Title
A genetic analysis of FMS regulation

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Publication Date
1990

Peer reviewed|Thesis/dissertation

A GENETIC ANALYSIS OF FMS REGULATION

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

GENETICS

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Many people have contributed to making this work possible. Their contributions have come in many forms: providing insight when I was blind; extricating me when I was stuck, and lifting me up when I was down. Without them I could not have survived, much less grown. Mike Bishop has provided guidance that I hope is not only reflected here, but which will persist and--I hope--be better rewarded in years to come. Harold Varmus has also provided guidance and, perhaps even more important, belief that I could make it. Among my past and present colleagues, Mike Simon and Josh Kaplan stand out as requiring special mention. A small group of individuals, particularly including David Levin, Martin McMahon and Kathy Weston, has also helped tremendously. Nancy Quintrell has been there from the beginning, listening tirelessly, supporting endlessly and helping thanklessly. Thank you. Finally my parents who, trite though it may seem, made contributions too numerous to mention. To all these people and the many unnamed, thank you all.

A Genetic Analysis of FMS Regulation

Alan Rogers

ABSTRACT

The regulation of the *fms* oncogene has been examined by genetic analysis. To identify potential activating lesions, a c-*fms* cDNA was isolated from human placenta and sequenced. Numerous amino acid substitutions distinguish the human protooncogene from SM-FeSV v-*fms*, attributable in large part to its derivation from the feline protooncogene, but the single striking lesion is a C-terminal truncation in v-*fms*.

Several site-directed mutants of c-*fms* were constructed to determine the influence of various domains on oncogenic activation. Neither N- nor C-terminal deletions are sufficient to activate the transforming potential of c-*fms*. An attempt was made to isolate additional transforming alleles of *fms* by random mutagenesis of the gene and screening for transforming capacity. No efficient transforming alleles were isolated, indicating that the set of lesions sufficient to produce oncogenic activation is either quite limited or requires multiple mutations of the protooncogene.

The role of autophosphorylation in transformation by *fms* was also investigated. Two other major sites of FMS autophosphorylation were identified by phosphate labelling of the protein, peptide mapping, and sequential Edman degradation. Mutations were introduced at two of the three major sites of autophosphorylation (Tyr-699 and

Tyr-809) in both c-*fms* and an activated allele of c-*fms*. Neither mutation substantially affects FMS kinase activity *in vitro*. Mutation of Tyr-699 to Phe slightly enhances the biological activity of normal c-*fms*, while mutation of Tyr-809 to Phe markedly reduces the focus formation activity of oncogenically activated *fms*.

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CHAPTER I: INTRODUCTION

The Genetic Basis of Cancer

To designate the diversity of states we call 'cancer' by a single name is perhaps more obscuring than illuminating. Conditions as distinct as neuroblastoma and small cell lung carcinoma, differing in age of onset, affected tissue, rate of progression and practically all other superficial phenomena are united only in being the result of disordered cell growth. The understanding of cancer thus requires an elucidation of its underlying causes so that both unifying and distinguishing themes can be appreciated.

The major unifying theme of cancer is its genetic basis. This theme was first recognized by Boveri (1914), but in retrospect studies of environmental causes of cancer 200 years ago provided the first clue when smoking (Hill, 1761) and chimney sweeping (Pott, 1775) were shown to dispose their practitioners to particular types of neoplasia. Although it could not have been appreciated at the time, both tobacco and coal tar contain potent mutagens, and it is this ability to damage DNA which makes them carcinogens.

Several additional lines of evidence cement the link between genes and cancer. That predisposition to cancer can be inherited is shown by pedigree analysis of a variety of human diseases including retinoblastoma (Knudson, 1971) and Bloom's syndrome (German, 1974), and is elegantly demonstrated by melanoma in platyfish-swordtail hybrids (reviewed in Schwab, 1987). Tumors were shown to be clonal by isozyme (Fialkow, 1974, 1976) and cytogenetic (Nowell, 1976) analysis, indicating the importance of stable intracellular changes

rather than simply a change in the extracellular environment for neoplastic growth. Finally, extensive analysis of cultured cells has shown that the transformed phenotype can be both acquired and lost in a manner similar to simple mutations (Barret and T'so, 1978; Varmus et al. 1981).

