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GENETIC AND BIOCHEMICAL ANALYSIS OF TRANSFORMATION BY pp60^{SRC}

by

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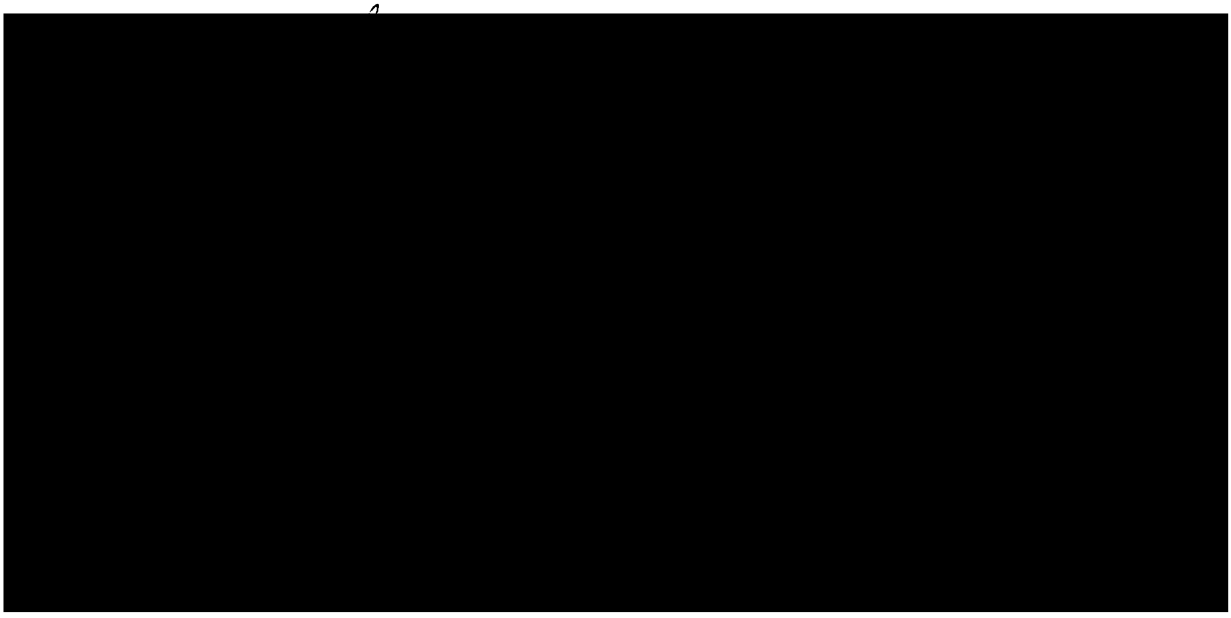

I have been very fortunate as a graduate student in that I had the guidance of Mike Bishop and Harold Varmus. Mike and Harold have provided the most exciting and supportive research environment imaginable, and both found ways to mitigate the ups and downs that inevitably characterize such endeavors. Many of my colleagues in the lab have been generous with their ideas and expertise. I would particularly like to thank Mike Simon for the interest he took in my work, David Morgan for being such an optimist as well as a first-rate collaborator, Jason Swedlow and the members of the Sedat lab for the impromptu microscopy tutorials, and Kathy Weston for enduring the agonies of reading early drafts of this dissertation (and providing remedial english lessons). Of all of my colleagues, I would like to give special thanks to Richard Parker. Richard was a close friend, a collaborator, and an inspiration -- I learned so much from him in the short time that I knew him. Although many of these people contributed greatly to the work described here, responsibility for all shortcomings lies with the author.

Genetic and Biochemical Analysis of Transformation by pp60^{src}.

Joshua M. Kaplan

ABSTRACT

Very little is known about the cellular proteins that either regulate the transforming activity or mediate the phenotypic effects of pp60^{src}. The goal of the work described in this dissertation was to identify these cellular proteins, first with genetic and subsequently with biochemical analysis.

In order to identify mutations that augment the transforming activity of c-*src*, cells containing 8 times the normal level of pp60$^{c\text{-}src}$ were mutagenized and transformed revertants were isolated. Sixteen genetic revertants were characterized, each one harboring transforming mutations in cellular genes that act independently of c-*src*.

Despite functional similarities to the receptor class of tyrosine kinases, pp60^{src} lacks functionally important domains found in the receptors: a transmembrane sequence, for mediating the association with membranes; a hydrophobic signal sequence for targeting the protein to the appropriate membranes; and a ligand-binding domain for regulating the kinase activity. In order to identify domains that perform these functions, mutant forms of pp60^{src} and proteins encoded by hybrid *src*-pyruvate kinase genes were analysed for association with membranes and subcellular localization. These experiments demonstrate: that amino acids 1-7 of pp60^{src} act as a

recognition sequence for N-terminal myristylation, and that lysine-7 is a critical component of this sequence; that N-terminal myristylation is not sufficient to cause membrane-association; that pp60^{src} contains multiple domains which together with N-terminal myristylation cause membrane-association; and that these membrane-anchoring domains target proteins to specific subcellular locations.

To determine whether membrane-anchoring domains act as binding sites for other cellular proteins, methods were developed for analyzing proteins that bind to pp60^{src}. These methods were used to identify a 97 kilodalton protein found in platelets which appears to bind to pp60^{src} specifically.

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CHAPTER 1: INTRODUCTION

CANCER IS A GENETIC DISORDER

Cancer comprises a bewildering array of diseases. Cancers afflict specific age groups, they affect specific tissues, and they respond to specific therapies, with various outcomes. Consequently, one must suspect that the underlying causes are equally diverse. How should one proceed in identifying the molecular origins of cancer? The first step was realizing that cancer is a genetic disorder (Boveri, 1914).

Several lines of evidence suggest that cancer is caused by changes in DNA. Like any other genetic trait, the neoplastic phenotype is faithfully transmitted from tumor cells to their progeny, and loss of this phenotype occurs at the rate of simple mutations (Varmus et al. 1981a and b). Secondly, naturally occurring tumors are typically clones of cells derived from a single abnormal precursor, as indicated by the expression of X-linked isozymes (Fialkow 1974,1976), by the focal pattern of tumor growth (Neiman et al. 1980; Teitelman et al.1988), and by cytogenetic abnormalities (reviewed in Varmus 1984). The clonality of tumors suggests that the events that initiate tumorigenesis are infrequent. The cytogenetic abnormalities often seen in tumors, such as translocations, deletions, and gene amplifications, are quite rare in normal tissues, and consequently may themselves initiate cancerous growth. Susceptibility to cancer clearly has a genetic basis, as indicated by human pedigrees with Bloom's syndrome (German 1974) and xeroderma pigmentosum (reviewed in Cleaver 1983). The latter is known to result in an increased rate of

DNA damage (Cleaver 1983). It is clear that experimental cancer can be caused by changes to DNA. Normal cells in culture can acquire the properties of cancer cells (Barrett and Ts'O 1978b), and this occurs at the rate of simple mutations. Furthermore, agents that induce mutations are also experimental carcinogens (Barret and Ts'O 1978a).

How many mutations must occur before cancer results? It is unlikely that a single mutation is sufficient to cause cancer. The incidence of human cancer increases exponentially with increasing age. Statistical analysis of the dependence of incidence upon age indicates that as many as 6 independent mutations must occur in a cellular lineage before cancer results (Armitage and Doll 1954;Peto 1978; Whittemore 1978). The natural history of tumors also suggests that tumorigenesis comprises a sequence of pathological changes. For instance in cervical carcinoma, a sequence of pre-malignant states, namely dysplasia and carcinoma *in situ*, precede progression to invasive carcinoma (Koss et al. 1963). Pre-malignant syndromes have been defined for human chronic myelogenous leukemia (Fialkow 1984) and avian bursal lymphomas (Cooper et al. 1968; Neiman et al. 1980). Presumably the multi-hit nature of human cancers is a manifestation of progression from pre-malignant to malignant states. It seems likely that each type of tumor is the consequence of several deleterious alterations. The etiology of cancer appears increasingly complex.

CANCER CAUSING GENES

Although Boveri proposed a genetic basis of cancer 75 years ago,

molecular identification of cancer-causing genes occurred in the last 20 years with the advent of molecular biology and, in particular, upon the characterization of RNA tumor viruses, which are also called retroviruses.

Cancer in animals can arise following infection by tumor viruses. Tumor viruses apparently encode a gene (usually one but sometimes more) which causes normal cells to become cancer cells. These genes are called oncogenes. Viral oncogenes (v-oncs) were originally defined by mutations which abolish the neoplastic effects of RNA tumor viruses (Toyoshima and Vogt 1969; Martin 1970). In the case of Rous Sarcoma Virus (RSV), these mutations have no ill effects on viral replication; consequently, transformation-defective deletion mutants tend to accumulate as RSV is passaged in culture. This fact, although puzzling, allowed investigators to define the v-onc of RSV as the segment of the viral genome that is consistently lacking in transformation-defective viruses (Duesberg and Vogt 1970;Wang et al. 1975). In a similar manner, the v-oncs of all the acutely transforming retroviruses were subsequently identified (reviewed in Weiss et al. 1982). Transformation defective viruses were used to generate molecular probes for viral oncogenes (Stehelin et al. 1976a). These molecular probes revealed a startling discovery: normal cells contain and express genes that are highly related to viral oncogenes (Stehelin et al. 1976b). These genes are called cellular oncogenes, or c-oncs (reviewed in Bishop 1983).

Tumor viruses are the products of viral capture of normal cellular genes. Cellular oncogenes, unlike v-oncs, bear the hallmarks

of normal cellular genes. Cellular oncogenes are never found within or linked to endogenous proviruses (Hughes et al. 1979). The chromosomal position of a c-onc is constant in a species (Hughes et al. 1979). Cellular oncogenes are phylogenetically conserved (Spector et al. 1978). In addition, the coding regions of c-oncs are interrupted by intervening sequences (Parker et al. 1981; Klempnauer et al. 1982). How is it that normal cellular genes cause cancerous growth? Viral capture must somehow activate the lethal potential of c-oncs, perhaps by causing inappropriate expression of the normal gene products or by creating a qualitatively abnormal gene product.

Activated oncogenes have been discovered in other contexts as well. When DNA from certain tumor cells is introduced into normal cells, some of the recipient cells acquire the neoplastic phenotype (Shih et al. 1979). This method, called genomic transfection, has been exploited to isolate activated oncogenes directly from tumor cell DNA (reviewed in Varmus 1984). In this way, multiple activated oncogenes can be recovered from a single tumor cell, which once again highlights the multi-hit nature of carcinogenesis (Fasano et al. 1984). Whether these mutations correspond to events that initiate tumorigenesis or to events that lead to malignant progression of a tumor is not known.

Analysis of abnormal chromosomes in tumor cells has also led to the discovery of c-oncs. Tumor cells often contain amplified regions of DNA, apparent either as a homogeneously staining region of a chromosome or as double minute chromosomes (reviewed in Cowell 1982). The mitotic stability of these abnormal chromosomes implies that they

are somehow beneficial to the tumor cell. These amplified sequences often contain c-oncs (reviewed in Varmus 1984), and consequently these cells grossly overexpress the products encoded by these genes. In some cases, amplification of c-oncs indicates malignant progression of the tumor and hence a poor clinical course (Brodeur et al. 1984; Little et al. 1983). Cellular oncogenes are often involved in the translocations and deletions that are observed in tumors (Rowley 1983; Yunis 1983). Translocations sometimes alter the coding sequence of the c-onc, giving rise to an enzymatically activated protein product (Konopka et al. 1984). Whether these rearrangements also activate the transcription of c-oncs is not known.

Cellular oncogenes can also be activated by more subtle genetic rearrangements. Integration of a tumor virus genome can activate neighboring c-oncs, by placing the c-onc under the influence of powerful viral transcriptional control elements (Neel et al. 1981, Payne et al.1982), by altering the coding region of the c-onc (Fung et al. 1983), or by both methods. Activation of c-oncs by viral insertion is a common mode of carcinogenesis among tumor viruses which lack viral oncogenes (reviewed in Varmus 1984).

Inactivation of cellular genes can also contribute to the genesis of cancer (reviewed by Ponder, 1988). This class of genes is called recessive oncogenes. For example, familial susceptibility to retinoblastoma (Knudson 1971) is caused by inactivation of the RB gene (Friend et al. 1986). How does loss of a gene cause cancer? Perhaps recessive oncogenes normally regulate the activity of c-oncs, hence loss of a recessive oncogene would activate the lethal potential of c-

oncs. On the other hand, activated oncogenes might promote growth by diminishing the growth-inhibiting activity of recessive oncogene products. This notion is supported by recent reports that the product of the RB gene forms a biochemical complex with proteins encoded by several viral oncogenes (Whyte et al. 1988). It is not known how commonly this mode of carcinogenesis occurs, or whether inactivation of recessive oncogenes corresponds to initiation or progression of tumorigenesis.

The malignant potency of oncogenes can also be demonstrated by introducing activated alleles into the germline of mice (reviewed by Hanahan 1987). Several investigators have generated such transgenic mice, which typically have an increased susceptibility to cancer. In general, the transgene is constructed so that the activated oncogene is expressed in a specific tissue. Although all of the cells in such a tissue express the activated oncogene, tumorigenesis in these mice nevertheless requires multiple steps. The resulting tumors are first apparent as hyperplastic focal lesions, a minority of which develop into frankly malignant tumors (Teitelman et al. 1988; Tsukomoto et al. 1988). Apparently, activation of a c-onc can initiate multi-step tumorigenesis.

A variety of methods have led to the discovery of over 40 genes, that are associated with the occurrence of human and experimental tumors. Some oncogenes were discovered independently by several of these methods. Activation of c-oncs may either initiate tumorigenesis, or provoke malignant progression of tumors. Although identifying activating lesions in c-oncs may prove useful in clinical

settings, the ultimate goal of rationally designed cancer therapy depends upon determining how activated oncogenes provoke cancerous growth.

THE ROLE OF ONCOGENES IN NORMAL GROWTH AND DEVELOPMENT

Normal cells express genes that cause cancerous growth in other contexts. Since these genes presumably perform some function in normal cells, perhaps c-oncs govern the proliferation and differentiation of normal tissues. Thus, cancer would result from exaggeration of the normal activity of oncogenes. In some cases, exaggerated expression of an otherwise normal c-onc is sufficient to cause neoplastic transformation of cells (Oskarsson et al. 1980; DeFeo et al. 1981). Conversely, activation of other c-oncs requires structural changes as well as exaggerated expression (Parker et al. 1984; Shalloway et al. 1984a and b; Hanafusa et al. 1984). The structural changes that are required for the latter to transform cells appear to act by releasing the product of these oncogenes from some form of negative regulation (Cartwright et al. 1987;Piwinca-Worms et al. 1987; and Kmiecik et al. 1987). Current evidence supports the model that activation of c-oncs corresponds to an excess of the normal activity of these genes.

One initially suspects that c-oncs normally perform vital functions because, in general, oncogenes are phylogenetically conserved. For instance, the *src* gene has been isolated from mammals, birds, fish, flies, and frogs. Perhaps c-oncs regulate the growth and

development of eukaryotes generally. Further support for this idea has emerged from biochemical analysis of the activities encoded by c-oncs, and from testing the physiological role of these activities genetically.

One would expect that the biochemical activities that are known to regulate growth, such as growth factors or their receptors, would be encoded by c-oncs. In so far as these factors have been defined, this appears to be true. The cellular homolog of the v-*sis* oncogene, c-*sis*, encodes the B chain of platelet derived growth factor (Doolittle et al. 1983; Waterfield et al. 1983). The c-*fms* gene encodes a hemopoietic growth factor receptor, the CSF-1 receptor, which is a transmembrane tyrosine kinase (Scherr et al. 1985). Likewise, the c-*erbB* gene encodes the epidermal growth factor (EGF) receptor, another transmembrane tyrosine kinase (Downward et al. 1984). There are also examples of c-oncs encoding factors that transmit growth control signals from membrane receptors to intracellular second messengers. The c-*ras* proteins control the activity of adenylate cyclase in budding yeast (Kataoka et al. 1985), although the role of c-*ras* in mammalian cells is likely to be different. Finally, the c-*fos* gene and the c-*jun* gene together encode 2 subunits of a transcriptional activator, AP-1, which mediates the effects of tumor-promoting phorbol esters (Angel et al. 1987; Lee et al. 1987). Further analysis of the biochemistry of growth and development will likely shed further light on the activity encoded by other c-oncs.

The vital role of c-oncs in development has also been demonstrated genetically. The dominant-white spotted locus of mice,

which controls the migration and proliferation of stem cells, encodes a transmembrane tyrosine kinase receptor (Geissler et al. 1988). The *Drosophila melanogaster sevenless* (Hafen et al. 1987) and *torso* (Sprenger et al. 1989) genes also encode transmembrane tyrosine kinases. *Sevenless* controls the development of a specific photoreceptor cell (Hafen et al. 1987), and *torso* controls the development of the anterior and posterior anatomy of embryos (Sprenger et al 1989). The c-*ras* genes of budding yeast control the differentiation of vegetatively growing cells into metabolically inactive spores (Kataoka et al. 1984; Tatchell et al. 1984). Further investigation of the genetic control of development will presumably define the physiological roles of other oncogenes as well.

Thus, various methods have defined a set of genes which are associated with neoplastic growth in a variety of settings. Furthermore, these genes encode ancient activities, and the physiological roles of some of these activities have been deduced by biochemically and genetic methods. Examples where these activities have been defined support the notion that cancer results from an excess of the activity normally encoded by these genes. Identifying the role of c-oncs in normal development is thus one instructive strategy for elucidating the mechanisms of carcinogenesis.

THE SRC ONCOGENE

Rous Sarcoma Virus induces fibrosarcomas in susceptible chickens and neoplastic transformation of susceptible cells in culture. A

single viral gene, the v-*src* gene, is responsible for these oncogenic activities. The v-*src* gene was originally defined by mutant viruses that are temperature sensitive for maintaining the transformed phenotype of infected cells (Toyoshima and Vogt, 1969; Martin, 1970).

The effects of v-*src* are most easily studied in cell culture. Infection of susceptible cells by RSV results in a complex set of phenotypic alterations, which as a whole is called the transformed phenotype. This phenotype has been studied most carefully in fibroblasts (reviewed in Hanafusa 1977); however, the effects of v-*src* have been determined in a variety of cell types. The hallmarks of a *src* transformed cell include:

1. **Growth factor independence.** Transformed cells grow *in vitro* in the absence of exogenous growth factors.

2. **Anchorage independent growth.** Fibroblasts will normally grow only when attached to a solid substrate, such as a plastic dish; however, transformed cells are capable of growing in semi-solid medium.

3. **Abnormal morphology.** Transformed cells are more rounded and refractile than normal cells.

4. **Altered hexose transport.** Transformed cells transport hexose across the plasma membrane at a greatly increased rate.

5.**Loss of contact inhibition of growth.** Transformed cells grow as foci on monolayers of normal cells. Fibroblasts normally are growth arrested when they come into contact with neighboring cells. As a consequence, normal fibroblasts form uniform cell monolayers of characteristically low cell densities in culture. Transformed cells, on the other hand, are capable of growing on top of neighboring cells, forming foci which actually continue to grow on an underlying monolayer of normal cells, and hence grow to much higher cell densities.

6.**Tumor formation.** Transformed cells will induce tumors when injected into syngeneic animals.

Expression of these phenotypes is not invariably linked. There are *src* mutants, called partially transformation-defective mutants (reviewed by Weber 1984), which produce some of these effects but not others.

In other contexts, *src* alters the differentiation state of cells. Infection with RSV blocks the ability of myoblasts to terminally differentiate into myotubes (Fiszman and Fuchs 1975). On the other hand, infection of PC12, a pheochromocytoma cell line, promotes differentiation of these cells into sympathetic neurons (Alema et al. 1985).

The biochemical and biological effects of RSV are obviously quite diverse, hence the *src* protein must simultaneously alter the activity of many different host proteins. There are two sorts of

models to explain how the *src* protein produces such diverse effects. Transformation by *src* results in the activation of 1000 host genes (Groudine and Weintraub,1980). Perhaps the *src* protein is a transcriptional activator. On the other hand, the *src* protein could directly regulate the activity of host proteins. These possibilities were distinguished by expressing v-*src* in enucleated cells (Beug et al. 1978). Several of the phenotypic changes that *src* produces in intact cells are also apparent in cells lacking nuclei, indicating that the *src* protein acts directly on host proteins. Obviously, tumorigenicity, serum independence, anchorage independence, and focus formation cannot be assayed in enucleated cells. Therefore, it is possible that these effects are mediated by changes in host gene expression provoked by v-*src*.

THE SRC PROTEIN IS A MEMBRANE ASSOCIATED TYROSINE KINASE

What sort of a protein could produce such pleiotropic effects? The protein encoded by v-*src* was first described as a transformation-specific antigen found in cells infected by RSV, but not in cells infected by transformation-defective viruses (Brugge and Erikson 1977). Rabbits injected with RSV develop tumors and subsequently produce antisera (TBR sera) that recognize a 60 kilodalton phosphoprotein (pp60^{src}). Since pp60^{src} is a phosphoprotein, several investigators suspected that pp60^{src} is a protein kinase. When ATP is incubated with pp60^{src}, isolated from cell extracts by immunoprecipitation, both pp60^{src} and the antibody molecule binding it

are phosphorylated (Collett and Erikson 1978; Levinson et al. 1978). Viral *src* protein purified by conventional chromatography also phosphorylates a number of proteins *in vitro* (Erikson et al. 1979; Levinson et al. 1980). A similar protein kinase is recognized by TBR sera in uninfected cells. This protein was subsequently shown to be encoded by c-*src* (Collett et al. 1979b; Oppermann et al. 1979).

The protein kinase activity of pp60^{src} was originally described as a threonine-specific kinase (Collett et al. 1979a). It was later discovered that immune-complexes containing the polyoma virus middle T antigen contained a protein kinase that phosphorylates middle T on tyrosine residues (Eckhart et al. 1979). The discovery of tyrosine kinases resulted from a serendipitous error in preparing a buffer, which allowed phospho-tyrosine to be resolved from phospho-threonine for the first time. Reconsideration of the kinase activity associated with pp60^{src} revealed that it too is a tyrosine kinase (Hunter and Sefton 1980). Ironically, it was later shown that the tyrosine kinase associated with middle T antigen is the c-*src* protein (Courtneidge and Smith 1983, 1984).

The *src* protein is a membrane protein. The subcellular localization of pp60^{src} has been analyzed by electron microscopy (Willingham et al. 1979), by biochemical fractionation (Courtneidge et al. 1980; Krueger et al. 1980), and by immunofluorescence (Rohrschneider 1980). The results of these analyses suggest that pp60^{src} tightly associates with the cytoplasmic face of intracellular membranes, especially at sites of contact between cells and the underlying substrate, which are called focal adhesions. The *src*

protein does not traverse the plasma membrane, since it is not detected by methods which specifically label the extra-cellular face of the plasma membrane. Chapters 3 and 4 of this thesis describe attempts to determine the sequences in pp60^{src} that are required for membrane localization and concentration in focal adhesions.

SUBSTRATES OF THE SRC KINASE

It is generally accepted that pp60^{src} provokes its phenotypic effects by phosphorylating host proteins. There are several reasons for believing that the tyrosine kinase activity of pp60^{src} is the transforming activity. Cells transformed by *src* have elevated levels of tyrosine-phosphorylated proteins (Sefton et al. 1980). Mutations which render pp60^{src} temperature sensitive for transformation are typically also temperature sensitive for tyrosine kinase activity (Collett and Erikson 1978; Levinson et al. 1978). With few exceptions (Verderame et al. 1989; Cross et al. 1984), defective versions of pp60^{src} are catalytically inactive (reviewed in Parsons and Weber 1989). Site specific alterations of the catalytic domain, such as inactivating the ATP binding site, abolish both the catalytic and transforming activities of pp60^{src} (Snyder et al.,1985). Furthermore, the transforming activity of pp60^{src} and other oncogenic kinases can be mimicked by treating cells with inhibitors of phosphotyrosine phosphatases, thereby increasing the abundance of tyrosine-phosphorylated proteins (Klarlund 1985).

The complexity of the transformed phenotype could indicate that

pp60^{src} phosphorylates several host proteins, or that pp60^{src} phosphorylates a single host protein which has diverse effects. Attempts have been made to distinguish these possibilities by the phenotypes of partially transformation defective *src* mutants (Weber 1984). These mutants demonstrate that the individual aspects of the transformed phenotype can be expressed in virtually any combination by specific *src* mutants. This fact eliminates the possibility that the transformed phenotype is the consequence of phosphorylating a single host protein. *Src* mutations which produce partially transformed phenotypes presumably alter the substrate specificity of pp60^{src}. Apparently, pp60^{src} must phosphorylate several host proteins to produce the fully transformed phenotype.

Investigators have used several methods to identify host proteins that are phosphorylated by pp60^{src} (reviewed by Cooper and Hunter 1984). Since pp60^{src} will phosphorylate non-physiological substrates *in vitro* (Graziani et al. 1983), most investigators have focussed on identifying *in vivo* substrates of pp60^{src} as potential mediators of the transformed phenotype. The pattern of total phosphorylated proteins in *src* transformed cells is very similar to that in normal cells because phosphotyrosine accounts for not more than 1% of total phosphoamino acids (Sefton et al. 1980). When methods are used to identify tyrosine-phosphorylated proteins specifically, differences between transformed cells and normal cells are apparent. Most investigators have identified tyrosine-phosphorylated proteins in extracts of cells labelled with $^{32}P_i$ by treating the crude phosphoproteins with strong alkali, which

selectively hydrolyses phosphoserine residues. Phosphoproteins which are resistant to alkali are subsequently subjected to phosphoamino acid analysis. Recently, anti-phosphotyrosine antibodies have been described which recognize tyrosine-phosphorylated proteins specifically (Frackelton et al. 1983; Comoglio et al. 1984; Wang 1985). These antibodies greatly simplify identification of tyrosine-phosphorylated proteins. The best estimate from these studies is that 50 host proteins are tyrosine-phosphorylated in *src* transformed cells. Since it is not known whether $pp60^{src}$ is directly responsible for phosphorylation of these proteins, they must be regarded as putative substrates of $pp60^{src}$.

Which of these 50 potential substrates mediate the neoplastic effects of $pp60^{src}$? If a substrate is responsible for an aspect of the transformed phenotype, then modification of this substrate should invariably accompany this phenotype. Several *src* mutants have proven useful in this analysis. Partially-transforming *src* alleles produce virtually all conceivable combinations of the phenotypic effects of wild type *src* (reviewed in Weber 1984). Analysis of these mutants suggests that none of the known substrate modifications is necessary or sufficient for producing specific phenotypes (Cooper et al. 1983a). A few of the non-transforming alleles of *src* encode catalytically active *src* proteins. Although these *src* genes do not transform cells, many of the known substrates are fully modified in cells expressing these *src* proteins (Kamps et al. 1986). While transformed cells typically express $pp60^{src}$ vigorously, cells can be transformed by sufficiently low quantities of $pp60^{src}$ that most substrate

modifications cannot be detected (Jakobovits et al. 1984). These results imply that none of the known substrate modifications contributes to transformation of cells. Chapter 2 of this thesis describes a genetic strategy for identifying physiologically important substrates of pp60^{src}.

POTENTIAL SUBSTRATES OF SRC

Despite the failure to assign specific physiological effects to known substrate modifications, it is instructive to consider what sort of substrates could mediate the transforming effects of pp60^{src}. For this reason many investigators have proceeded by analyzing the biochemical basis for specific phenotypic effects of *src*.

Serum independence of transformed cells. Although many of the biological effects of pp60^{src} are known to occur in enucleated cells (Beug et al. 1978), some investigators have analyzed activation of host gene expression by pp60^{src}. It is estimated that 1000 host genes are induced by *src* transformation (Groudine and Weintraub 1980). However, in cells transformed by temperature-sensitive alleles of *src*, a far simpler set of genes are induced following shift to the permissive temperature. One of the genes identified in this manner encodes a secreted mitogenic peptide growth factor (Sugano et al. 1987; Bedard et al. 1987). Perhaps activation of this gene explains why *src* transformed cells are serum independent.

Another possible explanation for the serum independence of *src* transformed cells is that pp60^{src} augments the response of cells to

other growth factors. This model arose from experiments demonstrating that the EGF receptor is stoichiometrically phosphorylated on tyrosine in *src* transformed cells (Wasilenko and Weber 1987). The kinase responsible for phosphorylation of the EGF receptor in *src* transformed cells has not been identified. If *src* transformed cells produce mitogenic EGF-like peptides (for which there is no evidence), the EGF receptor would be activated and hence phosphorylated. On the other hand, it is possible that $pp60^{src}$, or a tyrosine kinase activated by $pp60^{src}$, phosphorylates the EGF receptor. It has also been reported that overexpression of the c-*src* gene causes cells to be unusually sensitive to the mitogenic effects of EGF (Luttrell et al. 1988).

Alteration of the actin cytoskeleton causes morphological transformation. *Src* transformed cells are more rounded and refractile than normal cells. The underlying basis for morphological transformation appears to be loss of microfilament stress fibers. The morphological effects of *src* can be mimicked by treating cells with microfilament destabilizing drugs, such as cytochalasin B (Norberg et al. 1975; Weber et al. 1976). Conversely, restoring the stress fibers of *src* transformed cells, by growing them on dishes coated with fibronectin, also restores a normal cell morphology (Ali et al.,1977). Loss of stress fibers may be the consequence of decreased surface fibronectin on *src* transformed cells (Hynes 1974). Decreased surface fibronectin on transformed cells reflects decreased levels of the fibronectin mRNA (Tyagi et al. 1985).

The effect of $pp60^{src}$ on the actin cytoskeleton may also be due to tyrosine-phosphorylation of actin-binding proteins. Actin

filaments are coupled to the plasma membrane at specialized structures called focal adhesions (reviewed by Burridge et al. 1988). Since pp60^{src} is found predominantly in focal adhesions (Rohrschneider 1980), it is not surprising that 3 other proteins found in focal adhesions appear to be substrates of pp60^{src}: vinculin (Sefton 1981), talin (Pasquale et al. 1986; DeClue and Martin 1987), and the fibronectin receptor (Hirst et al. 1986). Tyrosine-phosphorylation of vinculin (Kamps et al. 1986) and talin (DeClue and Martin, 1987) are thought to be adventitious because non-transforming *src* proteins still phosphorylate them. On the other hand, tyrosine-phosphorylation of the fibronectin receptor has been proposed to dissociate actin filaments specifically from the plasma membrane by preventing talin from associating with the fibronectin receptor (personal communication from L. Rohrschneider). One must reserve judgment of these claims until it has been determined whether non-transforming *src* proteins also phosphorylate the fibronectin receptor. There may be other substrates of pp60^{src} in focal adhesions because most tyrosine-phosphorylated proteins are found in focal adhesions (Maher et al. 1985).

The abundance of the glucose transporter explains the rates of hexose uptake in transformed cells. Cells transformed by *src* have an increased rate of hexose transport across the plasma membrane. The increased rate of transport reflects an increased number of transporter molecules in the plasma membrane of transformed cells (Salter et al. 1982). Surprisingly, the underlying cause for the increased number of transporters depends upon the cell type. In

chicken embryo fibroblasts, the increase reflects a stabilization of the glucose transporter protein (Shawver et al. 1988). In rat fibroblasts, on the other hand, the increase is the consequence of an increase in the abundance of the glucose transporter mRNA (Flier et al. 1987). This discrepancy implies that the increase in the rate of hexose uptake is an indirect effect of pp60^{src}.

STRUCTURE OF THE SRC PROTEIN

Given the frustration of efforts to identify physiologically important substrates, many investigators have turned their attention studying the structure of pp60^{src}, as a first step toward determining how it is regulated.

The sequence of the *src* gene defines a gene family (Hanks et al. 1988). In the past several years, a number of other tyrosine kinases have been described, several of which are oncogene products and growth factor receptors (reviewed in Hunter and Cooper 1985). The genes encoding these proteins all share a region of strong homology, typically a 250 amino acid domain, corresponding to the C-terminal half of pp60^{src}, which is 30-50% identical with other family members. This domain is called the *src* homology 1 (SH1) domain.

Several lines of evidence suggest that the SH1 domain corresponds to the catalytic domain of tyrosine kinases. The SH1 domain also displays a weak homology with serine/threonine specific protein kinases (Hanks et al. 1988). Mutations of highly conserved residues in the SH1 domain impair catalytic activity. Controlled

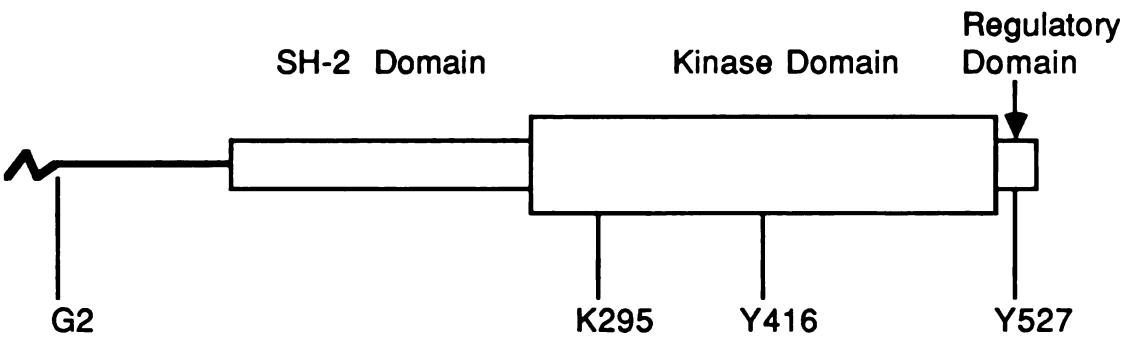
proteolytic digestion of pp60^{src} generates a C-terminal 30 kilodalton fragment which retains kinase activity (Levinson et al. 1981). Thus, SH1 is the catalytic domain of tyrosine kinases.

