2:   Partial DNA sequencing of wtFSV and mutant [FSV-S(1073);

FSV-T(1073); FSV-G(1073)] M13mp10 inserts.   The nucleo-

tide sequences encompassing the codon for tyrosine-1073

of wt FSV and the mutation sites of FSV-S(1073),

FSV-T(1073), and FSV-G(1073) DNAs were determined by the

dideoxynucleotide chain termination method.   The codons

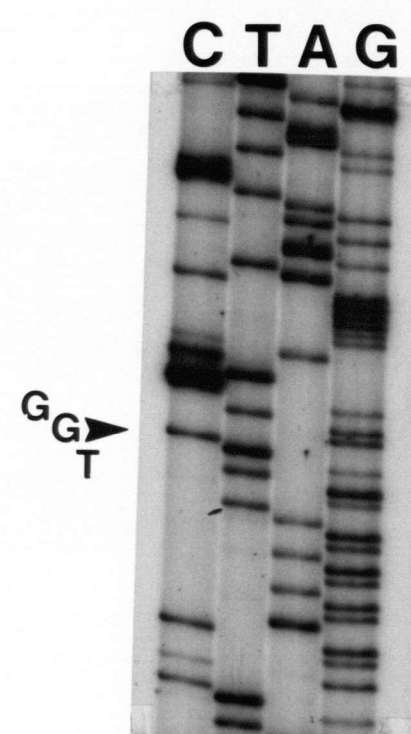for amino acid 1073 are indicated in each case.

C T A G   C T A G   C T A G   C T A G
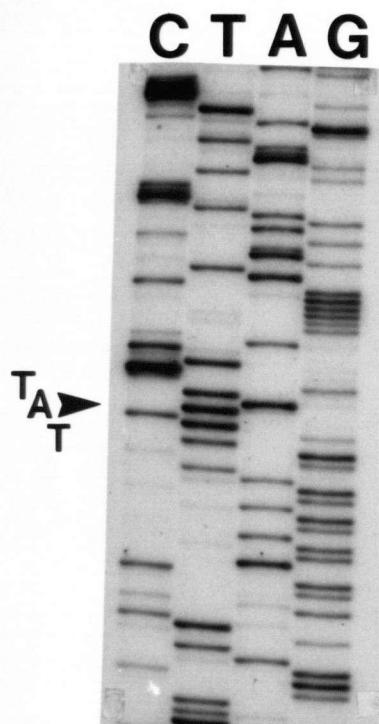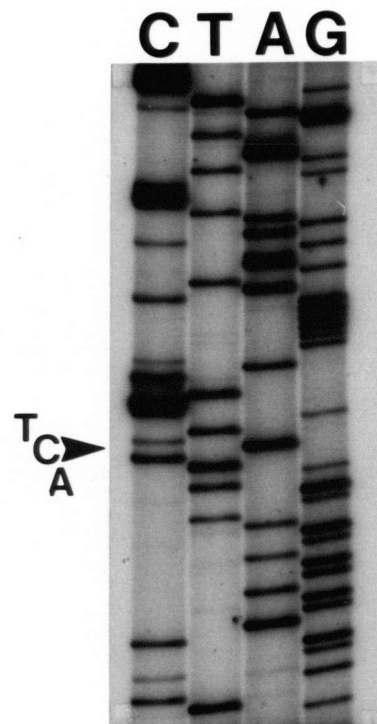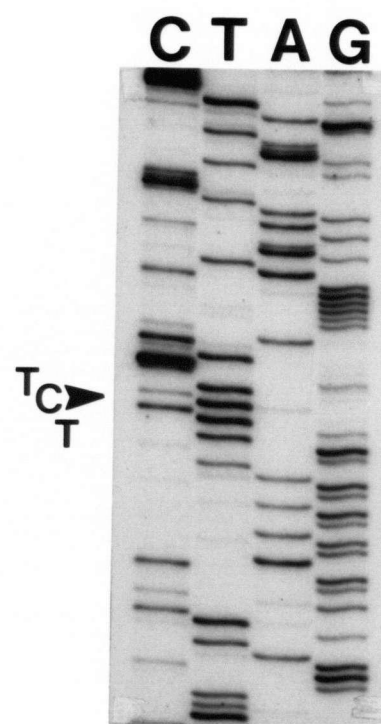
Y-1073   T-1073   S-1073   G-1073

5.2.2 Transforming Activity of Wild-type, Mutants and Their Revertant FSV DNAs.

The wild-type (wtFSV), revertants [FSV-Y(1073)] or mutated [FSV-S(1073), FSV-T(1073) and FSV-G(1073)] DNAs were isolated, purified as described in Chapter 4 and transfected onto rat-2 cells using the calcium phosphate coprecipitation technique (Graham and Van der Eb, 1973; Wigler, et al., 1979). Foci of transformed cells appeared approximately eight to ten days post-transfection with both wt and the revertant DNAs, but were not obvious until at least 30 days following transfection with the mutated DNAs. Cell lines cloned from foci induced by the FSV mutants were also not as overtly transformed by morphological criteria as cells expressing wt or revertant DNAs (Figure 5.3). Nonetheless, these cells demonstrated anchorage independent growth in soft agar unlike the normal rat-2 cells (data not shown). FSV-S(1073) transformed cells also induced tumors in syngeneic immuno-competent rats, but did so with a longer latent period than wt or revertant transformed cells. Therefore changes in the TAT codon for tyrosine-1073 to codons for serine, threonine or glycine resulted in mutant FSV DNAs that were still transformation-competent, but which transformed cells with a long latent period and induced a less tumorigenic phenotype compared with wt or revertant DNAs. The mutants were similar in biological activity to the previously isolated FSV-F(1073) mutant described in Chapter 4.

Figure 5.3:   Transformation of rat-2 cells following transfection with FSV DNAs. Phase-contrast photomicrographs of rat-2 cells (40x). Cells were transfected with wtFSV or with FSV genomes encoding substitutions at residue 1073. a, serine, FSV-S(1073); b, threonine, FSV-T(1073); c, glycine, FSV-G(1073); d, untransfected rat-2 cells; e, tyrosine, wtFSV.
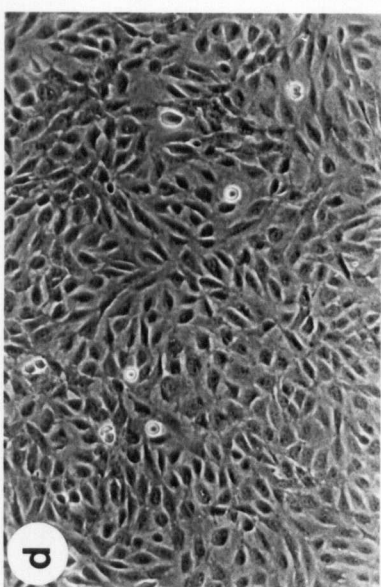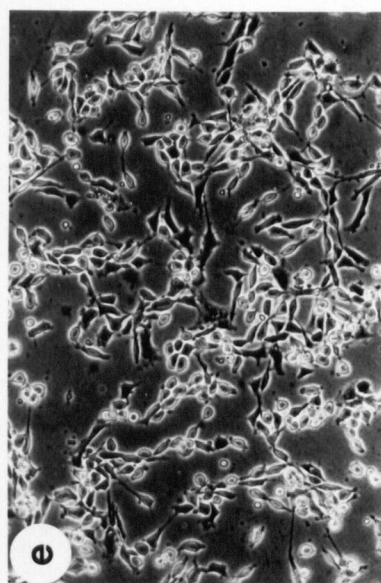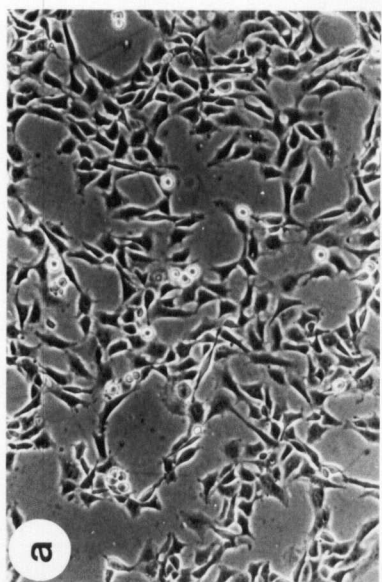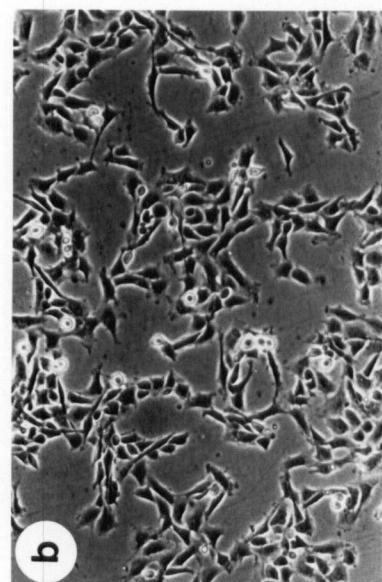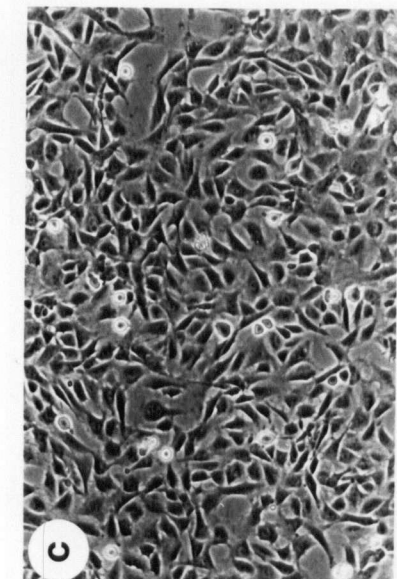
5.2.3   Expression, Structure and In Vitro Kinase Activities of wt and
Mutant Proteins

The transformed cells isolated following transfection with wt
or mutant FSV DNAs were examined for $P130^{gag-fps}$ expression, phos-
phorylation and associated kinase activity.   To identify $P130^{gag-fps}$
cells   were   metabolically   labelled   with $[^{35}S]$methionine   and
lysates were immunoprecipitated with a monoclonal antibody directed
against   avian $p19^{gag}$.   The   amount   of $[^{35}S]$methionine   incor-
porated   reflects   the   level   of $P130^{gag-fps}$ expression   which   varied
among   the   different   clones   of   wtFSV   and   mutant-transformed   cells
(Figure 5.4a).

A · number of· observations   suggested   that   the   long   latent
period for transformation and distinctive morphology associated with
cells transformed by mutant FSV DNAs were due to structural changes
introduced   into $P130^{gag-fps}$,   rather   than   decreased   expression
levels.   First, a clone of transformed cells isolated following trans-
fection with revertant FSV-Y(1073) DNA had approximately 5-fold less
$P130^{gag-fps}$ than   a   clone   of   mutant   transformed   cells,   yet   the
latter cells were not as overtly transformed as the revertant trans-
formed   cells · (data   not   shown).   Second,   a   clone   of   FSV-G(1073)
transformed cells had about 3-fold more $P130^{gag-fps}$ than a clone
of FSV-T(1073) transformed cells (Figure 5.4a), yet the FSV-G(1073)
transformed cells were flatter and less refractile than the FSV-T(1073)