While all neoplasia seems to result from genetic changes, the diversity of cancer may well be a reflection of the complexity of the underlying genetic changes. Several results indicate that single mutations are rarely sufficient to cause transformation. One is that the age profile of cancer incidence is far from linear, falling for many adult cancers closest to that expected if six independent mutagenic events are required for complete transformation (Armitage and Doll, 1954: Peto et al. 1975). Detailed analysis of a variety of specific tumor types has succeeded in identifying intermediate stages that precede fullblown malignancy in colorectal cancer (Fearon and Vogelstein, 1990), chronic myelogenous leukemia (Liu et al. 1988), and several other neoplasias (see Fearon and Vogelstein, 1990). Thus a simple identification of genes capable of contributing to transformation is not sufficient, but must be placed in a context that allows connections between them to be drawn and the contributions each makes to *in situ* malignancies assessed.

Cancer Genes

Identification of the genes involved in cancer is an important step towards its understanding. Obviously this is not a simple process and as is frequently the case the first clues came from

studies of viruses. In the past decade the evolution of DNA cloning technology has made possible a variety of additional approaches to the isolation of genes involved in cancer, known as oncogenes, so that now literally dozens are known.

The first progress towards a molecular understanding of cancer came from the now famous experiments of Rous. In 1911 he published two papers (Rous, 1911a,b) documenting the transmission of a sarcoma in chickens by a filterable agent. Although for technical and dogmatic reasons the study of Rous Sarcoma Virus (RSV) was not extensively pursued, work with mice in the Thirties demonstrated the presence of milk transmissible tumorigenic viruses in certain strains of mice, and in the Fifties transmission of leukemia by inoculation with cell extracts was achieved. In both these cases the onset of neoplasia was delayed by months, while the Rous virus was capable of causing sarcomas after an incubation period of only a few weeks. During the Sixties a similar rapidly-transforming murine sarcoma virus was found by Harvey (1964), Ha-MSV (Harvey murine sarcoma virus). It was isolated by passage of Moloney murine leukemia virus (Mo-MLV), a slowly transforming leukemia virus, through rats. Shortly thereafter several other acutely transforming murine viruses were isolated from mice, cats and chickens (see Bishop, 1985).

Finally in the Seventies the significance of these discoveries became clear. By this time a number of both DNA (adenovirus, SV40, Herpes Simplex Virus) and RNA viruses were known to be capable of causing transformation *in vivo* or *in vitro*. Mutational analysis had shown that these viruses contained genes necessary for transformation

(Fried, 1965), but it had not been possible to separate genetic functions required for transformation and viral replication. The discoveries that mutants of RSV could be isolated that lacked transforming capacity at high temperature yet retained replication competence (Martin, 1970) and that retained replication competence without transforming activity (Vogt, 1971) strongly suggested that at least some transforming viruses contain a gene whose sole function is to induce transformation.

Attempts to link the experimentally-approachable model of viral transformation to human cancers were finally nearing fruition. The 'oncogene hypothesis' of Todaro and Huebner (1972) had proposed that tumor viruses deposited potential transforming genes in the germ line in latent form, from which they were subject to activation. Fact was discovered to be both stranger and more enlightening than fiction. Stehelin et al. (1976) were able to show that *src*, the transforming gene of RSV, was related to genetic material found not only in its host, but in a broad range of other animals. Not only do normal cells contain a gene subject to mutagenic activation, its broad conservation indicated some role in normal cellular function. Peculiar properties of RSV had facilitated demonstration of a cellular *src* (proto-) oncogene, so a few years were required for the generalization of this finding but eventually it became clear that (practically) all acutely transforming viruses contain transforming genes related to the cellular repertoire.

Several lines of evidence prove conclusively that the viral oncogenes (v-oncs) are derived form cellular genes, rather than vice

versa. Cellular genes related to several v-oncs have been found in species as diverse as humans and flies (Shilo, 1987), even in one case yeast (Kutaoka et al. 1988). Proto-oncogenes have been shown to contain introns (Parker et al. 1981; Shalloway et al. 1981), a structural hallmark of cellular genes, thus excluding the possibility of rare infections distributing a viral gene across the phyla. Proto-oncogenes are not found associated with viral genes (Padgett et al. 1977) and, contrary to what would be predicted if they lay silent waiting to inflict cancer, they are widely expressed in normal tissues (Spector et al. 1978).