Among tyrosine kinases, there is a group which is clearly more closely related to pp60^{src} than to other tyrosine kinases (reviewed by Cooper 1989). The genes encoding this family of tyrosine kinases are probably products of gene duplications because they have a very similar pattern of intron-exon boundaries (Cooper et al. 1989). In addition to the SH1 domain, the members of this family of tyrosine kinases share 3 other regions of strong homology: the N-terminal 10 amino acids (Kaplan et al. 1988); a domain, called SH2, corresponding to amino acids 80-260 of pp60^{src} (Sadowski et al. 1986; Mayer et al. 1988); and a domain corresponding to amino acids 515-530 of pp60^{src} (Cooper 1989). Figure 1-1 schematically demonstrates the structure of pp60^{src}.

The amino terminal homology region acts as a recognition sequence for myristylation. The *src* protein is an unusual membrane protein. Unlike most membrane proteins, pp60^{src} has neither a hydrophobic signal sequence nor a hydrophobic membrane-spanning region. Association of pp60^{src} with the cytoplasmic face of cellular membranes is mediated in part by N-terminal fatty acylation of pp60^{src} with a 14 carbon saturated fatty acid, myristic acid (Buss et al. 1984; Schultz et al. 1985). Mutations that prevent myristylation of pp60^{src} encode catalytically active, cytosolic, and consequently non-transforming *src* proteins (Cross et al. 1984; Kamps et al. 1986). The sequences that are required for myristylation of pp60^{src} appear to be

Figure 1-1. Schematic representation of the structure of pp60^{src}. The functional domains of pp60^{src} are indicated as follows: amino acids 1-7, the myristylation signal; amino acids 80-260, the SH-2 domain; amino acids 261-514, the kinase domain (or SH-1); and amino acids 515-530, the regulatory domain. Functionally important amino acids in some of these domains have been indicated. Glycine-2 is the sit of N-terminal myristylation. Lysine-295 is involved in ATP-binding. Tyrosine-416 is the autophosphorylation site. And phosphorylation of tyrosine-527 inhibits both the catalytic and transforming activities of pp60^{src}.

Figure 1-1.


SH-2 Domain
Kinase Domain
Regulatory Domain
G2
K295
Y416
Y527

concentrated at the immediate N-terminus, hence it appears likely that other members of this family of tyrosine kinases will be myristylated and that this accounts for the conservation of the N-terminal sequences of these proteins. Chapter 3 of this thesis describes experiments that determine the sequences required for myristylation of pp60^{src}.

The SH2 domain is a regulatory domain. Several experiments support the notion that the SH2 domain regulates the catalytic activity of pp60^{src}. The intrinsic kinase activity of pp60^{src} is greatly enhanced by proteolytic digestions that sever the catalytic domain from the non-catalytic SH2 domain (Levinson et al. 1981). Mutations which alter the SH2 domain often activate the catalytic activity of pp60^{src} (Kato et al. 1986; Potts et al. 1988; Fox and Brugge, personal communication). Activation of pp60^{src} kinase activity *in vivo* (Ralston and Bishop 1985; Chackalaparampil and Shalloway 1988; Yonemoto et al. 1985) and *in vitro* (Collett et al. 1983) is often accompanied by new phosphorylations in the amino terminal domain of pp60^{src}. A further indication that the SH2 domain is the site of interaction between pp60^{src} and host proteins is that mutations in the SH2 domain of either v-*src* (Verderame et al. 1989) or v-*fps* (DeClue et al. 1987) restrict the transforming activity in rodent cells, but not in chicken cells. The nature of this host range dependence has not been determined, but could reflect host-dependent substrate specificity or regulation of tyrosine kinase activity.

In addition to tyrosine kinases, SH2 domains are found in the viral oncogene of the avian sarcoma virus CT10, v-*crk* (Mayer et al.

1988), the 148 kilodalton form of phospholipase C (Stahl et al. 1988; Suh et al. 1988), the GTPase activating protein GAP (Vogel et al. 1988), the actin binding protein α-spectrin (Lehto et al. 1988), and the yeast actin binding protein ABP1 (personal communication from D. Drubin). The function of the SH2 domain is not known in any of these cases. Chapter 4 of this thesis describes a strategy for determining the function of the SH2 domain.

The C-terminal homology region is also a regulatory domain. The first indication that the domain corresponding to amino acids 515-530 exerts a profound effect on pp60^{src} was the discovery that in all isolates of RSV this region was altered or lost when c-*src* was incorporated into the viral genome (Takeya and Hanafusa 1983; Swanstrom et al. 1983). This fact is provocative because several investigators had shown that both the transforming activity (Parker et al. 1984; Shalloway et al. 1984a and b; and Iba et al. 1984) and the kinase activity (Coussens et al. 1985) of the c-*src* protein are significantly attenuated compared to the v-*src* protein. The v-*src* and c-*src* proteins also differ in their sites of tyrosine-phosphorylation (Smart et al. 1981). While the v-*src* protein is phosphorylated on tyrosine-416, the c-*src* protein is phosphorylated on tyrosine-527, which falls in the C-terminal homology domain (Cooper et al. 1986). These facts suggest that phosphorylation of tyrosine-527 inhibits both the transforming and kinase activities of pp60^{src}. Apparently, loss of this phosphorylation site is at least partially responsible for the transforming activity of v-*src*.

Several subsequent experiments support the notion that

phosphorylation of tyrosine-527 inhibits the activities of pp60^{src}. Dephosphorylation of pp60^{src} *in vivo* (Courtneidge 1985) or *in vitro* (Cooper and King, 1986) activates the catalytic activity. Converting tyrosine-527 (or the analogous tyrosine in relatives of pp60^{src}) to a phenylalanine, thereby precluding phosphorylation, activates both the transforming and kinase activities of pp60^{src} (Cartwright et al. 1987; Kmiecik and Shalloway 1987; and Piwinca-Worms et al. 1987) and of other members of this family of tyrosine kinases (Amrein and Sefton 1988). It is not clear if phosphorylation of tyrosine-527 is the product of autophosphorylation or of phosphorylation by another kinase (Jove et al. 1987; Cooper and MacAuley 1988).

PROTEINS THAT REGULATE THE SRC PROTEIN.

Analysis of the structure of pp60^{src} has shed light on the regulation of its activities. Some of the proteins that mediate this regulation have been identified biochemically. Investigators have employed two basic strategies: identifying host protein kinases that phosphorylate pp60^{src}, and identifying host proteins that bind to pp60^{src}.

Phosphorylation of the src protein by host protein kinases. In addition to the kinase that phosphorylates tyrosine-527, several other protein kinases phosphorylate pp60^{src}. The cAMP-dependent protein kinase (A-kinase) phosphorylates serine-17 of pp60^{src} (Collett et al. 1979b), and the Ca^{2+} and phospholipid dependent protein kinase (C-kinase) phosphorylates serine-12 and serine-48 of pp60^{src} (Purchio et

al. 1985; Gould et al. 1985). Neither modification alters the catalytic activity nor the transforming activity of pp60^{src} (Cross and Hanafusa 1983; Gould et al. 1985); however it is possible that these modifications affect other properties of pp60^{src}. Phosphorylation by C-kinase cause a rapid internalization of the EGF receptor from the plasma membrane (Lin et al. 1986). Phosphorylation of the *lck* protein, a tyrosine kinase closely related to pp60^{src}, by C-kinase disrupts the association between the *lck* protein and the CD4 protein, a component of the T-lymphocyte antigen receptor complex (Hurley et al. 1989).

During mitosis, a novel form of pp60^{src} appears which has reduced mobility in SDS gels and which is phosphorylated on an N-terminal threonine residue (Chackalaparampil and Shalloway 1988). This modified form of pp60^{src} appears to have increased catalytic activity. The cdc2 protein (first identified in fission yeast), a serine and threonine specific protein kinase which is known to regulate the cell cycle (Lee and Nurse 1988), is a subunit of the mitosis specific kinase which phosphorylates pp60^{src} (Morgan et al. 1989). This implies that pp60^{src} is at least partially responsible for the cell cycle control exerted by the cdc2 protein kinase. On the other hand, a role for pp60^{src} in cell cycle control seems unlikely because pp60^{src} is normally expressed in non-proliferating tissues (Cotton and Brugge 1983; Golden et al. 1986) (discussed further below).

The *src* protein is also modified during the cellular response to PDGF (Ralston and Bishop 1985; Gould and Hunter 1988). Within minutes

of exposing intact cells to PDGF, a novel form of pp60^{src} appears which has slightly reduced mobility in SDS gels and slightly elevated catalytic activity *in vitro*. This modified form of pp60^{src} is phosphorylated on a novel N-terminal tyrosine residue. Although the tyrosine kinase responsible for this phosphorylation has not been identified, it is tempting to speculate that pp60^{src} is a substrate of the PDGF receptor tyrosine kinase, and that pp60^{src} is in part responsible for the mitogenic effects of PDGF.

Proteins that bind to the src protein. Newly synthesized pp60^{src} molecules are found associated with two host phosphoproteins, pp50 and pp90 (Oppermann et al. 1981a; Brugge et al. 1981). After 10 minutes, pp60^{src} leaves this complex and associates with the cytoplasmic face of the plasma membrane (Courtneidge and Bishop 1982; Brugge et al 1981). The 90 kilodalton protein has been identified as the heat shock protein hsp90 (Oppermann et al. 1981c). The 50 kilodalton protein is tyrosine-phosphorylated in avian cells transformed by *src* (Brugge and Arrow 1982; Oppermann et al. 1981a). At least two other tyrosine kinases, the v-*fps* and the v-*yes* proteins, also associate with pp50 and hsp90 (Lipsich et al. 1982). Although the function of this complex is not known, it has been speculated that pp50 and hsp90 are necessary to transport pp60^{src} from soluble polysomes to the plasma membrane (Courtneidge and Bishop 1982).

In cells transformed by polyoma virus, a proportion of pp60^{src} is found associated with middle T antigen (Courtneidge and Smith 1983, 1984; Bolen et al. 1984). This association explains the initial observation that middle T antigen is associated with a tyrosine kinase

(Ekhart and Hunter 1979). The pp60^{src} associated with middle T antigen has greatly enhanced catalytic activity and is phosphorylated on novel N-terminal tyrosine residues (Yonemoto et al. 1985; Cartwright et al. 1985). Association with middle T antigen appears to stabilize the form of pp60^{src} that is not phosphorylated on tyrosine-527 (Cartwright et al. 1986), which may account for activation of the kinase activity of pp60^{src} (Cooper and King 1986). At least two other tyrosine kinases are found associated with middle T antigen, the c-*yes* protein (Kornbluth et al. 1987) and the c-*fyn* protein (Kypta et al. 1988).

The complex that contains middle T antigen and pp60^{src} does not appear to be a simple heterodimer composed of these two subunits. The approximate molecular weight of this complex is 220 kilodaltons (Walter et al. 1982; Courtneidge and Smith 1983), which is larger than would be predicted for a complex between a single middle T molecule (a 55 kilodalton protein) and a single pp60^{src} molecule. Other potential subunits of this complex have been identified in immunoprecipitates that contain this complex. If ATP is added to purified middle T antigen, prepared by immunoprecipitation from crude cell extracts, 3 proteins are phosphorylated: middle T antigen (pp55), pp60^{src}, and a protein variously referred to as pp81 (Courtneidge and Heber 1987) and pp85 (Kaplan et al. 1987).

Although pp85 has not been purified, several lines of evidence suggest that it encodes a phosphatidylinositol kinase activity (Courtneidge and Heber 1987; and Kaplan et al. 1987). Transformation by polyoma appears to alter the phosphorylation and turnover of

phosphatidylinositol (Kaplan et al. 1986). Immunoprecipitates that contain middle T antigen also contain a phosphatidylinositol kinase activity (Whitman et al. 1985). Altered phosphorylation and turnover of phosphatidylinositol has been implicated in transformation by several other oncogenes (Sugimoto et al. 1984; Macara et al. 1984; Fry et al. 1985; and Jackowski et al. 1986). And phosphatidylinositol kinase activity is often found associated with activated tyrosine kinases (Kaplan et al. 1987). The significance of this association is pending, largely because the metabolite produced by this phosphatidylinositol kinase (which phosphorylates the 4' position of the inositol group) has no known function (Whitman et al. 1987).

Chapter 5 of this thesis describes a new strategy for identifying proteins that bind to pp60^{src}.

WHAT IS THE NORMAL PHYSIOLOGICAL ROLE OF THE SRC PROTEIN?

Despite detailed knowledge of the structure and activities of pp60^{src}, no biological function has been ascribed to pp60^{src}. This is largely due to the fact that mutants lacking pp60^{src} have not been isolated in any organism, despite heroic efforts (M. A. Simon, personal, and heartfelt, communication). Speculation about the function of pp60^{src} relies upon the temporal and spatial pattern of expression of c-*src* in normal development, and upon the precedents of other tyrosine kinases.

The normal pattern of expression of the c-src protein suggests a role in neurogenesis. The expression of pp60^{src} has been studied in

chicken, mouse, rat, and fruit flies. These analyses reveal that pp60^{src} is detectable in most tissues, but that unusually high levels are found in differentiated cell types with limited proliferative potential, such as neurons (Cotton and Brugge 1983; Simon et al. 1985), and blood platelets (Golden et al. 1986), implying that the presence of pp60^{src} is not sufficient to sustain the proliferation of a cell.

Careful analysis of the timing of pp60^{src} expression suggests that pp60^{src} performs a role in the differentiation of neural tissues, probably in establishing synaptic contacts. In the developing eye of the fruit fly, pp60^{src} expression is first apparent after differentiation of precursor cells into ommatidia (the subunit of the compound eye), but little pp60^{src} is found in the adult compound eye (Simon et al. 1985). A similar conclusion is reached from analysis of pp60^{src} expression in developing rat brains (Cartwright et al. 1988). Here the expression of pp60^{src} peaks either during embryogenesis (as in the developing striatum) or shortly after birth (as in the hippocampus and the cerebellum), with levels progressively declining with maturity. The sites of persistent expression of pp60^{src} in the adult brain are sites of persistent synaptic re-organization (Ross et al. 1988). A further indication that pp60^{src} plays a role in neurogenesis, is that in neurons pp60^{src} is found primarily in growth cones (Manness et al. 1988).

The form of pp60^{src} expressed in neurons is structurally distinct from that found in other tissues. The pp60^{src} found in neurons migrates more slowly in SDS gels and has a slightly elevated

catalytic activity (Brugge et al. 1985). The altered mobility of neuronal pp60^{src} is the consequence of a neuron specific alternative splicing of *src* mRNA (Martinez et al. 1987; Levy et al. 1987). This alternative splice introduces a novel exon, which encodes 6 amino acids (residues 114-119), at the junction between what are normally exons 3 and 4. This unique exon resides in the SH2 domain of pp60^{src}. Although the function of this novel exon is not known it is tempting to speculate that it acts as a binding site for a neuronal *src* binding protein.

The presence of enormously high levels of pp60^{src} in blood platelets (200x the level in the average fibroblast) (Golden et al. 1986) has led to speculation that pp60^{src} plays a role in either cell adhesion or secretion, the two differentiated functions of platelets. Consistent with this hypothesis is the finding that thrombin, an agonist that activates both platelet secretion and adhesion, activates an unidentified tyrosine kinase (Ferrel and Martin 1988; Golden et al. 1989). In both platelets and chromaffin cells, pp60^{src} is found mainly in specialized secretory vesicles: dense bodies in platelets (Rendu et al. 1989) and chromaffin granules in chromaffin cells (Parsons and Creutz 1986).

Is the src protein an intracellular subunit of a growth factor receptor? Although pp60^{src}, and its close relatives, are structurally very different from the growth factor receptor tyrosine kinases, it is very tempting to speculate that they have analogous functions. Both types of tyrosine kinases provoke similar alterations in the growth properties of cells. These tyrosine kinases phosphorylate many of the

same substrates (Kamps and Sefton 1988). Transmembrane tyrosine kinases have low basal kinase activity but can be activated either by mutation or by binding their cognate ligands. Similarly, the activity of the c-*src* protein is quite low compared to the v-*src* protein (Coussens et al. 1985), and is activated by mutations. Mutations that activate either type of tyrosine kinase are often deletions or point mutations in the N-terminal ligand-binding or SH2 domain. Which domain of pp60^{src} could be performing the analogous function of the ligand binding domain of the transmembrane tyrosine kinases? The SH2 domain is the best candidate for the ligand binding domain of pp60^{src}, because this domain is unique to tyrosine kinases that lack extracellular ligand binding domains, and because mutations in SH2 have analogous effects to mutations in ligand-binding domains.

Recently, a physical association between the *lck* protein tyrosine kinase and two components of the T-lymphocyte antigen receptor complex, CD4 and CD8, has been discovered (Rudd et al. 1988; Veillette et al. 1988). The *lck* protein is found in immune precipitates containing either CD4 or CD8. Crosslinking of CD4 causes an increase in the tyrosine kinase activity of the *lck* protein (Veillette et al. 1989). The CD4 and CD8 molecules are associated with the T-lymphocyte antigen receptor, and are thought to be involved in activation of T-lymphocytes by antigen. Perhaps activation of the *lck* tyrosine kinase mediates the response of T-lymphocytes to antigen. The role of the SH2 domain in the interaction of the *lck* protein and CD4 or CD8 has not been determined.

Chapter 5 of this thesis describes a strategy for identifying

molecules that regulate pp60^{src} in an analogous fashion to the regulation of the *lck* protein by CD4 and CD8.

CHAPTER 2:

GENETIC ANALYSIS OF HOST FACTORS INVOLVED IN RSV MEDIATED TRANSFORMATION

Rous Sarcoma Virus (RSV) has proved to be a powerful experimental system for studying the mechanism of neoplastic transformation. The biochemical and genetic simplicity of RSV has facilitated the analysis of the viral gene, v-*src*, that encodes the transforming activity. Conversely, the relative biochemical and genetic complexity of the host cell has hindered the analysis of its contributions to the neoplastic process. Efforts to identify host factors have thus far been limited to two sorts of biochemical strategies. Some investigators have asked how $pp60^{src}$ biochemically alters its host (Cooper and Hunter 1983a), while others have asked how the host biochemically alters $pp60^{src}$ (Krueger et al. 1983). Although both approaches have successfully identified such modifications, neither has convincingly identified either the physiological regulators of $pp60^{src}$, or the substrates that mediate the neoplastic effects of $pp60^{src}$, as summarized in chapter 1.

This chapter describes a genetic strategy for identifying host factors, pseudoreversion analysis of transformation defective *src* mutants.

Genetic Strategies for Identifying Host Factors

Functionally important protein-protein interactions can be inferred from observing phenotypic interactions between non-allelic genes (Botstein and Maurer 1982). These observations serve as a prelude to direct biochemical analysis of physical or regulatory interactions. One general approach is to select secondary mutations

(called suppressors) which reverse a specific mutant phenotype, such that the double mutant resembles the wild type more than the parental single mutant. In this way, it should be possible to isolate mutations in cellular genes which augment the transforming activity of transformation-defective *src* mutants. Mutations isolated in this way may identify cellular genes that encode either proteins that regulate $pp60^{src}$, or substrates that mediate the transforming effects of $pp60^{src}$.

When non-transformed cells that express a transformation-defective *src* allele are mutagenized, transformed cells (hereafter referred to as genetic revertants) should arise as the result of new mutations. Various methods have been used to enrich transformed cells selectively, including focus formation, formation of tumors in animals, and formation of colonies in soft agar. These assays rely on different properties of transformed cells, as summarized in chapter 1. Since the spontaneous incidence of focus formation can be quite high, and since animal assays are both awkward and costly, I decided to rely on growth in soft agar as the means to select transformed cells.

There will be three classes of genetic revertants. Some revertants will have mutations in the *src* gene (*src* revertants), and others will have mutations in cellular genes, which are called suppressors. There are two classes of suppressors, mutations that transform cells independent of the *src* gene (bypass suppressors), and mutations that augment the transforming activity of the mutant *src* gene (interaction suppressors). Mutations that activate a cellular oncogene, a c-*ras* gene for instance, are potential bypass suppressors.

Unfortunately, there are interesting classes of suppressors among the bypass suppressors. For instance mutations that activate something functionally downstream of pp60^{src} would also be classified as bypass mutants. Interaction suppressors are the most interesting class of revertants, because they may identify genes that are required for *src* to transform cells. Potential interaction suppressors are mutations that inactivate an inhibitor of pp60^{src}, such as a tyrosine phosphatase, or mutations that alter a substrate of pp60^{src}. Not all interaction suppressors are interesting. For instance, missense suppressors (or other informational suppressors) could activate the transforming potential of a mutant *src* gene, by causing the synthesis of a transforming *src* protein.

Distinguishing among these three classes of revertants requires the ability to segregate the suppressor genes from the *src* gene genetically. In yeast or *Drosophila*, one could simply genetically cross the revertant animal with a wild-type animal and observe segregation of genetic markers in the progeny. Since this is not possible in mammalian cell culture, other means must be found for segregating *src* genes from suppressor genes.

In prokaryotic systems, genetic markers are often segregated by exploiting transducing phages, rather than by carrying out genetic crosses. I have exploited recently described retroviral vectors to perform genetic crosses in mammalian cell culture (Cepko et al. 1984). These vectors encode replication-defective retroviruses. When provided retroviral helper functions, they replicate in mammalian cells as retroviruses. Generally, they can accommodate two cloned

genes, one of which usually encodes a selectable marker. The bacterial GPT gene uniquely suits this enterprise. In cells deficient for the HPRT gene (which encodes hypoxanthine phosphoribosyl transferase, an enzyme required for the utilization of guanine), one can select for expression of the GPT gene (with mycophenolic acid or methotrexate) or against the expression of the GPT gene (with 6-thioguanine, 6-TG) (Mulligan and Berg 1980; Tindall et al. 1984).

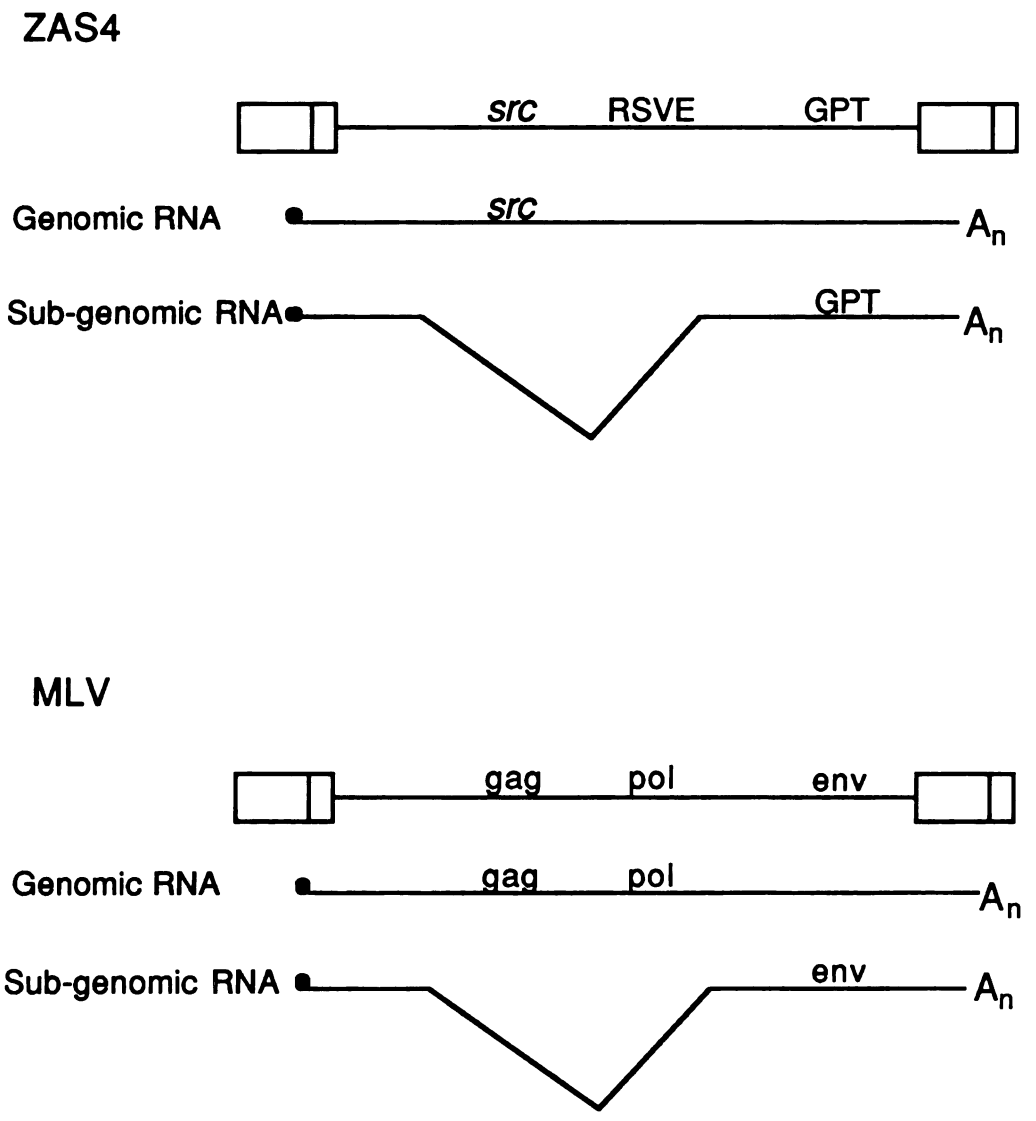
As a first step toward conducting a genetic screen for cellular genes that interact with the *src* gene, I constructed a retroviral vector that expresses the *src* gene and the GPT gene. Methods for exploiting these vectors in conducting genetic analyses will then be described.

Rat-1 fibroblasts were chosen as the genetic background for these experiments because they are euploid, and they have a very low incidence of spontaneous transformation ($<10^{-7}$ for growth in soft agar). I isolated an $hprt^-$ variant of rat-1 cells, RT, in which both forward and counter selections for GPT are possible, as described above.

A retroviral vector that expresses $pp60^{src}$ and GPT. The *gag* and *pol* genes of Moloney Murine Leukemia Virus (MLV) were replaced with the v-*src* gene, and the *env* gene was replaced with the GPT gene (see figure 2-1). The resulting viral DNA (ZAS4) encodes two RNAs, a genomic RNA, which corresponds to the *gag-pol* mRNA of MLV, and a spliced sub-genomic RNA, which corresponds to the *env* mRNA of MLV. The *src* gene is translated from the genomic RNA, while the GPT gene is translated from the spliced subgenomic RNA. When MLV replication

Figure 2-1. Structure of the ZAS4 retroviral vector. A 2 kb fragment (corresponding to nucleotides 7093-9238 of RSV) containing the v-*src* gene and the RSV enhancer was introduced into the ZIPVGPT vector (kindly provided by R. Cone and R. Mulligan). The predicted viral RNAs are indicated as wavy lines.

Figure 2-1.


ZAS4
src
RSVE
GPT
Genomic RNA
src
An
Sub-genomic RNA
GPT
An
MLV
gag
pol
env
Genomic RNA
gag
pol
An
Sub-genomic RNA
env
An

functions are provided, this vector replicates as an infectious retrovirus. ZAS4 viral DNAs encoding different *src* alleles are easily generated.

The resulting recombinant virus faithfully transduces both the *src* gene and the GPT gene. ZAS4 viral stocks typically have titers of 1000 infectious units per milliliter, when assayed by either mycophenolic acid resistance or by the transforming activity of *src*. After infection with a virus containing a transforming *src* gene, greater than 95% of mycophenolic acid resistant cells are transformed. The amount of $pp60^{src}$ produced varies a great deal (greater than 10-fold) among independently infected clones (see figure 2-3 for example). This variation presumably reflects the different chromosomal positions of these proviruses.

ZAS4 proviruses are rescued by superinfection with helper virus. Since some of the genetic revertants may contain mutations in the *src* gene which restore transforming activity, it would be useful to be able to recover the *src* gene from revertant cells and test its transforming activity in naive cells. This is possible because the *src* gene is carried by a retroviral vector. The ZAS4 DNA encodes a replication-defective retrovirus, lacking the trans-acting viral replication functions. These replication functions can be provided by infecting cells containing ZAS4 proviruses with wild-type MLV. The most effective method for superinfecting these cells is to co-cultivate ZAS4-infected cells with cells producing wild-type MLV. After 2-3 days of co-cultivation, the culture medium contains a mixed viral stock containing both MLV and ZAS4 genomes. ZAS4 virus rescued

in this manner can be assayed by infecting naive cells and either selecting mycophenolic acid resistance or analyzing *src* transforming activity. The titers of these rescued viral stocks vary (usually between 10-1000 infectious units/ml), depending upon the extent of superinfection, and upon the expression of the ZAS4 provirus being rescued.

Viral rescue is useful in distinguishing the types of genetic revertants. If a revertant contains a mutation in the *src* gene that restores transforming activity, then the rescued viral stock will transform normal rat-1 cells. Conversely, if the revertant contains a transforming mutation in a cellular gene, then the rescued viral stock will not transform normal rat-1 cells. Obviously, it is important to test the efficiency of viral rescue before beginning a genetic analysis.

Proviruses produced by infection with the ZAS4 virus are mitotically stable. While genes introduced into animal cells by transfection are genetically unstable and typically are maintained by constant selection (Perucho and Wigler 1981), mitotic stability is a hallmark of retroviral proviruses (Varmus et al. 1981a and b). I tested the mitotic stability of ZAS4 proviruses by selecting for 6-thioguanine resistant cells. Several independent clones of infected cells were subjected to 6-TG selections (see table 2-1). The apparent incidence of 6-TG resistance varied greatly among these clones, from virtually 100% to 0.001%. Although the basis of this variation is not known, it is likely that the differential resistance to 6-TG reflects differences in the expression of the GPT gene, and differences in

TABLE 2-1. The frequency of 6-TG resistance varies amongst clones.

Gene[a]	Clone	6-TG resistance (N)[b]	%TX[c]
v	1	10^{-5} (50)	0
v	2	10^{-5} (44)	16%
v	3	10^{-3}	10%
v	4	10^{-5}	50%
c	1	100%	NA
c	2	$5x10^{-6}$	NA
c	3	$3X10^{-5}$	NA
c	4	$5X10^{-4}$	NA
c	5	$3X10^{-4}$	NA

---

a) RT cells were infected with a ZAS4 virus carrying either a wild-type v-*src* gene (v) or c-*src* gene (c).

b) $5x10^5$ cloned infected cells were grown in 6-TG in 150mm dishes and resistant subclones appeared at the indicated frequency. The number (N) of 6-TG resistant clones isolated is indicated.

c) Since the cells infected by the virus containing v-*src* were morphologically transformed at the outset, it was possible to assess the fraction of the 6-TG resistant clones that remained morphologically transformed. Since c-*src* does not transform RT cells, this analysis is not applicable (NA) to these clones.

chromosomal position.

The src gene is often inactivated during conversion of ZAS4 infected cells to 6-TG resistance. It would be very useful if one could readily select for loss of the *src* gene from ZAS4 infected cells. This would allow one to distinguish between the classes of genetic revertants. Revertants that require the continued expression of *src* (*src* revertants and interaction suppressors) would phenotypically revert after loss of the *src* gene, while revertants that are phenotypically independent of *src* expression (bypass suppressors) would be unaffected by loss of the *src* gene.