Figure 5.4:   Expression of wtν and mutant P130$^{gag-fps}$ in trans-
formed rat-2 cells and quantitation of their respective
tyrosine protein kinase activities.   4a: Normal or
transformed rat-2 cells were labelled for 16 hours with
100 uci [^{35}S]methionine, immunoprecipitated with
anti-p19^{gag} serum and examined by gel electro-
phoresis to identify P130$^{gag-fps}$.   Lane A, rat-2
cells; lane B, FSV-T(1073); lane C, FSV-G(1073); land D,
FSV-S(1073); lane E, wtFSV. 4b: Cells from duplicate
unlabelled cultures were immunoprecipitated in an ident-
ical fashion and then incubated with 5 ug of acid-
denatured enolase in the presence of 10mM MnCl$_2$ and
2.5 uci [γ-^{32}P]ATP for 15 minutes at 30°C.
Samples were analysed by electrophoresis through a 7.5%
SDS-polyacrylamide gel and autoradiography.   Lane F,
rat-2 cells; lane G, FSV-T(1073); lane H, FSV-G(1073);
lane I, FSV-S(1073); lane J, wtFSV.
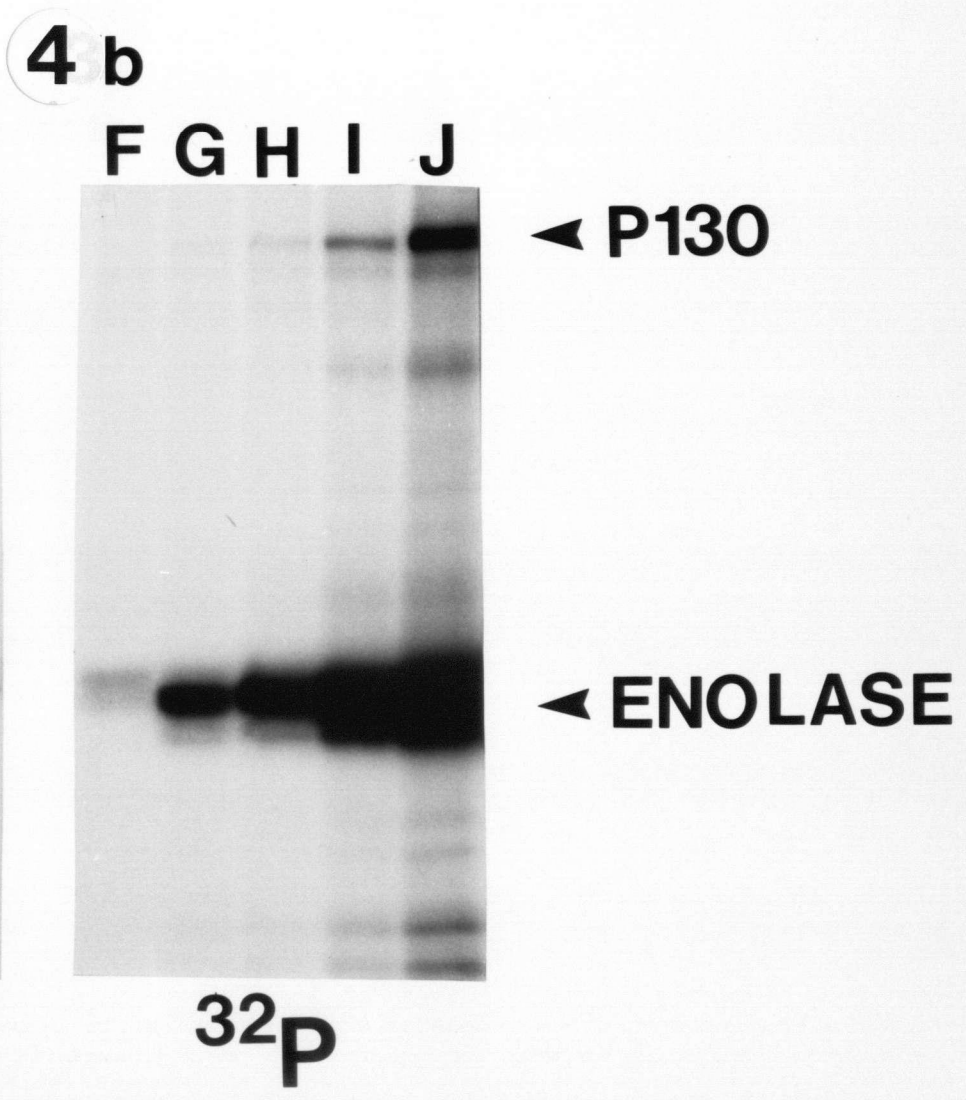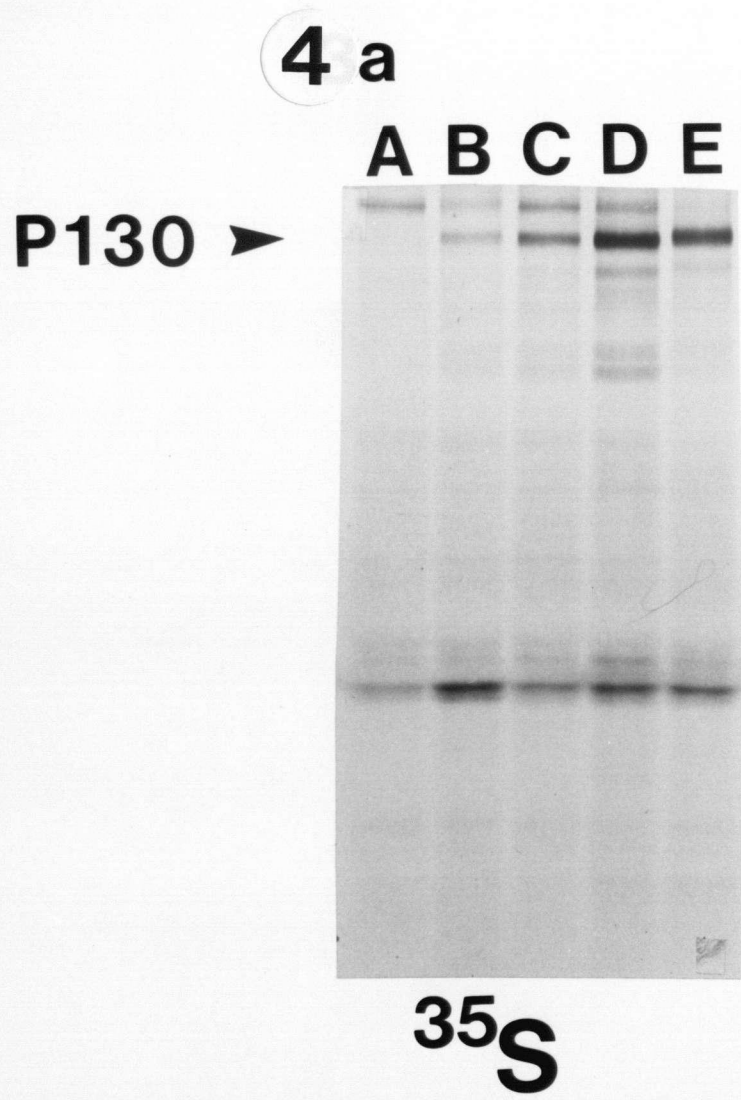
transformed cells (Figure 5.3).

In order to determine the relative kinase activities of the wt and mutant FSV P130$^{gag-fps}$ proteins, enolase was included in the immune complex kinase reactions as an exogenous substrate for tyrosine phosphorylation (Figure 5.4b). Quantitation of the extent of enolase phosphorylation by mutant and wt P130$^{gag-fps}$ proteins revealed that the kinase activities of FSV-S(1073), FSV-T(1073) and FSV-G(1073) proteins were 6.8, 4.7 and 6.3 fold lower, respectively, than that measured for wtP130$^{gag-fps}$. The tyrosine protein kinase activity of the wild-type and mutant proteins was quantitated by determining the ratio of ^{32}P-enolase, labelled by an _in vitro_ kinase reaction to that of the respective P130$^{gag-fps}$ metabolically labelled with [^{35}S]methionine (Figure 5.4). This ratio represents the extent of kinase activity per amount of P130$^{gag-fps}$ present in the reaction, and the values reported are an average of four independent experiments.

Cell lines that were cloned from each of the revertant FSV-Y(1073) transformed cells were shown to express P130$^{gag-fps}$ with similar _in vitro_ kinase activities and electrophoretic mobilities as wtFSV P130$^{gag-fps}$ (data not shown). Both biological and biochemical data indicated that the P130$^{gag-fps}$ proteins encoded by the revertant DNAs were structurally and functionally identical to

wtFSV P130$^{gag-fps}$ and therefore the revertant FSV-Y(1073) transforming proteins were not examined further.

Several lines of evidence show that the serine-1073 of FSV-S(1073) P130$^{gag-fps}$ and the threonine-1073 of FSV-T(1073) P130$^{gag-fps}$ do not become phosphorylated either in the rat-2 cells expressing these proteins or in vitro during immune complex kinase reactions. The electrophoretic mobilities of the mutant proteins were slightly greater than those of the wild-type P130$^{gag-fps}$, which may result from decreased phosphorylation (Figure 5.4). Autophosphorylation of the mutant proteins in vitro was reduced compared with that of wtFSV P130$^{gag-fps}$ (Figure 5.4b), consistent with the loss of the major autophosphorylation site. Conclusive evidence that residue 1073 was not phosphorylated in mutant proteins containing serine or threonine at this site was provided by comparing two-dimensional maps of ^{32}P-labelled tryptic phosphopeptides from mutant and wild type P130$^{gag-fps}$. Phosphorylated peptides 3a-3c of wtFSV P130$^{gag-fps}$ are derived from a single tryptic phosphopeptide containing residue 1073 (Chapters 3 and 4) and were clearly absent in tryptic peptide maps of FSV-S(1073) or FSV-T(1073) P130$^{gag-fps}$ phosphorylated in vitro (Figure 5.5). Similar analysis of P130$^{gag-fps}$ immunoprecipitated from cells metabolically labelled with ^{32}P-orthophosphate showed that residue 1073 was only phos-

Figure 5.5:   Tryptic phosphopeptide analysis of wt FSV and mutant FSV P130$^{gag-fps}$ proteins labelled <u>in vitro</u>. P130$^{gag-fps}$ encoded by wild-type and mutant FSVs were labelled in an immune complex kinase assay following immunoprecipitation with anti-p19^{gag} serum from transformed cells. The wt and mutant P130$^{gag-fps}$ proteins were gel purified, digested with trypsin and separated in two-dimensions on thin-layer cellulose plates. Electrophoresis at pH 2.1 was from left to right with the anode on the left, and chromatography was from bottom to top. An "O" indicates the sample origin. Tryptic digests were as follows: a, wtFSV; b, FSV-S(1073); c, FSV-G(1073); d, FSV-T(1073). The spot that is not numbered and occurs to the right of 3c is seen only <u>in vitro</u> and probably corresponds to the spot 2 found only <u>in vitro</u> with FSV-L5 P140$^{gag-fps}$.
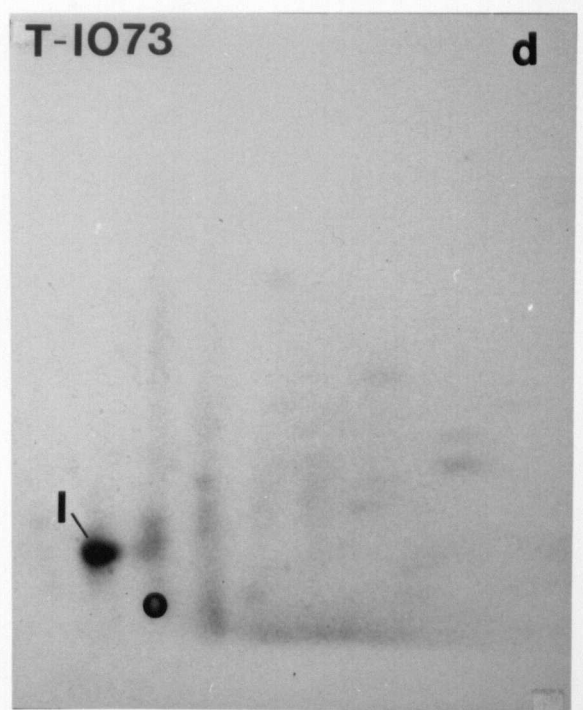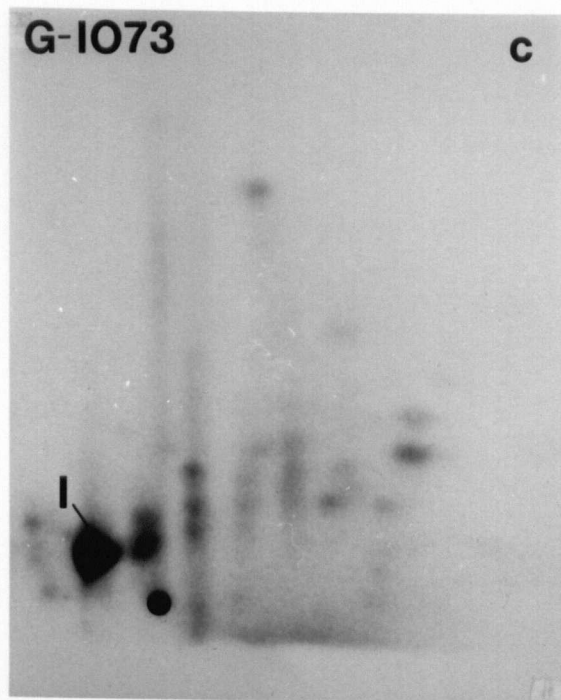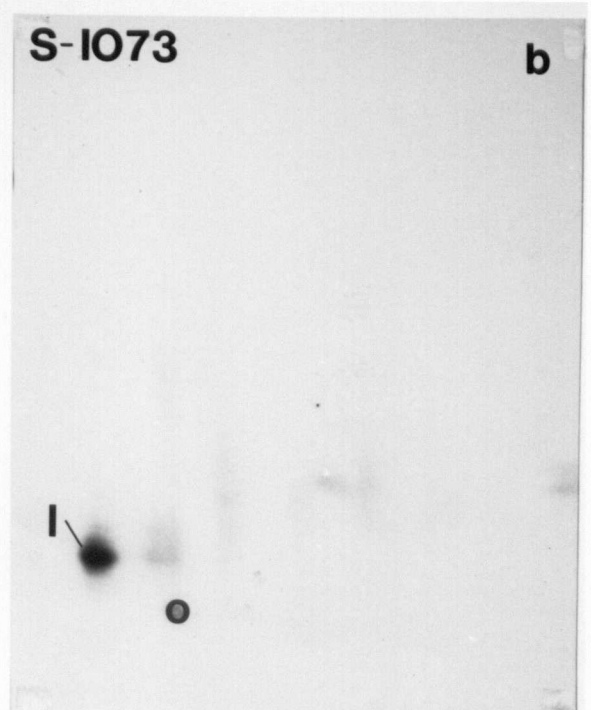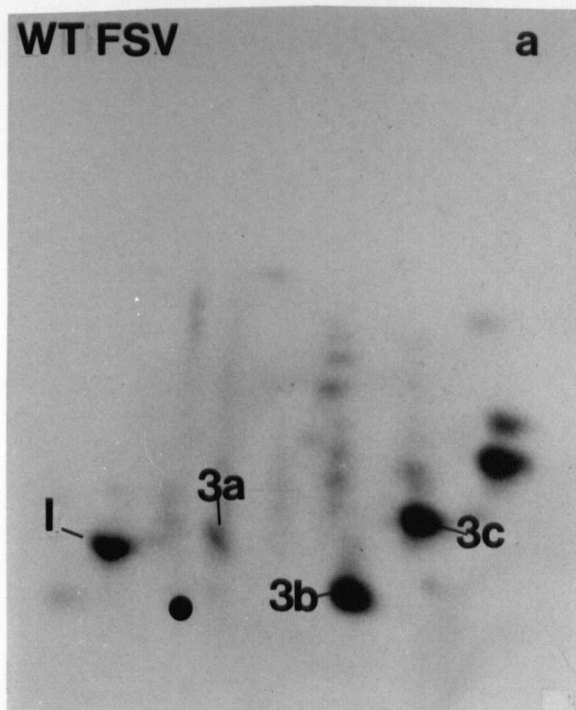
phorylated in the wild-type protein (Figure 5.6) consistent with the in vitro results. These tryptic peptide mapping studies support and confirm the assumption that neither serine nor threonine is phosphorylated when substituted for a tyrosine at residue 1073 within $P130^{gag-fps}$.

The retention of residual kinase activity and oncogenic potential by the mutant proteins suggests that they have not suffered gross conformational changes as a consequence of the amino acid substitutions at position 1073. However, the reduced kinase activity and oncogenic potential of these mutant proteins appears to correlate with a lack of phosphorylation at residue 1073 within FSV $P130^{gag-fps}$.