The derivation of v-oncs from proto-oncogenes provides an explanation for the generation of acutely transforming viruses from those with long latency. Hanafusa et al. (1977) partially recapitulated the transduction event by infecting chickens with a truncated, non-transforming version of RSV, recovered transforming variants and analyzed their structures. The recovered *src* genes contained sequences not present in the original innoculum and derived from the c-*src* gene (Wang et al. 1984). Finally, comparison of the structures of v- and c-oncs supports a general model for viral transduction according to which proviral insertion occurs upstream of a proto-oncogene, a (rare) chromosomal deletion links the proto-oncogene coding region to the viral 5′ LTR and the resulting transcript acquires a 3′ LTR during the imprecise process of reverse transcription (Swanstrom et al. 1983).

A golden age of retroviral cloning yielded about 20 distinct v-oncs. Only in recent and relatively rare cases, however, are viruses

associated with human neoplasia (Poiesz et al. 1980). The generally accepted model held that non-viral neoplasia involved the mutagenic activation of proto-oncogenes. Proof arrived in several forms, beginning with the demonstration in 1982 that DNA isolated from tumors and transfected into cultured cells could cause transformation (Shih et al. 1979), and that this DNA contained a gene homologous to the oncogene *ras* (Der et al. 1982; Parada et al. 1982). Other indictments of proto-oncogenes have been handed down by the study of chromosomal abnormalities and proviral insertions. Burkitt's lymphomas and mouse plasmocytomas are associated with specific chromosomal translocations (Klein, 1983). One partner in these translocations was found to carry the c-*myc* gene (Shen-Ong et al. 1982; Della-Favera et al 1982) such that the c-*myc* gene is directly apposed to an active Ig locus. The Philadelphia chromosome, observed in CML, truncates the c-*abl* gene and leads to its expression as a *bcr-abl* fusion. Relatives of c-*myc* were discovered as a result of their amplification in neuroblastoma (Schwab et al. 1983) and SCLC (Nau et al. 1985). A number of other oncogenes are modestly to substantially amplified in a variety of tumor types (see Bishop, 1989). Thus mutation, overexpression and amplification are all mechanisms by which proto-oncogenes contribute to neoplasia.

I have largely neglected an important emerging class of genes involved in cancer, the tumor suppressor genes, and will continue to do so. Briefly, tumor suppressors are defined as genes whose loss contributes to transformation. Their existence was long postulated on the basis of cytogenetic observations of consistent chromosome

loss (Mitelman, 1985) and cell fusion experiments which indicated that transformation was recessive (Sager, 1985). To date only two tumor suppressor genes have been isolated, the *Rb* and p53 genes, although multiple distinct genes are implied by cytogenetics (Stanbridge and Cavennee, 1989). Loss of the *Rb* gene is characteristic of retinoblastomas, and has been frequently observed in osteosarcoma, SCLC, bladder carcinomas and mammary carcinomas (*op cit*). Although clearly important in human cancer, tumor suppressor genes have been less well studied than oncogenes because their identification and analysis are complicated by their recessive action, and because activated oncogenes are generally capable of inducing dominant transformation even in their presence. Current progress is so rapid as to make this discussion obselete with the isolation of the NF-1 gene and the determination that GAP can supress transformation.

A final distinct class of oncogenes is that encoded by DNA tumor viruses. Unlike other oncogenes these have no close cellular counterparts. On that basis I will largely ignore them. Indications are that they transform cells through the subversion of pathways also employed by cell-derived oncogenes and tumor suppressor genes (see Eckart, 1989; Shenk, 1989). Several oncogenes of DNA tumor viruses appear to act at least in part by binding to and altering the activity of the Rb gene product and p53. At least one, the polyoma middle T gene product, associates with and stimulates the activity of the c-*src* protein (Courtneidge and Smith, 1983; Bolen et al. 1984).