Since the *src* gene is closely linked to the GPT gene, it seemed likely that some of the 6-TG resistant clones would have lost both genes. This was tested by selecting 6-TG resistant variants of cells infected by ZAS4 carrying a transforming *src* allele. If the *src* gene is lost during the conversion to 6-TG resistance, then the resulting clone of cells should phenotypically revert, and thus resemble uninfected cells. This analysis was conducted on several independent clones (see table 2-1). In general, clones that have a high incidence of 6-TG resistance produce mainly non-transformed drug resistant clones. On the other hand, clones that have a low incidence of 6-TG resistance (0.01-0.001%), produce roughly equal numbers of transformed and normal drug-resistant clones. Once again the basis of this variation is not known; however, it is clearly important to establish the behavior of a clone before initiating genetic analysis. Since the clones that have a high incidence of 6-TG resistance are likely to be phenotypically unstable, I have always chosen clones with a low

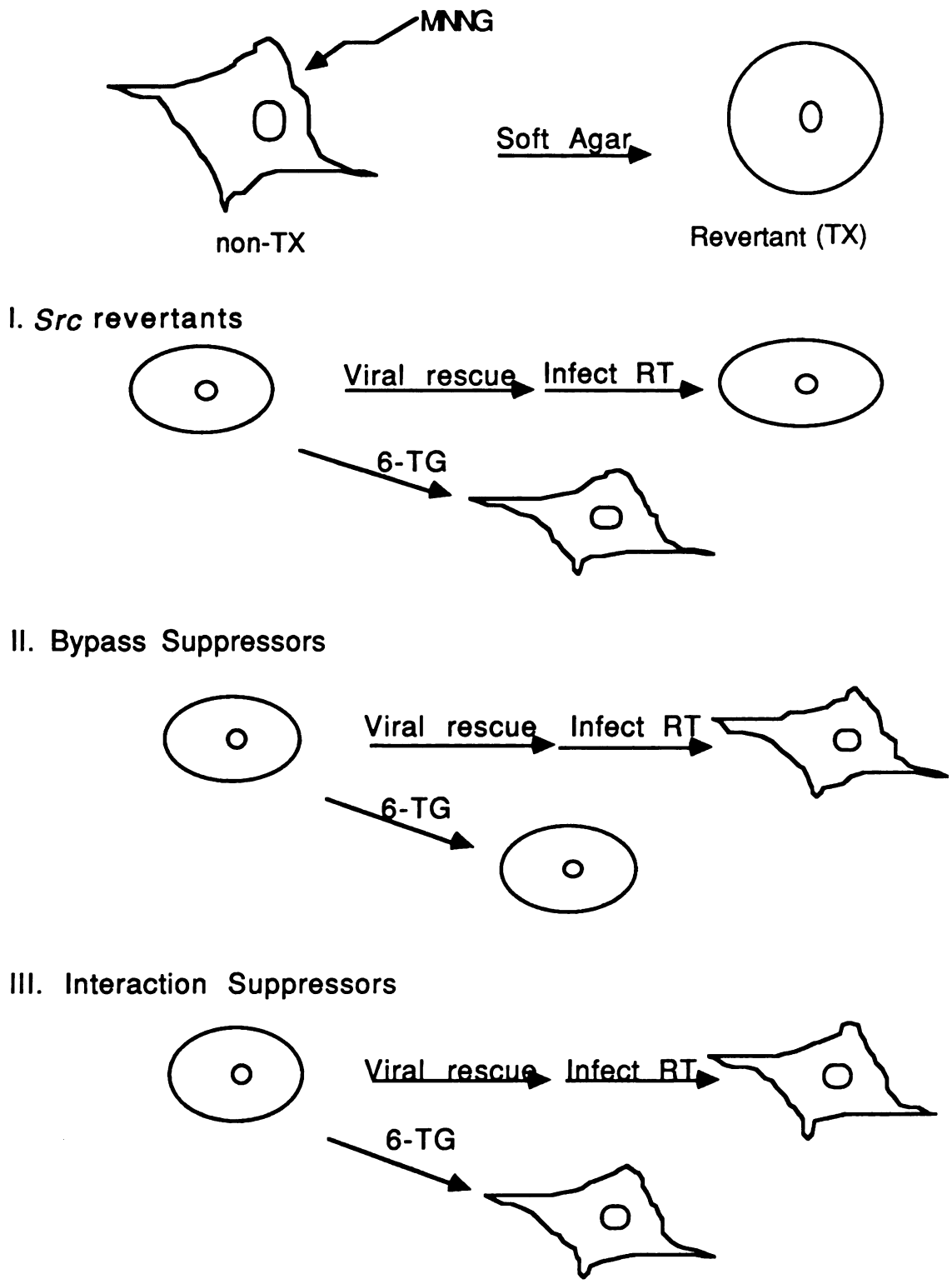
incidence of drug resistance.

The three classes of genetic revertants can be distinguished by rescuing the ZAS4 virus together with selecting 6-TG resistant clones. For the purposes of this discussion, let us assume that a ZAS4 virus containing a transformation-defective *src* gene has been introduced into rat-1 cells by infection, and a cloned infected cell has been isolated. This parental cell line is mutagenized and transformed revertants are isolated.

The methods described thus far allow one to distinguish the three classes of genetic revertants (see figure 2-2). If a revertant contains a transforming *src* gene due to intragenic reversion, then the rescued ZAS4 virus will transform normal rat-1 cells. The two classes of cellular mutations (bypass suppressors and interaction suppressors) are distinguished by selecting 6-TG resistant subclones. If a revertant contains a transforming mutation in a cellular gene, a bypass suppressor, then loss of the *src* gene will not affect the phenotype of this clone. Hence all of the 6-TG subclones derived from bypass suppressors will be transformed. On the other hand, if a revertant contains a mutation in a cellular gene that allows the mutant *src* gene to transform cells, then loss of the *src* gene will cause phenotypic reversion of the clone. Hence, some of the 6-TG resistant subclones derived from interaction suppressors will be non-transformed. If loss of the transformed phenotype is due to loss of the mutant *src* gene, and not loss of flanking cellular genes, then infecting non-transformed 6-TG resistant subclones with the parental ZAS4 virus should once again cause transformation.

Figure 2-2. Methods used to distinguish the 3 classes of genetic revertants. The parental non-transformed (non-TX) cell line is chemically mutagenized with nitrosoguanidine (MNNG) and transformed (TX) revertants are isolated by cloning in soft agar. Revertants that contain mutations in *src* which restore transforming (*src* revertants) should produce transforming rescued virus, and should give rise to some non-transformed 6-TG resistant subclones. Revertants that contain bypass suppressors should produce non-transforming rescued virus, and should produce only transformed 6-TG resistant subclones. Revertants that contain interaction suppressors should also produce non-transforming rescued virus, but in contrast to bypass suppressors will give rise to some non-transformed 6-TG resistant subclones.

Figure 2-2


MNNG
Soft Agar
non-TX
Revertant (TX)
I. *Src* revertants
Viral rescue
Infect RT
6-TG
II. Bypass Suppressors
Viral rescue
Infect RT
6-TG
III. Interaction Suppressors
Viral rescue
Infect RT
6-TG

The ZAS4 vector has been successfully adapted for expression of the c-*fos* gene (personal communication, J. Tuttleman), the v-Ha-*ras* gene, and the v-*myc* gene (my unpublished data). In principle, these vectors could be used to conduct genetic analyses for these oncogenes as well. The ZAS4 vector appears to be more adaptable to the expression of two genes than similar retroviral vectors. This may be because, together with the coding domain of *src*, the RSV transcriptional enhancer was incorporated into ZAS4 (see figure 2-1). Versions of ZAS4 that lack this enhancer do not express the GPT gene well (my unpublished data).

Choice of the transformation-defective *src* allele. The types of suppressors one anticipates depend upon the chosen starting allele. For instance, leaky tyrosine kinase mutants would be appropriate for isolating recessive mutations in negative regulators of $pp60^{src}$, such as tyrosine specific phosphatases, or the kinase responsible for phosphorylating tyrosine-527. Similarly, mutations affecting myristyl transferases, or host factors involved in membrane localization could be selected by suppressing alleles deficient in either of these respects. Mutants that are wild type in all of these respects, but are nonetheless transformation defective, might be suppressed by mutant substrates. Additionally, some alleles will yield higher frequencies of intragenic suppressors vis-a-vis host suppressors. One might expect that deletion mutants will revert intragenically at lower frequencies than simple missense alleles. To some extent, the facility with which revertants can be isolated and classified constrains the choice of starting alleles.

The remainder of this chapter describes attempts to identify mutations in cellular genes that activate the transforming potential of two transformation-defective *src* alleles, the L *src* gene and the c-*src* gene.

Analysis of the host range mutant L. In some systems, investigators have exploited the host dependence of a process in order to define its constituents biochemically. Avian and rodent fibroblasts, myoblasts, neuroretinal cells, and erythroid precursors are all transformed *in vitro* by $pp60^{src}$; however, avian macrophages are not -- despite producing catalytically active $pp60^{src}$ (Lipsich et al. 1984). Therefore, it is not surprising that some *src* mutants have novel host range properties.

RSV td SF/LO104, hereafter referred to as the mutant L, is a mutant RSV with a novel host range for transformation. The L mutant was isolated as a morphological revertant of an RSV transformed rat cell, B31 (Varmus et al. 1981b). Rat-1 cells infected with the L virus (rat-L) are morphologically normal, cannot grow in soft agar, and cannot form tumors in syngeneic animals (Oppermann et al. 1981b; Verderame et al. 1989). Surprisingly, virus rescued from rat-L cells transforms chicken embryo fibroblasts (CEF). CEF infected with the L virus (CEF-L) have a fusiform morphology, form colonies in soft agar, and produce sarcomas in chickens, albeit with a prolonged latency (Verderame et al. 1989). Further analysis demonstrated that L is a *src* mutant, having a 3 nucleotide deletion that eliminates phenylalanine-172, an invariant amino acid in the SH2 domain (Verderame et al. 1989). The biochemical basis of the host dependence

of the L *src* gene is unknown.

The host range restriction of the mutant L can be overcome by exaggerated expression. As a first step toward conducting the genetic screen, the L *src* gene was introduced into a retroviral GPT vector (creating MLV-Lgpt). Virus encoded by this vector was used to infect a rat-1 hprt$^-$ cell line (RT), and all of the infected cells appeared morphologically normal. Ten independently infected clones were chosen for further study. The relative amounts of the L *src* protein produced by these clones was analyzed by immune complex kinase reactions (see figure 2-3). There appears to be approximately a 10-fold variation in the expression of the L *src* gene among these clones. Many of these clones have an augmented ability to form colonies in soft agar, compared to the parental RT cells (see table 2-2). In general, the efficiency of colony formation in agar reflects the amount of the L *src* protein produced by the clone. For example clone-3 and clone-7 (figure 2-3, lanes 5 and 9) have low kinase activity and grow poorly in soft agar, while clone-6 (lane 8) and clone-8 (lane 10) produce significantly more pp60^{src} and grow well in soft agar.

The proviruses produced by infection with the MLV-Lgpt virus were rescued by co-cultivation with cells producing wild-type MLV. Rescued viral stocks were used to infect RT cells. The titer of the rescued viral stocks varied from clone to clone and correlated well with the expression of the provirus. For instance clone-3 produces relatively little L *src* protein (figure 2-3, lane 5) and no detectable rescued virus, while clone-8 (lane 10) produces similar amounts of pp60^{src} as B31 cells (lane 1) and produces a relatively high titer

Figure 2-3. Abundance of pp60^{src} in clones of RT cells infected with the MLV-Lgpt virus. The amount of pp60^{src} produced by 9 clones of RT cells infected with the MLV-Lgpt virus (clones 1-9) was compared to that produced by RT cells (lane 2) and the RSV transformed rat-1 cell line B31 (lane 1). *Src* proteins were isolated from detergent extracts of these cells by immunoprecipitation with TBR2 antisera, and subjected to immune complex kinase reactions. Under these conditions the heavy chain of IgG (IgH) and pp60^{src} are phosphorylated. The apparent mobility of IgH and of pp60^{src} (p60) are indicated by the arrows.

Figure 2-3

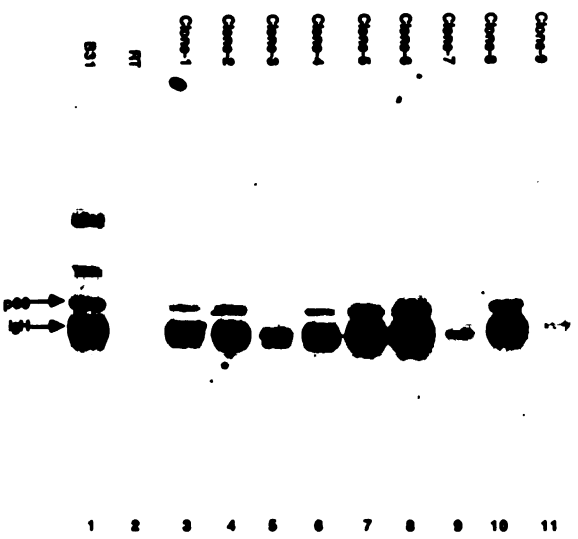


Table 2-2. RT cells expressing the L *src* gene grow in soft agar.

Clone	Growth in soft agar
RT[a]	$<10^{-6}$
B31[b]	10-50%
3[c]	10^{-5}
6[c]	10^{-3}-10^{-4}
7[c]	10^{-5}
8[c]	10^{-3}-10^{-4}

a) $3X10^5$ RT cells were seeded in 0.3% soft agar. After 14 days no macroscopic colonies were evident.

b) $3X10^5$ RT cells together with 1000 B31 cells were seeded in 0.3% soft agar. After 14 days, the macroscopic colonies were counted. The cloning efficiency of B31 in soft agar, in my experience, varies among experiments depending upon the density of cells and growth conditions.

c) $3X10^5$ cloned RT cells infected with MLV-L were seeded in 0.3% soft agar. After 14 days the number of macroscopic colonies were estimated. Since the cloning efficiency in soft agar was very high for some of the clones, these numbers can only be considered estimates. These clones were also analyzed for $pp60^{src}$ kinase activity, see figure 2-3.

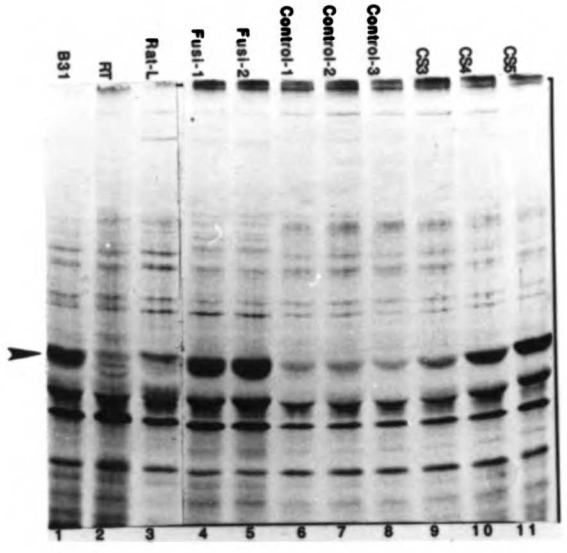
rescued viral stock.

Surprisingly, approximately 30% of the cells infected with virus rescued from clone-2 are morphologically fusiform. Two clones of fusiform transformed cells were isolated, fusi-1 and fusi-2, and virus produced by these clones was used to infect RT cells. Virus produced by fusi-1 and fusi-2 does not transform RT cells, implying that these clones do not contain a mutant L *src* gene with augmented transforming activity. Since the inability of the L *src* gene to provoke growth of rat cells in soft agar is overcome by exaggerated expression, it seemed likely that morphological transformation of fusi-1 and fusi-2 might also be the consequence of vigorous expression. I tested whether fusi-1 and 2 produce significantly more $pp60^{src}$ than non-transformed clones infected by the MLV-Lgpt virus. Fusi-1 and 2, and morphologically normal rat-L cells were labelled with L[^{35}S]methionine and the $pp60^{src}$ produced by these cells was analyzed by immunoprecipitation (see figure 2-4). As previously reported, rat-L (lane 3) produces about 25% as much $pp60^{src}$ as B31 cells, the parental transformed rat-1 cell (lane 1) (Verderame et al. 1989). On the other hand, fusi-1 (lane 4) and fusi-2 (lane 5) produce more $pp60^{src}$ than B31 cells. The expression of $pp60^{src}$ in RT cells infected with the virus produced by fusi-1 (lanes 6-8), which are morphologically normal, is similar to that in rat-L (lane 3).

These results suggest that exaggerated expression of the L *src* gene can overcome the apparent host dependence of its transforming activity. The host dependence the L *src* gene may simply reflect differences in the quantity of $pp60^{src}$ produced in the two hosts. If

Figure 2-4. Abundance of pp60^{src} in rat-1 cells transformed by the L *src* gene. The amount of pp60^{src} produced by RT cells (lane 2), rat-L cells (lane 3), B31 cells (lane 1), fusi-1 (lane 4), fusi-2 (lane 5), RT cells infected with the virus produced by fusi-1 (lanes 6-8), and RT cells infected with the ZAS4c-*src* virus, CS3 (lane 9), CS4 (lane 10), and CS5 (lane 11) are compared. These cells were labelled with L[^{35}S]methionine for 12 hours and pp60^{src} was isolated from detergent extracts by immunoprecipitation with TBR antisera as described in the methods. The apparent mobility of pp60^{src} is indicated by the arrow.

Figure 2-4.


B31
RT
Rat-L
Fusi-1
Fusi-2
Control-1
Control-2
Control-3
CS3
CS4
CS5
1
2
3
4
5
6
7
8
9
10
11

the transcription control elements of RSV are more potent in chicken cells than in rat cells, then any weakly defective *src* allele would appear host dependent. This scenario would make host range mutants much less attractive candidates for pseudoreversion analysis. Because the phenotype of RT cells expressing the L *src* gene is so sensitive to changes in the expression of $pp60^{src}$, I was unable to conduct a genetic screen for mutations that activate the transforming potential of L in rat cells.

Genetic analysis of the transforming activity of the c-src gene. The c-*src* gene is also a transformation defective *src* allele (Hanafusa et al. 1984; Parker et al. 1984; and Shalloway et al. 1984a and b). When the c-*src* gene is expressed at 50 times the normal level in fibroblasts, very subtle phenotypic changes occur. These cells are morphologically transformed, but cannot grow in soft agar or form tumors in syngeneic animals. Several point mutations have been described which potentiate the transforming activity of c-*src*. Converting tyrosine-527 to any other amino acid produces a transforming allele of *src*, as described in chapter 1 of this thesis. These mutations presumably activate the transforming activity by preventing phosphorylation of tyrosine-527, thereby enhancing the kinase activity. Several other activating mutations have been described (Levy et al. 1986; Kato et al. 1986), but the mechanism of activation is not known in these cases.

The chicken fibroblast c-*src* cDNA was subcloned into ZAS4, and viral stocks (ZAS4c-*src*) were produced by transfecting this proviral DNA into cell lines producing MLV replication functions. Three

independent clones of RT cells infected with the ZAS4c-*src* virus were isolated (CS3-5). These clones are morphologically normal and grow poorly in soft agar ($<10^{-7}$). The amount of $pp60^{src}$ produced by these clones was analyzed by immunoprecipitation (see figure 2-4, lanes 9-11). Since it produces the most $pp60^{src}$, the CS5 clone (lane 11) was chosen for further analysis. The amount and activity of $pp60^{src}$ produced by CS5 was carefully compared to RT cells (see figure 2-5) by labelling with L[^{35}S]methionine (lanes 1 and 2) and by kinase assays (lanes 3 and 4). CS5 cells (lane 2 and 4) produce 8-fold more $pp60^{src}$ than RT cells (lanes 1 and 3) by either assay.

The ZAS4c-*src* provirus in CS5 cells can be rescued by super-infection with wild-type MLV. These viral stocks have titers varying between 10 and 100 infectious units/ml, as assayed by mycophenolic acid resistance and have no apparent transforming activity.

The c-*src* gene is often lost during the conversion of CS5 cells to 6-TG resistance. CS5 cells are very sensitive to 6-TG, with the incidence of survival being approximately $5X10^{-6}$. Twelve independent 6-TG resistant clones were isolated, and the presence of the *src* gene was determined by assaying $pp60^{src}$ kinase activity (see figure 2-6). Three of the 11 clones (lanes 3,6, and 13) produce similar amounts of $pp60^{src}$ as the parental CS5 clone (lane 1). Eight of the 11 clones (lanes 4,5, and 7-12) produce similar amounts of $pp60^{src}$ as RT cells (lane 2), indicating that the c-*src* gene was inactivated during the conversion to 6-TG resistance. Thus, CS5 is a good candidate for conducting a genetic screen for mutations that activate the transforming potential of c-*src*.

Figure 2-5. Abundance and specific activity of pp60^{src} in CS5 cells. RT cells (lanes 1 and 3) and CS5 cells (lanes 2 and 4) were labelled with L[^{35}S]methionine. *Src* proteins were isolated from detergent extracts of these cells by immunoprecipitation with TBR antisera. Immune complexes were divided into 2 aliquots, one was analyzed directly for the abundance of pp60^{src} (lanes 1 and 2), the remainder was subjected to immune complex kinase reactions (lanes 3 and 4). The apparent mobility of pp60^{src} and of the heavy chain of IgG (IgH) are indicated by the arrows.

Figure 2-5.

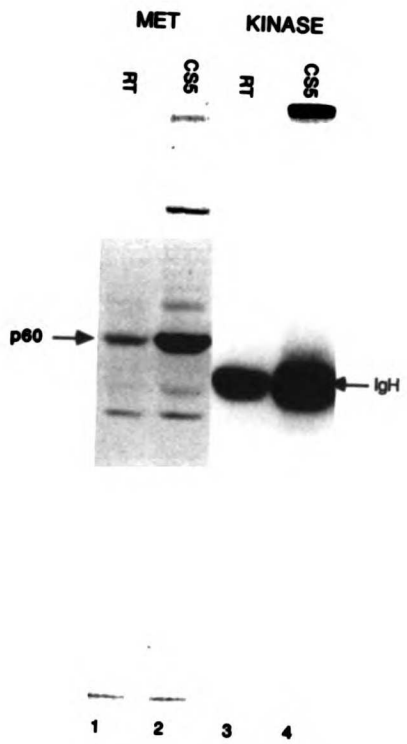
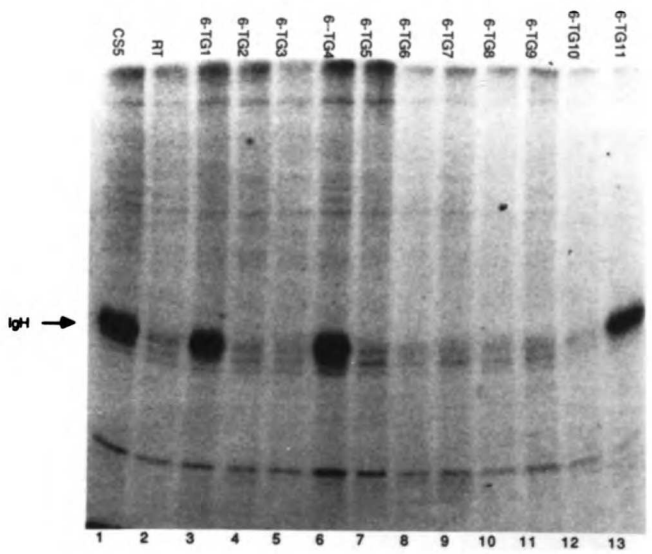


Figure 2-6. Abundance of pp60^{src} in 6-TG resistant subclones of CS5. *Src* proteins were purified from CS5 cells (lane 1), RT cells (lane 2), and 11 independent 6-TG resistant subclones of CS5 (lanes 3-13) by immunoprecipitation, using TBR antisera, from detergent extracts. The activity of pp60^{src} in these immune complexes was assayed by kinase reactions. Mobility of the IgG heavy chain (IgH) is indicated by the arrow.

Figure 2-6.


C55
RT
6-TG1
6-TG2
6-TG3
6-TG4
6-TG5
6-TG6
6-TG7
6-TG8
6-TG9
6-TG10
6-TG11
IgH
1 2 3 4 5 6 7 8 9 10 11 12 13

Mutagenesis of CS5 cells. Three independent mutagenesis experiments were conducted with CS5 (see table 2-3 for summary). Conditions of mutagenesis were chosen which gave 30-50% survival, as assayed by cloning efficiency following mutagenesis. Surviving cells were allowed to recover for 5 days, to allow expression of mutant phenotypes, and were then seeded in soft agar. Transformed genetic revertants were isolated by virtue of their growth in soft agar. In all 3 experiments, the apparent frequency of transformation was approximately $5X10^{-6}$. Twenty-eight revertants were picked from independently mutagenized cultures. To insure that the revertants represent clonal populations of cells, each was re-cloned at least 2 times in soft agar, and subsequently characterized further (summarized in table 2-4).

Revertants have heterogeneous morphologies and growth properties, which suggests that the mutations causing reversion are also heterogeneous. Four of the 28 revertants are morphologically transformed, the remainder are morphologically normal. Several of the revertants grow poorly in culture, with cloning efficiencies of 0.3-2%, compared to the 20-30% cloning efficiency of CS5 cells.

Reduced cloning efficiency made the subsequent viral rescue and isolation of 6-TG resistant subclones very difficult. Consequently, only 16 of the 28 clones were fully analyzed. 6-TG resistant subclones of these 16 revertants were isolated, loss of the c-*src* gene from these subclones was verified by kinase reactions, and the ability of these subclones to grow in soft agar was determined. All 16 revertants give rise to 6-TG resistant variants that have lost the c-

Table 2-3. Summary of the mutagenesis of CS5.

Expt.	Mutagen[a]	#Cells	#Revts.[b]	Survival[c]
I	MNNG	$1.35x10^7$	3	70%
II	MNNG	$2X10^7$	18	50%
III	EMS	$2.5X10^7$	7	10%

a) The indicated number of CS5 cells were exposed to either 1.5 μg/ml MNNG for 5 hours or 12.5 mM EMS for 2.5 hours. Cells were allowed to recover for 5 days and were then seeded into 0.3% soft agar.

b) Macroscopic soft agar colonies were picked from independently mutagenized cultures, and were then subjected to further analysis (see table 2-4).

c) 1000 CS5 cells were exposed to the indicated mutagen in parallel and the cloning efficiency on plastic relative to naive CS5 cells was determined as a measure of the survival.

Table 2-4. All of the revertants of CS5 that were analyzed contain bypass suppressors.

Revertant[A]	Morphology[B]	Viral Rescue[C]	6TG[D]	Cloning υ[E]
2-2	TX^+	tx^-	TX^+	2%
2-4	tx^-	ND	ND	
2-13	TX^+	tx^-	TX^+	
3-2	tx^-	ND	TX^+	
3-3	tx^-	tx^-	TX^+	0.3%
3-4	tx^-	ND	TX^+	
3-5	tx^-	tx^-	TX^+	
3-6	tx^-	ND	TX^+	
3-7	tx^-	ND	ND	
3-8	tx^-	ND	ND	
3-9	tx^-	ND	ND	
3-10	tx^-	ND	ND	
3-11	tx^-	tx^-	TX^+	0.7%
3-12	tx^-	ND	TX^+	
3-13	tx^-	ND	TX^+	
3-14	tx^-	ND	ND	
3-15	tx^-	ND	ND	
3-16	tx^-	ND	TX^+	
3-18	tx^-	ND	TX^+	
3-19	TX^+	tx^-	TX^+	

Table 2-4. All of the revertants of CS5 that were analyzed contain bypass suppressors.

Revertant[A]	Morphology[B]	Viral Rescue[C]	6TG[D]	Cloning υ[E]
3-20	tx^-	ND	TX^+	
E-2	tx^-	ND	ND	
E-5	tx^-	tx^-	ND	
E-10	tx^-	ND	ND	
E-11	tx^-	tx^-	TX^+	
E-12	tx^-	tx^-	TX^+	
E-13	tx^-	ND	ND	
E-15	TX^+	ND	ND	

A) Transformed revertants were cloned at least 3 times from soft agar. Revertant 2 series and 3 series were derived after mutagenesis with MNNG. Revertant E series was derived after EMS mutagenesis.

B) TX^+ = round or fusiform; tx^- = flat.

C) Pools of RT cells infected by rescued virus were seeded in soft agar. ND = not determined; tx^- = no growth in agar.

D) Multiple 6-TG resistant subclones were isolated. Clones shown to have lost the c-*src* gene by kinase assays were tested for growth in soft agar. ND = not determined; TX^+ = growth in soft agar.

E) The cloning efficiency of the indicated revertant clones was determined by seeding 500 cells on a 100 mm dish, and counting the resulting colonies after 14 days.

src gene, but nonetheless retain the ability to grow in soft agar. For this reason these revertants were classified as bypass suppressors. This conclusion was confirmed, for 8 of the 16, by demonstrating that virus rescued from these revertants is unable to transform RT cells, demonstrating that the suppressor gene is not linked to the *src* gene.

Since none of the clones characterized harbored either *src* revertants or interaction suppressors, none was characterized further.

DISCUSSION

In order to identify cellular genes that encode either regulators of $pp60^{src}$ or substrates of $pp60^{src}$, I designed a scheme for conducting pseudoreversion analysis of transformation-defective *src* mutants. A non-transformed fibroblast that contains 8 times the normal levels of $pp60^{c\text{-}src}$ was chemically mutagenized, and 16 transformed genetic revertants were isolated and characterized. The phenotype of all of the characterized revertants was shown to be independent of the *src* gene, and hence these revertants contain bypass suppressors. Although no mutants of interest were isolated, these experiments demonstrate that such a scheme is technically feasible. The following discussion will highlight the pitfalls of this strategy, and discuss other avenues for genetic analysis of *src* transformation.

Potential utility of host range mutants. The analysis of the L *src* gene indicates that, at least in some cases, the host dependent transforming activity of a *src* gene is merely the consequence of

differences in the expression of the *src* gene in the two hosts. The differences in the amount of pp60^{src} produced could reflect host-dependent transcription, translation, or protein stability. Another possible explanation is that the dose of wild-type pp60^{src} required to transform the two hosts differs. In either case, weakly transforming *src* alleles would appear host dependent. The extreme sensitivity of the phenotype of cells expressing the L *src* protein to changes in the expression of pp60^{src} makes them unsuitable for pseudoreversion analysis.

Problems encountered in the screen for mutations in cellular genes that activate the transforming potential of c-*src*. The analysis of genetic revertants of CS5 was very time-consuming. Isolating the CS5 cell line, conducting the mutagenesis, isolating and re-cloning revertants, and classifying the revertants represents 8 months of work. Obviously, the likelihood of success would improve if one could analyze significantly more than the 16 revertants that I analyzed here. The number of revertants isolated is not a limitation; one could isolate hundreds, if desired.

The major difficulties encountered were the time consuming nature of the analysis required to classify the revertants, and the predominance of bypass suppressors (16/16 analyzed). All of the revertants must be propagated in culture or stored as frozen stocks until they have been analyzed, which creates the bulk of the work. If the analysis of the revertants were streamlined, then many more revertants could be analyzed. Likewise, if one could somehow increase the proportion of interesting revertants, then fewer would have to be

analyzed.

The most time-consuming step in the analysis of revertants was isolating and characterizing 6-TG resistant subclones, which under the best of circumstances requires 6 weeks. Because many revertants grow poorly in culture, it often takes longer. Multiple 6-TG resistant subclones must be analyzed, because only those clones which have lost the *src* gene are informative. Except for the few morphologically transformed revertants (4/28), the 6-TG resistant subclones must be assayed by growth in soft agar, a two week affair. Time would be saved if the frequency of inactivation of the *src* gene were higher. For this reason, it may have been a mistake to focus on the cell lines that have more stable proviruses. Furthermore, if a higher percentage of the resistant clones had lost the *src* gene (as opposed to the 50-80% observed), then one would no longer need to analyze individual clones of 6-TG resistant cells, saving 2-3 weeks of work.

Another potential improvement would be to create a simple biochemical or phenotypic screen to identify the interesting classes of revertants. For instance, one could screen for elevated levels of phosphotyrosine, thereby identifying revertants in which the catalytic activity of $pp60^{src}$ had been augmented. Such a screen could be performed as a "dot western" utilizing anti-phosphotyrosine antibodies. Once revertants with increased levels of phosphotyrosine have been identified they could be genetically characterized as described above. Similarly, one could perform simple phenotypic screens for interesting classes of revertants. In addition to neoplastic transformation, revertants may acquire novel phenotypic

properties. For instance, revertants may be conditional for growth, depending upon temperature or exogenous growth factors, properties which could be exploited in the physiological and molecular analysis of the suppressor gene.

Alternatively, one might be able to bias the experiment in favor of the interesting classes of revertants by starting with partially transformed cells. For this reason *src* alleles that produce partially transformed phenotypes may be of greater interest. Although it is difficult to predict the prevalence of the different classes of revertants, the choice of *src* mutant will undoubtedly determine the outcome.

Recessive oncogenes as potential regulators of pp60^{src}. Activation of pp60^{src} has recently been implicated in the genesis of some human tumors. Elevated levels of pp60^{src} kinase activity have been reported in colon carcinomas (Bolen et al. 1987; Cartwright et al. 1989), breast adenocarcinomas (Rosen et al. 1986), and neuroblastomas (Bolen et al. 1985). The biochemical nature of the activation has not been determined in any of these cases, although in neuroblastomas a novel form of p60 that is phosphorylated on N-terminal tyrosines has been reported. In 2/3 of colon carcinoma cell lines and tumors, there is 5-8 fold more pp60^{src} kinase activity than in normal human colonic mucosal cells, and an increased abundance of tyrosine-phosphorylated proteins (Cartwright et al. 1989). The increase in pp60^{src} kinase activity cannot be explained by increased abundance of pp60^{src}, nor by changes in the phosphorylation of pp60^{src}. Since inactivation of cellular genes, that is a recessive

oncogenes, is thought to play a role in the genesis of colon carcinomas, breast carcinomas, and neuroblastomas (reviewed by Ponder 1988), it is tempting to speculate that activation of $pp60^{src}$ in these tumors is the consequence of inactivating a cellular gene that encodes an inhibitor of $pp60^{src}$.

CHAPTER 3:

The first seven amino acids encoded by the v-*src* oncogene act as a myristylation signal: lysine-7 is a critical determinant.

ABSTRACT

The transforming protein of Rous Sarcoma Virus, $pp60^{v\text{-}src}$, is covalently coupled to myristic acid by an amide linkage to glycine-2. Myristylation promotes the association of $pp60^{v\text{-}src}$ with cellular membranes, and this subcellular location is essential for transforming activity. Findings presented here, in conjunction with the previous reports of others, imply that the seventh amino acid encoded by v-*src* might be important in the myristylation reaction. Replacement of lysine-7 by asparagine greatly reduced myristylation, membrane association, and transforming activity of $pp60^{v\text{-}src}$. In contrast, substitution of arginine at residue seven had no effect upon any of these properties of $pp60^{v\text{-}src}$. Addition of amino acids 1-7 encoded by v-*src* are sufficient to cause myristylation of a *src*-pyruvate kinase fusion protein. We conclude that that the recognition sequence for myristylation of $pp60^{v\text{-}src}$ comprises amino acids 1-7, and that lysine-7 is a critical component of this sequence.

INTRODUCTION

The v-*src* oncogene of Rous Sarcoma Virus (RSV) encodes a protein tyrosine kinase, $pp60^{v\text{-}src}$ (Bishop and Varmus 1982). Association with membranes is among the properties of $pp60^{v\text{-}src}$ that are required for transforming activity (Krueger et al. 1983). Variants of $pp60^{v\text{-}src}$ that are weakly associated with membranes have attenuated oncogenic activities (Courtneidge and Bishop 1982;Krueger et al. 1982). Since

the subcellular location of $pp60^{v\text{-}src}$ plays a pivotal role in transformation by RSV, we have explored the mechanism by which $pp60^{v\text{-}src}$ associates with membranes.