5.2.4 Tyrosine Phosphorylation of wtFSV and Mutant FSV $P130^{gag-fps}$ from Transformed Cells

Phosphoamino acid analysis of mutant $P130^{gag-fps}$ proteins isolated from ^{32}P-labelled cells showed that the mutant proteins contained substantially less phosphotyrosine than wt $P130^{gag-fps}$ (Figure 5.7). This is consistent with the tryptic phosphopeptide mapping results of the mutant proteins isolated from ^{32}P-metabolically labelled transformed cells and further confirms that the major site of tyrosine phosphorylation (Figure 5.6; spots 3a and 3c) was destroyed in these mutated $P130^{gag-fps}$ proteins. However, the loss of tyrosine-1073 did not appear to qualitatively

Figure 5.6: Tryptic phosphopeptide analysis of $P130^{gag-fps}$ encoded by wild-type and mutant FSVs. FSV $P130^{gag-fps}$ was labelled with ^{32}P <u>in vivo</u> by incubation of wtFSV transformed cells with ^{32}P-orthophosphate for 12 hours and mutant FSV transformed cells with ^{32}P-orthophosphate for 4 hours. The ^{32}P-labelled $P130^{gag-fps}$ proteins were isolated by subsequent immunoprecipitation of the labelled protein with anti-p19^{gag} serum. Gel-purified $P130^{gag-fps}$ was then digested with trypsin and separated by electrophoresis at pH 2.1 in the first dimension and chromatography in the second dimension. The anode is to the left and the cathode to the right. Tryptic digest were as follows: A, wtFSV; B, FSV-S(1073); C, FSV-G(1073); and D, FSV-T(1073).
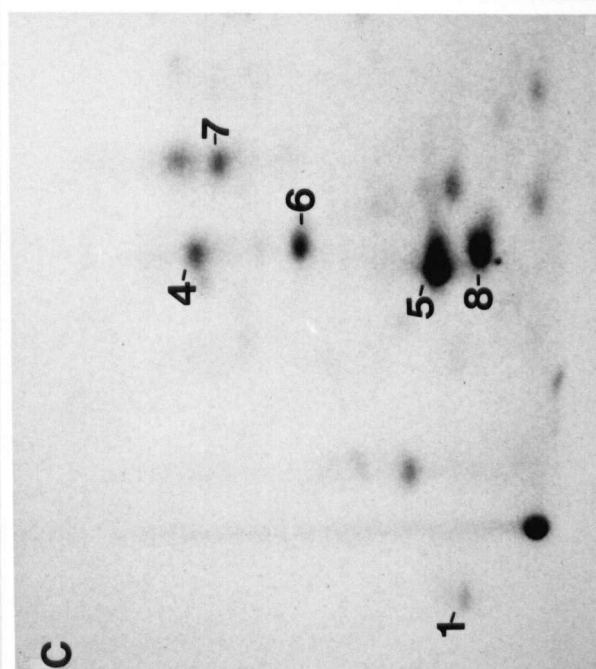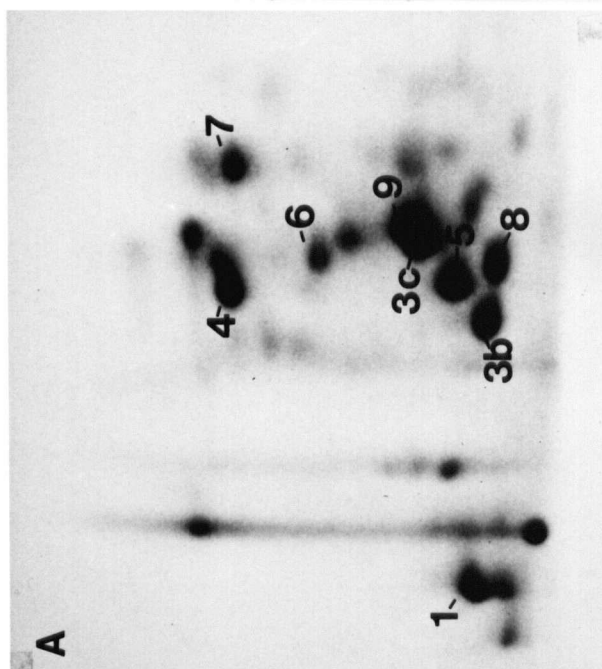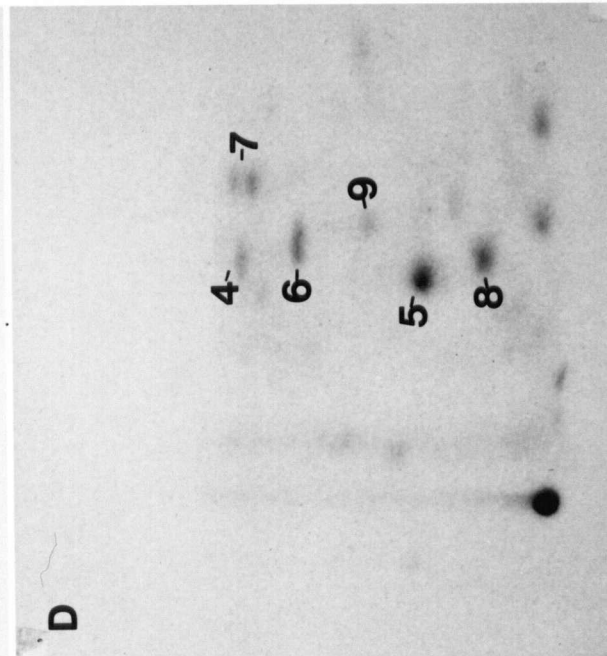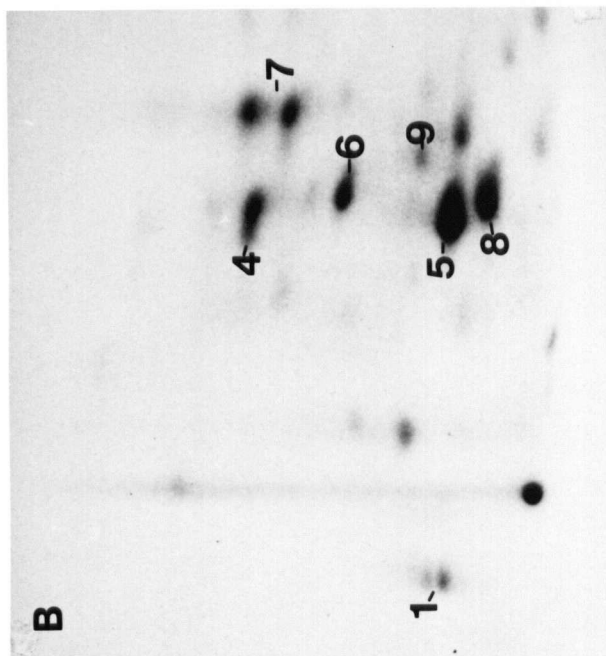
Figure 5.7:   Phosphoamino acid analysis of wtFSV and mutant FSV P130$^{gag-fps}$ proteins.   Rat-2 cells transformed with wtFSV or mutant FSV [FSV-S(1073); FSV-T(1073); FSV-G (1073)] were labelled with ^{32}P$_i$ for 12 hours, lysed, immunoprecipitated with anti-p19^{gag} serum and the immunoprecipitates separated by gel electro- phoresis. ^{32}P-labelled P130$^{gag-fps}$ was recov- ered from the gels, acid-hydrolyzed and analysed for phosphoamino acids by two-dimensional electrophoresis at pH 1.9 and pH 3.5 followed by autoradiography. The positions of marker phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) as revealed by ninhydrin staining are indicated.   A, wtFSV; B, FSV-S(1073); C, FSV-G(1073); and D, FSV-T(1073). The number of Cerenkov counts loaded were as follows: wtFSV, 1300 cpm; FSV-S(1073), 1100 cpm; FSV-G(1073), 540 cpm; and FSV-T(1073), 308 cpm.
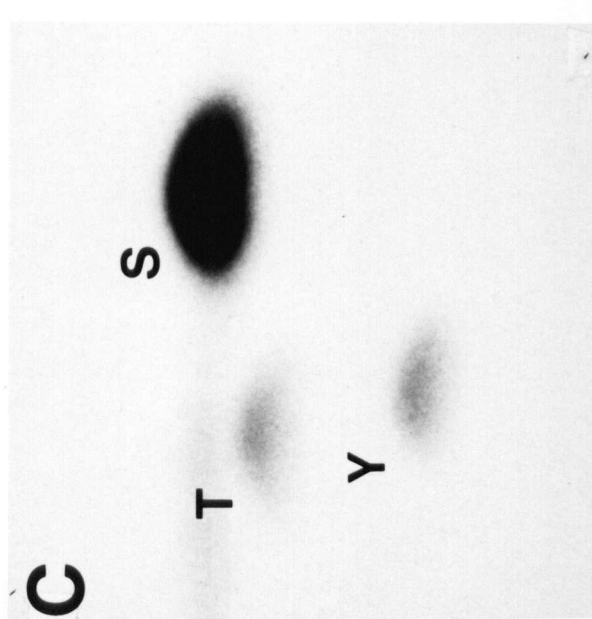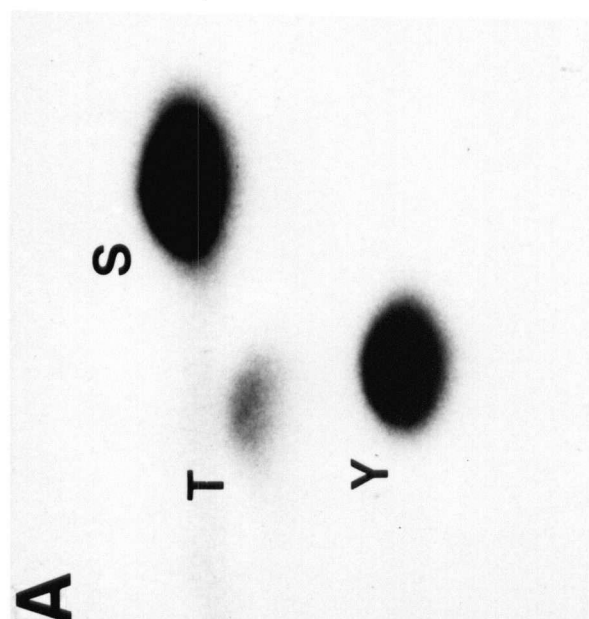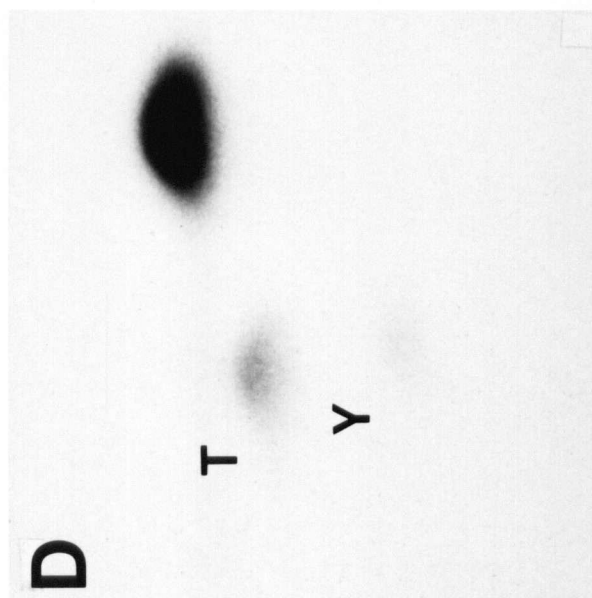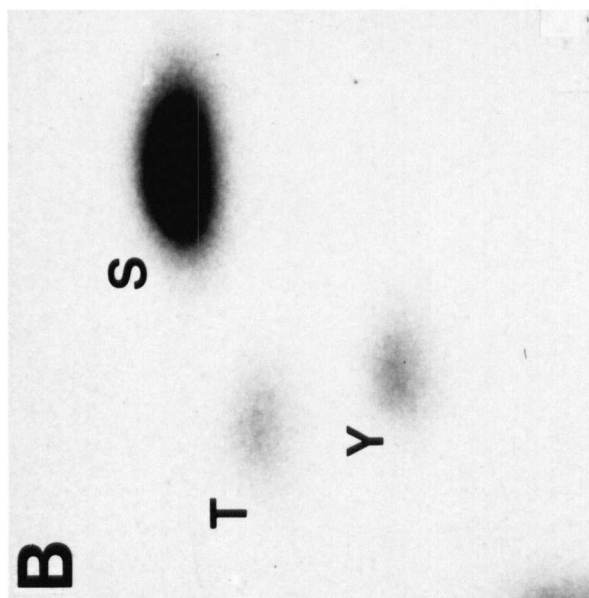
affect the phosphorylation of other tyrosine and serine residues within $P130^{gag-fps}$. The mutant $P130^{gag-fps}$ proteins were still phosphorylated at tryptic phosphopeptide 4, which represents the other major tyrosine site in wtFSV $P130^{gag-fps}$ phosphorylated only in vivo. Also, the minor and variable gag tyrosine site (peptide 1) was phosphorylated in the mutant proteins, although this phosphopeptide was not obvious in the map of FSV-T(1073) $P130^{gag-fps}$ shown in Figure 5.6. In addition, the uncharacterized phosphotyrosine containing peptide 6 and the major phoshoserine sites (peptides 5 and 8) were demonstrably phosphorylated in the mutant proteins. In fact, in a series of independent tryptic digests, metabolically labelled for 4 hours or 12 hours, with $^{32}P_i$, the only consistent difference between the tryptic phosphopeptides of wtFSV and mutated $P130^{gag-fps}$ proteins was the absence of 3a-3c from the mutant proteins [FSV-S(1073); FSV-T(1073); and FSV-G(1073)]. These tryptic phosphopeptide maps are similar to those reported for the previously isolated FSV-F(1073) mutant $P130^{gag-fps}$ described in the preceding chapter. Taken together these data suggest that the gross structure of these mutant proteins was probably maintained in the presence of the changes at position 1073 within $P130^{gag-fps}$

5.3 Discussion

The fact that neither serine nor threonine were phosphory-lated when placed at a tyrosine kinase recognition site is a satisfying

demonstration of the specificity of the P130$^{gag-fps}$ protein kinase activity for tyrosine residues. While it seems probable that the amino acids surrounding tyrosine-1073 of wt P130$^{gag-fps}$ and other tyrosines are important in targeting the kinase to a particular residue (Hunter, 1982; Pike, et al., 1982; Wong and Goldberg, 1983b; Braun, et al, 1984), it is apparent that there is a strict requirement for tyrosine at the site of phosphorylation.

It is interesting to speculate as to the reasons for this specificity. The mechanisms for transferring a phosphate group to the phenolic hydroxyl of a tyrosine residue may be quite different from those required to phosphorylate the less reactive aliphatic hydroxyls of serine or threonine. Although as discussed previously in Chapter 1, there is an obvious sequence relationship between the enzymatic domains of tyrosine protein kinases such as FSV P130$^{gag-fps}$, and those kinases specific for serine and threonine residues, such as the catalytic subunit of cAMP-dependent protein kinase. This suggests that the basic biochemical events involved in phosphorylation may be similar for all the hydroxyamino acids. In fact, kinetic studies suggest similar mechanisms of phosphate transfer whether the nucleophilic hydroxyl is on a serine or tyrosine residue (Erneux, et al., 1983; Bolen, et al., 1980; Wong and Goldberg, 1984). An alternative explanation might be that the active site of P130$^{gag-fps}$ may be folded such that only a tyrosine at residue 1073 can be positioned to accept a phosphate group. Substitutions at position 1073 might also disrupt a conformational signal formed by the surrounding recognition sequence.

Amino acid substitutions at the conserved tyrosine-1073 residue of P130$^{gag-fps}$ did not completely abolish its activity. I have changed tyrosine-1073 to serine, threonine, glycine and phenylalanine residues and have found that all of these mutant FSV genomes induced cells of nearly identical transformed phenotype albeit after a long latent period. In addition, these transformed cells expressed P130$^{gag-fps}$ with a similar reduced biochemical activity. These data indicate that phosphorylation of this highly conserved tyrosine does not have an essential function in catalysis, but rather may play a regulatory role.