Mechanisms of Oncogene Activation

With the case for proto-oncogene involvement in neoplasia established, much attention has been focused on the nature and biochemical consequences of activating lesions. These have provided some clues to the controls which normally inhibit proto-oncogenes and the pathways by which their activated counterparts cause transformation. From a theoretical standpoint there are two general classes of mutations that might activate proto-oncogenes, expression mutants and structural mutants. Several proto-oncogenes are capable of transforming fibroblasts when efficiently expressed, yet oncogenes uncovered in retroviruses and transfection assays inevitably bear mutations in their coding sequence, suggesting a requirement for structural changes for full oncogenic activation. Most proto-oncogenes are incapable of inducing a transformed state even when expressed at extremely high levels. Apparently structural mutations are required either to release them from some efficient inhibitor or allow them to act along a novel pathways.

An examination of the set of proto-oncogenes capable of inducing transformation in their pristine state is only somewhat informative. C-*jun*, c-*fos*, c-*myc*, c-*mos* and c-*ras* are among the few genes which fall into this category (*neu* is a special case; see Lehvaslaiho et al. 1989; Lee et al. 1989; DiMarco et al. 1990). Regulation of the abundance of their gene products must be an important mechanism for controlling cell growth, and inhibitors of function must be either stochiometrically inadequate or affect

primarily their abundance. Support for this notion comes from the observation that c-*jun*, c-*fos*, c-*myc* and c-*mos* are all extremely tightly regulated at the level of mRNA abundance. Interestingly, expression of the c-*fos*, c-*myc* and c-*jun* genes is dramatically induced in response to a variety of mitogens. None the less, all these genes bear mutations in their naturally occurring oncogenic isolates and transform more efficiently when mutated, implying that additional controls may serve to tame their function in normal cells.

The primary structural basis of activating mutations has been determined for practically all oncogenes. In several cases multiple independent activated alleles have been isolated, sometimes emphasizing the critical role of a particular protein domain (eg *erbB*, *neu*; Bargmann et al. 1986; Nilsen et al. 1985; Gamett et al. 1986), and sometimes revealing the diversity of important regulatory domains (eg *src* and *trk*; Jove and Hanafusa, 1987; Coulier et al. 1990). In either case very little light has been cast, absent knowledge of a biochemical function for the gene product.

Biochemical Functions of Oncogene Products

Oncogene products can be divided into three broad classes, the GTP binding proteins, the protein kinases and the nuclear oncogenes. Members of the first two classes share extensive primary sequence homology, facilitating their assignment, and extensive analysis of mutants has confirmed the relevance of their biochemical activity to transformation. Nuclear oncogenes form a somewhat more diverse

group, though most have been assigned a role in modulation of transcription.

In all cases two major questions have been the focus of study of the biochemical properties of oncogene products: (1) what structures are involved in the control of their activity and how, and (2) what are the downstream mediators of their transforming functions and how do oncogenes exert their effects on them. Striking advances have been made over the last few years in identifying both upstream modulators and downstream effectors of oncogene action, yet a definitive assessment of their importance remains problematic. Even for the few cases in which it has been possible to suggest that a single downstream target may be of primary importance, interactions between oncogene product and target remain murky and identification of secondary effectors difficult (see Hall, 1990). Perhaps this should come as no surprise. Any single effector sufficient for producing transformation would itself be a likely target for oncogenic activation. The cell has a propensity for employing multiple pathways to control critical events, a point emphasized by the multiple hits required to produce neoplasia. If an oncogene product is capable of producing signals along three independent pathways, it is easy to envision a scenario in which no one pathway is either necessary or sufficient to induce transformation yet any pair is adequate. Alternately, one pathway may be necessary but not generally sufficient, requiring either reinforcement by a distinct signal or exceedingly high signalling on its own. Tools for separating out the effects of individual downstream effectors are

still in their infancy. Gene disruption by homologous recombination, injection of neutralizing antibodies, antisense RNA, *in vitro* systems and specific enzyme inhibitors all promise to provide some assistance in eventually unraveling the tangled web. Up to this point most progress has come in the areas of identifying potentially-important substrates, delineating pathways and dissecting the effects of upstream regulators and downstream targets by mutation of oncogenes themselves.