Unlike most membrane proteins, $pp60^{v\text{-}src}$ is synthesized on soluble polyribosomes (Lee et al. 1979) and lacks a conventional hydrophobic signal sequence. These peculiarities are explained, in part, by the covalent coupling of $pp60^{v\text{-}src}$ to a 14 carbon saturated fatty acid, myristic acid (Buss and Sefton 1984; Schultz et al. 1985). Myristylation promotes the association of $pp60^{v\text{-}src}$ with membranes in that non-myristylated variants of $pp60^{v\text{-}src}$ are not associated with membranes and are consequently transformation-defective (Kamps et al. 1985; Pellman et al. 1985b). Several other myristylated proteins have been described, but $pp60^{v\text{-}src}$ is a rare example for which a function of the myristyl moiety is known. Myristylation is not, however, a sufficient explanation for the localization of $pp60^{v\text{-}src}$, because several unrelated myristylated proteins are cytosolic (Olson et al. 1985), and because myristylated yet cytosolic variants of $pp60^{v\text{-}src}$ have been described (Buss and Sefton 1984;Garber et al. 1985).

Myristylation occurs during or immediately after synthesis of $pp60^{v\text{-}src}$ (Buss and Sefton 1984;Garber et al. 1985). Upon removal of the N-terminal methionine residue from the nascent chain by an as yet uncharacterized amino peptidase (Schultz et al. 1985), the resulting N-terminal glycine residue is coupled to myristic acid by a myristyl transferase, which has been partially purified and characterized by Towler et al. (Towler et al. 1986; Towler et al. 1987a; Towler et al. 1987b). This sequence of events has also been documented for other

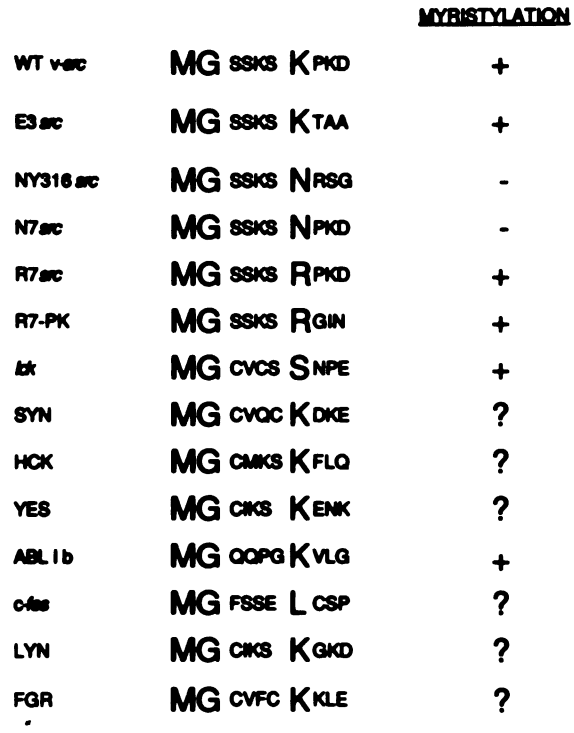
myristylated proteins (Magee and Courtneidge 1985; McIlhinney et al. 1985; Olson and Spizz 1986; Wilcox et al. 1987). The sequences required for myristylating $pp60^{v\text{-}src}$ are confined to its extreme N-terminus, the first 14 amino acids being sufficient (Pellman et al. 1985a). Of these 14 amino acids, only glycine-2 has been directly implicated in the myristylation process. *Src* proteins containing glutamic acid-2 in place of glycine-2 are not cleaved by the amino terminal peptidase, while those containing alanine-2 are cleaved but are not coupled to myristic acid by the transferase (Buss et al. 1986). Additional residues clearly play a role because some *src* alleles, such as the NY316 v-*src* mutant, encode N-terminal met-gly, but the products are nontheless poor substrates for the myristyl transferase (Garber et al. 1985). We report here that lysine-7 is an important component of the signal for myristylating $pp60^{v\text{-}src}$,and that the N-terminal 7 amino acids of $pp60^{v\text{-}src}$ are sufficient to cause myristylation when transferred to a novel context.

RESULTS

Codons 8-37 of v-src are not required for myristylation and transforming activity of $pp60^{v\text{-}src}$. We have isolated a naturally occuring variant of B77 v-*src*, E3 *src*, which has suffered an in-frame deletion of codons 8-37 (unpublished data of G. Mardon) creating the novel amino terminal sequence depicted in figure 3-1. The deletion in E3 *src* overlaps the deletions in several mutants previously described by Pellman et al. (Pellman et al. 1985b). The most closely related of

Figure 3-1. Amino terminal sequences of protein tyrosine kinases related to $pp60^{v\text{-}src}$. Amino acids 1-10 of the wild type (Schwartz et al. 1983), E3 (our unpublished data), and NY316 (Pellman et al. 1985b) *src* proteins, and the R7-PK, mouse *lck* (Marth et al. 1985), human SYN (Semba et al. 1986), human HCK (Quintrell et al. 1987), human YES (Sukegawa et al. 1987), human ABL Ib (Shtivelman et al. 1986, and personal communication from P. Jackson), feline c-*fes* (Roebroek et al. 1987), human LYN (Yamanashi et al. 1987), and human FGR (Katamine et al. 1987) encoded proteins are depicted by the single letter amino acid code. Those proteins which are presently or have previously been shown to be myristylated are indicated.

Figure 3-1.


MYRISTYLATION
WT v-src MG SSKS KPKD +
E3 src MG SSKS KTAA +
NY316 src MG SSKS NRSG -
N7 src MG SSKS NPKD -
R7 src MG SSKS RPKD +
R7-PK MG SSKS RGIN +
lck MG CVCS SNPE +
SYN MG CVQC KDKE ?
HCK MG CMKS KFLQ ?
YES MG CIKS KENK ?
ABL Ib MG QQPG KVLG +
c-fes MG FSSE LCSP ?
LYN MG CIKS KGKD ?
FGR MG CVFC KKLE ?

these mutants is NY316 *src*, in which codons 7-15 were replaced by four unrelated amino acids, creating the amino terminal sequence shown in figure 3-1. The NY316 *src* protein is recognized by the amino peptidase but not by the myristyl transferase (Garber et al. 1985). Therefore, we expected that the E3 *src* gene would also encode a non-myristylated and consequently transformation-defective *src* protein. We tested the biological potency of E3 *src* by incorporating it into a Moloney Murine Leukemia Virus expression vector and using the resulting plasmid to transfect rat-1 fibroblasts. Rat-1 cells expressing the E3 *src* protein are morphologically transformed (data not shown) and are anchorage independent for growth (1% cloning efficiency in soft agar). Since virtually all known biologically active alleles of v-*src* encode myristylated *src* proteins, we expected that the E3 *src* protein wouldalso be efficiently acylated. This was tested by comparing the incorporation of methionine (Figure 3-2, lanes 1-3) and myristic acid (Figure 3-2, lanes 4 and 5) into the wild type and E3 *src* proteins. The E3 and wild type *src* proteins are myristylated to the same extent.

We conclude from these experiments that amino acids 8-37 are not required for proper acylation and transforming activity of $pp60^{v\text{-}src}$. Furthermore, the simplest explanation for the behavior of the NY316 *src* protein is that amino acid-7 is a critical component of the recognition sequence for the myristyl transferase.

Transforming activity of the N7 and R7 src proteins. We directly tested the role of lysine-7 in myristylation of $pp60^{v\text{-}src}$ by replacing it with either an asparagine (N7) or an arginine (R7) (see

Figure 3-2. Myristylation of the E3 *src* protein. Rat-1 cells expressing either the wild type (lanes 1 and 4) or the E3 (lanes 3 and 5) *src* proteins were labelled for 18 hours with either L[^{35}S]methionine (lanes 1-3) or [^{3}H]myristic acid (lanes 4 and 5). Labelled cells were solublized in lysis buffer and *src* proteins were immunoprecipitated with TBR serum as described in the methods.

Figure 3-2.

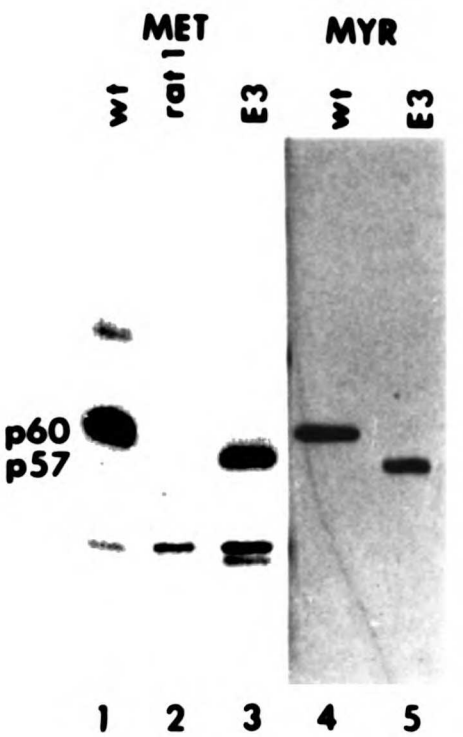


figure 3-1). These mutant *src* genes were incorporated into a RSV vector, and virus was produced by transfecting CEF with these viral DNAs. After ten days to allow virus spread, CEF infected with either the wild type (Figure 3-3, panel B) or the R7 (panel C) RSV viruses were morphologically transformed, while CEF infected with the N7 (panel D) RSV virus were indistinguishable from uninfected CEF (panel A). Viral stocks derived from these cultures were assayed for their ability to induce transformed foci on monolayers of CEF, and for their ability to produce $pp60^{v\text{-}src}$ protein kinase activity in acutely infected CEF (see Table 3-1). Although the N7 virus stock was at least 1000 fold less active than either the R7 or wild type viruses in the focus assay, it produced equivalent $pp60^{v\text{-}src}$ kinase activity in acutely infected cells. These experiments demonstrate that N7 encodes an active protein kinase that is defective for morphological transformation of chicken cells.

Myristylation of the N7 and R7 src proteins. We next asked whether the attenuated transforming activity of N7 reflects an underlying defect in the myristylation of the N7 *src* protein. The extent to which the wild type, R7, and N7 *src* proteins are acylated was determined by comparing the incorporation of methionine (Figure 3-4, panel A) and myristic acid (Figure 3-4, panel B) into these proteins. The N7 *src* protein (lane 3) is apparently myristylated 80-90% less well than either the wild type (lanes 2) or the R7 (lanes 4) *src* proteins, in general agreement with their respective transforming activities.

Defective acylation of the N7 *src* protein could be due to

Figure 3-3. Morphology of CEF infected with wild type, N7, and R7 RSV viruses. Untransfected CEF (panel A), or CEF transfected 10 days previously with DNA encoding wild type (panel B), R7 (panel C), or N7 (panel D) RSV virus as described in the text. Viral stocks derived from these cultures have equivalent relative kinase titers.

Figure 3-3.

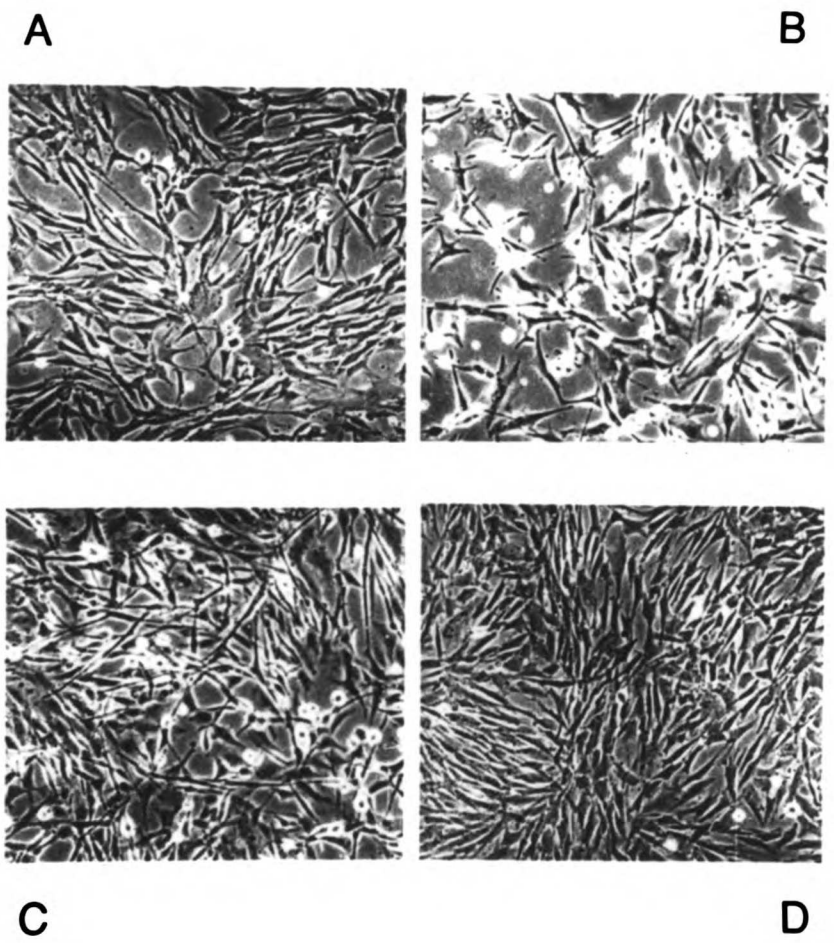


Table 3-1. Titers of mutant RSVs.

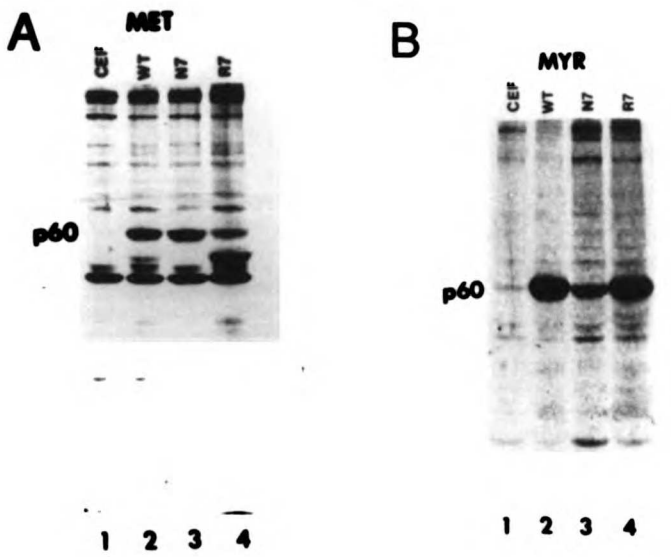
Virus	Titer (FFU/ml[a])	Relative kinase titer[b]
Wild type	$2x10^5$	1.00
R7	$5x10^5$	1.25
N7	$<1x10^5$	1.12

[a] CEF were infected with dilutions of virus and overlaid with agar. Foci of transformed cells were counted after 14 days.

[b] Relative kinase titers were determined as described in the text. These results represent the average of triplicate cultures.

Figure 3-4. Myristylation of the N7 and R7 *src* proteins. Infected CEF were labelled for 18 hours with either L[^{35}S]methionine (panel A) or [^{3}H]myristic acid (panel B). Labelled cells were solublized in lysis buffer and *src* proteins were immunoprecipitated with monoclonal anti-pp60$^{v\text{-}src}$ 127. Panel A was exposed for 20 hours, and panel B was exposed for 30 days. CEF had previously been infected with wild type (lane 2), N7 (lane 3), or R7 (lane 4) RSV viruses.

Figure 3-4.

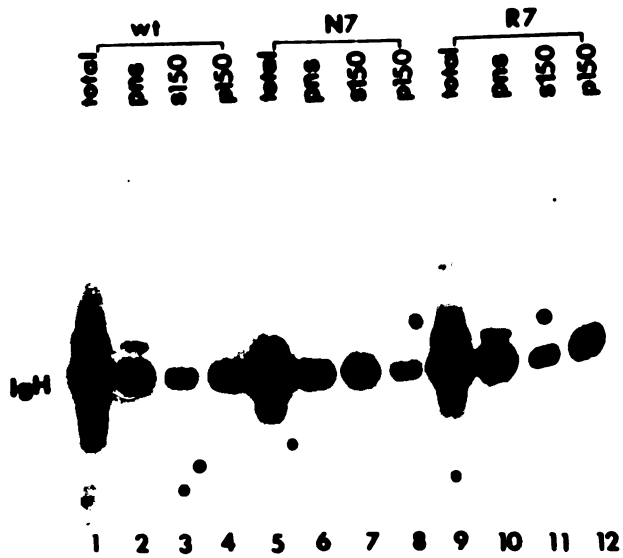


altered recognition by either the amino peptidase or the myristyl transferase. These two possibilities can be distinguished by the presence or absence of methionine-1 in the mature N7 *src* protein. We tested this by asking whether we could detect [^{35}S]-methionine-labelled amino terminal 18 and 16 kilodalton V8 proteolytic fragments of pp60$^{v\text{-}src}$, since these fragments contain methionine only if the amino peptidase fails to remove methionine-1. We determined that methionine-1 is not present in the mature N7 *src* protein, which implies that asparagine-7 affects recognition by the myristyl transferase, but not by the amino peptidase (data not shown).

Membrane association of the N7 and R7 src proteins. The extents to which these proteins are myristylated also correlates well with their association with total cellular membranes in subcellular fractionations (see Figure 3-5). Crude membrane (lanes 4, 8, and 12) and cytosolic fractions (lanes 3, 7, and 11) were isolated by differential centrifugation, and the amount of pp60$^{v\text{-}src}$ in these fractions was determined by immune complex kinase reactions. In agreement with previous reports (Courtneidge et al. 1980;18), 70-80% of the wild type (lane 4) and R7 (lane 12) pp60$^{v\text{-}src}$ kinase activity was recovered with total cellular membranes; however, only 10% of the N7 pp60$^{v\text{-}src}$ kinase activity (lane 8) was recovered with total cellular membranes. These experiments demonstrate that the seventh amino acid can determine whether pp60$^{v\text{-}src}$ is myristylated, and whether it is associated with cellular membranes. These results also provide an explanation for the behavior of the NY316 *src* protein, which also contains asparagine-7.

Figure 3-5. Membrane association of the N7 and R7 *src* proteins. CEF infected with wild type (lanes 1-4), N7 (lanes 5-8), or R7 (lanes 9-12) RSV viruses were subjected to subcellular fractionation as described in the methods. Equivalent amounts of the total extract (lanes 1,5,and 9), the supernatant of a 1000xg spin (pns, lanes 2,6, and 10), the supernatant of a 150,000xg spin (s150, lanes 3, 7, and 11), and the pellet of a 150,000xg spin (p150, lanes 4,8, and 12) were analyzed by immune complex kinase assays utilizing TBR sera. In subsequent experiments these assays were quantitated as described in the methods.

Figure 3-5.



Amino acids 1-7 of the R7 src gene are sufficient to cause myristylation in a novel context. Previous reports have demonstrated that amino acids 1-14 of $pp60^{v\text{-}src}$ are sufficient to direct the myristylation of a *src*-globin fusion protein (Pellman et al. 1985a). The simplest explanation for the behavior of the E3 *src* protein, however, is that amino acids 1-7 constitute a sufficient myristylation signal. We tested the role of sequences C-terminal to amino acid 7 by fusing codons 1-7 of the R7 *src* gene to a gene encoding a normally non-myristylated protein, the chicken muscle pyruvate kinase gene. The resulting fusion gene, R7-PK (see figure 3-1), was incorporated into a SV40 expression vector, and used to transfect COS7 cells (see Figure 3-6). The R7-PK fusion protein (lanes 3 and 6) incorporated myristic acid, while the native PK protein (lanes 2 and 4) did not. These results demonstrate that amino acids 1-7 constitute a sufficient myristylation signal.

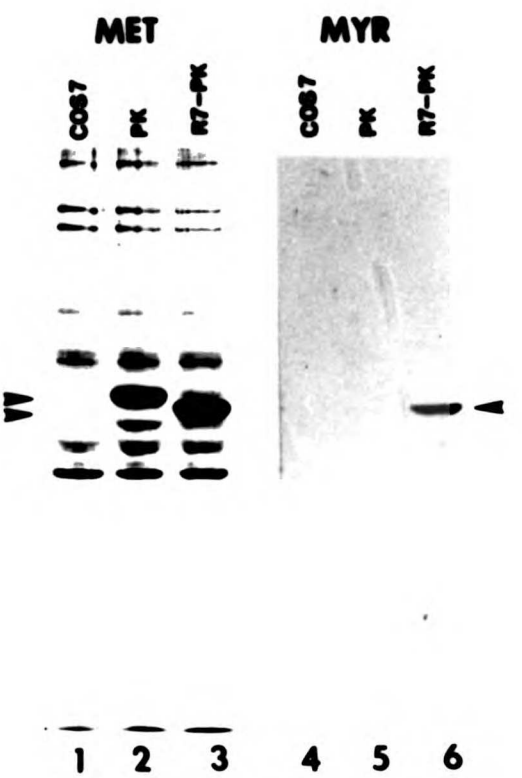
DISCUSSION

As a first step toward elucidating the mechanism of the membrane localization of $pp60^{v\text{-}src}$, we have identified a new determinant of the myristylation signal, lysine-7, and we have shown that amino acids 1-7 of $pp60^{v\text{-}src}$ are sufficient to cause myristylation in a novel context.

What is the role of lysine-7 in the myristylation of $pp60^{v\text{-}src}$? Replacing lysine-7 with asparagine largely abolished myristylation of $pp60^{v\text{-}src}$. It is formally possible that the residual labelling of the N7 *src* protein by myristic acid represents metabolic conversion of the

Figure 3-6. Myristylation of a hybrid *src*-pyruvate kinase protein. Untransfected COS7 cells (lanes 1 and 4) or COS7 cells transfected with either RL142PK10X encoding native PK (lanes 2 and 5), or pR7-PK encoding the R7-PK fusion protein (lanes 3 and 6) as described in the methods were labelled with either L[^{35}S]methionine (lanes 1-3) or [^{3}H]myristic acid (lanes 4-6). Labelled cells were solubilized with lysis buffer and PK proteins were immunoprecipitated with rabbit anti-chicken M1 pyruvate kinase antibody. Lanes 1-3 were exposed for 20 hours; lanes 4-6 were exposed for 30 days.

Figure 3-6.



label into amino acids precursors. We think this is unlikely because 10-20% of the N7 *src* protein pellets with microsomal membranes, which is consistent with residual myristylation.

Our conclusions are also supported by in vitro myristylation studies. A myristyl transferase partially purified from yeast acylates the octapeptide Gly-Ser-Ser-Lys-Ser-Asn-Pro-Lys (K_m=1000μM, V_{max}=8%), derived from the N-terminal sequence of the N7 *src* protein, substantially less well than Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys (K_m=40μM, V_{max}=43%), derived from the wild-type *src* protein (Towler et al. 1988). This result demonstrates that asparagine-7 directly affects recognition by myristyl transferase. Furthermore, the octapeptide derived from the amino terminal sequence of the N7 *src* protein has substantial residual activity as a substrate for myristyl transferase, which is consistent with the the residual myristylation that we see in vivo. Since asparagine-7 had no effect upon recognition of either the N7 or the NY316 *src* proteins by the amino peptidase (Garber et al. 1985, and our unpublished observations), we conclude that lysine-7 is a critical determinant of recognition by the myristyl transferase.

Another possible interpretation of these data is that asparagine-7 is a substitution that uniquely alters recognition by the myristyl transferase. This interpretation implies that most substitutions for lysine-7 would be active, and therefore that lysine-7 would not be conserved among myristylated proteins generally. There is very poor conservation of amino acid-7 among these proteins (not shown). However, if one compares the N-terminal sequences of protein tyrosine kinases related to $pp60^{v\text{-}src}$, lysine-7 is found in most of

them (see Figure 3-1). Other than glycine-2, this is the most conserved of the first 10 amino acids of these proteins. Another surprising feature of these sequences is that none contains arginine-7, which we have shown to be active in $pp60^{v\text{-}src}$ and which we predict would be an active substitution in other proteins related to $pp60^{v\text{-}src}$.

In summary, it seems likely that lysine-7 promotes recognition of a family of proteins by the myristyl transferase. Alternatively, lysine-7 might be conserved if this domain performs some function, other than recognition by the myristyl transferase, that these proteins have in common. The dissimilarity between the amino terminal sequence of $pp60^{v\text{-}src}$ and that of other known myristylated proteins might reflect recognition by distinct myristyl transferases.

What are the other sequence determinants of the myristylation signal? It seems very likely that amino acids 1-7 of $pp60^{v\text{-}src}$ constitute a sufficient myristylation signal. This putative signal works in three different sequence contexts, namely the wild-type and E3 *src* proteins and the R7-PK protein. Furthermore, the sequences C-terminal to lysine-7 in $pp60^{v\text{-}src}$ bear no resemblance to those in its relatives. Thus, sequences C-terminal to lysine-7 play a minor role, if any, in the myristylation of $pp60^{v\text{-}src}$. Since methionine-1 must be removed and glycine-2 provides the site for joining to myristate, amino acids 3-6 are the remaining potential determinants of this signal. Towler et al. have previously reported that the yeast myristyl transferase is sensitive to amino acids-3 and 6 of its substrates, utilizing octapeptides derived from the catalytic subunit

of the cAMP dependent protein kinase. Leucine, phenylalanine, tyrosine, and aspartate at position 3 (Towler et al. 1987a) and aspartate at position 6 are inactivating substitutions, whereas serine at position 6 strongly favors binding of the octapeptides to the transferase (Towler et al. 1986). However, these substitutions have not been tested in octapeptides derived from $pp60^{v\text{-}src}$. It is interesting to note that the c-*fes* protein, which is at most loosely associated with membranes (Young et al. 1984), contains phenylalanine-3 and glutamate-6, both of which should be inactive substitutions. It has not been reported whether the c-*fes* protein is myristylated.

What is the function of the myristyl moiety? The myristyl moiety has been hypothesized to promote the association of $pp60^{v\text{-}src}$ with membranes (Buss et al. 1986; Garber et al. 1985). The behavior of the N7 *src* protein supports this hypothesis. The N7 *src* protein, like the previously reported non-myristylated variants (Kamps et al. 1985; Pellman et al. 1985b), is enzymatically active, not associated with membranes, and biologically impotent. These similarities, which result from various alterations of the amino terminal sequence, suggest a common underlying cause -- the lack of myristylation. We cannot, however, conclude from these data that the myristyl moiety is sufficient, or even necessary, for the membrane association and biological activity of $pp60^{v\text{-}src}$. The transformation defective tsNY68 (Buss and Sefton 1984) and NY18-3 (Garber et al. 1985) *src* proteins, like several myristylated proteins unrelated to *src*, are not associated with membranes despite their myristylation. This behavior implies the presence of a second prerequisite for localization,

perhaps a tertiary or quaternary structure. On the other hand, the non-myristylated *src* proteins of the recovered Avian Sarcoma Virus strains 157 and 1702 are both biologically active and membrane associated, albeit weakly (Krueger et al. 1982). This behavior implies that alternative amino terminal sequences (derived in these cases from the signal sequence of $gp85^{env}$) can substitute functionally for the myristyl moiety (Garber and Hanafusa 1987). Thus, covalently bound myristate probably plays no part in other properties of $pp60^{v-src}$ --such as concentration within adhesion plaques (Rohrschneider 1980), or substrate recognition.

In summary, the myristyl moiety contributes to the membrane localization of $pp60^{v-src}$, which is a prerequisite for its transforming activity. Additional studies should reveal whether $pp60^{v-src}$ has a second signal that is required in conjunction with myristylation for membrane association and localization within membranes.

CHAPTER 4:

The src protein contains multiple membrane-anchoring domains.

ABSTRACT

The transforming protein of Rous Sarcoma Virus, pp60^{src}, is tightly associated with both plasma membranes and intracellular membranes. This association is in part due to the N-terminal myristylation of pp60^{src}; however, several lines of evidence suggest that N-terminal amino acid sequences also mediate the membrane association and subcellular localization of pp60^{src}. Experiments reported here suggest that pp60^{src} contains at least 3 domains which in conjunction with N-terminal myristylation are capable of mediating membrane association, and of determining subcellular localization. Amino acids 1-14 of pp60^{src} are sufficient to cause a hybrid *src*-pyruvate kinase protein to associate with cytoplasmic granules. In contrast, amino acids 38-111 appear to mediate association with the plasma membrane and perinuclear membranes, while amino acids 204-259 appear to mediate association with just perinuclear membranes. We conclude from these experiments that the membrane association and subcellular localization of pp60^{src} result from independent domains which target the protein to specific subcellular locations.

INTRODUCTION

The transforming protein of Rous Sarcoma Virus (RSV), pp60^{src}, is a membrane associated tyrosine kinase. Association of pp60^{src} with membranes is essential for its transforming activity (reviewed by

Krueger et al. 1983). In various cell types, pp60^{src} is found associated with plasma membranes (Courtneidge et al. 1982; Willingham et al. 1979), with perinuclear membranes (Resh and Erikson 1985), with chromaffin granules (a type of secretory vesicle) (Parsons and Creutz 1986), and with the growth cones of developing neurons (Maness et al. 1988; Sobue and Kanda 1988). Very little is known about how pp60^{src} is specifically targeted to these subcellular locations.

Several lines of evidence implicate the N-terminus of pp60^{src} in stabilizing its association with membranes. The N-terminus of pp60^{src} is covalently coupled to a 14 carbon fatty acid, myristic acid (Buss and Sefton 1985; Schultz et al. 1985). The myristyl moiety has been hypothesized to promote the association of pp60^{src} with membranes (Buss et al. 1986; Garber et al. 1985). On the other hand, not all myristylated *src* proteins are associated with membranes (Buss et al. 1984; Garber et al. 1985), and some non-myristylated *src* proteins are membrane associated, albeit weakly (Krueger et al. 1982). These results suggest that pp60^{src} contains amino acid sequences which, in conjunction with N-terminal myristylation, mediate association with membranes.

The membrane anchoring domain was previously localized to the N-terminal 13 kilodaltons (kd) of pp60^{src} (Levinson et al. 1981). Membrane vesicles containing pp60^{src} were digested with protease and subsequently the association of proteolytic fragments of pp60^{src} with membranes was analyzed. While a C-terminal 52 kd fragment (p52^{src}) remained associated with membranes, a 47 kd C-terminal fragment was released from the vesicles. These experiments suggest that the N-

terminal 13 kd of pp60^{src} act as a membrane anchoring domain. Since p52^{src} is membrane-associated, despite lacking the N-terminal myristyl moiety, these results also suggest that internal sequences are sufficient to sustain the association of pp60^{src} with membranes. The experiments reported here test the role of N-terminal amino acid sequences in the membrane association and subcellular localization of pp60^{src}.

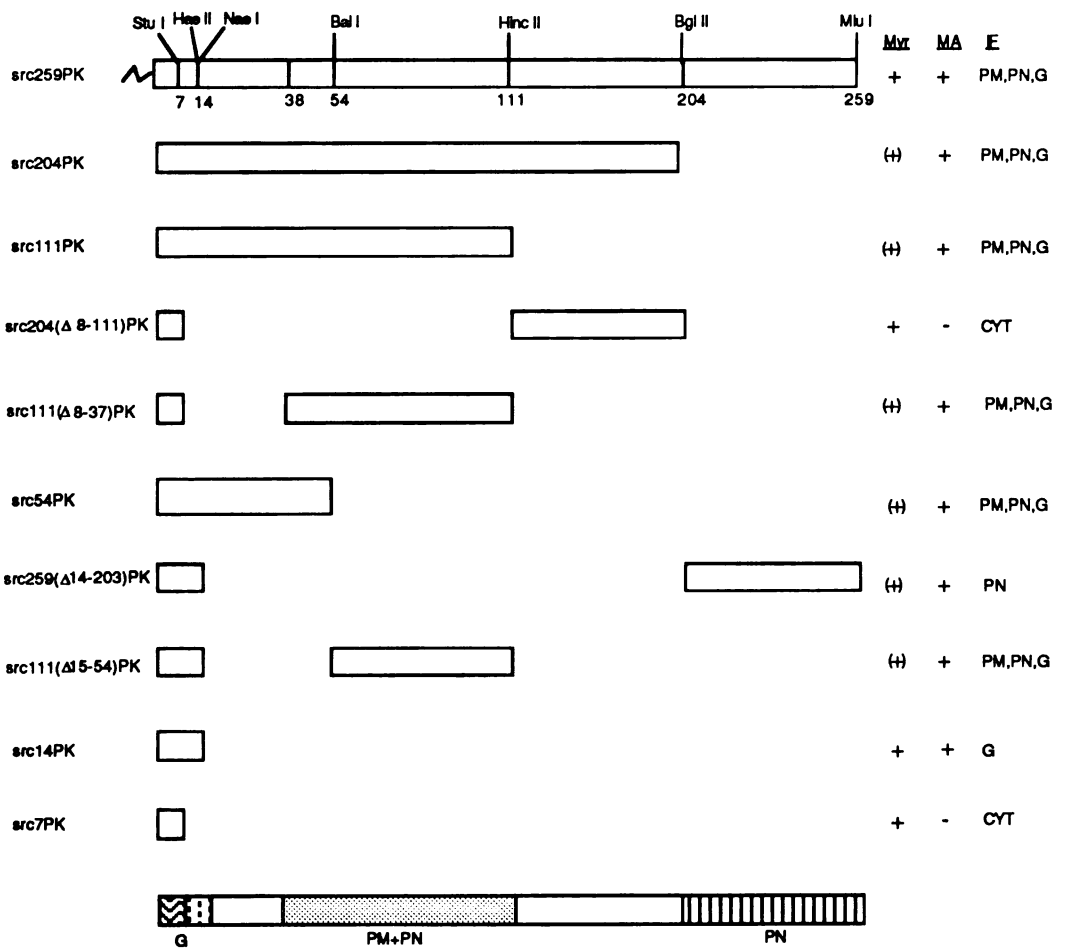
RESULTS

In order to test the role of N-terminal amino acid sequences of pp60^{src} in association with membranes and in subcellular localization, a set of genes encoding hybrid *src*-PK proteins were constructed. Since myristylation is normally required for membrane-association, all of these hybrid genes contain sequences encoding the myristylation signal, which comprises the first 7 amino acids of pp60^{src} (Kaplan et al. 1988). The association of these hybrid proteins with membranes was first analyzed by a crude biochemical fractionation, and subsequently by immunofluorescence. The structure of these hybrid proteins and the results of these analyses are summarized in figure 4-1.