The similar effects of these mutations was surprising considering that the R-groups of the substituting amino acids are quite different; phenylalanine possesses a hydrophobic aromatic ring, serine and threonine have polar aliphatic side chains, while glycine lacks a side chain and can introduce greater flexibility into the polypeptide. These considerations imply that the important characteristic of tyrosine-1073 is its ability to become phosphorylated. I have therefore concluded that the reduced kinase activity and transforming potential of the mutant proteins are not due to disruptive changes in protein conformation, but are a consequence of the absence of phosphorylation.

Although tyrosine-1073 appears to be important for the regulation of P130$^{gag-fps}$ tyrosine kinase activity, there are

additional tyrosine, serine and threonine phosphorylation sites within the $P130^{gag-fps}$ of FSV transformed cells which may also have a regulatory function.   Therefore, the activity and function of the FSV transforming protein may be subject to modulation by a number of cellular kinases, each with different amino acid substrate specifities.

CHAPTER 6

6.0 Site-directed Mutagenesis of Lysine-950 Within P130$^{gag-fps}$

Eliminates Both Its Kinase Activity and Transforming Ability.

6.1 Introduction

To understand more fully the biological roles of tyrosine protein kinases, it is important to identify the specific structural domains or amino acid residues that contribute to the enzymatic activities of these proteins. The catalytic domains of p60^{src}, P140$^{gag-fps}$ and the EGF receptor have been partially defined by limited tryptic digestion (Levinson, et al., 1981; Weinmaster et al., 1983; Brugge and Darrow, 1984; Basu et al., 1984). Since these cleavage fragments possess tyrosine kinase activity, they must, by definition, contain the active center which functions in ATP binding and phosphotransfer.

Barker and Dayhoff (1982) have shown that the cyclic-AMP dependent serine protein kinase (cAPK) and the p60^{src} tyrosine protein kinase are distantly related, based on sequence homology within their respective catalytic domains. More specifically, this homology includes a lysine residue that is proposed to function in ATP-binding.

The identity and putative function of this lysine was determined by studies using the reactive ATP analogue, p-fluorosulfonylbenzoyl 5' - adenosine (FSBA). Affinity labels such as FSBA are compounds that bind specifically and reversibly to a protein due to their complementarity with the active site and then react covalently with one or more amino acid residues within the active site by virtue of a small reactive group (Singer, 1977). The structure of FSBA is similar to that of ATP, except that the triphosphates have been replaced by a side chain of comparable size that contains a reactive sulfonylfluoride group in the position of the γ-phosphate (Figure 6.1). FSBA can react with the free electron pair of lysine, tyrosine, histidine (Zoller, et al., 1981) and cysteine (Togaski and Reisler, 1982), yielding an adduct of the sulfonylbenzoyladenosine moiety of FSBA and the amino acid. The use of affinity labels such as FSBA provides a means for locating specific regions of the protein that are potentially involved in binding or catalysis.

FSBA was shown to bind specifically to lysine-71 within cAPK (Zoller, et al., 1981) and lysine-295 within p60[src] (Kamps, et al., 1984). Covalent modification of these kinases with FSBA results in enzymatically inactive proteins. FSBA also inactivates the serine-specific cGMP-dependent protein kinase (cGPK), by covalently modifying the equivalent lysine in its ATP binding site (Hashimoto, et al., 1982). FSBA has also been useful in studies concerning the

Figure 6.1:   Comparison of the structure of p-fluorosulfonylbenzoyl-

5'-Adenosine (FSBA) and Adenosine 5'-triphosphate (ATP).
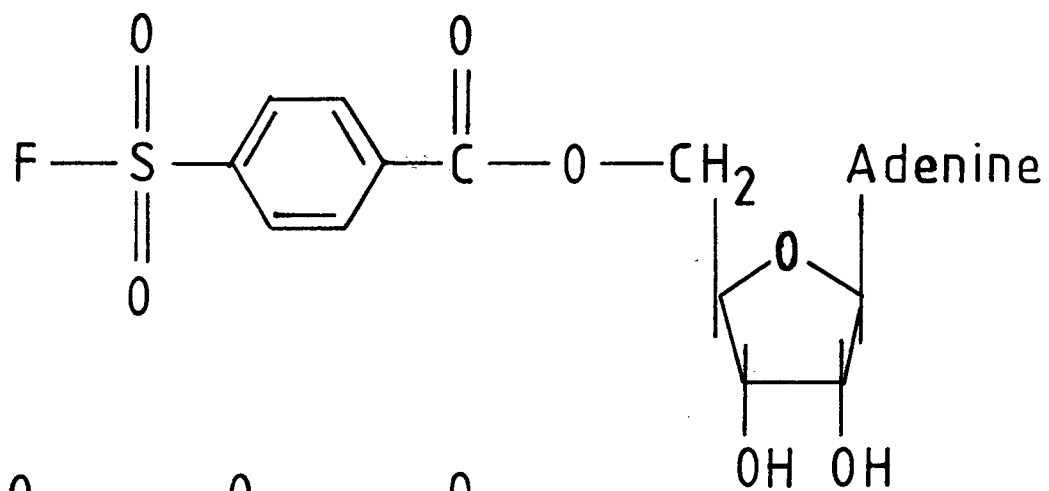
FSBA
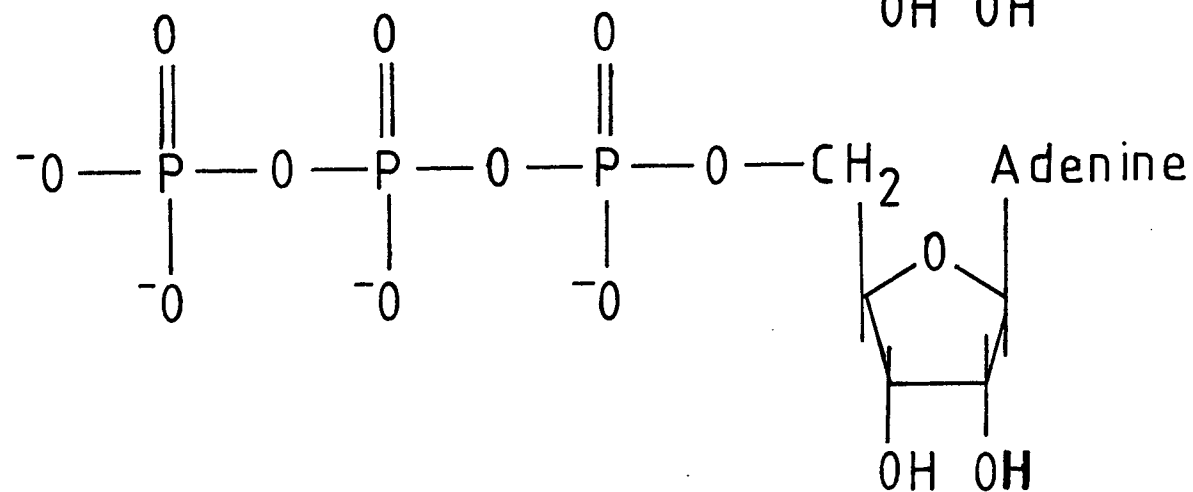
ATP

nucleotide binding sites of casein kinase II (Hathaway, et al., 1981) and the EGF receptor (Buhrow, et al., 1982; Buhrow, et al., 1983; Basu, et al., 1984).

Therefore, it appears that the tertiary structures of the ATP binding regions within the cAMP-dependent and cGMP-dependent serine kinases, as well as the p60^{src} tyrosine protein kinase all orient a homologous lysine residue such that it reacts with FSBA. These finding provide strong evidence that the sequence homology found between p60^{src} and the catalytic subunit of cAPK, reflect structural as well as functional homology and support the idea that protein kinases, irrespective of their amino acid substrate specificity, share a common ancestry.

Similarly, the C-terminal kinase domain of P130$^{gag-fps}$ contains a lysine at position 950 which is homologous to the highly conserved lysine-295 of p60^{src} (Shibuya and Hanafusa, 1982), which has been proposed to function as an ATP binding site in p60^{src} (Kamps, et al., 1984). To assay for the presence of amino acid residues which participate in ATP binding, I have used FSBA to covalently modify the ATP binding site within P140$^{gag-fps}$.

In addition, to investigate more specifically the requirement for lysine-950 within P130$^{gag-fps}$, I have used the technique of

oligonucleotide-directed mutagenesis. In collaboration with M. Zoller, lysine-950 within P130$^{gag-fps}$ has been mutated in order to evaluate the contribution of this residue in the phosphotransfer reaction. If the kinase activity intrinsic to P130$^{gag-fps}$ is crucial for its transforming function, then alterations in its ATP binding site should eliminate not only its kinase activity, but also its transforming potential.

6.2 Results

6.2.1 Inactivation of P140$^{gag-fps}$ Kinase Activity by Treatment with FSBA

In order to measure the inactivation of P140$^{gag-fps}$ kinase activity by FSBA over time, immunoprecipitates prepared from FSV-L5 transformed CEF were incubated at 37°C with 1.7 mM FSBA. Aliquotes were removed at timed intervals following the addition of FSBA, placed on ice, incubated in the presence of [γ-^{32}P]ATP as described in section 2.4 and the ^{32}P-labelled proteins resolved by SDS-PAGE (Figure 6.2A, lanes 1-6). To control for nonspecific loss of P140$^{gag-fps}$ kinase activity during incubation at 37°C, P140$^{gag-fps}$ immunoprecipitates were not treated with FSBA, but were incubated at 37°C, sampled at the start and finish of the experiment and assayed as for the FSBA treated samples (Figure 6.2A; lanes 7

Figure 6.2   Inactivation of P140$^{gag-fps}$ kinase activity by treatment with FBSA A. FSBA inactivation of P140$^{gag-fps}$ kinase activity over time: Immunoprecipitates from FSV-L5 transformed cells were incubated at 37°C in the presence or absence of 1.7 m$\underline{M}$ FSBA, sampled at various times, placed on ice, kinased and the ^{32}P-labelled proteins resolved by electrophoresis through a 15% SDS-polyacrylamide gel. Lanes 1 through 6 represent incubation times of 0, 10, 30, 45, 60 and 90 minutes respectively. Lane 7, immunoprecipitates left on ice for 90 minutes in the absence of FSBA; lane 8, immunoprecipitates incubated at 37°C for 90 minutes in the absence of FSBA; lane 9, purified catalytic subunit of cAPK (CAT) incubated at 37°C for 90 minutes in the absence of FSBA and kinased in the presence of histones; lane 10, CAT incubated in the presence of 1.7 m$\underline{M}$ FSBA for 90 minutes at 37°C and kinased in the presence of histones. B. Dose response of P140$^{gag-fps}$ kinase activity for FSBA inactivation: Immunoprecipitates from FSV-L5 transformed cells were incubated in the presence of various concentrations of FSBA at 37°C for 60 minutes, kinased, and analysed by 7.5% SDS-PAGE followed by autoradiography. Lanes 1, immunoprecipitates untreated with FSBA and incubated on ice for 60 minutes. Lanes 2 through 8 represent immunoprecipitates treated with final concentrations of 0, .1, .2, .5, 1.0, 1.5, and 2.0 m$\underline{M}$ FSBA respectively.
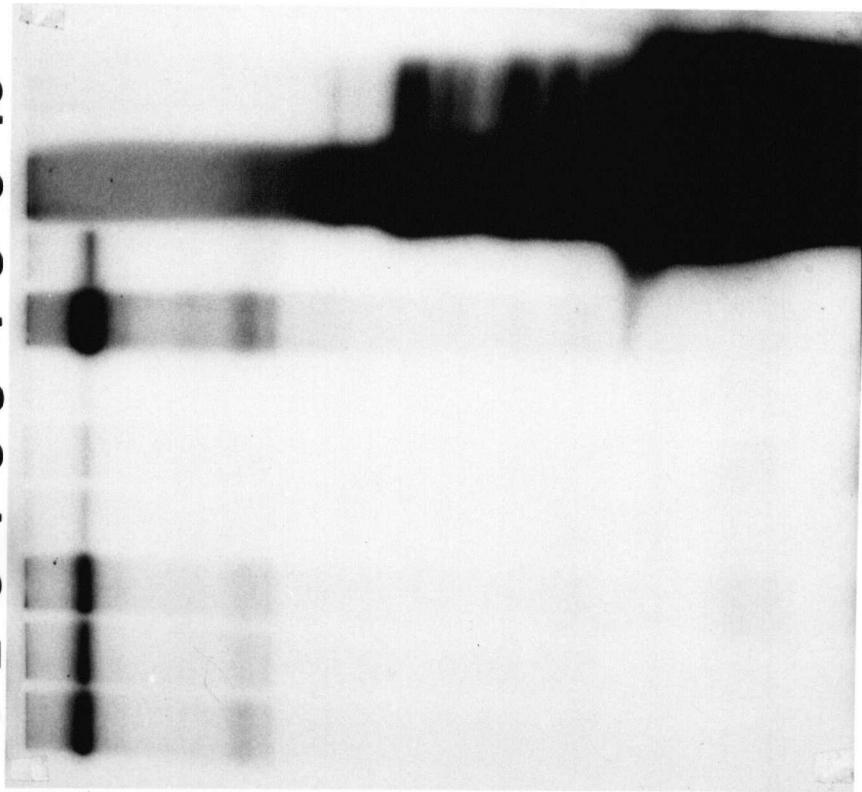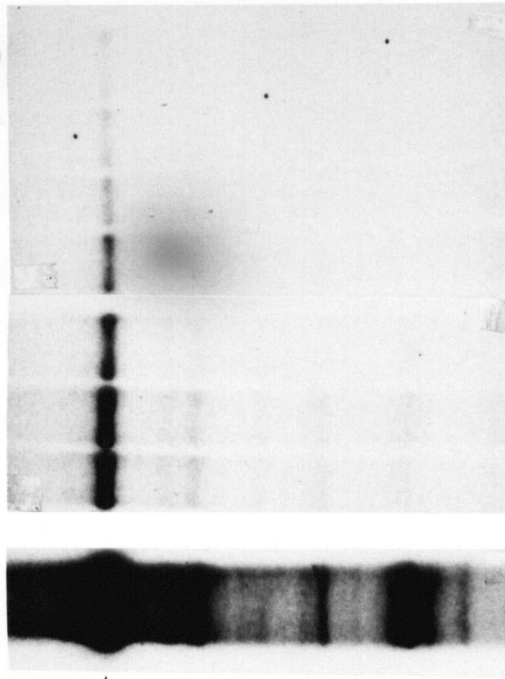
and 8). To test whether the synthesized FSBA used in these experiments was chemically reactive, purified type II catalytic subunit from porcine heart muscle (CAT) (obtained from M. Zoller) was treated with FSBA and its residual phosphotransferase activity was measured by the addition of histone type II (Sigma) to the kinase assay and compared with the kinase activity intrinsic to untreated CAT (Figure 6.2A; lanes 9 and 10). To quantitate the extent of enzyme inactivation resulting from treatment of $P140^{gag-fps}$ with FSBA, ^{32}P-labelled proteins were excised from the gel and the amount of ^{32}P-incorporated was determined by Cerenkov counting. Pre-incubation of $P140^{gag-fps}$ with FSBA for 90 minutes at 37°C results in only 8% residual kinase activity, while 35% residual activity was detected with $P140^{gag-fps}$ that had not been treated with FSBA. This represents a 4.3-fold decrease in $P140^{gag-fps}$ kinase activity that can probably be attributed to FSBA inactivation. The inactivation of CAT by FSBA was not quantitated since there was an obvious and significant decrease in incorporation of ^{32}P into histones following treatment of CAT with FSBA. Therefore, pre-incubation of $P140^{gag-fps}$ with 1.7 m$\underline{M}$ FSBA decreased the phosphotransferase activity in a time-dependent fashion.