The src7pk protein is cytosolic. We have previously shown that the first 7 amino acids of pp60^{src} act as a recognition sequence for myristylation (Kaplan et al. 1988). Fusing sequences encoding the first 7 amino acids of pp60^{src} to the coding region of PK creates a hybrid gene, *src*7PK, which encodes a myristylated protein. If

Figure 4-1. Summary of the membrane-association of *src*-PK proteins. The sequences derived from pp60^{src} in all of the *src*-PK proteins are depicted. The restriction sites used to create internal deletions of *src* sequences and to fuse the *src* sequences to the coding region of PK are indicated. Residue numbers correspond to the normal pp60^{src} sequence (Schwartz et al. 1983). Myristylation (Myr) of hybrid proteins, where analyzed, is indicated by a "+." When *src*-PK proteins associated with membranes, myristylation was assumed, indicated by (+), but was not tested. Membrane-association (MA) of hybrid proteins, as determined by biochemical fractionations is indicated by a "+." The intracellular distribution of hybrid proteins was determined by immunofluorescence (IF). Association of hybrid proteins with plasma membranes (PM), with the cytoplasm (CYT), with perinuclear membranes (PN), or with cytoplasmic granules (G) is indicated. At the bottom of the figure the N-terminal 259 amino acids of pp60^{src} are divided into functional domains. The first 7 amino acids of pp60^{src} (indicated by wavy lines) act as a recognition sequence for myristylation. Amino acids 1-14 cause association with cytoplasmic granules (G). Amino acids 38-111 (indicated by the stippled boxes) together with the myristylation signal cause association with both the plasma membrane and perinuclear membranes (PM+PN). Amino acids 204-259 (indicated by the vertically cross-hatches) together with the first 14 amino acids causes association solely with perinuclear membranes (PN). The myristyl moiety is indicated by the crooked line.

Figure 4-1


Stu I
Hae II
Nae I
Bal I
Hinc II
Bgl II
Mlu I
Myr
MA
E
src259PK
7 14
38
54
111
204
259
+ + PM,PN,G
src204PK
(+) + PM,PN,G
src111PK
(+) + PM,PN,G
src204(Δ 8-111)PK
+ - CYT
src111(Δ 8-37)PK
(+) + PM,PN,G
src54PK
(+) + PM,PN,G
src259(Δ14-203)PK
(+) + PN
src111(Δ15-54)PK
(+) + PM,PN,G
src14PK
+ + G
src7PK
+ - CYT
G
PM+PN
PN

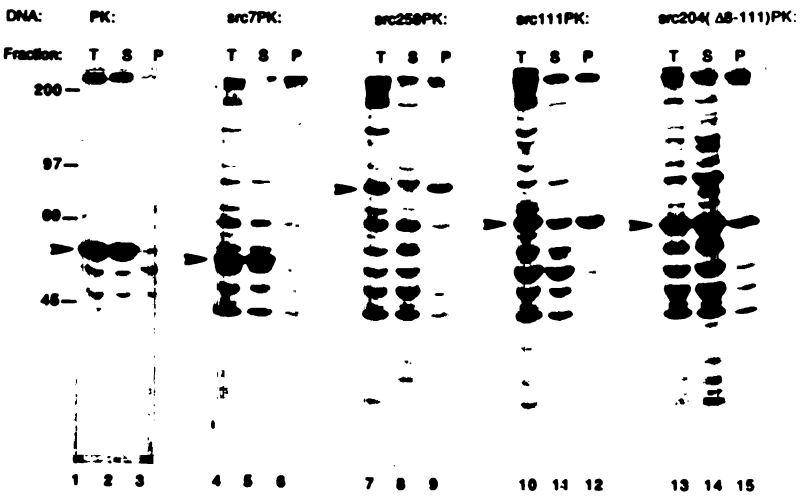
myristylation is a sufficient cause for membrane association, then the *src*7PK protein ought to be membrane-associated. Cells expressing either the native PK protein or the *src*7PK protein were fractionated into soluble and membrane fractions and these fractions were assayed for PK proteins (see figure 4-2). Virtually all of the native PK protein (lanes 1-3) and the *src*7PK protein (lanes 4-6) was recovered in the soluble fraction (lane 2 and 5 respectively), implying that a second domain of pp60^{src} is required in conjunction with myristylation to cause membrane association.

The src259pk protein associates with membranes. Since previous reports had implicated N-terminal sequences as membrane anchoring domains (Levinson et al. 1981; Krueger et al. 1982; Cross et al. 1984; Garber et al. 1985) sequences encoding the first 259 amino acids of pp60^{src} were fused to the coding sequences of PK, creating a hybrid gene called *src*259PK. Cells expressing the *src*259PK protein were fractionated into cytosol and membrane fractions, and these fractions were assayed for the *src*259PK protein (see figure 4-2, lanes 7-9). Most of the *src*259PK protein was recovered in the membrane fraction (lane 9). This result implies that sequences in the N-terminal half of pp60^{src}, together with myristylation, are sufficient to mediate association with membranes.

The src259PK protein and pp60^{src} associate with membranes in a similar manner. Before further characterizing the membrane-anchoring domains comprised by the N-terminal 259 amino acids of pp60^{src}, we tested whether the *src*259PK and pp60^{src} proteins associate with membranes in a similar manner. If *src*259PK and pp60^{src} associate with

Figure 4-2. The first 111 amino acids of pp60^{src} contain a membrane anchoring domain. COS7 cells were transfected with plasmids encoding native PK (lanes 1-3), *src*7PK (lanes 4-6), *src*259PK (lanes 7-9), *src*111PK (lanes 10-12), or *src*204(Δ8-111)PK (lanes 13-15) as described in the methods. Transfected cells were labelled with L-[^{35}S]methionine for 12 hours. Crude extracts (T, lanes 1,4,7,10, and 13) were fractionated into cytosol (S, lanes 2,5,8,11, and 14) and a membrane pellet (P, lanes 3,6,9,12, and 15) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The positions of 200 kilodalton (kd), 97 kd, 69 kd, and 45 kd molecular weight markers are indicated. The *src*-PK proteins are indicated by the arrow heads.

Figure 4-2.


DNA:
PK:
src7PK:
src258PK:
src111PK:
src204(Δ8-111)PK:
Fraction:
T S P
200
97
69
46
1 2 3
4 5 6
7 8 9
10 11 12
13 14 15

membranes in a similar manner, then destroying the myristylation signal should prevent the association of *src*259PK with membranes. We have previously shown that replacing lysine-7 with an asparagine largely abolishes myristylation of pp60^{src} (Kaplan et al. 1988). The myristylation signal of *src*259PK was destroyed by converting lysine-7 to an asparagine, and cells expressing this protein were fractionated as before (see figure 4-3, lanes 4-6). The protein encoded by this gene, N7-259PK, is found predominantly in the soluble fraction of cells (lane 5), implying that the membrane association of *src*259PK, like that of pp60^{src} itself, requires N-terminal myristylation. A second hallmark of the association of pp60^{src} with membranes is that, like other peripheral membrane proteins, pp60^{src} can be removed from membranes by alkaline extractions (see figure 4-4, lanes 4 and 5). Similarly, the *src*259PK protein is extracted from membranes following extraction with base (lanes 9 and 10). On the other hand, neither pp60^{src} (lanes 2 and 3) nor *src*259PK (lanes 6 and 7; and figure 4-3, lanes 1-3) are extracted from membranes by 0.3 M NaCl. By these three criteria *src*259PK and pp60^{src} appear to associate with membranes in a similar manner.

The first 111 amino acids of the src protein are sufficient for membrane association. Previous reports indicate that the N-terminal 13 kd of pp60^{src} act as a membrane anchoring domain (Levinson et al. 1981). This possibility was tested by fusing the coding region of PK to sequences encoding either the first 204 amino acids of pp60^{src} (creating the *src*204PK gene), the first 111 amino acids of pp60^{src} (creating the *src*111PK gene), or the first 7 amino acids of pp60^{src}

Figure 4-3. The N7-259PK protein is cytosolic. COS7 cells were transfected with plasmids encoding either the *src*259PK (lanes 1-3) protein or the N7-259PK protein (lanes 4-6). Transfected cells were labelled with L[^{35}S]methionine for 12 hours. Crude extracts (T, lanes 1,4) were fractionated into cytosol (S, lanes 2,5) and a membrane pellet (P, lanes 3,6) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The apparent *SRC*259PK protein is indicated by the arrow. The positions of 97 kd and 69 kd molecular weight markers are indicated.

Figure 4-3.

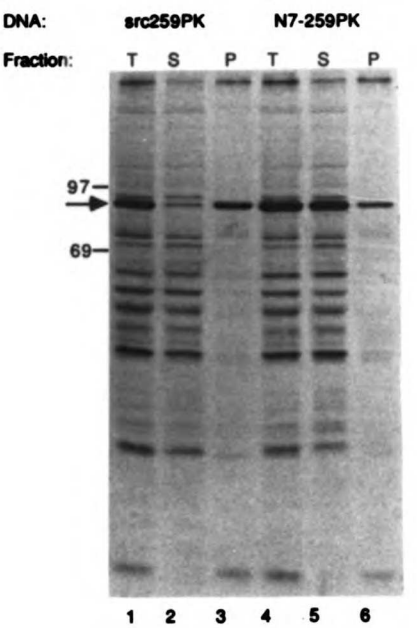
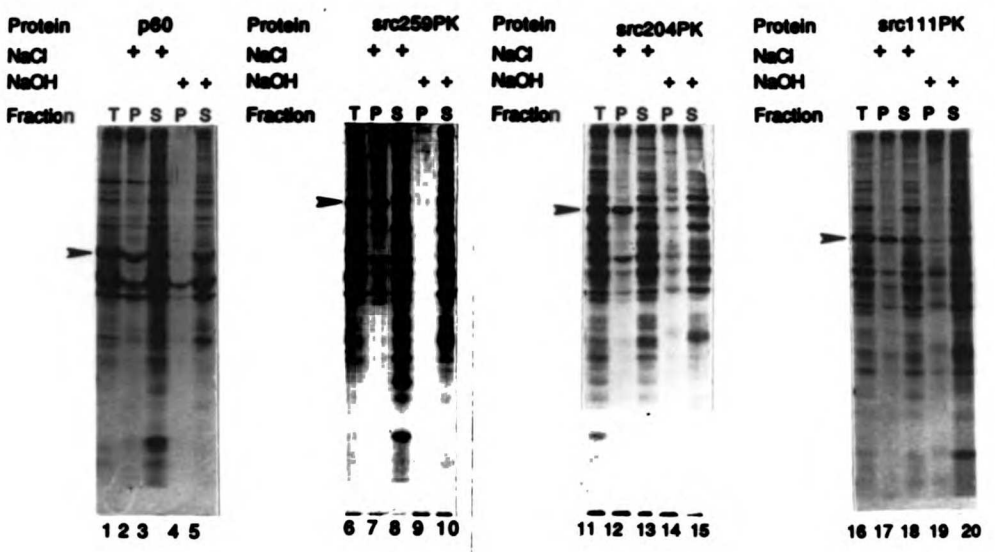


Figure 4-4. The *src*-PK proteins and pp60^{src} associate with membranes in a similar manner. B31 cells (lanes 1-5) or COS7 cells transfected with plasmids encoding *src*259PK (lanes 6-10), *src*204PK (lanes 11-15), or *src*111PK (lanes 16-20) proteins were labelled with L-[^{35}S]methionine for 12 hours. Crude extracts (T, lanes 1,6,11, and 16) were adjusted to either 0.3 M NaCl (lanes 2,3,7,8,12,13,17, and 18) or 0.1 M NaOH (lanes 4,5,9,10,14,15,19, and 20), and then fractionated into cytosol (S, lanes 3,5,8,10,13,15,18, and 20) and a membrane pellet (P, lanes 2,4,7,9,12,14,17, and 19) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with either mAb327 antibody (lanes 1-5) or a polyclonal rabbit anti-PK antibody (lanes 6-20). The positions of pp60^{src} and the *src*-PK proteins are indicated by the arrow heads.

Figure 4-4.


Protein
p60
NaCl
NaOH
Fraction
T P S P S
1 2 3 4 5
Protein
src259PK
NaCl
NaOH
Fraction
T P S P S
6 7 8 9 10
Protein
src204PK
NaCl
NaOH
Fraction
T P S P S
11 12 13 14 15
Protein
src111PK
NaCl
NaOH
Fraction
T P S P S
16 17 18 19 20

fused in-frame to amino acids 112-204 [creating the *src*204(Δ8-111)PK gene]. Cells expressing these proteins were fractionated as before (see figure 4-2). While most of the *src*204(Δ8-111)PK protein (lanes 13-15) was recovered in the cytosol (lane 14), both the *src*204PK (not shown, but see figure 4-4 lanes 11-15) and the *src*111PK (lanes 10-12) proteins were recovered primarily with the membrane pellet (lane 12). Both the *src*204PK (see figure 4-4, lanes 11-15) and the *src*111PK (lanes 16-20) proteins associated with membranes in a manner similar to pp60^{src} because they were not extracted from membranes by 0.3 M NaCl, but were extracted by base. We conclude that the first 111 amino acids of pp60^{src} are sufficient to cause association with membranes, and that the association is similar to that found with native pp60^{src}. By contrast, amino acids 111-204 cannot cooperate with N-terminal myristylation to cause membrane-association.

The first 111 amino acids of the src protein contain at least 2 independent membrane anchoring domains. The domain represented by the first 111 amino acids of pp60^{src} was divided into several segments and the sequences encoding these segments were fused to the coding region of PK. The association of these hybrid proteins with membranes was analyzed by biochemical fractionation as before.

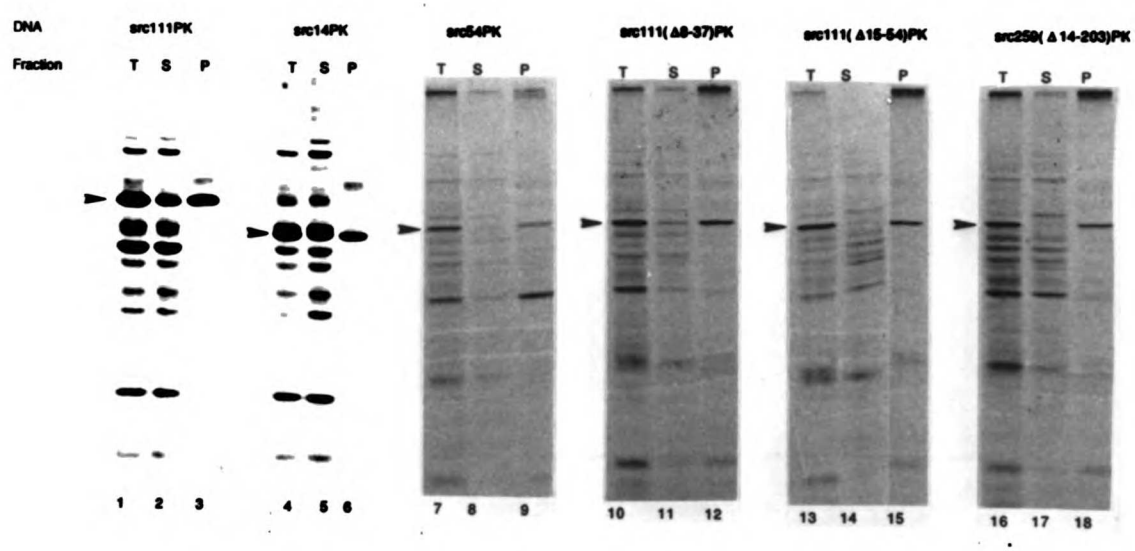
(i) The first 14 amino acids of the src protein contain a membrane anchoring domain. It was previously shown that fusing the first 14 amino acids of pp60^{src} to any one of several other proteins causes these proteins to associate with membranes in crude biochemical fractionations (Pellman et al. 1985a; Brooks-Wilson et al. 1989). This result was confirmed by fusing sequences encoding the first 14

amino acids of pp60^{src} to the PK coding region, creating *src*14PK. Most of the *src*14PK protein (figure 4-5, lanes 4-6) was recovered with the membrane pellet (lane 6) after subcellular fractionation. Similarly, other hybrid proteins that contained the first 14 amino acids of pp60^{src} also associated with membranes: for example *src*54PK (lanes 7-9), *src*111(Δ15-54)PK (lanes 13-15), and *src*259(Δ14-203)PK(lanes 16-18). Consequently, we cannot use this analysis to test whether amino acids 15-54, 55-111, and 204-259 also contain membrane-anchoring domains. Subsequent analyses will demonstrate that these sequences target proteins to distinct subcellular locations (see figure 4-7), implying that they contain additional membrane-anchoring domains. Since the *src*7PK protein is cytoplasmic, amino acids 8-14 must encode at least a portion of a membrane-anchoring domain.

(ii) Amino acids 38-111 of the src protein also contain a membrane anchoring domain. We have previously shown that the protein encoded by the deletion mutant E3 *src*, which lacks amino acids 8-37, is transforming, catalytically active, and membrane-associated (Kaplan et al. 1988). This suggests that amino acids 8-14 of pp60^{src} are not necessary for membrane association of pp60^{src}, and therefore that pp60^{src} must contain additional membrane-anchoring domains. The presence of a second membrane-anchoring domain is also indicated by the association of a 52 kilodalton C-terminal proteolytic fragment of pp60^{src}, p52^{src}, with membranes (Levinson et al. 1981). A second membrane anchoring domain was identified by fusing the sequence encoding the first 81 amino acids of the E3 *src* protein to the coding region of PK, creating the *src*111(Δ8-37)PK gene. Most of the

Figure 4-5. The first 111 amino acids of pp60^{src} contain at least two membrane anchoring domains. COS7 cells were transfected with plasmids encoding the *src*111PK (lanes 1-3), the *src*14PK (lanes 4-6), the *src*54PK (lanes 7-9), the *src*111(Δ8-37)PK (lanes 10-12), the *src*111(Δ15-54)PK (lanes 13-15), or the *src*259(Δ14-203)PK (lanes 16-18) proteins. Transfected cells were labelled with L-[35S]methionine for 12 hours. Crude extracts (T, lanes 1,4, 7,10,13, and 16) were fractionated into cytosol (S, lanes 2,5,8,11,14, and 17) and a membrane pellet (P, lanes 3,6,9,12,15, and 18) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The *src*-PK proteins are indicated by the arrow heads.

Figure 4-5.


DNA
Fraction
src111PK
T S P
1 2 3
src14PK
T S P
4 5 6
src54PK
T S P
7 8 9
src111(Δ9-37)PK
T S P
10 11 12
src111(Δ15-54)PK
T S P
13 14 15
src259(Δ14-203)PK
T S P
16 17 18

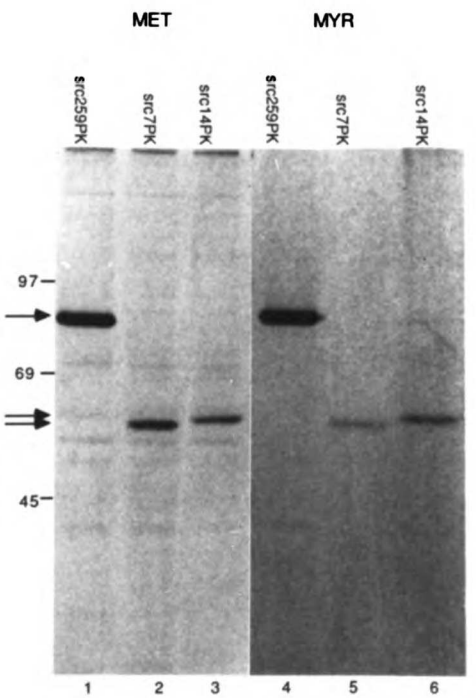
*src*111(Δ8-37)PK protein (figure 4-5, lanes 10-12) is recovered with the membrane pellet (lane 12) following subcellular fractionation. This implies that amino acids 38-111 of pp60^{src} also contain a membrane-anchoring domain.

Similar proportions of the src7PK, the src14PK, and the src259PK molecules are myristylated. An alternative explanation for the behavior of the cytoplasmic *src*7PK protein and the membrane-associated *src*259PK and *src*14PK proteins is that they differ not by the presence of a membrane-anchoring domain, but rather in the stoichiometry of myristylation. If the association of pp60^{src} with membranes were mediated solely by the myristyl moiety, then only the myristylated molecules would associate with membranes. This possibility was tested by comparing the stoichiometry of myristylation (see figure 4-6) of the *src*7PK (lanes 2 and 5), the *src*14PK (lanes 3 and 6), and the *src*259PK (lanes 1 and 4) proteins. Cells expressing these proteins were labelled with either L[^{35}S]methionine (lanes 1-3) or with [^{3}H]myristic acid (lanes 4-6), and the PK proteins were analyzed by immunoprecipitation. Comparison of the labelling of these proteins by methionine and myristate suggests that differences in the stoichiometry of myristylation of these proteins cannot explain the difference in their association with membranes.

This possibility was also tested by analyzing the membrane association of myristate labelled proteins. If the only relevant difference between the cytoplasmic *src*7PK and *src*204(Δ8-111)PK proteins and the membrane associated *src*259PK and *src*14PK proteins is the proportion of the molecules that are myristylated, then all of the

Figure 4-6. The stoichiometry of myristylation of the *src*259PK, the *src*7PK, and the *src*14PK proteins are similar. COS7 cells were transfected with plasmids encoding either the *src*259PK (lanes 1 and 4), the *src*7PK (lanes 2 and 5), or the *src*14PK (lanes 3 and 6) proteins. Transfected cells were labelled with either L[^{35}S]methionine (lanes 1-3) or [^{3}H]myristic acid (lanes 4-6) for 12 hours. Labelled cells were solublized with lysis buffer, and PK proteins were immunoprecipitated with a polyclonal anti-PK antibody. The positions of 97 kd, 69 kd, and 45 kd molecular weight markers are indicated. The PK proteins are indicated by the arrows.

Figure 4-6.


MET
MYR
src259PK
src7PK
src14PK
src259PK
src7PK
src14PK
97
69
45
1
2
3
4
5
6

myristylated molecules should be membrane-associated. Cells expressing these proteins were labelled with [^{3}H]myristic acid and subsequently divided into membrane and cytosol fractions. Virtually all of the myristate labelled *src*7PK protein was recovered in the cytoplasmic fraction, while most of the *src*259PK protein and the *src*14PK protein was recovered with the membrane pellet (data not shown). Similarly, most of the myristate labelled *src*204(Δ8-111)PK protein was cytoplasmic (data not shown). For these reasons, the inability of the *src*7PK protein and the *src*204(Δ8-111)PK protein to associate with membranes cannot reflect inefficient myristylation.

Localization of src-PK proteins by immunofluorescence. The biochemical fractionation procedure utilized to analyze the *src*-PK proteins relies upon the differential sedimentation of soluble and membrane-associated proteins. Rapid sedimentation does not unambiguously demonstrate that a protein is membrane-associated, but could be the consequence of the protein's association with a large protein complex. This method also does not distinguish plasma membranes from intracellular membranes. In order to confirm that the sedimentation of *src*-PK proteins is due to membrane association, and to determine which membrane a particular protein associates with, the distribution of these proteins within cells was analyzed by immunofluorescence.

(i) pp60^{src} associates with plasma membranes, perinuclear membranes, and cytoplasmic granules in transfected COS7 cells. The intracellular distribution of pp60^{src} was previously analyzed by immunofluorescence and electron microscopy (Rohrschneider 1980;

Willingham et al. 1979; Resh and Erikson 1985; Parsons and Creutz 1986; and Sobue and Kanda 1988). These studies indicate that in various cell types pp60^{src} is found associated with plasma membranes (typically at sites of focal adhesion), perinuclear membranes, and secretory granules. The distribution of pp60^{src} in COS7 cells following transfection with a plasmid containing the c-*src* gene was determined by immunofluorescence. The intracellular distribution of pp60^{src} in COS7 cells appeared to be a composite of these subcellular locations. The protein was found in plasma membranes, in perinuclear membranes, and in cytoplasmic granules (figure 4-7, panel A).

(ii) The PK protein, the src7PK protein, and the src204(Δ8-111)PK protein are cytoplasmic. The intracellular distribution of the PK (figure 4-7, panel B), *src*7PK (panel C), and the *src*204(Δ8-111)PK (panel G) proteins was analyzed by staining fixed cells with a polyclonal anti-PK antibody. These proteins had a distinctive cytoplasmic distribution. The margins of cells expressing these proteins were poorly visualized after staining with anti-PK antibody, and in general the intensity of the staining was proportional to the thickness of the cell, that is to the volume of underlying cytoplasm. These results confirm the interpretation of the biochemical fractionations described above.

(iii) Most of the membrane-associated src-PK proteins have an intracellular distribution similar to pp60^{src}. The *src*259PK protein (figure 4-7, panel D), the *src*204PK protein (panel E), the *src*111PK protein (panel F), the *src*111(Δ8-37)PK protein (panel H), the *src*54PK protein (panel I), and the *src*111(Δ15-54)PK protein (panel J), had a

Figure 4-7. Membrane-anchoring domains target proteins to specific subcellular locations. COS7 cell were transfected with plasmids encoding pp60^{src} (A), PK (B), *src*7PK (C), *src*259PK (D), *src*204PK (E), *SRC*111PK (F), *src*204(Δ8-111)PK (G), *src*111(Δ8-37)PK (H), *src*54PK (I), *src*111(Δ15-54)PK (J), *src*259(Δ14-203)PK (K), or *src*14PK (L) proteins. 48 hours after exposure to these DNAs, the cells were seeded onto glass coverslips. After an additional 24 hours, cells were fixed with paraformaldehyde, permeablized with triton X-100, and stained with either anti-pp60^{src} mAb327 or a polyclonal anti-PK antibody, as described in the methods.

Figure 4-7.

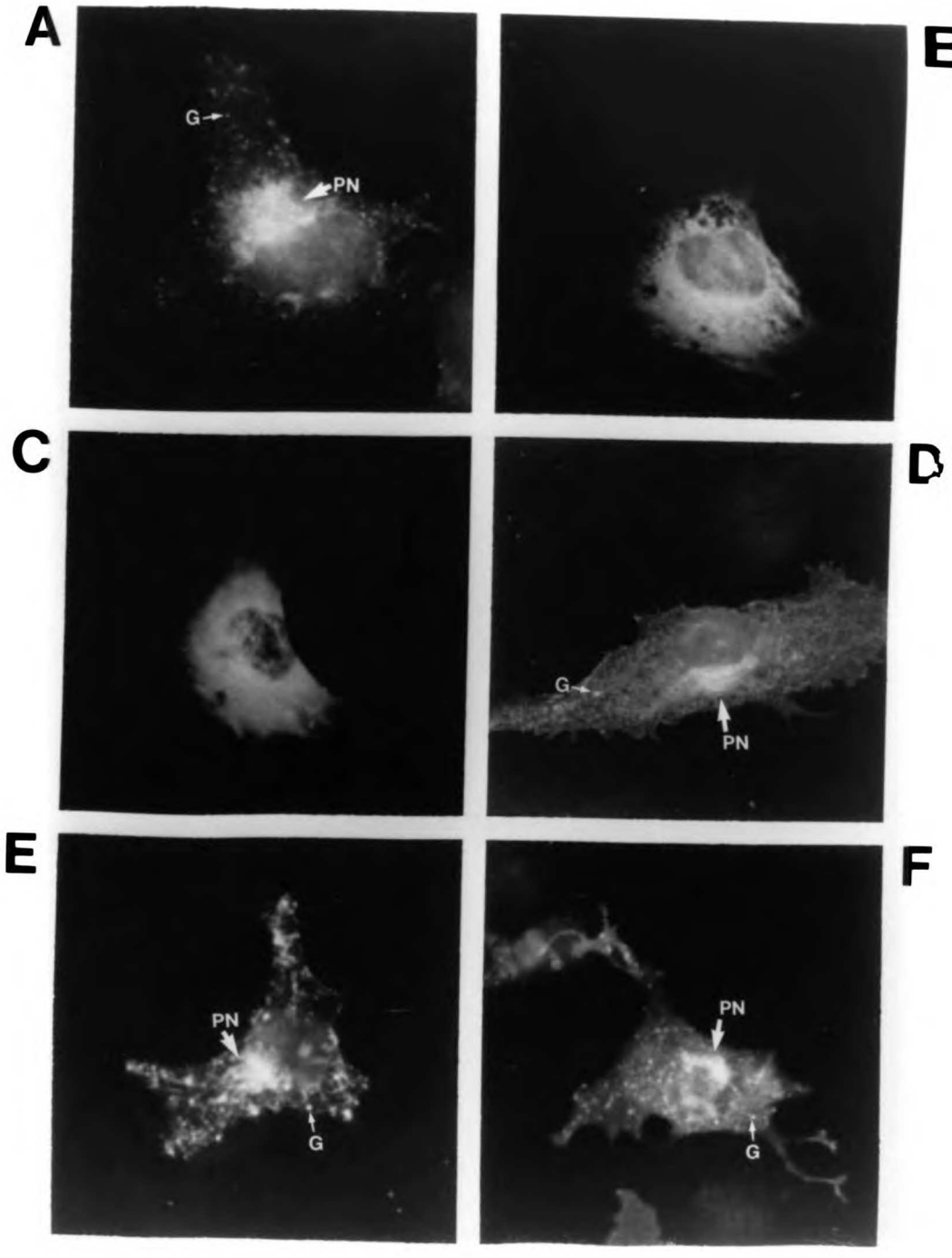
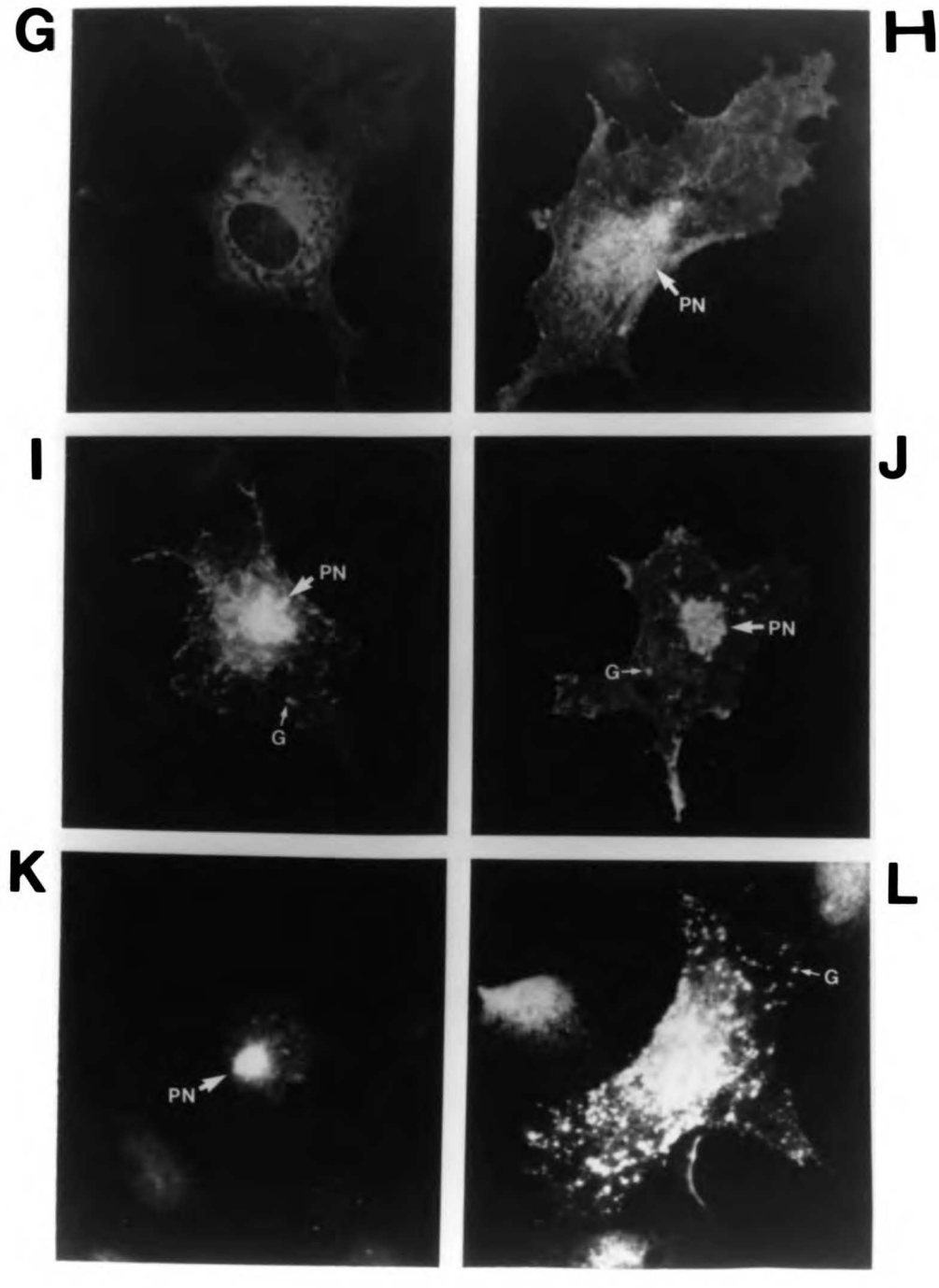


Figure 4-7.



distribution similar to pp60^{src} itself. The margins of cells expressing these proteins were clearly visualized after staining with anti-PK antibody, indicating association with the plasma membrane. These proteins were also found in perinuclear membranes and cytoplasmic granules.

Often cells expressing *src*-PK proteins that associate with the plasma membrane appeared to have a filamentous component to the fluorescent signal (see panel E, for instance). The filamentous structures that contain these *src*-PK proteins also contain actin, as demonstrated by staining with rhodamine-conjugated phalloidin, an actin specific stain (data not shown). It is not clear if these filamentous structures correspond to sites of adhesion.

(iv) The src259(v14-203)PK protein associates specifically with perinuclear membranes. Interestingly, the *src*259(Δ14-203)PK protein associated with perinuclear membranes but not with either plasma membranes or cytoplasmic granules (panel K). Since the distribution of the *src*259(Δ14-203)PK and the *src*l4PK (see below) proteins were different, we conclude from this result that amino acids 204-259 contain a membrane-anchoring domain that specfically targets proteins to perinuclear membranes.

(v) The srcl4PK protein associates with cytoplasmic granules. Cells expressing the *src*l4PK protein had a punctate pattern of cytoplasmic fluorescence after staining with anti-PK antibody (figure 4-7, panel L). These fluorescent spots had an apparent diameter of 0.2 microns, and were uniformly distributed throughout the cytoplasm. We suspect that this distribution corresponds to a membranous

organelle, rather than a large proteinaceous complex, because the *src*14PK protein does not sediment rapidly after dissolution of membranes with 1% Triton X-100 (data not shown).

The *src*14PK is the only hybrid protein that associates solely with cytoplasmic granules. Although most of the *src*-PK proteins that contain amino acids 1-14 are also found in cytoplasmic granules, proteins that contain additional portions of pp60^{src} associate with other membranes as well. For instance, both the *src*54PK protein (panel I) and the *src*111(Δ15-54)PK protein (panel J) associate with the plasma membrane and perinuclear membranes, in addition to cytoplasmic granules. Because the distribution of these proteins differs from *src*14PK, amino acids 15-54 and 55-111 must contain additional sorting information. These results imply that multiple domains of pp60^{src} can affect the intracellular distribution of a protein.

DISCUSSION

Several lines of evidence suggest that the association of pp60^{src} with membranes is mediated in part by the recognition of pp60^{src} by a membrane protein, and that this membrane protein may be a receptor for an extracellular ligand. First, pp60^{src} is not uniformly distributed in cellular membranes. In fibroblasts, pp60^{src} is found in plasma membranes (Courtneidge et al. 1980; Kreuger et al. 1980; Willingham et al. 1979) and perinuclear membranes (Resh and Erikson 1985), while in platelets (Rendu et al. 1989) and chromaffin cells

(Parsons and Creutz 1986) it is found in cytoplasmic granules. Furthermore, the pp60^{src} found in plasma membranes is concentrated at sites of adhesion, such as focal contacts (Rohrschneider 1980) or neuronal growth cones (Maness et al. 1988; Sobue and Kanda, 1989). The specificity of the subcellular localization of pp60^{src} suggests that pp60^{src} is recognized by specific proteins in these locations. Second, reconstitution of purified pp60^{src} into lipid vesicles *in vitro* requires the addition of membrane proteins (Resh 1988). Third, recently it was shown that the *lck* protein, which is an intra-cellular tyrosine kinase related to pp60^{src}, associates with two transmembrane glycoproteins, the CD4 and CD8 antigens of T-lymphocytes (Rudd et al. 1988; Veillette et al. 1988), and that the catalytic activity of *lck* can be modulated by crosslinking the CD4 antigen (Veillette et al. 1989). It is tempting to speculate that pp60^{src} is part of an analogous complex.

If pp60^{src} were part of a multimeric protein complex, and association of the component subunits of this complex mediates membrane association and subcellular localization of pp60^{src}, then one would expect: 1. that pp60^{src} would behave like a peripheral membrane protein; 2. that myristylation would not be sufficient to cause membrane association of pp60^{src}; 3. that together with myristylation specific domains of pp60^{src} would mediate its association with membranes; and 4. that these membrane-anchoring domains might target pp60^{src} to specific subcellular locations. In order to refine our understanding of the mechanism of transformation by pp60^{src}, we have explored the role of N-terminal amino acid sequences in promoting

association with membranes and in targeting the protein to specific subcellular locations.

The src protein is a peripheral membrane protein. Previous reports have demonstrated that the association of pp60^{src} with membranes cannot be disrupted by chelating agents or by extreme salt concentrations, while non-ionic detergents completely extract pp60^{src} from membrane vesicles (Levinson et al. 1981; Krueger et al. 1980). These results led some investigators to conclude that pp60^{src} is an integral membrane protein. We have shown that pp60^{src} is a peripheral membrane protein, because it can be extracted from membrane vesicles with alkali. Similarly, hybrid *src*-PK proteins that are associated with membranes are also extracted with alkali. Perhaps extraction from membranes with alkali will be a general feature of myristylated proteins .

Myristylation is not sufficient to cause membrane association. Myristylation is neither necessary or sufficient for the association of proteins with membranes. Several myristylated proteins are cytoplasmic (Olson et al. 1985), including several forms of pp60^{src} (Buss et al 1984; Garber et al. 1985). Conversely, non-myristylated forms of pp60^{src} have been described which still associate with membranes (Krueger et al. 1982; Garber and Hanafusa 1987). A C-terminal proteolytic fragment of pp60^{src} that lacks the myristyl moiety (so-called p52^{src}) remains associated with membranes (Levinson et al. 1981). However, since the non-myristylated membrane-associated forms of pp60^{src} are extracted from membranes by salt, myristic acid probably contributes to the affinity of the association with

membranes. Our results provide further support for the notion that the myristyl moiety does not solely mediate the association of pp60^{src} with membranes. The myristylated *src*7PK and *src*204(Δ8-111)PK proteins are cytoplasmic as revealed by both biochemical fractionation and by immunofluorescence.

The first 111 amino acids of pp60^{src} contain 2 membrane-anchoring domains. We have identified two membrane-anchoring domains of pp60^{src}. Together with myristylation amino acids 1-14 or amino acids 38-111 are sufficient to cause proteins to associate with membranes by crude biochemical fractionation. These results agree well with previous reports mapping the membrane anchoring domain to the N-terminal 13 kd of pp60^{src} (Levinson et al. 1980), and may explain the association of p52^{src} with membranes.

Together with previous reports, the results presented here suggest that the association of pp60^{src} with membranes is mediated by both myristylation and N-terminal membrane-anchoring domains. The initial association of pp60^{src} with membranes is normally mediated by the myristic acid. Hence, non-myristylated mutants are typically cytosolic. However, when membranes that contain pp60^{src} are digested with protease, the N-terminal 8 kd can be cleaved from pp60^{src}, creating p52^{src}, without disrupting the association with membranes (Levinson et al. 1981). Hence, once associated with membranes, the association of pp60^{src} with membranes is further stabilized by the membrane-anchoring domains.

Membrane-anchoring domains target molecules to distinct subcellular locations. Previous reports have demonstrated that

pp60^{src} is found in plasma membranes, perinuclear membranes, and cytoplasmic granules. COS7 cells are peculiar in that pp60^{src} is found in each of these locations. This allowed us to demonstrate that specific domains of pp60^{src} are responsible for targeting the protein to each subcellular location. Amino acids 1-14 cause proteins to associate with cytoplasmic granules. Perhaps the first 14 amino acids of pp60^{src} specifically target proteins to this compartment of neurons and platelets. Amino acids 38-111 cause proteins to associate with both plasma membranes and perinuclear membranes. We suspect that amino acids 38-111 comprise multiple membrane-anchoring domains, because in conjunction with amino acids 1-14 either amino acids 15-54 or 55-111 are sufficient to cause association with plasma membranes, perinuclear membranes, as well as cytoplasmic granules. Amino acids 204-259 together with amino acids 1-14 cause a protein to associate with primarily perinuclear membranes. We conclude from these results suggest that the subcellular localization of pp60^{src} is mediated by multiple independent membrane-anchoring domains.

Is the subcellular distribution of pp60^{src} controlled by phosphorylation? These experiments imply that pp60^{src} contains information that could potentially target it to multiple subcellular locations. How is it that in some cell types pp60^{src} is localized to one subcellular compartment and not to others? It is tempting to speculate that the activity of membrane-anchoring domains is regulated by phosphorylation. Together with previous reports, these experiments indicate that amino acids 1-14 of pp60^{src} act as a membrane anchoring domain, targeting molecules specifically to cytoplasmic granules.

Interestingly, two nearby serines are known to be phosphorylated by cellular kinases, serine-12 by C-kinase (Gould et al. 1985; Gentry et al. 1986) and serine-17 by the cAMP-dependent protein kinase (Collett et al 1979a; Karess and Hanafusa 1981; Cross and Hanafusa 1983). Phosphorylation of these sites may potentially regulate the subcellular location of pp60^{src}. Phosphorylation of the EGF-receptor by C-kinase is known to cause internalization of receptor molecules (Lin et al. 1986).

What is the site of the transforming activity of pp60^{src}? The transforming activity of pp60^{src} requires association with membranes, presumably because the crucial substrates of pp60^{src} are also membrane-associated. Since pp60^{src} is capable of associating with various membranes, it is not clear whether transformation is the consequence of the activity of pp60^{src} at a single or at multiple subcellular locations. No mutant *src* gene has been reported in which all of the membrane anchoring domains have been deleted. The most 5' breakpoint involved in deletion mutations previously reported corresponds to codon 14. Thus, these *src* proteins all contain the membrane-anchoring information residing in the first 14 amino acids of pp60^{src}, and all, except the NY18-3 *src* protein, are membrane-associated. The NY18-3 *src* gene lacks the sequences that normally encode amino acids 169-264, and it encodes a myristylated cytosolic protein. Our results do not explain the behavior of this *src* protein. Perhaps the conformation of this *src* protein precludes association with membranes. Surprisingly, many of these deletions starting at codon 15 retain transforming activity; however, none of these proteins

has been analyzed by immunofluorescence. Our results indicate that these transforming *src* proteins would associate with primarily perinuclear membranes and cytoplasmic granules.

CHAPTER 5:

ANALYSIS OF PROTEINS THAT BIND TO $pp60^{src}$.

INTRODUCTION

The previous chapters describe attempts to identify cellular proteins that interact with pp60^{src} by genetic screens (chapter 2), and to identify the domains of pp60^{src} that are recognized by cellular proteins (chapters 3 and 4). This chapter describes a strategy for purifying cellular proteins that bind to pp60$^{c\text{-}src}$. Among these proteins should be those responsible for the subcellular localization of pp60$^{c\text{-}src}$, and perhaps for the regulation of pp60$^{c\text{-}src}$.

Several cellular proteins are known to associate with pp60$^{v\text{-}src}$, as reviewed in chapter 1. Newly synthesized pp60$^{v\text{-}src}$ associates with 2 cellular proteins, pp50 and hsp90, which are thought to transport pp60^{src} from soluble polysomes to the cyptoplasmic face of the plasma membrane. In polyoma transformed cells, pp60$^{c\text{-}src}$ associates with middle T antigen, the transforming protein of polyoma virus (Courtneidge and Smith 1983, 1984; Bolen et al. 1984), and p85 (Courtneidge and Heber 1987; Kaplan et al. 1987), a cellular protein that is thought to be a phosphatidyl inositol kinase. Recent evidence suggests that pp60$^{v\text{-}src}$ also associates with a phosphatidyl inositol kinase (Fukui and Hanafusa 1989). Association of these proteins with pp60^{src} was demonstrated by co-immunoprecipitation. In a similar manner, several cellular proteins, including the product of the RB gene, were shown to associate with an adenovirus transforming protein, the E1a protein (Whyte et al. 1988).

An alternative strategy for identifying protein-protein

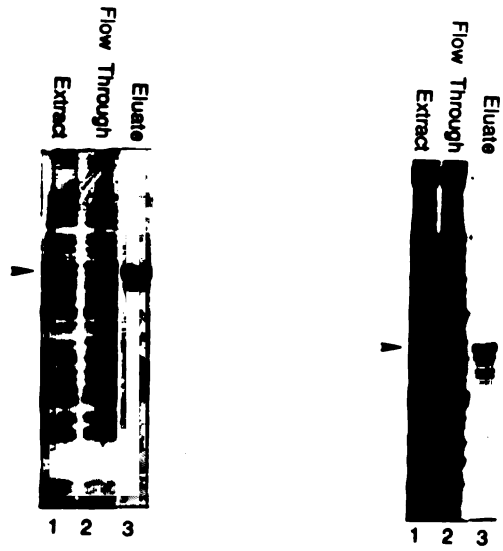
interactions, protein-affinity chromatography, has been applied to the study of phage T4 DNA replication proteins (Formosa et al. 1983), and of cytoskeletal proteins (Drubin et al. 1989; K. Miller personal communication). The purified protein of interest, pp60^{src} for instance, is chemically cross-linked to a solid matrix. This resin is used as a reagent for purifying proteins that specifically bind to pp60^{src}. The sensitivity of this method is determined by the concentration of pp60^{src} on the affinity matrix; consequently, this strategy relies upon the availability of purified pp60^{src}. If the affinity matrix has 0.5 mg/ml (equivalent to 9 μM), then one should be able to detect protein interactions that have dissociation constants of 9 μM. This represents a big advantage over co-immunoprecipitation, a technique which relies upon the stability of pre-formed complexes during purification, and hence dilution.

This chapter describes preliminary attempts to identify cellular proteins that bind to pp60^{src} by protein-affinity chromatography.

Purification of pp60^{src}. As starting material for purification of pp60^{src}, I took advantage of overexpression of pp60^{src} by baculovirus vectors (Morgan et al. 1989), and natural overexpression of pp60^{src} in human platelets (Golden et al. 1986). Insect cells infected with a recombinant baculovirus vector carrying a *src* cDNA are an abundant source of pp60^{src}, which accounts for 1% of total soluble proteins (see figure 5-1 panel A, lane 1). In detergent extracts prepared from human platelet concentrates, pp60^{src} accounts for approximately 0.1% of the soluble proteins (data not shown). Using either starting material, pp60^{src} can be substantially purified (1000

Figure 5-1. Purification of pp60^{src} from insect cell and platelet extracts. Detergent extracts were prepared from either insect cells infected with a recombinant baculovirus (panel A, lane 1) or human platelet concentrates (panel B, lane 1) as described in the methods. These extracts were passed through a mAb327 immunoaffinity column, the column was washed and the bound material was eluted with diethylamine pH11. Aliquots of the crude extracts (lane 1), the flow through (lane 2), and the eluted material (lane 3) were analyzed on SDS-PAGE gels. The position of pp60^{src} is indicated by the arrow.

Figure 5-1.


Extract
Flow Through
Eluate
1 2 3
Extract
Flow Through
Eluate
1 2 3

fold) in a single step of immuno-affinity purification (see figure 5-1). The pp60^{src} purified from baculovirus infected cells is virtually homogeneous (panel A, lane 3), while that purified from platelets is approximately 50% homogeneous (panel B, lane 3). The major contaminants in the material purified from platelets correspond to C-terminal proteolytic fragments of pp60^{src}, and the prevalence of these contaminants varies. In this manner, 10 mg of pp60^{src} can be purified from a liter of infected insect cells, and 2 mg of pp60^{src} can be purified from 50 units of human platelet concentrates.

These two starting materials were chosen because it seemed likely that the pp60^{src} purified from them would have different modifications, and hence different activity. The pp60^{src} purified from either source is very active as a protein kinase. The purified pp60^{src} has not lost activity in the course of the purification (unpublished data of J. Kaplan, D. Morgan, and D. Feder). The pp60^{src} purified from insect cells is catalytically more active than that purified from platelets, or from fibroblasts (unpublished data of J. Kaplan and D. Morgan). The difference in the kinase activity of these preparations probably reflects differences in the extent of phosphorylation of tyrosine-527. While others have shown that platelet pp60^{src} is phosphorylated on tyrosine-527 (Gould et al. 1985), the pp60^{src} purified from insect cells is not phosphorylated on tyrosine-527 (unpublished data of D. Morgan). The pp60^{src} purified from insect cells is myristylated (data not shown). Presumably, the platelet pp60^{src} is also myristylated, although this has not been demonstrated. These preparations of pp60^{src} were used to prepare a

affinity matrix for purification of cellular proteins that bind to pp60^{src}.

Methods for coupling pp60^{src} to a solid matrix. The manner in which pp60^{src} is coupled to the matrix will probably determine whether pp60^{src}-binding proteins will bind to the matrix. To determine the best method for identifying pp60^{src}-binding proteins, I compared two methods of coupling pp60^{src} to the matrix.

In one method, pp60^{src} is randomly coupled to the matrix via covalent modification of primary amines. Since pp60^{src} contains 31 lysine residues, this matrix should be heterogeneous for the conformation of pp60^{src} molecules. Another factor that will determine the conformation of pp60^{src} on the resin is whether each molecule is coupled via a single, or multiple primary amines. Molecules that are multiply coupled are likely to be denatured, and hence will probably not be recognized by pp60^{src}-binding proteins. The extent of coupling was controlled by varying the time of the coupling reaction. Affinity matrices prepared in this way were tested by comparing the pattern of fibroblast protein bound by the pp60^{src}-resin and by a BSA-resin. Although these matrices bind similar amounts of fibroblast proteins (estimated to be 0.2%, see figure 5-2 lanes 12-16), the pp60^{src}-resin appears to bind some proteins selectively (data not shown, but see figure 5-3). The binding capacity of pp60^{src}-resins for these specific bands depends on the extent of the coupling. Resins prepared with 66% (figure 5-3, lanes 10-13) of the pp60^{src} bound bind less specific proteins than those prepared with 25% (lanes 6-9) of the pp60^{src} bound to the resin.

Figure 5-2. Analysis of proteins bound to pp60^{src}-resins. CEF were labelled with L[^{35}S]methionine and a detergent extract was prepared. Equivalent amounts of the crude extract (lane 1) were bound to either BSA-resin (lanes 2-6), pp60^{src} bound to a resin via mAb2-17 (p60-mAb, lanes 7-11), or pp60^{src} bound to affigel (p60-affi, lanes 12-16). The resins were washed and the bound material was serially eluted with 0.25 M NaCl (lanes 2,3,7,8,12, and 13), 0.5 M NaCl (lanes 4,5,9,10,14, and 15), and boiling in 2% SDS (lanes 6,9, and 16). Equivalent amounts of each eluate was analyzed by SDS-PAGE gel.

Figure 5-2.

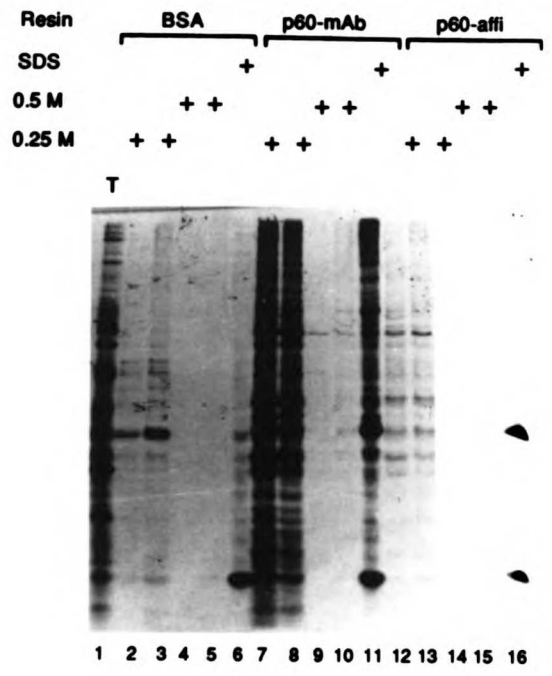
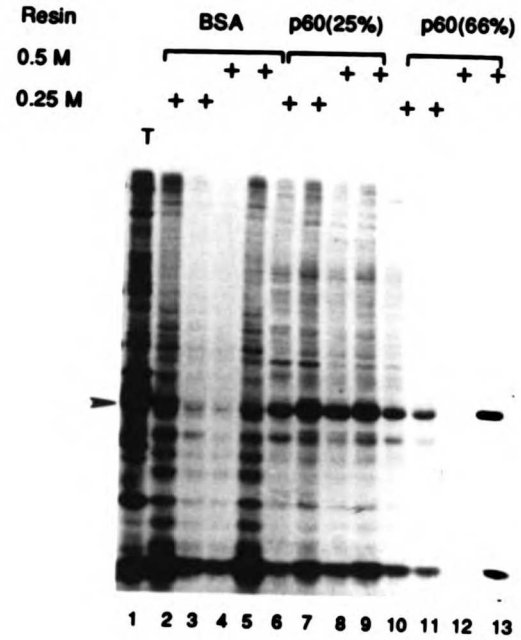

Resin
BSA
p60-mAb
p60-affi
SDS
0.5 M
0.25 M
T
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 5-3. The polyoma middle T antigen binds to pp60^{src}-resins. Polyoma transformed 3T3 cells were labelled with L[^{35}S]methionine and a detergent extract was prepared. Equivalent amounts of the crude extract was bound to either BSA-affigel (lanes 2-5); p60-affi in which 25% of the pp60^{src} was bound to the resin, p60(25%) (lanes 6-9); or p60-affi in which 66% of the pp60^{src} was bound to the resin, p60(66%) (lanes 10-13). The resins were washed and the bound material was serially eluted with 0.25 M NaCl (lanes 2,3,6,7,10, and 11), and with 0.5 M NaCl (lanes 4,5,8,9,12, and 13). The polyoma middle T antigen was immune precipitated from equivalent amounts of each eluate. The position of the polyoma middle T antigen is indicated by the arrow.

Figure 5-3.



The binding capacity of pp60^{src}-resin for pp60^{src}-binding proteins could be greatly increased if all of the molecules of pp60^{src} had a favorable orientation on the resin. For this reason, I prepared pp60^{src}-resins in which pp60^{src} is bound to the resin via a monoclonal antibody that binds to amino acids 2-17 of pp60^{src}. Affinity resins prepared in this way have significantly higher binding capacity for specific proteins, binding 1% of the total protein exposed to the resin (see figure 5-2, lanes 7-11), while pp60^{src} bound to affigel bound only 0.2% of the protein (lanes 12-16). For this reason, I relied on this method for preparing pp60^{src}-resins.

The polyoma middle T antigen binds specifically to pp60^{src}-resins. Since in polyoma transformed cells pp60^{src} is found associated with middle T antigen, it seemed possible that middle T antigen would bind specifically to pp60^{src}-resins. This was tested by binding detergent extracts prepared from polyoma transformed cells labelled with L[^{35}S]methionine to either pp60^{src}-resin (figure 5-3, lanes 6-13) or a control resin, BSA-resin (lanes 2-5). After washing the resins, proteins bound to these resins were serially eluted with 0.25 M and 0.5 M NaCl, and eluted proteins were assayed for the abundance of the middle T antigen by immunoprecipitation. Significantly more middle T antigen is bound by the pp60^{src}-resin than the BSA-resin. Additionally, the pp60^{src}-resin prepared with 25% of the pp60^{src} (lanes 6-9) binds significantly more middle T antigen than that prepared with 66% of the pp60^{src} bound (lanes 10-13). These results demonstrate that these methods can be used to demonstrate specific recognition of pp60^{src} by pp60^{src}-binding proteins.

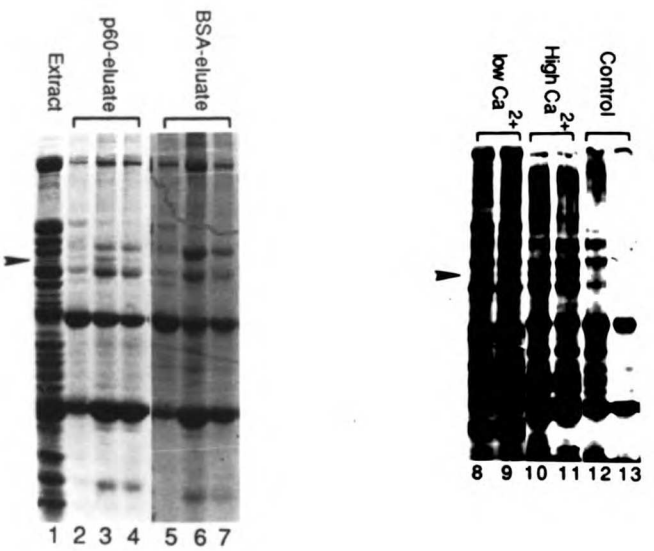
Platelet extracts contain a 97 kilodalton pp60^{src}-binding protein. Since platelets express 200 time the amount of pp60^{src} found in a normal fibroblast (Golden et al. 1985), extracts prepared from platelets should also be a good source of pp60^{src}-binding proteins. Resting platelets can be activated by various agonists, resulting in secretion of specific proteins and increased adhesiveness. The cytoplasmic states of resting and activated platelets can be mimicked by low calcium (250 nM) or high calcium (1 mM) concentrations respectively. Platelet extracts, prepared with either low or high calcium concentrations, were bound to either pp60^{src}-resin or a control resin. The proteins bound to these resins were eluted by increasing salt concentrations (see figure 5-4). In the high calcium conditions (lanes 1-6,10, and 11), a 97 kilodalton protein binds to the pp60^{src}-resin (lanes 2-4) but not to the control resin (lanes 6-7). Significantly less p97 binds to the pp60^{src}-resin in the low calcium conditions (lanes 8 and 9). The 97 kilodalton pp60^{src}-binding protein is not a glycoprotein, because it is not recognized by several different lectins (data not shown).

DISCUSSION

This chapter describes methods for purifying pp60^{src}-binding proteins by affinity chromatography. These methods were utilized to identify a 97 kilodalton (p97) non-glycosylated, hence presumably cytoplasmic, pp60^{src}-binding protein found in platelet extracts. The p97 protein is not α-actinin or hsp90, as determined by western

Figure 5-4. A 97 kd platelet protein binds to pp60^{src}-resins. A detergent extract was prepared from washed human platelets in either high Ca^{2+} (i.e. 2mM $CaCl_2$) (lanes 1-7, 10-13) or low Ca^{2+} (i.e. 1 mM EGTA) (lanes 8 and 9) conditions. Equivalent amounts of the crude extract (lane 1) was bound to either pp60^{src}-resin (lanes 2-4,8-11) or the control BSA-resin (lanes 5-7,12, and 13). The resins were washed and the bound material was eluted with 0.2 M NaCl. Negligible amounts of p97 remained associated with the resin following this elution (data not shown). The position of p97 is indicated by the arrow.

Figure 5-4.


Extract
p60-eluate
BSA-eluate
1 2 3 4 5 6 7
low Ca2+
High Ca2+
Control
8 9 10 11 12 13

blotting (data not shown). Obviously, these experiments are only a preliminary investigation of p97. Validation of the importance of any pp60^{src}-binding protein will depend upon further experiments: purification of p97 for the purpose of protein sequencing or raising antibodies; demonstration of colocalization of p97 and pp60^{src} in platelets, and perhaps other cell types, by immunolocalization; and assaying the effect of p97 on the catalytic activity of pp60^{src}. Interestingly, one of the proteins that is phosphorylated on tyrosine following thrombin stimulation of platelets is a 97 kd protein.

Since these experiments have identified only 1 candidate pp60^{src}-binding protein (p97), it is important to consider potential improvements in these methods. Since platelets are such a rich source of pp60^{src}, it seems likely that it should also be a good source of pp60^{src}-binding proteins. These proteins should co-purify with pp60^{src} in the immunoaffinity chromatography. Thus, the salt washes of the anti-pp60^{src} column should be enriched for pp60^{src}-binding proteins. In preliminary experiments, I showed that these fractions contain 1% of the total protein exposed to this column. The pp60^{src}-binding proteins in these fractions should be further purified by binding them to pp60^{src}-resins. Since pp60^{src}-resins also bind 1% of the total protein bound to them, the combined two step purification should yield substantially purified preparation of pp60^{src}-binding proteins.

If pp60^{src} were a subunit of a growth factor receptor complex, you would expect that some pp60^{src}-binding proteins would be transmembrane glycoproteins. None of the experiments I have conducted

indicate that there are glycoproteins that specifically bind to pp60^{src}-resin. Perhaps a better method for detecting these proteins would be to purify glycoproteins, on lectin resins, and subsequently apply these purified glycoproteins to the pp60^{src}-resin.

One might also expect that since neurons express a modified form of pp60^{src}, which is encoded by a tissue specific mRNA, neurons might contain tissue specific pp60^{src}-binding proteins. For this reason, it might be a good idea to prepare neuronal pp60^{src}-resins and test their binding capacity for neuronal versus fibroblast proteins.

CHAPTER 6: FUTURE DIRECTIONS

The goal of the work described in this thesis was to identify components of the cell that cooperate with pp60^{src} to produce cancer. Several approaches were taken. First, a strategy was devised for identifying cellular genes that either regulate pp60^{src} or mediate its transforming activity. Second, since several lines of evidence suggested that the membrane-association and subcellular localization of pp60^{src} are mediated by interaction with cellular proteins, the sequences in pp60^{src} that promote membrane-association were analyzed. Third, preliminary experiments were conducted to isolate cellular proteins that bind to pp60^{src}, in order to identify candidate proteins that either regulate pp60^{src} or control its subcellular localization. Potentially interesting future directions for each of these approaches will be discussed below.

Genetic analysis of transformation by pp60^{src}. The strategy described in chapter 2 failed to identify cellular genes that cooperate with *src* to transform cells for 2 reasons: 1. the difficulty of using the genetic analysis as the primary screen for interaction suppressors; and 2. the high background of bypass suppressors. The analysis of genetic revertants could be greatly facilitated by devising a preliminary biochemical screen to eliminate bypass suppressors. For instance, both *src* revertants and interaction suppressors might be expected to augment the kinase activity of pp60^{src}, thereby increasing the abundance of tyrosine-phosphorylated proteins generally, whereas bypass suppressors would not be expected to do so. Therefore, one could enrich for interesting mutants by screening the revertants for high levels of tyrosine-phosphorylated

proteins, by a "dot western" procedure for instance.

On the other hand, the prevalence of bypass suppressors in the experiments described in chapter 2 might simply have been the consequence of an unfortunate choice of a starting allele, that is c-*src*. Subsequent experiments have shown that c-*src* genes that have been genetically activated by converting tyrosine-527 to a phenylalanine do not transform rat-1 fibroblasts, suggesting that multiple events may be required to activate the transforming potential of c-*src* in these cells (unpublished data of H. Hirai). Determining whether other alleles produce lower backgrounds of bypass suppressors will await some other brave soul.

An alternative approach for identifying genes that activate the transforming potential of c-*src* is to survey naturally occurring tumors for mutations that enhance the activity of pp60^{src}. Given the multi-hit nature of human tumors, it is possible that the sorts of mutations that I was unable to isolate experimentally can be found in naturally occurring tumors. Activated forms of pp60^{src} have been described in neuroblastomas (Bolen et al. 1985), breast adenocarcinoma (Rosen et al. 1986), and colon carcinoma (Bolen et al. 1987; Cartwright et al. 1989). The biochemical basis of the activation of pp60^{src} in these tumors is not known. The methods described in chapter 2 could be used to determine if the augmented activity of pp60^{src} results from a mutation in pp60^{src} or in another gene. Since many of the genetic changes (both dominant and recessive) involved in the genesis of these tumors have been identified, there is the possibility of isolating the relevant gene.

Subcellular localization of pp60^{src}. It seems likely that membrane-anchoring domains act as recognition sequences for other proteins which bind to pp60^{src}, and that these proteins probably also regulate the activity of pp60^{src}. Further characterization of the membrane-anchoring domains of pp60^{src} should allow one to test: 1. whether transformation is the consequence of the activity of pp60^{src} at a specific or at multiple subcellular locations; 2. whether pp60^{src} produces specific phenotypic effects at specific subcellular locations; and 3. whether membrane-anchoring domains also act as sites of regulation of either the transforming activity or the kinase activity of pp60^{src}.

Since pp60^{src} associates with various cellular membranes, it is not known whether transformation is the consequence of the activity of pp60^{src} at a single or at multiple subcellular locations. It would not be surprising, given the pleiotropic effects of pp60^{src} if the fully transformed phenotype requires localization of pp60^{src} to multiple sites, which would suggest that pp60^{src} must phosphorylate multiple substrates in order to transform a cell.

Chapters 3 and 4 describe a strategy for identifying sequences that are involved in the membrane-association and subcellular localization of pp60^{src}. If upon further analysis the specific residues involved in subcellular localization are identified, it should be possible to design forms of pp60^{src} which are localized to a single subcellular location. Will a pp60^{src} protein that associates only with plasma membranes transform cells? If only a partially transformed phenotype is produced, then one can ascribe specific

phenotypic effects to the activity of pp60^{src} at the plasma membrane. It would then be informative to localize the known substrates of pp60^{src}, and hence potentially define the phenotypic effect that a specific substrate modification mediates.

Identification of the specific residues involved in membrane-anchoring should allow one to test whether the membrane-anchoring domains also mediate regulation of either the catalytic or transforming activities of pp60^{src}. For instance if a mutation inactivates a membrane-anchoring domain and augments the kinase activity of pp60^{src}, then the protein that binds to this sequence probably both localizes pp60^{src} and represses its activity.