To demonstrate that the loss in $P140^{gag-fps}$ kinase activity was actually due to inactivation of the enzyme by FSBA, the dose response for FSBA inactivation of $P140^{gag-fps}$ kinase activity was

determined. Immunoprecipitates were prepared as described above and incubated at 37°C for 60 minutes in the presence of various FSBA concentrations (Figure 6.2B; lanes 2 through 8). Lane 1 represents an untreated P140$^{gag-fps}$ containing immunoprecipitate that was left on ice during the incubation period. The results indicate that an increase in FSBA concentration can be correlated with a decrease in kinase activity, which suggests that FSBA inactivates P140$^{gag-fps}$ by reacting with a portion of the protein that functions in ATP binding. Although the inactivation may be due to a nonspecific modification of the protein by FSBA it is important to note that the kinase activity of P140$^{gag-fps}$ is stable in the presence of phenylmethylsulfonylfluoride (PMSF) (data not shown), a nonspecifically reactive compound that contains the same phenylsulfonylfluoride moiety as FSBA. The failure of PMSF to destroy kinase activity suggests that inactivation due to random reactions of the enzyme with the sulfonylfluoride group of FSBA probably does not occur. These preliminary experiments provided strong indirect evidence that P140$^{gag-fps}$ contains an ATP binding site that may be modified by FSBA. However, to conclusively prove that FSBA reacts with the active site of P140$^{gag-fps}$ one would have to demonstrate that the inactivation could be specifically inhibited by ATP.

6.2.2 Site-directed Mutagenesis of Lysine-950 Within the Putative ATP-binding Site of P130$^{gag-fps}$

I have expanded the mutagenesis studies of FSV to include the lysine (K) at position 950 within P130$^{gag-fps}$, proposed to function in ATP binding. Lysine-950 within P130$^{gag-fps}$ was changed to arginine (R) or glycine (G) using oligonucleotide-directed mutagenesis of the M13mp10-FSV clone (in collaboration with M. Zoller).

These mutations were chosen for the following reasons. Since the positive charge carried by the ε-amino group of lysine-950 may be important in ATP binding and/or catalysis, the amino acid arginine was chosen as an analogous substitute, since its side chain is also positively charged. However, if the size and shape of lysine-950 is absolutely critical to produce the required tertiary structure for a functional active site, then the substitution of arginine for lysine may result in an enzymatically inactive tyrosine protein kinase. On the other hand, the glycine mutation represents a control for ATP binding at residue 950. Since its substitution for a lysine residue results in an amino acid that lacks an R-group which can participate in ATP binding, it should yield a protein deficient in phosphotransferase activity.

The oligonucleotides designed to direct the desired changes within the FSV genome are shown in Figure 6.3. Mutagenic oligo-

Figure 6.3:   The synthetic oligonucleotides used to mutate the codon for lysine-950 of FSV P130$^{gag-fps}$. Oligonucleotides 17 long were designed to alter the codon for residue 950 by one or two nucleotides to code for an arginine or a glycine as indicated. The mutagenic oligonucleotide encoding the wild-type codon AAA was used to revert FSV-R(950) to FSV-K(950). The figure displays nucleotides 3212-3236 of the wt FSV nucleotide sequence (Shibuya and Hanafusa, 1982).

5' ... ACCCCCGTGGCGGTG<u>AAA</u>TCCTGC ... 3'   LYS Wild-type FSV

      5'-CCGTGGCGGTG<u>AGA</u>TCC-3'   ARG   FSV-R (950)

      5'-CCGTGGCGGTG<u>GG</u>GATCC-3'   GLY   FSV-G (950)

      5'-CCGTGGCGGTG<u>AAA</u>TCC-3'   LYS   FSV-K (950)

nucleotides 17 nucleotides long were synthesized to alter the AAA codon for lysine-950 to an AGA codon for arginine and a GGA codon for glycine. Apart from the single base substitution of a G for A in the case of FSV-R(950), and the two base change of GG for AA in the case of FSV-G(950), the synthetic mutagenic primers are identical with nucleotides 3216-3233 of the FSV genome as defined by Shibuya and Hanafusa (1982). The M13mp10-FSV clone, mutagenesis procedure, screening and propagation of mutants was exactly as described in the preceding two chapters. Once again to control for second-site mutations that could have occurred during the mutagenesis procedures and/or the propagation of mutant phage, the FSV-R(950) genome was reverted using an oligonucleotide which encoded the wild-type sequence (Figure 6.3).

6.2.3 Transfection of Rat-2 Cells with Wild-type, Mutant or Revertant FSV DNAs

To test whether the FSV mutated genomes possessed transforming activity and also to ensure that the revertant FSV-K(950) DNA had the same transforming potential as the wild-type FSV DNA, rat-2 cells were transfected with the wtFSV, FSV-R(950), FSV-G(950), or FSV-K(950) DNAs. These transfected cells were cultured in DMEM supplemented with 5% calf serum and 0.5 uM dexamethasone, and were examined daily for the appearance of foci. Approximately 10 days following transfection of rat-2 cells with wtFSV DNA or revertant FSV-K (950) DNA, foci containing transformed cells with a round refractile morphology were obvious

on a background of normal flat rat-2 cells. However, at 10 weeks post-transfection, rat-2 cells transfected with mutant FSV-G(950) or FSV-R(950) DNAs did not appear to be different from control cultures transfected with rat-2 carrier DNA only, which suggested that these mutated FSV DNAs were nontransforming. The revertant FSV-K(950) DNA had the same transforming potential as the wtFSV DNA, which indicated that alterations in the codon for lysine-950 eliminated the transforming activity of FSV DNA.

Foci of transformed rat-2 cells that appeared after transfection with wtFSV or revertant FSV-K(950) DNAs were picked, cloned in soft agar, and expanded in mass culture. The morphological phenotype of the revertant transformed cell lines was indistinguishable from that of the wild-type FSV cell line (Figure 6.4; panel B and D), and both of these FSV transformed cell lines formed large colonies in soft agar (data not shown). As expected, the cells cloned from these two FSV transformed cell lines were very round and refractile, unlike the normal rat-2 cells which were characteristically very flat and somewhat transparent (Figure 6.4; panel A and panel C).

Since foci were selected for transformed phenotype and since the changes at residue 950 had apparently eliminated the transforming activity of P130$^{gag-fps}$, I cotransfected the mutated FSV genomes with an alternative selectable marker. Rat-2 thymidine kinase (TK) minus cells were cotransfected with the Herpes simplex type 1 thymidine

Figure 6.4:   Morphological phenotypes of rat-2 cells following trans-

fection with FSV DNAs. Phase-contrast photomicrographs

of rat-2 cells (40x). Cells were transfected with wt

FSV, revertant FSV-K(950) FSV genomes or cotransfected

with mutant FSV-R(950) DNA plus pTK_1. Following

selection in HAT-DMEM containing 10% FBS or DMEM plus 5%

CS and .5uM dexamethasone cells were cloned and grown in

mass culture. Panel A, normal rat-2 cells; panel B, wt

FSV transformed cells; panel C, FSV-R(950) transfected

rat-2 cells; and panel D, revertant FSV-K (950)

transformed cells.
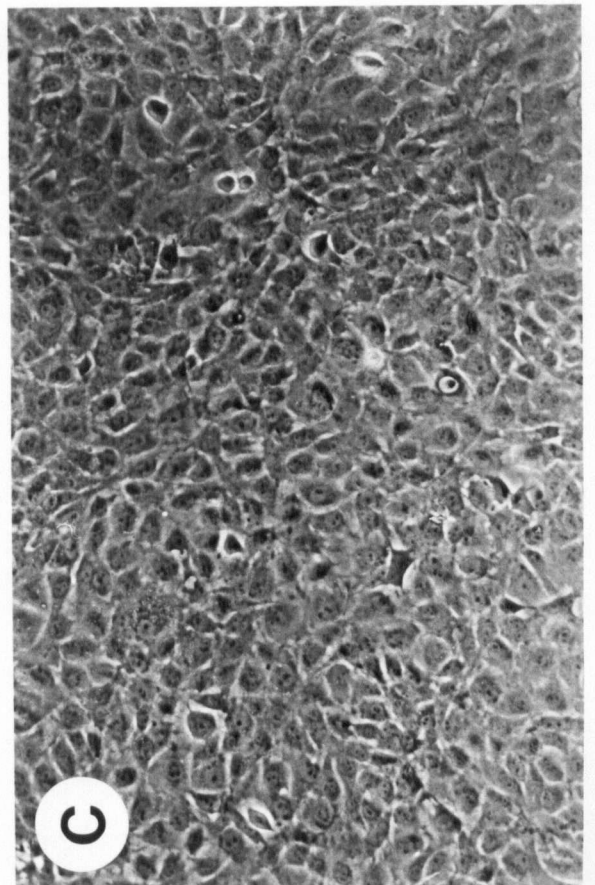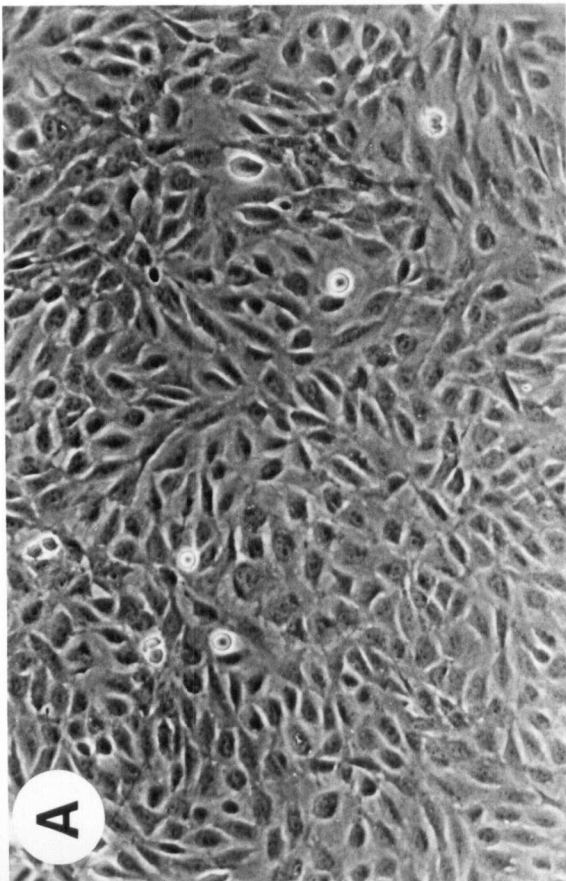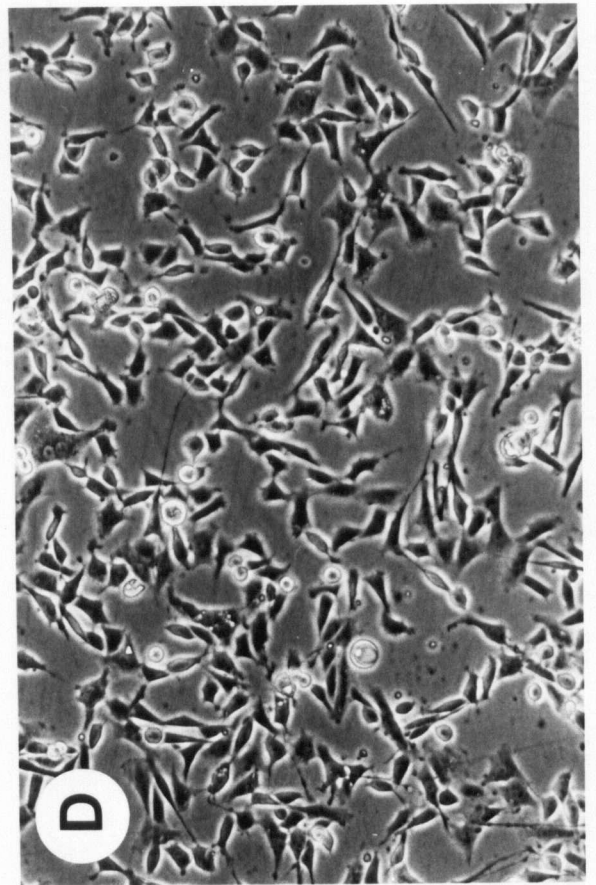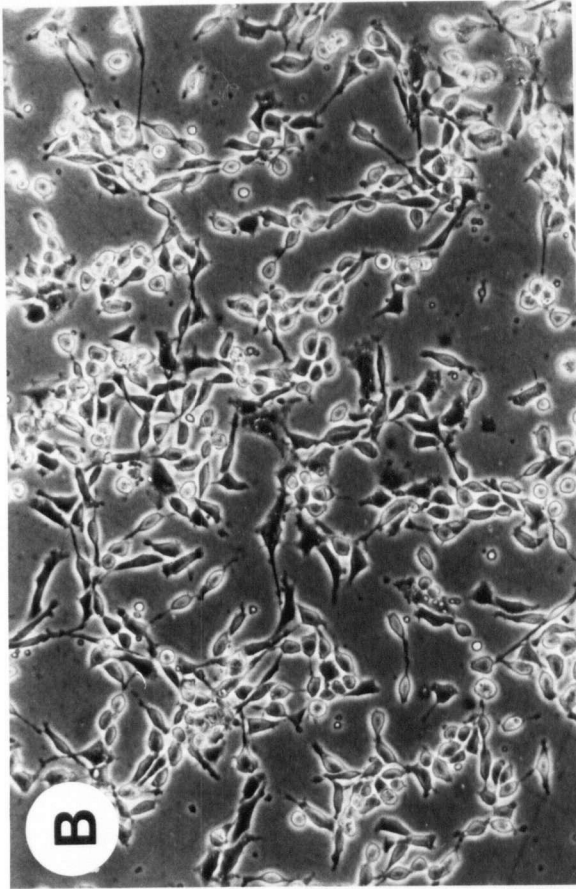
kinase gene (pTK$_1$) together with wild-type FSV, mutant [FSV-R(950) or FSV-G(950)] or revertant [FSV-K(950)] 4.7 kb inserts as described in section 2.9 and placed under HAT selection 48 hours post-transfection. The resulting HAT selected colonies were cloned from the original plates and grown in 35 mm wells for further analysis. The HAT resistant cells transfected with the mutant DNAs were similar in appearance to rat-2 cells, however, approximatley 50% of the HAT selected clones from cells transfected with wt FSV or revertant FSV DNAs had a transformed phenotype.