Ultimately, understanding the mechanism underlying the subcellular localization, and potentially regulation, of pp60^{src} will require identifying the proteins that specifically bind to the membrane-anchoring domains. Hopefully, approaches such as the one described in chapter 5 will eventually lead to the identification of these proteins. The sorts of experiments described above should suggest whether these proteins also regulates the activity of pp60^{src}, and which specific phenotypic effects of pp60^{src} it might be mediating. Obviously, the identification of these proteins is the most difficult task among the experiments discussed; however, these proteins may shed light on the mechanism of transformation by pp60^{src}, and hence may provide potential sites for therapeutic intervention.

LITERATURE CITED

Aitkin, A., P. Cohen, S. Santikarn, D. H. Williams, A. G. Calder, A. Smith, and C. B. Klee. (1982) FEBS Lett. 150, 314-318.

Alema, S., Casalbore, P., Agostini, E. and Tato, F. (1985) Nature 316, 557-559.

Ali, I. V., Mautner, V., Lanza, R. and Hynes, R. O. (1977) Cell 11, 115-126.

Amrein, K. E. and Sefton, B. M. (1988) Proc. Natl. Acad. Sci. USA 85, 4247-4251.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Jerrlich, P. and Karin, M. (1987) Cell 49, 729-739.

Armitage, P. & Doll, R., Stochastic models for carcinogenesis. In Proceedings of the 4th Berkeley Symposium on Mathermatical Statistics and Probability (ed. J. Neyman), pp. 19-38, University of California Press, Berkeley.

Barret, J. C. and T'so, P. O. P. (1978a) Proc. Natl. Acad. Sci. USA 75, 3297-3301.

Barrett, J. C. and Ts'O, P. O. P. (1978b) Proc. Natl. Acad. Sci. USA 75, 3761-3765.

Bedard, P.-A., Alcorta, D., Simmons, D. L., Luk, K.-C. and Erikson, R. L. (1987) Proc. Natl. Acad. Sci. USA 84, 6715-6719.

Beug, H., Claviez, M., Jockusch, B. M. and Graf, T. (1978) Cell 14, 843-856.

Bishop, J. M. (1983) Ann. Rev. Biochem. 52, 301-354.

Bishop, J.M. & Varmus, H.E., Functions and origins of retroviral transforming genes. In Molecular Biology of Tumor Viruses, Part III: RNA Tumor Viruses (R. Weiss, N. Teich, H. E. Varmus and J. Coffin, Eds.), pp. 999-1108, Cold Spring Harbor Press, New York, 1982.

Boettiger, D. and Durban, E. M. (1979) CSH Symp. Quant. Biol. 44, 1249-1254.

Bolen, J. B., Thiele, C. J., Israel, M. A., Yonemotot, W., Lipsich, L. A. and Brugge, J. S. (1984) Cell 38, 767-777.

Botstein, D. and Maurer, D. (1982) Ann. Rev. Gen. 16, 61-83.

Boveri, T. (1914) Jena: Fischer.

Brodeur, G., Seeger, C., Schwab, M., Varmus, H. E. and Bishop, J. M. (1984) Science 224, 1121-1124.

Brooks-Wilson, A. R., Ball, E. and Pawson, T. (1989) Mol. Cell. Biol. 9, 2214-2219.

Brugge, J. S. and Erikson, R. L. (1977) Nature 269, 346-347.

Brugge, J. S., Cotton, P. C., Queral, A. E., Barrett, J. N., Nonner, D. and Keane, R. W. (1985) Nature 316, 554-557.

Bryant, D., and J. T. Parsons. (1982) J. Virol. 44, 683-691.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988) Ann. Rev. Cell Biol. 4, 487-525.

Buss, J. E., and B. M. Sefton. (1985) J. Virol. 53, 7-12.

Buss, J. E., M. P. Kamps, and B. M. Sefton. (1984) Mol. Cell. Biol. 4, 2697-2704.

Buss, J. E., M. P. Kamps, K. Gould, and B. M. Sefton. (1986) Science 232, 468-474.

Carr, S. A., K. Biemann, S. Shogi, D. C. Parmelee, and K. Titani. (1982) Proc. Natl. Acad. Sci. USA 79, 6128-6131.

Cartwright, C. A., Hutchinson, M. A. and Eckhart, W. (1985) Mol. Cell. Biol. 5, 2647-2652.

Cartwright, C. A., Kaplan, P. L., Cooper, J. A., Hunter, T. and Eckhart, W. (1986) Mol. Cell. Biol. 6, 1562-1570.

Cartwright, C. A., Kaplan, P. L., Cooper, J. A., Hunter, T. and Eckhart, W. (1987) Cell 49, 83-91.

Cartwright, C. A., Simantov, R., Cowan, W. M., Hunter, T. and Eckhart, W. (1988) Proc. Natl. Acad. Sci. USA 85, 3348-3352.

Cepko, C. L., Roberts, B. E. and Mulligan, R. C. (1984) Cell 37, 1053-1062.

Chackalaparampil, I. and Shalloway, D. (1988) Cell 52, 801-810.

Chow, M., J. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlans, and F. Brown. (1987) Nature 327, 482-486.

Cleaver, J. E., Xeroderma Pigmentosum. In The Metabolic Basis of Inherited Disease (J. Stanbury, J. Wyngaarden, D. Fredrickson, J. Goldstein, and M. Brown, eds.), pp. 1227-1248, McGraw Hill, New York, 1983.

Collett, M. S. and Erikson, R. L. (1978) Proc. Natl. Acad. Sci. USA 75, 2021-2024.

Collett, M. S., Erikson, E. and Erikson, R. L. (1979a) J. Virol. 29, 770-781.

Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S. and Erikson, R. L. (1979b) Proc. Natl. Acad. Sci. USA 76, 3159-3163.

Collett, M. S., Wells, S. K. and Purchio, A. F. (1983) Virology 128, 285-297.

Comoglio, P. M., Di Renzo, M. F., Tarone, G., Giancotti, F. G., Naldini, L. and Marchisio, P. C. (1984) EMBO J. 3, 483-489.

Cooper, J.A., The *src* family of protein-tyrosine kinases. In Peptides and protein phosphorylation (B. Kemp and PF Alewood eds.), CRC Press Inc, in press.

Cooper, J. A., Gould, K. L., Cartwright, C. A. and Hunter, T. (1986) Science 231, 1431-1434.

Cooper, J. A. and Hunter, T. (1984) Curr. Top. Microbiol. Immunol. 107, 125-162.

Cooper, J. A. and King, C. S. (1986) Mol. Cell. Biol. 6, 4467-4477.

Cooper, J. A. and MacAuley, A. (1988) Proc. Natl. Acad. Sci. USA 85, 4232-4236.

Cooper, J. A., Nakamura, K. D., Hunter, T. and Weber, M. J. (1983a) J. Virol. 46, 15-28.

Cooper, J. A., Reiss, N. A., Schwartz, R. J. and Hunter, T. (1983b) Nature 302, 218-222.

Cooper, M. D., Payne, L. N., Dent, P. B., Burmester, B. R. and Good, R. A. (1968) J. Natl. Cancer Inst. 41, 373-389.

Cotton, P. C. and Brugge, J. S. (1983) Mol. Cell. Biol. 3, 1157-1162.

Courtneidge, S. A. (1985) EMBO J. 4, 1471-1477.

Courtneidge, S. A. and Bishop, J. M. (1982) Proc. Natl. Acad. Sci. USA 79, 7117-7121.

Courtneidge, S. A., Levinson, A. D. and Bishop, J. M. (1980) Proc. Natl. Acad. Sci. USA 77, 3783-3787.

Courtneidge, S. A. and Smith, A. E. (1983) Nature 303, 435-439.

Courtneidge, S. A. and Smith, A. E. (1984) EMBO J. 3, 585-591.

Coussens, P. M., Johnson, P. J. and Shalloway, D. (1985) Mol. Cell.

Biol. 5, 2753-2763.

Cowell, J. K. (1982) Ann. Rev. Genet. 16, 21-59.

Cross, F. R. and Hanafusa, H. (1983) Cell 34, 597-607.

Cross, F. R., Garber, E. A., Pellman, D. and Hanafusa, H. (1984) Mol. Cell. Biol. 4, 1834-1842.

DeClue, J. and Martin, G. S. (1987) Mol. Cell. Biol. 7, 371-378.

DeClue, J. E., Sadowski, I., Martin, G. S. and Pawson, T. (1987) Proc. Natl. Acad. Sci. USA 84, 9064-9068.

DeFeo, D., Gonda, M. A., Young, H. A., Chang., E. H., Lowy, D. R. and Ellis, R. W. (1981) Proc. Natl. Acad. Sci. USA 78, 3328-3332.

Deusberg, P. H. and Vogt, P. K. (1970) Proc. Natl. Acad. Sci. USA 67, 1673-1680.

Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., DeVare, S. G., Robbins, K. C., Aaronson, S. A. and Antoniades, H. N. (1983) Science 221, 275-276.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. D. (1984) Nature 307, 521-527.

Drubin, D. G., Miller, K. G. and Botstein, D. (1988) J. Cell Biol. 107, 2551-2561.

Dunn, J. E. (1966) Proc. R. Soc. Med. 59, 1198-1204.

Erikson, R. L., Collett, M. S., Erikson, E. and Purchio, A. F. (1979) Proc. Natl. Acad. Sci. USA 76, 6260-6264.

Eckhart, W., Hutchinson, M. A. and Hunter, T. (1979) Cell 18, 925-933.

Fasano, O., Birnbaum, D., Edlund, L., Fogh, J. and Wigler, M. (1984) Mol. Cell. Biol. 4, 1605-1612.

Ferrel, J. E. and Martin, G. S. (1988) Mol. Cell. Biol. 8, 3603-3610.

Fialkow, P. J. (1974) New Engl. J. Med. 291, 26-35.

Fialkow, P. J. (1976) Biochem. Biophys. Acta 458, 283-321.

Fialkow, P. J., Clonal evolution of human myeloid leukemias. In Genes and Cancer - UCLA Symposia on Molecular and Cellular Biology (J. M. Bishop, J. D. Rowley, and M. Greaves, eds.), vol. 17, pp. 215-226, Alan R. Liss Inc., New York, 1984.

Fiszman, M. Y. and Fuchs, P. (1975) Nature 254, 429-431.

Flier, J. S., Mueckler, M. M., Usher, P. and Lodish, H. F. (1987) Science 235, 1492-1495.

Formosa, T., Burke, R. L. and Alberts, B. M. (1983) Proc. Natl. Acad. Sci. USA 80, 2442-2446.

Frackelton, A. R., Jr., Ross, A. H., and Eisen, H. N. (1983) Mol. Cell. Biol. 3,1343-1352.

Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M. and Dryja, T. P. (1986) Nature 323, 643-646.

Fry, M. J., Gebhardt, A., Parker, P. J. and Foulkes, G. (1985) EMBO J. 4, 3173-3178.

Fukui, Y. and Hanafusa, H. (1989) Mol. Cell. Biol. 9, 1651-1658.

Fung, Y. K. T., Lewis, W. G., Kung, H. J. and Crittenden, L. B. (1983) Cell 33, 357-368.

Garber, E. A., F. R. Cross, and H. Hanafusa. (1985) Mol. Cell. Biol. 5, 2781-2788.

Garber, E. A., and H. Hanafusa. (1987) Proc. Natl. Acad. Sci. USA 84, 80-84.

Geissler, E. N., Ryan, M. A. and Housman, D. E. (1988) Cell 55, 185-192.

Gentry, L. E., Chaffin, K. E., Shoyab, M. and Purchio, A. F. (1986) Mol. Cell. Biol. 6, 735-738.

Golden, A. and Brugge, J. S. (1989) Proc. Natl. Acad. Sci. USA 86, 901-905.

Golden, A., Nemeth, S. P. and Brugge, J. S. (1986) Proc. Natl. Acad. Sci. USA 83, 852-856.

Gould, K. L. and Hunter, T. (1988) Mol. Cell. Biol. 8, 3345-3356.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D. and Hunter, T. (1985) Cell 42, 849-857.

Graham, F. L., and A. J. Van der Eb. (1973) Virology 52, 456-467.

Graziani, Y., Erikson, E. and Erikson, R. L. (1983) J. Biol. Chem. 258, 2126-2131.

Groudine, M. and Weintraub, H. (1980) Proc. Natl. Acad. Sci. USA 77, 5351-5354.

Hafen, E., Basler, K., Edstroem, J.-E. and Rubin, G. M. (1987) Science 236, 55-63.

Hanafusa, H., Comprehensive Virology (eds. H. Fraenkel-Conrat and R.R. Wagner), pp. vol. 10:401-438, Plenum Press, New York, 1977.

Hanafusa, H., Iba, H., Takeya, T. and Cross, F. R. (1984) Cancer Cells 2, 1-7.

Hanahan, D., Oncogenes and growth control (P. Kahn and T. Graf, eds.), pp. 349-363, Springer-Verlag, Berlin, 1986.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) Science 241, 42-52.

Henderson, L. E., H. C. Krutzsch, and S. Oroszlan. (1983) Proc. Natl. Acad. Sci. USA 80, 339-343.

Hirst, R., Horwitz, A., Buck, C. and Rohrschneider, L. (1986) Proc. Natl. Acad. Sci. USA 84, 6470-6474.

Hughes, S. H., Payvar, F., Spector, D., Schimke, R. T., Robinson, H. L. and et al. (1979) Cell 18, 347-359.

Hunter, T. and Cooper, J. A. (1985) Ann. Rev. Biochem. 54, 897-930.

Hunter, T. and Sefton, B. M. (1980) Proc. Natl. Acad. Sci. USA 77, 1311-1315.

Hurley, T. R., Luo, K. and Sefton, B. M. submitted.

Hynes, R. O. (1974) Cell 1, 147-156.

Iba, H., Takeya, T., Cross, F. R., Hanafusa, T. and Hanafusa, H. (1984) Proc. Natl. Acad. Sci. USA 81, 4424-4428.

Jackowski, S., Rettenmeier, C. W., Scherr, C. J. and Rock, C. O. (1986) J. Biol. Chem. 261, 4978-4985.

Jakobovits, E. B., Majors, J. E. and Varmus, H. E. (1984) Cell 38, 757-765.

Jha, K. K., Siniscalco, M., and Ozer, H. L. (1980) Somatic Cell Genetics 6, 603-614.

Jove, R., Kornbluth, S. and Hanafusa, H. (1987) Cell 50, 937-943.

Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. (1984) Cell 39, 499-509.
Kamps, M. P. and Sefton, B. M. (1988) Oncog. Res. 3, 105-115.

Kamps, M. P., Buss, J. E. and Sefton, B. M. (1986) Cell 45, 105-112.

Kamps, M. P., J. E. Buss, and B. M. Sefton. (1985) Proc. Natl. Acad. Sci. USA 82, 4625-4629.

Kaplan, D. R., Whitman, M., Schaffhausen, B., Raptis, L., Garcea, R. L., Pallas, D., Roberts, T. M. and Cantley, L. (1986) Proc. Natl. Acad. Sci. USA 83, 3624-3628.

Kaplan, J. M., Mardon, G., Bishop, J. M. and Varmus, H. E. (1988) Mol. Cell. Biol. 8, 2435-2441.

Karess, R. E. and Hanafusa, H. (1981) Cell 24, 155-164.

Katamine, S., V. Notario, C.D. Rao, T. Miki, M.S.C. Cheah, S.R. Tronick, and K.C. Robbins. (1987) Molec. Cell. Biol. 8, 259-266.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J. and Wigler, M. (1984a) Cell 40, 19-26.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J. and Wigler, M. (1984b) Cell 37, 437-445.

Kato, J.-Y., Takeya, T., Grandori, C., Iba, H., Levy, J. B. and Hanafusa, H. (1986) Mol. Cell. Biol. 6, 4155-4160.

Klarlund, J. K. (1985) Cell 41, 707-717.

Klempnauer, K. H., Gonda, T. J. and Bishop, J. M. (1982) Cell 31, 453-463.
Kmiecik, T. E. and Shalloway, D. (1987) Cell 49, 65-73.

Knudson, A. G. (1971) Proc. Natl. Acad. Sci. USA 68, 820-.

Konopka, J. B., Watanabe, S. M. and Witte, O. N. (1984) Cell 37, 1035-1042.

Kornbluth, S., Sudol, M. and Hanafusa, H. (1987) Nature 325, 171-173.

Koss, L. G. (1963) Cancer 16, 1160-1211.

Krueger, J. G., Garber, E. A. and Goldberg, A. R. (1983) Curr. Top. Micro. and Immuno. 107, 51-124.

Krueger, J. G., Garber, E. A., Goldberg, A. R. and Hanafusa, H. (1982) Cell 28, 889-896.

Krueger, J. G., Wang, E. and Goldberg, A. R. (1980) Virology 101, 25-40.

Kypta, R. M., Hemming, A. and Courtneidge, S. A. (1988) EMBO J. 7, 3837-3844.

Lee, J. S., H. E. Varmus, and J. M. Bishop. 1979. J. Biol. Chem. 245:8015-8022.

Lee, M. and Nurse, P. (1988) Trends in Genet. 4, 287-290.

Lee, W., Mitchell, P. and Tjian, R. (1987) Cell 49, 741-752.

Lehto, V.-P., Wasenius, V.-M., Salven, P. and Saraste, M. (1988) Nature 334, 388-.

Levinson, A. D., Courtneidge, S. A. and Bishop, J. M. (1981) Proc. Natl. Acad. Sci. USA 78, 1624-1628.

Levinson, A. D., Opperman, H., Levintow, L., Varmus, H. E. and Bishop, J. M. (1978) Cell 15, 561-572.

Levinson, A. D., Oppermann, H., Varmus, H. E. and Bishop, J. M. (1980) J. Biol. Chem. 255, 11973-11980.

Levy, J. B., Dorai, T., Wang, L.-H. and Brugge, J. S. (1987) Mol. Cell. Biol. 7, 4142-4145.

Levy, J. B., Iba, H. and Hanafusa, H. (1986) Proc. Natl. Acad. Sci. USA 83, 4228-4232.

Lin, C. R., Chen, W. S., Lazar, C. S., Carpenter, C. D., Gill, G. N., Evans, R. M. and Rosenfeld, M. G. (1986) Cell 44, 839-848.

Lipsich, L. A., A. J. Lewis, and J. S. Brugge. (1983) J. Virol. 48, 352-360.

Lipsich, L. A., Cutt, J. R. and Brugge, J. S. (1982) Mol. Cell. Biol. 2, 875-880.

Lipsich, L., Brugge, J. S. and Boettiger, D. (1984) Mol. Cell. Biol. 4, 420-1424.

Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A. F. and Minna, J. D. (1983) Nature 306, 194-196.

Lonberg, N., and Gilbert, W. (1983) Proc. Natl. Acad. Sci. USA 80, 3661-3665

Luttrell, D. K., Luttrell, L. M. and Parsons, S. J. (1988) Mol. Cell. Biol. 8, 497-501.

Macara, I. G., Marinetti, G. V. and Balduzzi, P. C. (1984) Proc. Natl. Acad. Sci. USA 81, 2728-2732.

Magee, A. I., and S. A. Courtneidge. (1985) EMBO J. 4, 1137-1144.

Maher, P. A., Pasquale, E. B., Wang, J. Y. J. and Singer, S. J. (1985)

Proc. Natl. Acad. Sci. USA 82, 6576-6580.

Maness, P. F., Aubry, M., Shores, C. G., Frame, L. and Pfenninger, K. H. (1988) Proc. Natl. Acad. Sci. USA 85, 5001-5005.

Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Cell 33, 153-159.

Marchildon, G. A., J. E. Casnelli, K. A. Walsh, and E. G. Krebs. (1984) Proc. Natl. Acad. Sci. USA 81, 7679-7682.

Marth, J. D., R. Peet, E. G. Krebs, and R. M. Perlmutter. (1985) Cell 43, 393-404.

Martin, G. S. (1970) Nature 227, 1021-1023.

Martinez, R., Mathey-Prevot, B., Bernards, A. and Baltimore, D. (1987) Science 237, 411-415.

McIlhinney, R. A. J., S. J. Pelly, J. K. Chadwick, and G. P. Cowley. (1985) EMBO J. 4, 1145-1152.

Miller, A. D., Law, M. -F., and Verma, I. M. (1985) Mol. Cell. Biol. 5, 431-437.

Morgan, D. O., Kaplan, J. M., Bishop, J. M. and Varmus, H. E. (1989) Cell in press

Mulligan, R. C. and Berg, P. (1980) Science 209, 1422-1427.

Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. and Astrin, S. M. (1981) Cell 23, 323-334.

Neiman, P., Jordan, L., Weiss, R. A. and Payne, L. N. (1980) Cold Spring Harbor Conf. Cell Proliferation 7, 519-528.

Norberg, R., Lidman, K. and Fagraeus, A. (1975) Cell 6, 507-512.

Olson, E. N., and G. Spizz. (1986) J. Biol. Chem. 261, 2458-2466.

Olson, E. N., D. A. Towler, and L. Glaser. (1985) J. Biol. Chem. 260, 3784-3790.

Oppermann, H., Levinson, W. and Bishop, J. M. (1981c) Proc. Natl. Acad. Sci. USA 78, 1067-1071.

Oppermann, H., Levinson, A. D., Levintow, L., Varmus, H. E., Bishop, J. M. and Kawai, S. (1981a) Virology 113, 736-751.

Oppermann, H., Levinson, A. D. and Varmus, H. E. (1981b) Virology 108, 47-70.

Oppermann, H., Levinson, A. D., Varmus, H. E., Levintow, L., and

Bishop, J. M. (1979) Proc. Natl. Acad. Sci. USA 76, 1804-1808.

Oskarsson, M., McClements, W. L., Blair, D. G., Maizel, J. V. and Vanderwoude, G. F. (1980) Science 207, 1222-1224.

Ozols, J., S. A. Carr, and P. Strittmatter. (1984) J. Biol. Chem. 259, 13349-13354.

Parker, R. C., Varmus, H. E. and Bishop, J. M. (1981) Proc. Natl. Acad. Sci. USA 78, 5842-5846.

Parker, R. C., Varmus, H. E. and Bishop, J. M. (1984) Cell 37, 131-139.

Parsons, J. T. and Weber, M. J. (1989) Curr. Top. Microbiol. and Immunol , in press.

Parsons, S. J. and Creutz, C. E. (1986) Biochem. Biophys. Res. Comm. 134, 736-742.

Pasquale, E. B., Maher, P. A. and Singer, S. J. (1986) Proc. Natl. Acad. Sci. USA 83, 5507-5511.

Payne, G. S., Courtneidge, S. A., Crittenden, L. B., Fadley, A. M., Bishop, J. M. and Varmus, H. E. (1981) Cell 23, 311-322.

Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa. (1985a) Nature 314, 374-377.

Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa. (1985b) Proc. Natl. Acad. Sci. USA 82, 1623-1628.

Persing, D. H., H. E. Varmus, and D. Ganem. (1987) J. Virol. 61, 1672-1677.

Perucho, M. and Wigler, M. (1981) Cold Spring Harbor Symp. Quant. Biol. 45, 829-838.

Peto, R. (1977) Cold Spring Harbor Conf. Cell Proliferation 4, 1403-1428.

Piwinca-Worms, H., Saunder, K. B., Roberts, T. M., Smith, A. E. and Cheng, S. H. (1987) Cell 49, 75-82.

Ponder, B. (1988) Nature 335, 400-402.

Potts, W. M., Reynolds, A. B., Lansing, T. J. and Parsons, J. T. (1989) Oncogene Res. in press.

Purchio, A. F., Shoyab, M. and Gentry, L. E. (1985) Science 229, 1393-1395.

Quintrell, N., R. Lebo, H. Varmus, J. M. Bishop, M. J. Pettenati, M. M. Le Beau, M. O. Diaz, and J. D. Rowley. (1987) Mol. Cell. Biol. 7, 2267-2275.

Ralston, R. and Bishop, J. M. (1985) Proc. Natl. Acad. Sci. USA 82, 7845-7849.

Rendu, F., Lebret, M., Danielian, S., Fagard, R., Levy-Toledano, S., and Fischer, S. (1989) Blood 73, 1545-1551.

Resh, M. D. (1988) Mol. Cell. Biol. 8, 1896-1905.

Resh, M. D. and Erikson, R. L. (1985) J. Cell Biol. 100, 409-417.

Roebroek, A. J. M., J. A. Schalken, C. Onnekink, H. P. J. Bloemers, and W. J. M. Van de Ven. (1987) J. Virol. 61, 2009-2016.

Rohrschneider, L. R. (1980) Proc. Natl. Acad. Sci. USA 77, 3514-3518.

Ross, C. A., Wright, G. E., Resh, M. D., Pearson, C. A. and Snyder, S. H. (1988) Proc. Natl. Acad. Sci. USA 85, 9831-9835.

Rowley, J. D. (1983) Nature 301, 290-291.

Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L. and Schlossman, S. F. (1988) Proc. Natl. Acad. Sci. USA 85, 5190-5194.

Sadowski, I., Stone, J. C. and Pawson, T. (1986) Mol. Cell. Biol. 6, 4396-4408.

Salter, D. W., Baldwin, S. A., Lienhard, G. E. and Weber, M. J. (1982) Proc. Natl. Acad. Sci. USA 79, 1540-1544.

Scherr, C. J., Rettenmeir, C. W., Sacca, R., Roussel, M. F., Look, A. T. and Stanley, E. R. (1985) Cell 41, 665-675.

Schultz, A. M., Henderson, L. E., Oroszlan, S., Garber, E. A. and Hanafusa, H. (1985) Science 227, 427-429.

Schwartz, D. E., R. Tizard, and W. Gilbert. (1983) Cell 32, 853-869.

Seeburg, P. H., W. W. Colby, D. J. Capon, D. V. Goeddel, and A. D. Levinson. (1984) Nature 312, 71-75.

Sefton, B. M., Hunter, T., Ball, E. H. and Singer, S. J. (1981) Cell 24, 165-174.

Sefton, B. M., Hunter, T., Beemon, K. and Eckhart, W. (1980) Cell 20, 807-816.

Semba, K., M. Nishizawa, N. Miyajima, M. C. Yoshida, J. Sukegawa, Y. Yamanashi, M. Sasaki, T. Yamamoto, and K. Toyoshima. (1986) Proc.

Natl. Acad. Sci. USA 83, 5459-5464.

Shalloway, D., Coussens, P. M. and Yaciuk, P. (1984a) Cancer Cells 2, 9-17.

Shalloway, D., Coussens, P. M. and Yaciuk, P. (1984b) Proc. Natl. Acad. Sci. USA 81, 7071-7075.

Shawver, L. K., Olson, S. A., White, M. K. and Weber, M. J. (1987) Mol. Cell. Biol. 7, 2112-2118.

Shtivelman, E., B. Lifshitz, R. P. Gale, B. A. Roe, and E. Canaani. (1986) Cell 47, 277-284.

Simon, M., Drees, B., Kornberg, T. and Bisho, J. M. (1985) Cell 42, 831-840.

Smart, J. E., Oppermann, H., Czernilofsky, A. P., Purchio, A. F., Erikson, R. L. and Bishop, J. M. (1981) Proc. Natl. Acad. Sci. USA 78, 6013-6017.

Snyder, M. A., Bishop, J. M., McGrath, J. P. and Levinson, A. D. (1985) Mol. Cell. Biol. 5, 1772-1779.

Sobue, K. and Kanda, K. (1988) Biochem. Biophys. Res. Comm. 157, 1383-1389.

Spector, D. H., Varmus, H. E. and Bishop, J. M. (1978) Proc. Natl. Acad. Sci. USA 75, 4102-4106.

Sprenger, F., Stevens, L. M. and Nusslein-Vollhard, C. (1989) Nature 338, 478-483.

Stahl, M. L., Ferenz, C. R., Kelleher, K. L., Kriz, R. W. and Knopf, J. L. (1988) Nature 332, 269-272.

Stehelin, D., Guntaka, R. V., Varmus, H. E. and Bishop, J. M. (1976a) J. Mol. Biol. 101, 349-365.

Stehelin, D., Varmus, H. E., Bishop, J. M. and Vogt, P. K. (1976b) Nature 260, 170-173.

Streuli, C. H., and B. E. Griffin. (1987) Nature 326, 619-622.

Sugano, S., Stoeckle, M. Y. and Hanafusa, H. (1987) Cell 49, 321-328.

Sugimoto, Y., Whitman, M., Cantley, L. C. and Erikson, R. (1984) Proc. Natl. Acad. Sci. USA 81, 2117-2121.

Suh, P.-G., Ryo, S. H., Moon, K. H., Suh, H. W. and Rhee, S. G. (1988) Cell 54, 161-169.

Sukegawa, J., K. Semba, Y. Yamanashi, M. Nishizawa, N. Miyajima, T. Yamamoto, and K. Toyoshima. (1987) Mol. Cell. Biol. 7, 41-47.

Swanstrom, R., Parker, R. C., Varmus, H. E. and Bishop, J. M. (1983) Proc. Natl. Acad. Sci. USA 80, 2519-2523.

Takeya, T. and Hanafusa, H. (1983) Cell 32, 881-890.

Tatchell, K., Chaleff, D. T., DeFeo-Jones, D. and Scolnick, E. M. (1984) Nature 309, 523-527.

Teitelman, G., Alpert, S. and Hanahan, D. (1988) Cell 52, 97-105.

Temin, H. M., and H. Rubin. (1958) Virology 6, 669-688.

Tindall, K. R., Stankowski, L. F., Machanoff, R. and Hsie, A. W. (1984) Mol. Cell. Biol. 4, 1411-1415.

Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. (1987a) Proc. Natl. Acad. Sci. USA 84, 2708-2712.

Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. (1988) J. Biol. Chem. 263, 1784-1790.

Towler, D. A., S. R. Eubanks, D. S. Towery, S. P. Adams, and L. Glaser. (1987b) J. Biol. Chem. 262, 1030-1036.

Towler, D., and L. Glaser. (1986) Proc. Natl. Acad. Sci. USA 83, 2812-2816.

Toyoshima, K. and Vogt, P. K. (1969) Virol. 39, 930-931.

Tsukamoto, A. S., Grosscchedl, R., Guzman, R. C., Parslow, T. and Varmus, H. E. (1988) Cell 55, 619-625.

Tyagi, J. S., Hirano, H. and Pastan, I. (1985) Nucl. Acids Res. 13, 8275-8284.

Varmus, H. E. (1984) Ann. Rev. Genet. 18, 553-612.

Varmus, H. E., Quintrell, N. and Ortiz, S. (1981a) Cell 25, 23-36.

Varmus, H. E., Quintrell, N. and Wyke, J. (1981b) Virology 108, 28-46.

Veillette, A., Bookman, M. A., Horak, E. M. and Bolen, J. B. (1988) Cell 44, 301-308.

Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E. and Bolen, J. B. (1989) Nature 338, 257-259.

Verderame, M. F., Kaplan, J. M. and Varmus, H. E. (1989) J. Virol. 63, 338-348.

Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M. and Gibbs, J. B. (1988) Nature 335, 90-93.

Vogt, P., In Fundamental Techniques in Virology (K. Habel and N.P. Salzman, Eds.), pp. 198-211, Academic Press, New York/London, 1969.

Walter, G., Hutchinson, M. A., Hunter, T. and Eckhart, W. (1982) Proc. Natl. Acad. Sci. USA 79, 4025-4029.

Wang, J. Y. J. (1985) Mol. Cell. Biol. 5, 3640-3643.

Wang, L. H., Duesberg, P., Beemon, K. and Vogt, P. K. (1975) J. Virol. 16, 1051-1070.

Wasilenko, W. J., Shawver, L. K. and Weber, M. J. (1987) J. Cell. Physiol. 131, 450-457.

Waterfield, M. D., Scrace, G. J., Whittle, N., Stroobant, P., Johnson, A., Wasteson, A., Werstermark, B., Heldin, C. H., Huang, J. S. and Deuel, T. F. (1983) Nature 304, 35-38.

Weber, K., Rathke, P. C., Osborn, M. and Franke, W. W. (1976) Exp. Cell. Res. 102, 285-297.

Weber, M. J. (1984) Adv. in Viral Onc. 4, 249-268.

Weiss, R., Teich, N., Varmus, H. E. and Coffin, J. (1982) Cold Spring Harbor, NY: Cold Spring Harbor.

Whitman, M., Kaplan, D. R., Schaffhausen, B. S., Cantley, L. and Roberts, T. M. (1985) Nature 315, 239-242.

Whitman, M., Kaplan, D., Roberts, T. and Cantley, L. (1987) Biochem. J. 247, 165-174.

Whittemore, A. S. (1978) Adv. Cancer Res. 27, 55-88.

Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. and Harlow, E. (1988) Nature 334, 124-129.

Wilcox, C., J. Hu, and E.N. Olson. (1987) Science 238, 1275-1278.

Willingham, M. C., Jay, C. and Pastan, I. (1979) Cell 18, 125-134.

Yamanashi, Y., S. Fukushige, K. Semba, J. Sukegawa, N. Miyajima, K. Matsubara, T. Yamamoto, and K. Toyoshima. (1987) Mol. Cell. Biol. 7, 237-243.

Yonemoto, W., Jarvis-Morar, M., Brugge, J. S., Bolen, J. B. and

Israel, M. A. (1985) Proc. Natl. Acad. Sci. USA 82, 4568-4572.

Young, J. C., and G. S. Martin. (1984) J. Virol. 52, 913-918.

Yunis, J. J. (1983) Science 221, 227-235.