6.2.4 Expression and Characterization of Mutant FSV-R(950) and FSV-G(950) P130$^{gag-fps}$ Proteins

Fifteen HAT resistant clones originating from rat-2 cells cotransfected with pTK$_1$ and FSV-R(950) or FSV-G(950) DNAs were selected and tested for expression of P130$^{gag-fps}$. Equal numbers of cells were labelled with [^{35}S]methionine for 16 hours, lysed, immunoprecipitated with anti-p19^{gag} serum and examined for the presence of P130$^{gag-fps}$ by SDS-PAGE and fluorography. Of the 15 different clones tested from each set of FSV-R(950) or FSV-G(950) transfected cells, only one clone from each set was found to express P130$^{gag-fps}$ (Figure 6.5; panel A). The figure also shows two TK positive rat-2 lines [R-2(tk)] which are negative for P130$^{gag-fps}$ expression and a wt FSV P130$^{gag-fps}$ expressing cell line (K-950). The cells expressing FSV-R(950) and FSV-G(950) P130$^{gag-fps}$ pro-

Figure 6.5: Analysis of wild-type and mutant FSV transfected rat-2 cells for FSV P130$^{gag-fps}$ synthesis and tyrosine protein kinase activity. R-950 and R-2 (tk) indicate rat-2 cells that were transfected with pTK$_1$ plus FSV-R (950) DNA and HAT selected. G-950 and R-2 (tk) indicate rat-2 cells that were cotransfected with pTK$_1$ plus FSV-G(950) DNA and selected in HAT-DMEM, K-950 indicates wt FSV transformed rat-2 cells. R-2 indicates normal rat-2 cells. A. Rat-2 cells cotransfected with pTK$_1$ plus mutant FSV-R(950) or FSV-G(950) DNAs were selected in HAT-DMEM. HAT resistant clones were grown in 35 mm wells, labelled for 16 hours with 100 uCi [^{35}S]-methionine, immunoprecipitated with anti-p19^{gag} serum and examined by gel electrophoresis and fluoro-graphy to detect P130$^{gag-fps}$. B. Normal rat-2 cells and FSV-R(950) cells were labelled for 16 hours with 1 mCi of ^{32}P-orthophosphate, processed as described in section 2.3 and analysed by SDS-PAGE (7.5%). C. Cells from unlabelled cultures were immuno-precipitated as described above and then incubated with 5 ug of acid-denatured enolase in the presence of 10 mM MnCl$_2$ and 2.5 uCi [γ-^{32}P]ATP for 15 minutes at 30°C. Samples were analysed by electrophoresis through a 7.5% SDS-polyacrylamide gel and autoradiography.

A

R-950 R-2(tk) G-950 K-950

◄ PI30 ►

^{35}S

B

R-2 R-950

^{32}P

C

R-2 R-950 G-950 K-950

◄ ENOLASE

KINASE

– 178 –
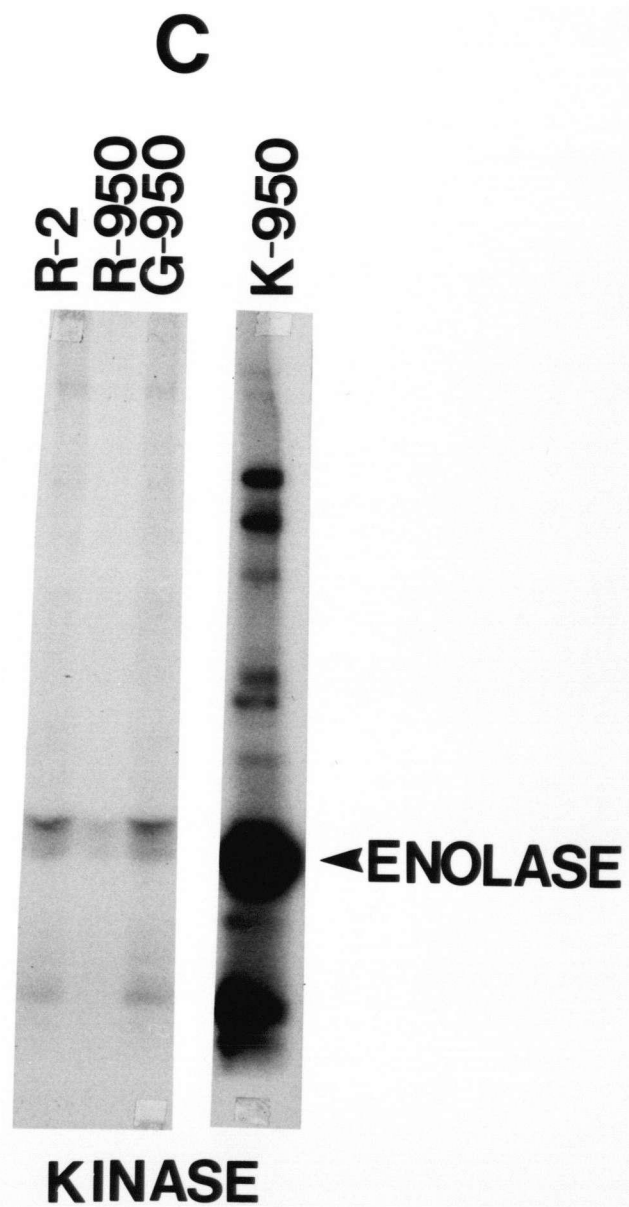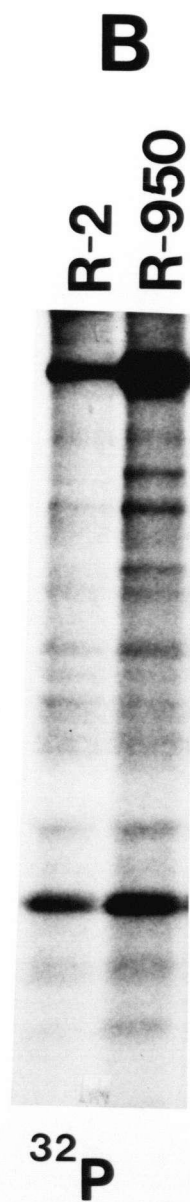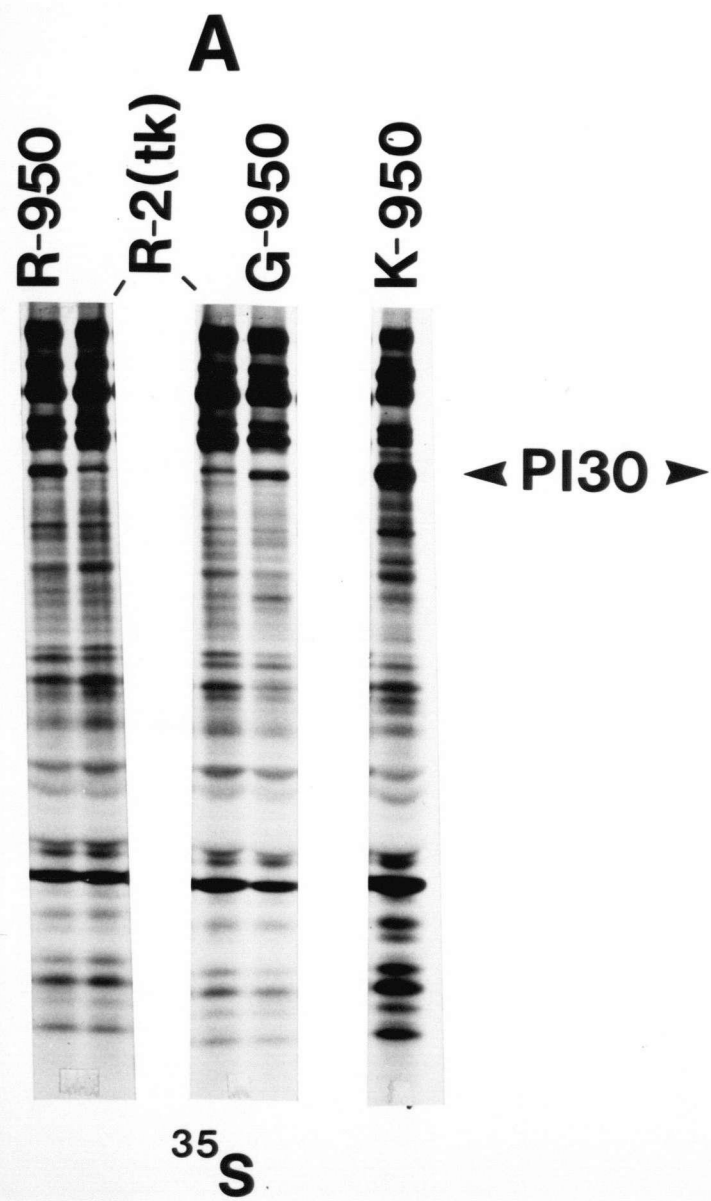
teins had a normal morphological phenotype similar to that of rat-2 cells (Figure 6.4; panels A and C). Consistent with this flat normal morphological phenotype, neither FSV-R(950) nor FSV-G(950) rat-2 cells displayed anchorage independent growth, as judged by their inability to grow in soft agar (data not shown).

To determine whether these mutant $P130^{gag-fps}$ proteins had kinase activity, wild type FSV and mutant [FSV-R(950) or FSV-G (950)] $P130^{gag-fps}$ proteins were immunoprecipitated from equal numbers of unlabelled cells and the immune complexes were incubated with $MnCl_2$, [$\gamma-^{32}P$]ATP, and soluble denatured rabbit muscle enolase (Figure 6.5; panel C). In this reaction wtFSV $P130^{gag-fps}$ (K-950) is autophosphorylated and also phosphorylates enolase·at a single tyrosine residue (Cooper, et al., 1984a). However, neither of the mutant cell lines (R-950 nor G-950) demonstrated detectable autophosphorylation of $P130^{gag-fps}$ or phosphorylation of the exogenous substrate enolase; the background from both of these reactions is similar to that found with normal rat-2 cells (Figure 6.5; panel C). This experiment indicates that alterations in the codon for lysine-950 within $P130^{gag-fps}$ yields an enzymatically inactive tyrosine protein kinase.

In FSV-transformed cells, $P130^{gag-fps}$ is phosphorylated mainly at serine and tyrosine residues, and to a lesser degree at threonine residues (see Figure 5.7; panel A). In order to determine

whether mutant FSV-R(950) P130$^{gag-fps}$ was phosphorylated in cells expressing this protein, both normal rat-2 cells (R-2) and mutant FSV-R (950) rat-2 cells (R-950) were metabolically labelled with ^{32}P-orthophosphate for 12 hours, lysed, immunoprecipitated with anti-p19^{gag} serum, and the labelled proteins resolved by SDS-PAGE. The data show that mutant R-950 P130$^{gag-fps}$ was found to be phosphorylated in intact cells (Figure 6.5; panel B). Since the mutant protein is both stably expressed and phosphorylated these data suggest that the substitution of arginine for lysine-950 does not substantially disrupt the conformation of the protein.

To investigate the phosphorylation sites of the mutant protein, purified in vivo ^{32}P-labelled FSV-R(950) P130$^{gag-fps}$ was subjected to tryptic peptide analysis. Inspection of the FSV-R-(950) P130$^{gag-fps}$ tryptic map indicates that it is missing the major site of tyrosine phosphorylation, tyrosine-1073 (Figure 6.6; panel C). This site is contained within tryptic phosphopeptides 3b and 3c of wtFSV P130$^{gag-fps}$ (Figure 6.6, panel A). In fact, the FSV-R (950) P130$^{gag-fps}$ tryptic map is similar to the tryptic phospho-peptide map of the mutant FSV-S(1073) P130$^{gag-fps}$ (Figure 6.6; panel B) in which the major tyrosine phosphoaceptor site (tyrosine-1073) has been destroyed (Chapter 5). Interestingly, FSV-R (950) P130$^{gag-fps}$ appears to be phosphorylated at tryptic phosphopeptide 4, which represents the other major tyrosine site which is phosphory-lated exclusively in vivo in both wt FSV P130$^{gag-fps}$ and in all

Figure 6.6: Comparison of tryptic phosphopeptides from P130$^{gag-fps}$ encoded by wild-type FSV, mutant FSV-S(1073) and mutant FSV-R (950). Cells were labelled with 1 mCi ^{32}P-orthophosphate for 12 hours, lysed, immunoprecipitated and P130$^{gag-fps}$ was identified as described in the legend for Figure 6.5. Gel-purified P130$^{gag-fps}$ was then digested with trypsin and separated by electrophoresis at pH2.1 on thin-layer cellulose plates in the first dimension and chromatography in the second dimension. Tryptic digests were as follows: A, wt FSV; B, FSV-S(1073); and C, FSV-R(950).
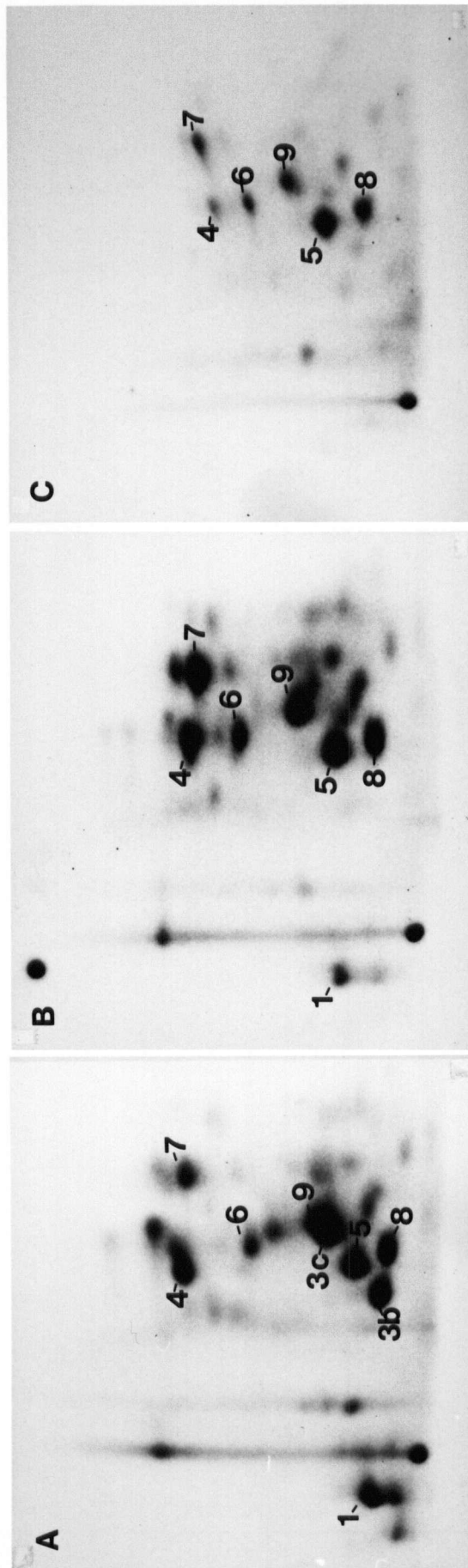
the FSV P130$^{gag-fps}$ proteins mutated at tyrosine-1073: FSV-S(1073) (Figure 6.6; panel B), FSV-G(1073) (Figure 5.6, panel C) and FSV-T (1073) (Figure 5.6; panel D). Little is known about the other sites of phosphorylation: spots 5, 6, 7, 8 and 9 (Figure 6.6). Except for spots 5 and 8, the other spots represent variable and minor sites of phosphorylation. However, spot 5, and probably spot 8, contain phosphoserine, and spot 6 is known to be a minor site of tyrosine phosphorylation (Chapter 3).