APPENDIX ONE:

MATERIALS AND METHODS

Materials. Monoclonal anti-pp60^{src} antibodies 327 (mAb327) and 127 (mAb127) were provided by Dr. J. Brugge (Lipsich et al. 1983). Rabbit anti-chicken M1 pyruvate kinase antibody was provided by Dr. B. Roberts. Anti-pp60^{src} tumor bearing rabbit sera were prepared as described (Brugge and Erikson 1977). A circularly permuted clone of the Prague A strain of RSV, pSL102, was provided by Dr. T. Parsons (Bryant and Parsons 1982). Simian virus 40 (SV40) expression vectors containing the chicken M1 PK cDNA, RL142PK10X, RL18PK8X, and RL18PK12X (which differ by the reading frame at the polylinker sequence), were provided by B. Roberts (Kalderon et al. 1984). A plasmid, p5H, containing the chicken fibroblast c-*src* cDNA was provided by H. Hanafusa (Levy et al. 1986). A replication defective MLV-gpt vector, ZIPVGPT, was provided by R. Mulligan. A mouse fibroblast cell line, clone-1, that produces wild-type MLV virus was provided by B. Bowerman. An HPRT$^-$ derivative of Balb/c 3T3 cells, 2TGOR cells, was provided by H. Ozer (Jha et al. 1980). The psi-2 ecotropic packaging cell line was provided by R. Mulligan (Mann et al. 1983), and the PA12 amphotropic packaging cell line was provided by D. Miller (Miller et al. 1985). Reagents for detecting proteins immobilized on nitrocellulose (western blots) were purchased from Vector labs, and used according to the manufacturer's specifications.

Reagents were obtained as follows: protease inhibitors, paraformaldehyde, Bovine Serum Albumin (fraction V), hemoglobin, all chemicals, and detergents, the Sigma Chemical Co.; fluorescein conjugated goat anti-mouse IgG, goat anti-rabbit IgG, and normal goat serum, the Jackson Immunochemicals Co.; protein A conjugated to

sepharose CL-4B, Pharmacia Fine Chemicals; rhodamine conjugated phalloidin, Molecular Probes Inc.; affi-gel activated agarose affinity matrix and reagents for silver staining SDS-PAGE gels, BioRad; L[^{35}S]methionine, ICN.; [^{3}H]myristic acid, 22.4 Ci/mmol, (50 mCi/ml in DMSO), New England Nuclear; restriction enzymes, E. coli DNA polymerase I, and T4 DNA ligase, New England Biolabs Inc.; human serum fibronectin, Collaborative Research Inc.; gold seal glass coverslips (18X18 mm, thickness 1.5), VWR; staphylococcal V8 endoproteinase, Boehringer Mannheim Biochemicals; human platelet concentrates, Irwin Memorial Blood Bank; semi-dry electroblotter (used according to the manufacturer's specifications), E&K; polyclonal rabbit anti-mouse IgG, Dako; monoclonal anti-pp60^{src} antibody that recognizes amino acids 2-17 of pp60^{src} (anti-2-17), Microbiological Associates (NCI repository hybridoma number 203-7D10).

Cell culture and viral infections. Cultures of primary chicken embryo fibroblasts (CEF) were prepared and propagated as described (Vogt 1969). Cultures of SF9 insect cells, the host for baculoviruses, were prepared and propagated as described (Summers and Smith 1987; Luckow and Summers 1989). Recombinant baculoviruses were constructed and produced as described (Summers and Smith 1987). The recombinant baculovirus encoding pp60^{src} (pVLc-*src*) was previously described (Morgan et al. 1989). Recombinant helper-free MLV viral stocks were produced by transfection of packaging cell lines, and used to infect mouse or rat fibroblasts as described (Cepko et al 1984). Since psi-2 cells contain the gpt gene, I found it most convenient to produce ZAS4 virus in PA12 cells. MLV viral stocks were stored as

aliquots at -80°C.

Cloning transformed cells in soft agar. The cloning efficiency of *src* transformed cells varies depending on the type of agar used, and on the number of cells seeded in each dish. I have always used Difco bacto-agar which seems to be the most permissive for growth. A stock solution of 1.4% agar (in ddH_2O) is autoclaved, microwaved just before use, and adjusted to 47°C in a water bath. A 2x DME is prepared from concentrated components. I find that the 2x DME cannot be stored for long periods at 4°C (i.e. >7 days), so I always prepared this the day of the experiment. Bottom agar (5 mls/60mm dish and 10 mls/100mm dish) is prepared by mixing equal parts of the 1.4% agar (47°C) and 2x DME (37°C), and is kept at 47°C. The appropriate number of cells is resuspended in 1 volume of DME (room temperature) and is quickly mixed with 1 volume of bottom agar (47°C) and then poured on top of the hardened bottom agar. I usually used 5ml of top agar/60mm dish and 10 ml/100mm dish. Cloning efficiency of *src* transformed cells should be 20-50%, with higher efficiencies obtained at higher cell densities.

Metabolic labelling, immunoprecipitation, and subcellular fractionation. COS7 cells, Rat-1 cells, and CEF were labelled with L[^{35}S]methionine for 18 hours in Dulbecco's Modified Eagle's medium (DME) containing 10% the normal concentration of L-methionine, 10% dialyzed fetal calf serum, and 200 μCi/ml L[^{35}S]methionine. COS7 cells, Rat-1 cells, and CEF were labelled with [^{3}H]myristic acid for 18 hours in DME with 10% fetal calf serum, 1% DMSO, and 500 μCi/ml [^{3}H]myristic acid. Labelled cell extracts were prepared with lysis

buffer (20 mM Tris-Cl pH8.0, 150 mM NaCl, 1% NP-40, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mg/ml fraction V BSA). Immunoprecipitations with TBR, polyclonal rabbit sera, and monoclonal antibodies were performed as described (Krueger et al. 1980) with extracts containing equal amounts of TCA precipitable radioactivity. Immune complex kinase reactions were performed as described (Krueger et al. 1980). In cases where these reactions were quantitated, the immune complexes were washed with lysis buffer supplemented with 0.2% SDS and 300 mM NaCl and then counted in a Beckman LS7800 scintillation counter. Crude cell extracts prepared by Dounce homogenization of hypotonically swelled cells were fractionated into cytosol and membrane fractions by differential centrifugation, as previously described (Krueger et al. 1980). This method differs from that described by Courtneidge et al. (1980) in the addition of 1 mM EDTA and 0.1% BME to the swelling buffer. These conditions significantly reduce the loss of plasma membranes in the nuclear pellet. In some cases, the crude homogenate was first adjusted to either 300 mM NaCl or 100 mM NaOH, and incubated at 0°C for 15 minutes, before differential centrifugation.

Proteolytic digestion by V8 endoproteinase. When pp60^{src} is partially digested with V8 protease 4 fragments are generated (V1-V4). The V1 fragment corresponds to the N-terminal 34 kd of pp60^{src}; the V2 fragment corresponds to the C-terminal 30 kd; while the V3 and V4 fragments correspond to the N-terminal 18 and 16 kd respectively. *Src* proteins are first immunoprecipitated and run on preparative 10% SDS-PAGE gels, which is washed in dH_2O (to remove SDS and BME, both of

which inhibit V8 protease) and dried on Whatman filter paper as usual. The gels must be marked with either radioactive ink or fluorescent dye in order to align the film with the gel. The pp60^{src} band is excised from the preparative gel. Try to cut slices which are the same width as the lanes of the V8 gel. Then with a scalpel carefully scrape the whatman paper off of the gel slice. Pour a 12 or 14% SDS-PAGE gel with a 2 cm stacking gel (the V8 gel). Place the dry gel slice in a dry sample well of the V8 gel, taking care not to trap any air between the slice and the stacking gel. I find it convenient to use the needle of a Hamilton syringe to guide the slice into the well. Swell the gel slice, now in the sample well, with running buffer. Force out any trapped air bubbles with the Hamilton syringe. Remove the running buffer with the hamilton syringe, and let the slice swell for an additional 5 minutes. Finally remove the residual running buffer. To each lane add 40 μl of V8 digestion buffer (for 1 ml --100 μl Laemmli sample buffer without BME, 200 μl 50% glycerol, 700 μl H_2O, and 100-500 ng V8 protease). Overlay the samples as usual with running buffer. Run the samples through the stacking gel at 15 mA constant current, then run through the running gel as usual (150 V constant voltage). The digestion occurs while the samples are stacking.

Isolating HPRT$^-$ rat-1 cells, RT. An HPRT$^-$ derivative of rat-1 cells, RT cells, was isolated by selecting spontaneous 6-TG resistant subclones of rat-1 cells. Briefly, $5x10^5$ rat-1 cells were seeded into DME supplemented with 6 μg/ml 6-TG in 150 mm dishes, and resistant clones were picked after 14 days. The spontaneous frequency of 6-TG resistance was usually 10^{-5} to 10^{-6}. The success of 6-TG selections

strictly depends upon the density of the cells when the selection is imposed. As cells become more crowded, $HPRT^+$ cells will convert the 6-TG to the toxic nucleoside and poison their $HPRT^-$ neighbors. Thus, the recovery of 6-TG resistant cells increases as the cell density decreases. In reconstruction experiments, I determined that at $5x10^5$ cells/150 mm dish 50% of the 6-TG resistant cells are actually recovered, whereas at 10^6/dish only 10% are recovered.

The 6-TG resistant clones vary in the stability of their phenotype. Some clones revert to $HPRT^+$ at unacceptably high frequencies; therefore clones were tested for the stability of the $HPRT^-$ phenotype. I have worked mainly with 2 $HPRT^-$ derivatives of rat-1, RT cells and B1a cells. Both are stably $HPRT^-$, are morphologically normal, and grow poorly in soft agar ($<10^{-7}$ cloning efficiency).

Chemical mutagenesis of CS5 cells. CS5 cells were mutagenized with either nitrosoguanidine (MNNG) (1.5 μg/ml) or ethyl methane sulfonate (EMS) (12.5 mM). The exposure to mutagen must be done in serum free conditions, because serum proteins inactivate the mutagen. In fact, when handling several independent cultures, it is convenient to stop the mutagenesis by adding serum to 10% final concentration. The survival of cells was monitored by mutagenizing dishes containing 1000 cells and counting the resulting colonies after 14 days. The dose of mutagen was selected to give about 20-50% survival.

The time period following the mutagenesis that precedes selection for the desired phenotype is called the expression time, and it is an important variable in performing these experiments.

Recessive mutations are phenotypically expressed only after the wild-type gene product has been lost due to turn-over or dilution through cell division. Thus, recovery of recessive mutations is favored by longer expression times. For instance, recovery of HPRT$^-$ mutants is optimal after expression times of 5-7 days. Shorter expression times strongly bias the experiment toward dominant mutations. In some cases, the efficacy of the mutagenesis was determined by comparing the induced frequency of 6-TG resistance, that is loss of the gpt gene, to the spontaneous frequency. There should be a 100x increase following mutagenesis.

In the experiments described in chapter 2, subconfluent CS5 cells were exposed to either MNNG for 4 hours, or EMS for 2 hours at 37^oC, washed extensively. After 5 days, $5x10^5$ mutagenized cells were seeded into 100 mm dishes of 0.35% soft agar. In order to avoid picking sibling revertants, each soft agar dish corresponded to an independently mutagenized culture, and only 1 colony was picked from each dish. There were typically 1-10 soft agar colonies in each dish, and these were picked after 3 weeks. Spontaneous soft agar colonies were never observed.

Construction of recombinant MLVsrc-gpt viruses. The MLVgpt vector ZIPVGPT was modified by eliminating the *Sph I* site of pBR322 and the *Nco I* site at nucleotide 7227 of MLV. A 2.1 kb fragment containing the *src* coding region and the enhancer of the SRA strain of RSV (nucleotides 7093-9238) was cloned into ZIPVGPT at the *Bgl II* site. This recombinant plasmid contains two *Sph I* sites, both in the sequences derived from RSV (at nucleotides 8567 and 9151). In order

to facilitate switching *src* alleles, the *Sph I* site corresponding to nucleotide 9151 of RSV was destroyed. The resulting plasmid, ZAS4, contains unique *Nco I* and *Sph I* sites which are used to switch *src* alleles. A similar plasmid, ZAS2, in which the sequences derived from RSV end at the *Nru I* site at nucleotide 8793, does not express the gpt gene well, perhaps because this plasmid lacks the RSV enhancer.

In an attempt to improve expression of the *src* gene, several modified forms of ZAS4 were constructed. In ZAS5, the right hand LTR of MLV was replaced with an MSV LTR. In ZAS6, the gpt gene (which occupies the 3' cloning site) is expressed from the HSV TK promoter, while in ZAS7 the gpt gene is expressed from the SV40 early promoter. In ZAS8, the order of the gpt and *src* genes is reversed, and the *src* gene is expressed from the SV40 early promoter. The ZAS6 virus has been used succesfully, and was also adapted for the expression of *fos* (unpublished data of J. Tuttleman). These plasmids and the corresponding maps are available from N. Quintrell.

Oligonucleotide directed mutagenesis. Codon 7 of v-*src* from the B77 strain of RSV was mutated by the method of Seeburg et al. (Seeburg et al. 1984). Nucleotide coordinates of RSV are as described by Schwartz et al. (Schwartz et al. 1983). Synthetic oligonucleotides partially complementary to nucleotides 7135-7155 of RSV were used to convert lysine-7 to either asparagine (AGCAAGAGC**AA**TCCTAAGGAC) or arginine (AGCAAGAGC**AGG**CCTAAGGAC), the latter creating a novel *Stu* I restriction site at nucleotide 7138.

Isolation of the E3 src gene. The E3 *src* gene was isolated by selecting transforming back-mutants of a transformation-defective v-

src allele, the E *src* gene (derived from the tdSF/LO302 strain of RSV), which we have previously described (Oppermann et al. 1981b; Varmus et al. 1981b). The DNA sequences of the E and E3 *src* genes reveal a single nucleotide deletion in codon 28 of E *src*, and a compensating 89 nucleotide deletion in E3 *src* that restores the reading frame (unpublished data of G. Mardon). The sequences deleted in E3 *src* normally encode amino acids 8-37 of $pp60^{v\text{-}src}$.

Construction, transfection, and assays of replicating recombinant RSV viruses. A circularly permuted clone of the Prague A strain of RSV, pSL102, was used to construct two plasmids, pPR-REP and pPRΔ*Bam*. pPR-REP is a pBR322 clone containing the 7 kb *Pvu* II (8671) - *Sal* I (6031) fragment of pSL102. pPRΔ*Bam* is a pBR322 clone containing the 4 kb *Sal* I (6031) - *Bam* HI (530) fragment of pSL102. pPRΔ*Bam*N7 and pPRΔ*Bam*R7 are identical to pPRΔ*Bam* except for the mutation introduced in codon 7 of v-*src*. The pPRΔ*Bam* subclones and pPR-REP were linearized with *Sal* I and ligated together (200 μg/ml, 20 minutes at 25^{o}C). The resulting ligation products were ethanol precipitated and then used to transfect CEF as described (Graham and Van der Eb 1973). Viral stocks were harvested 10 days after transfection. In order to confirm that the transfections had worked equivalently, the extent to which the cultures were infected was determined by $pp60^{v\text{-}src}$ immune complex kinase assay. Viral stocks derived from transfections with pPRΔ*Bam*, pPRΔ*Bam*N7, and pPRΔ*Bam*R7 are referred to as wild type, N7, and R7 RSV viruses respectively. Viral stocks were titered either by focus assays, as previously described (Temin and Rubin 1958), or by relative kinase titers. Relative kinase

titers were determined by infecting equal numbers of CEF with equal volumes of virus-free medium or medium conditioned by cells infected with wild type RSV, N7 RSV, or R7 RSV; 48 hours post-infection, before viral spread had occurred, infected cultures were lysed in lysis buffer and subjected to immune complex kinase assays, and quantitated as described above.

Metabolic labelling, immunoprecipitation, and subcellular fractionation. COS7 cells were labelled with L[^{35}S]methionine for 18 hours in Dulbecco's Modified Eagle's medium (DME) containing 10% the normal concentration of L-methionine, 10% dialyzed fetal calf serum, and 200 μCi/ml L[^{35}S]methionine. COS7 cells were labelled with [^{3}H]myristic acid for 18 hours in DME with 10% fetal calf serum, 1% DMSO, and 500 μCi/ml [^{3}H]myristic acid. Labelled cell extracts were prepared with lysis buffer (20 mM Tris-Cl pH8.0, 150 mM NaCl, 1% NP-40, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mg/ml fraction V BSA). Immunoprecipitations with polyclonal rabbit sera, and monoclonal antibodies were performed as described (Krueger et al. 1980). Crude cell extracts prepared by Dounce homogenization of hypotonically swelled cells were fractionated into cytosol and membrane fractions by differential centrifugation, as previously described (Krueger et al. 1980).

Construction of recombinant src-pyruvate kinase genes. The N-terminal amino acid sequence of the hybrid *src*-PK proteins is derived from pp60^{src} (B77 strain of RSV), while the C-terminal amino acids are derived from PK. The *src*-PK proteins are designated by the C-terminal boundary of the domain of pp60^{src} that is fused to PK, and in cases

where this domain contains internal deletions, the amino acids that are lost are indicated in parentheses. Residue numbers reported correspond to the sequence of the Prague-C strain of RSV (Schwartz et al. 1983). The amino acids derived from the chicken M1 PK (residues 120-xx) are the same in all cases. The nucleotide sequence encoding PK were derived from either RL142PK10X, RL18PK8X, or RL18PK12X whichever was required to maintain an open reading frame. The *src*7PK plasmid (previously known as R7-PK) was described previously (Kaplan et al. 1988). Sequences encoding various N-terminal amino acids of pp60^{src} were fused to the coding region of PK by cutting both genes with restriction endonucleases, repairing the ends with E. coli DNA polymerase I, and fusing the resulting blunt ends. Internal deletions of *src* sequences were constructed by cutting the *src* gene with restriction endonucleases, and fusing the repaired ends. The restriction sites utilized in these constructions are indicated in figure 7. The *Stu I* site at codon 7 is a restriction polymorphism which was created by converting lysine-7 to an arginine (Kaplan et al. 1988). An SV40 vector expressing the chicken fibroblast c-*src* cDNA was constructed by replacing the PK gene of RL142PK10X with the 2 kb *Nco I-Bgl II* fragment of p5H.

Transfection of recombinant src-pyruvate kinase genes. COS7 cells were exposed to calcium phosphate precipitates containing 20 μg of recombinant *src*-PK DNA for 18 hours, then subjected to shock with Tris-buffered saline (0.8% NaCl, 0.1% glucose, 0.038% KCl, 0.2% Tris-Cl, 0.06% Tris Base, and 4.5 mg/l Phenol Red) containing 25% DMSO for 2 minutes. Transfected COS7 cells were labelled for 18 hours with

either L[^{35}S]methionine or [^{3}H]myristic acid starting 36 hours after the transfection had begun.

Immunofluorescence. Glass coverslips were coated with human serum fibronectin (100μg/ml in PBS) for 1 hour at 37^{o}C in a humidified chamber. Transfected COS7 cells were seeded onto glass coverslips 24 hours after the transfection had begun. After 8-12 hours, coverslips were washed with Dulbecco's Phosphate Buffered Saline (PBS), cells were fixed with 3.5% paraformaldehyde in PBS for 20 minutes, cells were permeablized with 0.1% TritonX-100 in PBS, and coverslips were coated with 10% normal goat serum (NGS) in PBS to block non-specific binding sites. Primary antibodies used were monoclonal anti-pp60^{src} 327 (10 μg/ml in PBS) and polyclonal rabbit anti-PK (1/200 dilution in PBS). Fluorescein conjugated antibodies were used at a dilution of 1/200 in PBS. Rhodamine conjugated phalloidin was used according to the manufacturer's suggestions. Stained coverslips were washed several times with PBS, then with absolute ethanol, and finally embedded in glycerol containing 2% propyl gallate, an anti-bleaching reagent. Microscopy was done either with a Zeiss Photomicroscope III, or an inverted Olympus fluorescence microscope.

Production of ascites fluids. Either Balb/c (for mAb 327) or nude (for anti-2-17) mice were primed for production of ascites fluid by injection of 0.2 ml of pristane into the peritoneum (ip.) at least 7 days before injection of hybridoma cells. Each primed animal was injected ip. with 10^{7} washed hybridoma cells. Ascites fluid was collected through 16 guage needles as necessary. In the case of mAb 327, it often took 2-3 months for ascites to be produced. In the

future, I would suggest producing mAb 327 ascites in nude mice, in which the anti-2-17 ascites appeared within 7 days. If care is taken, you can recover 5-10 mls of ascites from each mouse.

Purification of IgGs from ascites fluids. Ascitic fluid was diluted in 10 volumes of binding buffer (1.5 M glycine pH 8.9, 3.0 M NaCl). The diluted ascitic fluid was adsorbed (at 10 ml/hour) to a protein A sepharose CL-4B column that had been equilibrated with binding buffer. The column was then washed with 20 column volumes of binding buffer. The bound antibody was eluted with 0.1 M citrate pH 5, and eluates were collected in 1 ml fractions. The peak of eluted protein was pooled and neutralized by dialysis with 0.1 M borate pH 8.2. Ascitic fluids produced by mAb 327 hybridoma cells generally contain 2-3 mg/ml, while those produced by anti-2-17 hybridoma cells contain 5-6 mg/ml. Complete recovery of IgGs from either ascites requires multiple rounds (at least 3) of purification by protein A sepharose chromatography. Recovery should be monitored by SDS-PAGE gels. After elution of IgGs, the protein A sepharose column was regenerated with 20 column volumes of 0.1 M citrate pH 3, and then equilibrated with binding buffer before use.

Preparation of an anti-pp60^{src} immunoaffinity matrix. For the purposes of purifying pp60^{src}, an anti-pp60^{src} immunoaffinity matrix was constructed largely as described (Schneider et al. 1982). Because monoclonal anti-p60 327 antibody binds very poorly to protein A, 10 mg of a polyclonal rabbit anti-mouse IgG antibody was bound to each ml. of protein A sepharose CL-4B in 0.1 M Borate pH 8.2. After 1 hour of binding at 25^{o}C, this resin was washed repeatedly with borate buffer.

10 mg of mAB 327, purified from ascites fluid, was bound to each ml. of this resin for 1 hour at 25°C also in borate buffer. After both antibodies are bound, this resin is washed in 0.1 M triethanolamine pH 8.2. Finally, the antibodies are chemically cross-linked to the resin by resuspending the resin in 20 volumes of 0.1 M triethanolamine pH 8.2, 20 mM dimethyl pimelimidate and agitating for 30 minutes at 25°C. The coupling reaction is stopped by adding ethanolamine pH 8.2 to 50 mM. The coupled resin is washed with the most stringent elution buffer to be used, in this case 10 mM diethylamine pH 11, to remove any non-covalently bound material. Affinity matrices produced in this manner have far higher binding capacities than matrices prepared by randomly coupling IgG to resins such as affi-gel 10, presumably because all of the antibody molecules have a conformation that allows recognition of antigen.

Purification of pp60^{src} from infected insect cells. Detergent lysates of insect cells were prepared 2 days after infection with the VLc-s virus (at a multiplicity of infection of 5-10). Infected cells were washed twice with hepes buffered salin (50 mM hepes pH 7.4, 150 mM NaCl), then lysed in lysis buffer (50 mM hepes pH 7.4, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, and 5 μg/ml Leupeptin). All subsequent steps were performed at 4°C. The crude lysate was clarified by centrifugation (20,000Xg, 30 minutes), then adjusted to 10% glycerol, and then adsorbed overnight to the mAb 327 affinity column. The column was subsequently washed successively with 5 column volumes of a salt wash (50 mM hepes pH 7.4, 500 mM NaCl, 1% sodium cholate, 10% glycerol) and then a KSCN wash (1 M KSCN pH 8, 1% sodium cholate, 10%

glycerol). The bound pp60^{src} was eluted with elution buffer (10 diethylamine pH 11, 150 mM NaCl, 1% sodium cholate, 10% glycerol). The peak of eluted protein was pooled and then neutralized by adjusting to 50 mM tricine pH 8.5. At neutral pH or above 1 mg/ml pp60^{src} is insoluble, creating a pretty white powder which cannot be resolublized in boiling 2% SDS. On a good day, 10 mg of p60 can be recovered from 1 liter of infected cells.

Purification of pp60^{src} from human platelets. Pooled human platelet concentrates were adjusted to 10 mM citrate pH 6.4 and 1 mM EDTA. Red blood cells were removed from the platelet concentrates by centrifugation (200xg, 20 minutes). In general, this low speed spin was repeated 2 times. The platelets were next washed 3-4 times with platelet wash buffer (20 mM citrate pH 6.4, 150 mM KCl, 10 mM glucose, 1 mM EDTA, 1 μM cytochalasin D) by centrifugation (JS4.2 rotor 4200 RPM, 20 minutes, no brake). Washed platelets (typically 1ml/unit) were lysed in lysis buffer (50 mM hepes pH 7.4, 1% triton X-100, 50 mM NaF, 100 μM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 1 μM cytochalasin D, 1 mM EDTA, 100 μg/ml leupeptin, and 10 μg/ml aprotinin) by Dounce homogenization. The crude extracts was clarified by centrifugation (100,000Xg, 3 hours). Clarified extracts were adjusted to 10% glycerol, and then adsorbed to the mAb 327 affinity column overnight. The column was washed and eluted as before, with the exception that the salt and KSCN washes and the elution buffer were supplemented with 50 mM NaF, 1 mM DTT, 100 μM sodium orthovanadate, and protease inhibitors.

Preparation of pp60^{src}-affinity resins. Two methods were used

for preparing these pp60^{src}-resins. Affi-gel 10 was used according to the manufacturers suggestions to randomly couple pp60^{src} to an agarose matrix. Briefly, purified pp60^{src} in 50 mM tricine pH 8.5, 150 mM NaCl, 1% sodium cholate, and 1 mM DTT was incubated with the washed affi-gel 10 resin overnight at 4^{o}C with agitation. The coupling reaction was stopped by adding ethanolamine pH 8.2. The extent of coupling could be increased to 80% by coupling in the presence of 80 mM $CaCl_2$, presumably by counteracting the electrostatic repulsion of p60 by the matrix; however, I found that matrices produced in the absence of $CaCl_2$, in which 25% of the p60 added to the matrix was bound, had a higher binding capacity for cellular proteins, as described in chapter 5. Control resins used were 1 mg/ml BSA coupled to affi-gel 10.

The pp60^{src}-affinity resins were also prepared by binding pp60^{src} to the matrix via the monoclonal anti-2-17 antibody. This method has the advantage that all of the pp60^{src} molecules have the same orientation on the matrix, and resins prepared in this way appear to have a higher binding capacity for cellular proteins than those prepared with affi-gel 10, as described in chapter 5. Briefly, 3 mg of anti-2-17, purified from ascites fluid, was bound to each ml of protein A sepharose CL-4B in 0.1 M Borate pH 8.2 for 1 hour at 25^{o}C. The resin was then washed extensively in 50 mM Tricine pH 8.5, 150 mM NaCl, 1% sodium cholate, and 1 mM DTT. Purified pp60^{src} was incubated with the washed resin for 2-4 hours at 4^{o}C with agitation, and then washed with the same buffer. The pp60^{src} was chemically coupled to the resin by resuspending the washed resin in 20 volumes of 50 mM

Tricine pH 8.5, 150 mM NaCl, 1% sodium cholate, 1 mM DTT, and 20 mM dimethyl pimelimidate. After 1 hour of agitation at 4°C, the coupling reaction was stopped by adding ethanolamine pH 8.2 to 50 mM. The coupled resin was next washed with the most stringent elution buffer to be used. Control resins used were 3mg/ml anti-2-17 coupled to protein A sepharose CL-4B.

The $pp60^{src}$-resins typically have 300-500 μg/ml of $pp60^{src}$, and 3 ml columns were normally used.

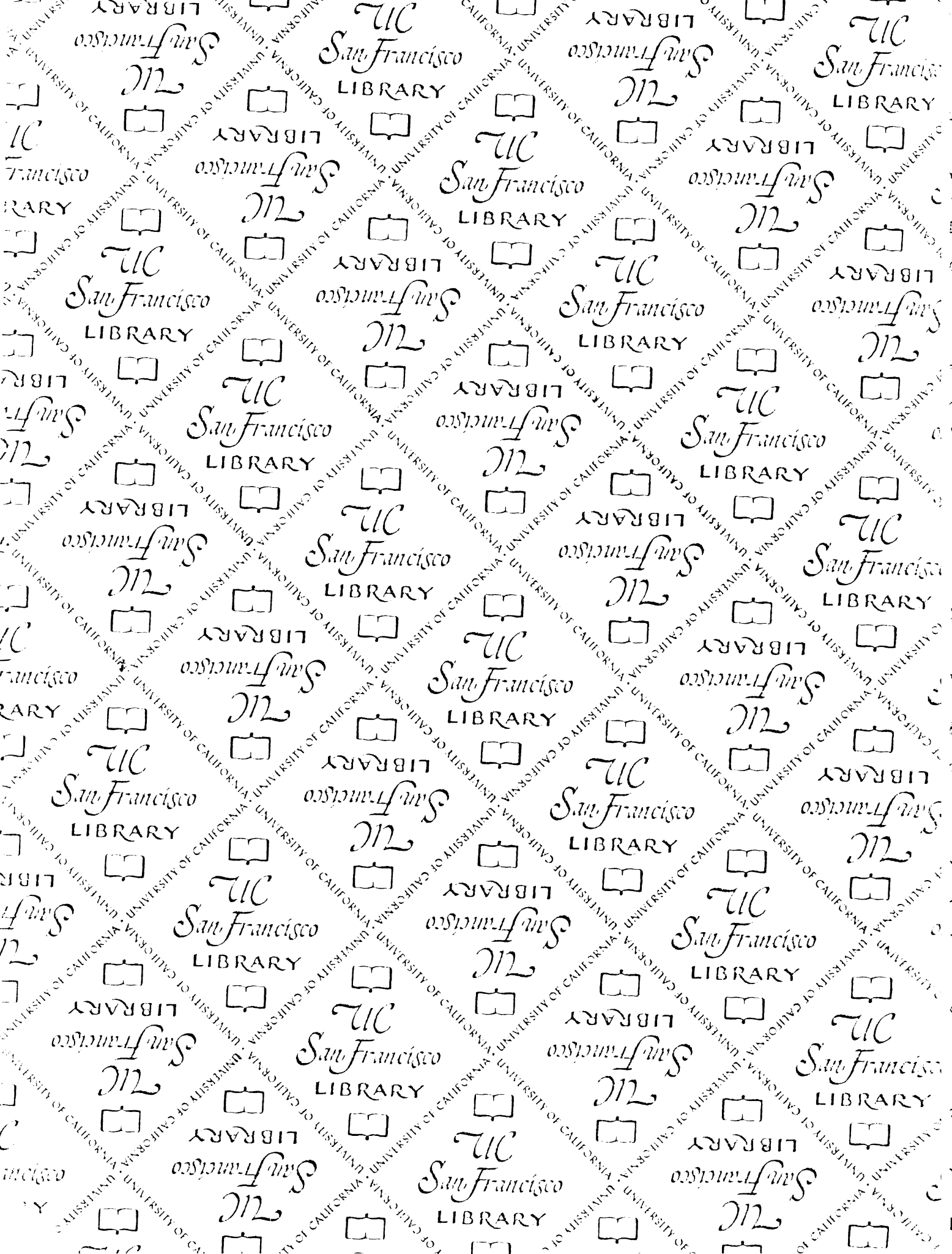
Affinity chromatography using $pp60^{src}$-resins. Detergent extracts were prepared from L[^{35}S]methionine labelled mouse fibroblasts (NIH 3T3 cells), polyoma transformed 3T3 cells (V4 cells), or unlabeled washed human platelets (1 ml packed platelets/10 ml of lysis buffer) by lysing in lysis buffer. Low calcium lysis buffer is composed of 50 mM hepes pH 7.4, 1% triton X-100, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EGTA, 10% glycerol, 100 μM sodium orthovanadate, 100 μM ATPλS, 100 μg/ml leupeptin, 1 mM PMSF, and 10 μg/ml aprotinin. High calcium lysis buffer has the same components with the exception of EGTA, and it is supplemented with 2 mM $CaCl_2$. Crude lysates were clarified by centrifugation (100,000Xg, 1 hour). Platelet extracts prepared this way typically had protein concentrations of 10 mg/ml. Affinity columns (p60-resin, and a control anti-2-17 resin) were equilibrated with 10 void volumes of lysis buffer, and then non-specific binding sites were saturated with lysis buffer supplemented with 1% BSA.

The tissue culture cell extracts (500 μl/reaction) were bound to 100μl of either $pp60^{src}$-resin or control resin for 2-4 hours at 4°C

with agitation. The resins were then washed successively (3 times each) with 500 μl of wash buffer (same components as lysis buffer except 0.05% triton X-100), and wash buffer containing the indicated salt concentrations. The percentage of protein recovered in each fraction was determined by scintillation counting. Each wash was pooled and then concentrated by TCA precipitation. Equivalent amounts of material were then analyzed by SDS-PAGE gels.

Two mls of platelet extract was applied to both pp60^{src} and control resins (3 ml columns), at 3 ml/hour. Columns were then washed with wash buffer (at 10 ml/hour) as above except that 1 ml fractions were collected. Fractions containing the peaks of eluted proteins were concentrated by TCA precipitation and analyzed by SDS-PAGE gels.

When indicated these gels were analyzed for the presence of glycoproteins by probing Western Blots with biotin-conjugated lectins, and avidin-conjugated alkaline phosphatase (reagents purchased from Vector Labs, and used according to manufacturer's suggestions). It was found that the background signal on these lectin blots was reduced if they were pre-incubated with blocking buffer (0.1 M sodium acetate pH 5, 1 M NaCl, 1mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1% hemoglobin). Blocking buffers containing BSA produce high backgrounds in lectin blots. The divalent cations in blocking buffer are required for Con A to bind to glycoproteins.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