Presumably the majority of these uncharacterized sites of phosphorylation contain phosphoserine, and possibly some phosphothreonine, since phosphoamino acid analysis of FSV-R(950) P130$^{gag-fps}$ yields mainly phosphoserine, minor amounts of phosphothreonine and trace levels of phosphotyrosine (data not shown). However, the use of vanadate as an inhibitor of phosphotyrosine specific phosphatases during in vivo labelling of FSV-R(950) rat-2 cells increases the detection of phosphotyrosine comparable to that of phosphothreonine, confirming that this mutant protein is phosphorylated at tyrosine (I. MacLaren, personal communication).

Except for the phosphorylation of tryptic peptide 1, which is a variable site of autophosphorylation, the tryptic phosphopeptides from in vivo ^{32}P-labelled FSV-S(1073) P130$^{gag-fps}$ and FSV-R (950) P130$^{gag-fps}$ have similar mobilities (Figure 6.6). This indicates that FSV-R(950) P130$^{gag-fps}$ is only missing the sites of autophosphorylation consistent with its lack of kinase activity when

assayed in the immune complex reaction (Figure 6.5, panel C). The fact
that FSV- R(950) P130$^{gag-fps}$ contained sites of phosphorylation
related to both wtFSV P130$^{gag-fps}$ and mutant FSV-S(1073)
P130$^{gag-fps}$ argues that the mutation of residue 950 does not have
a global effect on the protein's native structure. However, the lack
of detectable kinase activity exhibited by the mutant FSV-R(950) pro-
tein suggests that changes at position 950 within P130$^{gag-fps}$
affect the protein's ability to function in the phosphotransfer
reaction.

6.2.5 Trans-Phosphorylation of FSV-R(950) P130$^{gag-fps}$ by
       FSV P140$^{gag-fps}$

Since tyrosine-1073 is the major site of autophosphorylation
it is not surprising that this site is not phosphorylated in an enzyma-
tically deficient P130$^{gag-fps}$ protein. However, to ensure that
the lack of autophosphorylation within this mutant protein was actually
due to an inactive kinase rather than a change in protein structure
which prevented phosphorylation at this site, I determined whether
FSV-R(950) P130$^{gag-fps}$ could be phosphorylated in trans by the
enzymatically active FSV P140$^{gag-fps}$. Cleared cell lysates from
FSV-L5 transformed rat-1 cells and FSV-R(950) rat-2 cells were pre-
pared. One-half of each lysate was combined and the mixture was immun-
oprecipitated, while the remainder of each lysate was immunopreci-
pitated separately. The precipitated immune complexes were kinased in
the presence of MnCl$_2$ and [γ-^{32}P]ATP at 20°C for 15 minutes,

following which the ^{32}P-labelled proteins were separated by SDS-PAGE and visualized by autoradiography. For comparative controls, in vitro kinase reactions of wtFSV P130$^{gag-fps}$ and mutant FSV-S(1073) P130$^{gag-fps}$ were also separated on the same gel, while a kinased rat-2 immunoprecipitate was included as a background control (Figure 6.7).

When FSV P140$^{gag-fps}$ and FSV-R(950) P130$^{gag-fps}$ were coimmunoprecipitated and incubated in the presence of [γ -^{32}P]ATP both proteins were labelled with ^{32}P (Figure 6.7; lane 1). However FSV-R(950) P130$^{gag-fps}$ was not labelled in the same ATP:phosphotransferase reaction when immunoprecipitated and kinased alone (Figure 6.7; lane 3), while FSV P140$^{gag-fps}$ was labelled under the same conditions (Figure 6.7; lane 2). These data indicate that the incorporation of ^{32}P into FSV-R(950) P130$^{gag-fps}$ is a result of trans-phorphorylation by the enzymatic activity intrinsic to FSV P140$^{gag-fps}$.

In order to identify the sites within FSV-R(950) P130$^{gag-fps}$ that were phosphorylated in trans by FSV P140$^{gag-fps}$ the ^{32}P-labelled protein was excised, eluted, concentrated and subjected to tryptic peptide analysis. The major tryptic phosphopeptides found in FSV-R(950) P130$^{gag-fps}$ phosphorylated in trans were those which correspond to the major autophosphorylation site of wtFSV P130$^{gag-fps}$ (Figure 6.8; 3a and 3b), which is known to contain tyrosine-1073 (Chapter 3 and 4). These data

Figure 6.7:   <u>In vitro</u> phosphorylation of FSV-R(950) P130$^{gag-fps}$ by FSV P140$^{gag-fps}$.   Cell lysates from unlabelled cells were either immunoprecipitated together or separately as indicated below, kinased in the presence of 10 mM MnCl$_2$ plus 5 uCi [γ-^{32}P]ATP and analysed by electrophoresis through a 7.5% polyacrylamide gel followed by autoradiography.   The kinase reactions were from the following immunoprecipitated cell lysates: Lane 1, FSV-L5 (P140$^{gag-fps}$) transformed cells plus FSV-R(950)   rat-2   cells;   lane   2,   FSV-L5 (P140$^{gag-fps}$)   transformed   cells;   lane 3,   FSV-R(950) rat-2 cells;   lane 4, wt FSV (P130$^{gag-fps}$)   transformed cells; lane 5, FSV-S(1073) transformed cells; lane 6, normal rat-2 cells.
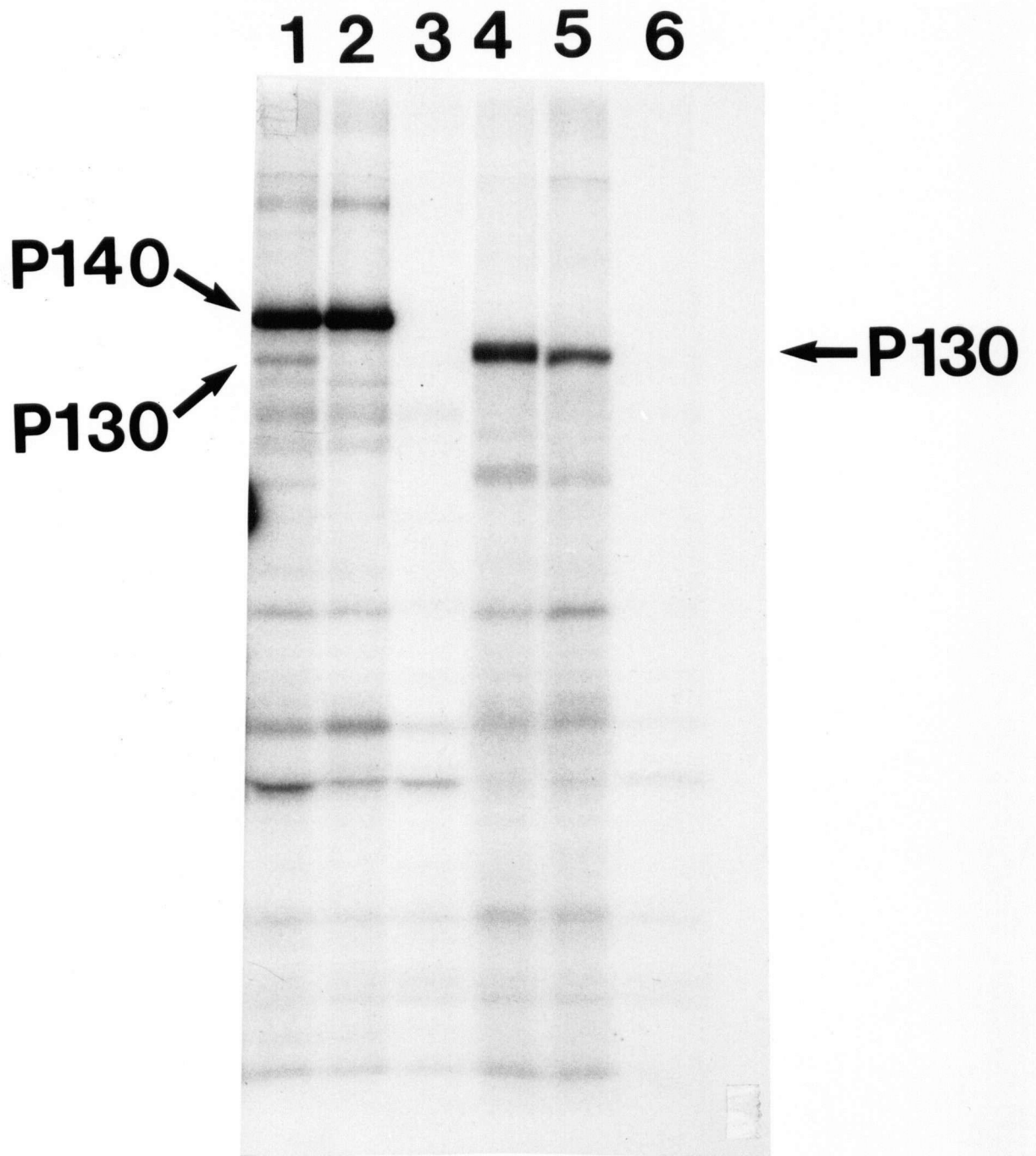
Figure 6.8:   Tryptic phosphopeptide analysis of <u>in vitro</u> phosphor-
ylated wt FSV P130$^{gag-fps}$ and <u>in vitro</u> trans-
phosphorylated FSV-R(950) P130$^{gag-fps}$.
P140$^{gag-fps}$ encoded by FSV-L5 and mutant FSV-R
(950) P130$^{gag-fps}$ were coimmunoprecipitated and
labelled in an immune complex kinase assay following
immunoprecipitation with anti-p19^{gag} serum from
FSV-L5 transformed cells and FSV-R(950) rat-2 cells. wt
FSV P130$^{gag-fps}$ from wt FSV transformed cells was
immunoprecipitated and kinased in an identical fashion.
The ^{32}P-labelled gel purified proteins were
digested with trypsin and separated in two dimensions on
thin-layer cellulose plates. Electrophoresis at pH 2.1
was from left to right with the anode on the left, and
chromatography was from bottom to top. Tryptic digests
were as follows: A, wt FSV P130$^{gag-fps}$; B, FSV-R
(950) P130$^{gag-fps}$ trans-phosphorylated by FSV
P140$^{gag-fps}$.
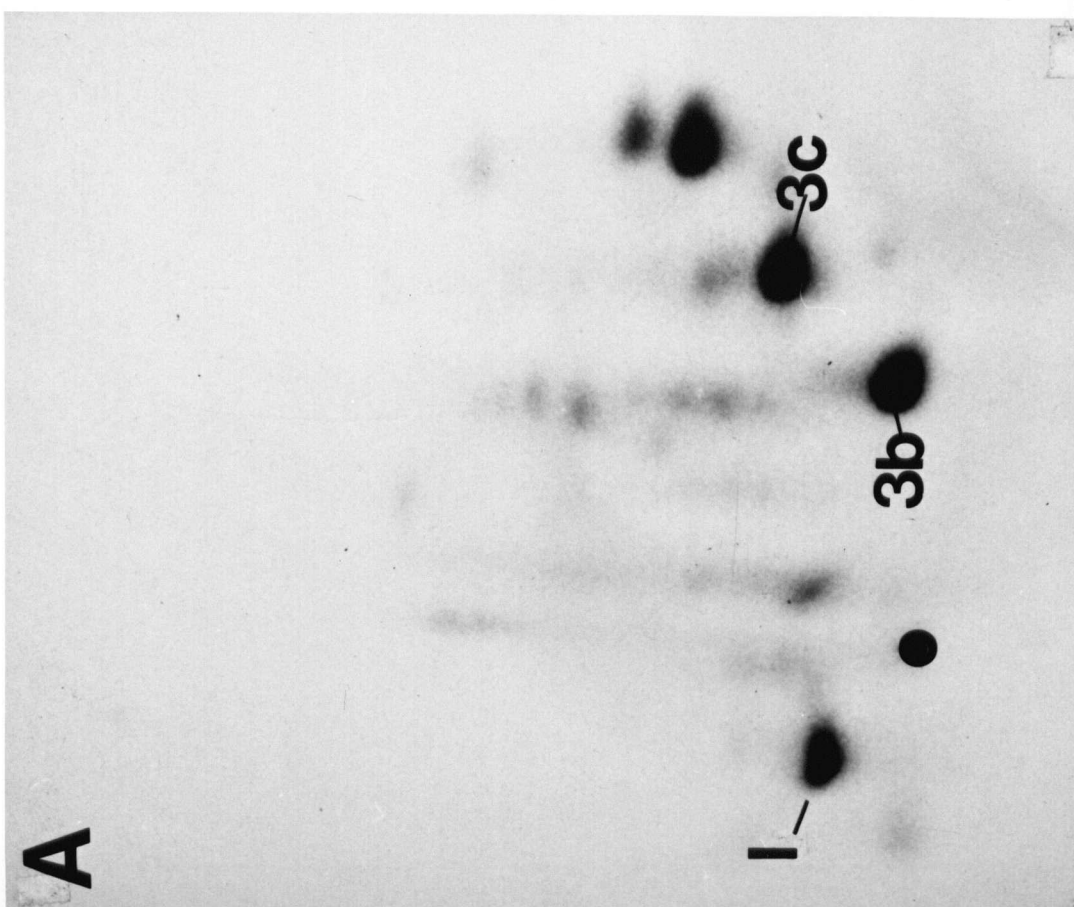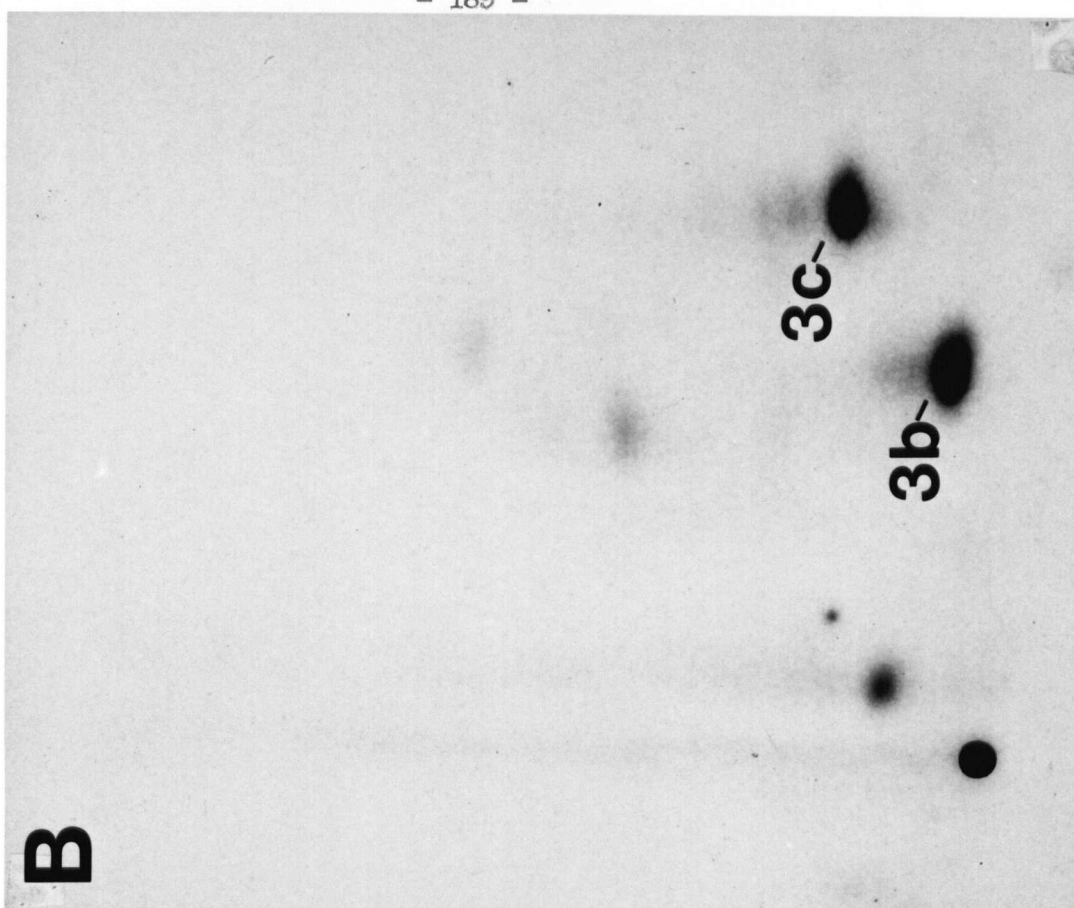
suggest that the mutant FSV-R(950) P130$^{gag-fps}$ has maintained a conformation around the major tyrosine phosphoacceptor site which permits phosphorylation <u>in trans</u> by FSV P140$^{gag-fps}$, and indicates that the phosphotransferase reaction of the FSV transforming protein can be an intermolecular event as previously described (Mathey-Prevot, 1984).

6.3 <u>Discussion</u>

The ATP analogue, FSBA, inactivated the tyrosine specific protein kinase activity intrinsic to the FSV transforming protein in a concentration and time dependent manner. FSBA is also known to specifically inactive the kinase activities associated with the cyclic-nucleotide dependent protein kinases cAPK (Zoller, <u>et al.</u>, 1981) and cGPK (Hashimoto, <u>et al.</u>, 1982), and the tyrosine specific protein kinase p60^{src} (Kamps, <u>et al.</u>, 1984). These studies show that inactivation occurs when FSBA reacts with a homologous lysine residue within the putative ATP binding site of these proteins. Therefore, by analogy, one would predict that FSBA also reacts with a residue located in the ATP binding site of the FSV transforming protein. Inspection of the FSV P130$^{gag-fps}$ amino acid sequence indicates that lysine-950 is the likely target for FSBA binding, since this residue is homologous to the lysine residues known to be covalently modified by FSBA in cAPK, cGPK, and p60^{src}. Although the FSBA inactivation data demonstrates the presence of an ATP binding site within P130$^{gag-fps}$, a detailed understanding of this binding site and the spatial relation-

ships between localized residues such as lysine-950 and ATP binding will only emerge from x-ray crystallography.

Although, the tertiary structure of a protein kinase has yet to be determined, the crystal structure of a number of dinucleotide binding proteins is known. These include lactate dehydrogenase (LDH) (Adams, et al., 1973) and nucleotide binding proteins such as adenylate kinase (AK) (Pai, et al., 1977). A comparison of the amino acid sequence surrounding lysine-950 of P130$^{gag-fps}$ with the sequence known to comprise the adenosine binding sites of these enzymes reveals the homologous sequence: Gly X Gly XX Gly, sixteen residues on the N-terminal side of lysine-950 (Rossmann, et al, 1974; Pai, et al., 1977). X-ray crystallographic studies show that this sequence of glycines is found between two B-pleated sheets that form the adenine dinucleotide binding pocket of lactate dehydrogenase (Adams, et al., 1971; Rossmann, et al., 1974). Apparently the Gly X Gly XX Gly forms a loop which is involved in the proper positioning and binding of the pyrophosphate moiety of NAD. A topological comparison of adenylate kinase with several dehydrogenases suggests that the substrate-binding sites in AK are equivalent to the NAD-binding sites in the dehydro-genases, with ATP corresponding to the adenosine containing half of NAD (Pai, et al., 1977). The cluster of glycines has probably been conserved to provide the flexibility needed to accommodate changes in protein structure induced by NAD or ATP binding. In fact, X-ray analysis indicates that the phosphate-binding loop in both AK (Pai,

et al., 1977) and LDH (Adams, et al., 1973) is the epicenter of a large
conformational change. Alcohol dehydrogenase, glyceraldehyde 3-phos-
phate dehydrogenase and glutathione reductase also bind nucleotides as
cofactors and these enzymes all contain the Gly X Gly XX Gly in a homo-
logous position (Wierenga and Hol, 1983). It is interesting to note
that this array of glycines is also found in the GTP binding protein of
$p21^{c-ras}$, and mutations in the second gly have been implicated in
the oncogenic activation of normal cellular ras (Wierenga and Hol,
1983).

As discussed in Chapter 1, FSV $P130^{gag-fps}$ belongs to a
family of tyrosine kinases that are all related to $p60^{src}$ by a
high degree of sequence homology within the domains which catalyze
tyrosine phosphorylation. It is interesting that the characteristic
sequence of glycine residues found in the nucleotide binding proteins
discussed above is also present in all the oncogenic proteins belonging
to the src family of tyrosine kinases (see Figure 1.1). In addition,
proteins that do not display tyrosine protein kinase activity also
contain this sequence. For example, the serine protein kinase cAPK
contains the Gly X Gly XX Gly on the N-terminal side of lysine-71, the
lysine covalently modified by FSBA (Zoller, et al., 1981). Whether or
not the presence of the Gly X Gly XX Gly sequence in these protein
kinases is a result of the divergent evolution of these proteins from a
primordial adenine nucleotide-binding protein (Schulz and Schirmer,
1974, Eventoff and Rossmann, 1975), or reflects an essential sequence

of evolutionarily conserved amino acids is not clear. The ubiquity and absolute conservation of the Gly X Gly XX Gly and the neighbouring lysine residue in every protein kinase characterized to date further strengthens the argument that lysine-950 within P130$^{gag-fps}$ plays an essential role in catalysis. As more direct evidence, the site-directed mutagenesis of the P130$^{gag-fps}$ lysine-950 destroys the tyrosine protein kinase activity intrinsic to FSV P130$^{gag-fps}$. In addition, mutations of lysine-950 also eliminate the transforming activity of P130$^{gag-fps}$ and support the idea that tyrosine protein kinase activity is crucial for transformation by FSV.

It has been proposed that the positive charge carried by the ϵ-amino group of lysine may activate or position the γ-phosphate of ATP, which would consequently activate this phosphate for nucleophilic attack by the OH group of a serine, threonine or tyrosine residue (Kamps, et al., 1984). However, other characteristics of this lysine residue must also be important for enzymatic activity since the positively charged arginine residue could not substitute for lysine-950 within P130$^{gag-fps}$.

The stable expression of the mutant FSV-R(950) P130$^{gag-fps}$ protein, with an arginine substituted for lysine-950 and the ability of this protein to be phosphorylated both in vivo and

in vitro suggests that this single amino acid change does not grossly affect the structure around the phosphorylation sites within the protein. However, the lack of kinase and transforming activities exhibited by the FSV-R(950) mutant suggests that this conservative amino acid change may perturb the local conformation of the ATP-binding site.

It is interesting that the in vivo phosphorylation pattern of FSV-R(950) P130$^{gag-fps}$ is similar to other FSV P130$^{gag-fps}$ proteins which have mutations at residue 1073. Since tyrosine-1073 is normally the major site of tyrosine phosphorylation within P130$^{gag-fps}$ both in vitro (autophosphorylation) and in vivo, the lack of phosphorylation at tyrosine-1073 within FSV-R(950) P130$^{gag-fps}$ is consistent with its inability to undergo autophosphorylation or to phosphorylate the exogenous substrate enolase in vitro.

P130$^{gag-fps}$ isolated from FSV transformed cells is phosphorylated not only at tyrosine, but also at serine and threonine residues. This suggests that other cellular kinases are also involved in cellular transformation by FSV. Either the cyclic-nucleotide-dependent protein kinases or protein kinase C may be responsible for the phosphorylation of the serine and threonine residues. Although it had been assumed that phosphotyrosine was the product of the tyrosine kinase activity intrinsic to the FSV transforming protein, phosphoamino

acid analysis and tryptic phosphopeptide mapping of FSV-R(950) P130$^{gag-fps}$ indicated that this mutant protein was phosphorylated at tyrosine residues in intact cells. The phosphorylation of tyrosine residues within FSV-R(950) P130$^{gag-fps}$, which is devoid of tyrosine protein kinase activity, suggests that this protein may be phosphorylated by a cellular protein kinase specific for tyrosine residues. On the other hand, the possibility exists that the tyrosine-phosphorylation detected in the FSV-R(950) P130$^{gag-fps}$ may be the product of a unique type of autophosphorylation.

CHAPTER 7

7.0 SUMMARY

The transforming protein of FSV has an intrinsic tyrosine specific protein kinase activity and is itself phosphorylated at multiple tyrosine and serine residues. Thus, phosphorylation of the FSV transforming protein is complex and may well affect its activity and function. Since the kinase and transforming activities of FSV $P140^{gag-fps}$ are related, I have investigated the phosphorylation of the FSV transforming protein in detail.

The major fps-specific phosphorylation sites of FSV $P140^{gag-fps}$, which include two phosphotyrosine residues and a phosphoserine residue, have been localized to the C-terminal region of the protein, which contains the kinase domain. Comparative tryptic phosphopeptide analysis has indicated that the phosphotyrosine residues are contained within a region that is highly conserved between the tranforming proteins of different FSV variants. This suggests that these phosphorylation sites are important for the activity of the protein.

The relationship between the phosphorylation of the FSV transforming protein and its enzymatic and biological activities has been investigated. The strategy involved mutations at the major tyrosine phosphorylation site within FSV $P130^{gag-fps}$, tyrosine-1073. Oligonucleotide-directed mutagenesis was used to change the codon for

tyrosine-1073 to a codon for phenylalanine, serine, threonine or glycine. These amino acid substitutions at position 1073 within P130$^{gag-fps}$ allowed evaluation of the effect of phosphorylation on the protein's activity and function. In addition the specificity of phosphorylation was determined by examining the serine and threonine replacements at residue 1073.

All of the FSV-tyrosine-1073 mutant genomes transformed rat-2 cells and induced cells of nearly identical morphological phenotype; however, only after a long latent period. In addition these different mutant transformed cells expressed P130$^{gag-fps}$ proteins with reduced kinase activities. The similar effects of these mutations was surprising considering that the R-groups of the substituting amino acids are quite different. The results obtained with the different mutations imply that the important characteristic of tyrosine-1073 is its ability to become phosphorylated. Therefore, given the caveat that mutations at residue 1073 do not have a global effect on protein con- formation, I have interpreted the reduced kinase activity and trans- forming potential exhibited by the mutant proteins to be a direct consequence of a lack of phosphorylation at residue 1073.

The fact that neither serine nor threonine were phosphory- lated when placed at a tyrosine kinase recognition site demonstrates the specificity of the P130$^{gag-fps}$ protein kinase activity for

tyrosine residues. While it seems probable that the amino acids surrounding tyrosine-1073 of wtFSV P130$^{gag-fps}$ are important in targeting the kinase to a particular residue, it is apparent that there is a strict requirement for tyrosine at the site of phosphorylation.

Amino acid substitutions at tyrosine-1073 of P130$^{gag-fps}$ did not completely abolish its activity. This suggests that phosphorylation of tyrosine-1073 may not have a crucial function in catalysis, but may play a regulatory role. However, the results do not exclude the possibility that some activity of FSV P130$^{gag-fps}$ other than tyrosine phosphorylation is involved in its transforming ability. Although, these data strongly suggest that tyrosine phosphorylation of P130$^{gag-fps}$ can modulate its activity and strengthen the case for the involvement of tyrosine phosphorylation in transformation by FSV.

Mutations within the putative ATP-binding site of P130$^{gag-fps}$ at lysine-950 destroy both its kinase and transforming activities. These results support the idea that the tyrosine kinase activity intrinsic to P130$^{gag-fps}$ is essential for its tranforming function. A conservative amino acid change to arginine at residue 950 allowed for the stable expression of the mutant protein. Tryptic phosphopeptide mapping and phosphoamino acid analysis of the mutant protein indicated that it was not phosphorylated at tyrosine-1073, but was phosphorylated at a second tyrosine site previously identified in wt P130$^{gag-fps}$ as a site exclusively phosphorlyated in vivo. The

phosphorylation of tyrosine residues within a mutant protein devoid of intrinsic tyrosine protein kinase activity suggested that FSV P130$^{gag-fps}$ may be a target for phosphorylation by a cellular protein kinase specific for tyrosine residues. The activity and function of the FSV transforming protein may be subject to modulation by a number of cellular kinases, each with different amino acid substrate specificities.

The possible participation of a number of cellular kinases during FSV transformation is consistent with many investigations to date which reveal that cellular transformation by FSV occurs by a complex and as yet undefined mechanism. Nonetheless, the use of oligonucleotide-directed mutagenesis to specifically alter key amino acid residues within FSV P130$^{gag-fps}$ provides a powerful technique that may help to unravel this complex mechanism.

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