Nuclear Oncogenes

This is the most diverse group of oncogenes and the only one not united by any biochemical function. Since transcriptional regulation is one of the fundamental determinants of cell state it was long believed that some regulators of transcription might be capable of oncogenic activation. Several members of the set of nuclear oncogenes have been shown to bind DNA in a sequence specific fashion and affect transcription of adjacent genes, and growing evidence indicates a role for the *myc* family (Eilers, personal communication) and *ets* (Gunther et al. 1990; Wasylyk et al. 1990) in transcriptional regulation.

Three families of nuclear oncogenes, *myc*, *fos* and *jun*, belong to the set of 'Early Response' genes whose transcription is rapidly induced when quiescent cells are exposed to proliferative stimuli (Kelly et al. 1983; Greenberg and Ziff, 1984; Lamph et al. 1988). Each is tightly transcriptionally regulated and induced by both proliferative stimuli and the actions of cytoplasmic oncogene

products (Curran 1988; Cole, 1986; Cohen and Curran, 1988). High level expression of their unmutated cellular genes is sufficient to drive transformation (Small et al. 1988, Lee et al. 1988; Miller et al. 1984; Schutte et al. 1989). Fos and Jun are clearly involved in transcription as components of the AP-1 transcription factor. Myc contains both a leucine zipper and a helix-loop-helix domain and appears to directly regulate the expression of at least some genes (Eilers, personal communication). All this points to a function of *myc*, *fos* and *jun* genes as nuclear mediators of cell proliferation which act by inducing expression of genes.

The *myb* and *erbA* oncogenes are also transcription factors Sokura et al. 1989; Weston and Bishop, 1989; Sap et al. 1986; Weinberger et al. 1986). Unlike *myc*, *fos* and *jun*, expression of c-*myb* and c-*erbA* is not ubiquitous. Activated alleles affect the growth of only a very limited set of hematopoetic cells, and have been suggested to function by blocking differentiation (Kahn et al 1986; Zenke et al. 1988; Clarke et al. 1988; Ness et al. 1987).

The Ras Oncogenes

The *ras* family consists of three highly related members: Harvey *ras* (Ha-*ras*), Kirsten *ras* (Ki-*ras*) and N-*ras*. A set of at least nine related genes has been found in mammalian cells. *Ras* proteins are characterized by GTP binding activity. On the basis of their plasma membrane location and similarity to G proteins it is thought that *ras* proteins serve as second messenger molecules to propagate proliferative signals from outside the cell. An important

advance in understanding of *ras* transformation was made with the discovery of the GTPase Activator Protein (GAP; Trahey and McCormick, 1987). GAP stimulates the intrinsic GTPase activity of normal *ras* proteins but not activated *ras* proteins. Not only does GAP stimulate what is thought to be the inactivating event of GTP hydrolysis, it is also vital for transformation by activated *ras* as mutations effecting the domain required for GAP interaction (the effector binding domain) also lose their transforming activity (Adari et al. 1988).

The Protein Kinase Oncogenes

A few broad themes unite this largest class of oncogenes. Most prominent is that numerous studies using site-directed mutagenesis have shown kinase activity to be indispensable for transformation, implying that these proteins function by modifying substrate proteins. The predominant class of kinase oncogenes is composed of those whose products catalyze phosphorylation of proteins on tyrosine residues, the protein tyrosine kinases (PTKs). Tyrosine phosphorylation was initially described as a novel protein modification carried out by the *src* and *abl* products (Hunter and Sefton, 1980; Witte et al. 1980), but was soon found to be catalyzed by the epidermal growth factor (EGF) receptor as well. Since then almost all oncogenic protein kinases have been shown to phosphorylate tyrosine residues.

The initial discovery of kinase activity associated with the *src* gene product (Collet and Erikson, 1978; Levinson et al. 1978) indicated a mechanism by which cytoplasmic proteins could promulgate

their transforming activity. Kinases had been recognized for years as carrying out regulatory functions (Krebs and Beavo, 1979), but had only been seen to modify serine and threonine residues. Tyrosine phosphorylation constitutes a tiny fraction of total cellular protein phosporylation (Hunter and Sefton, 1980) and seems to be specifically associated with proteins capable of affecting cell growth (Hunter, 1987). Transformation by PTK oncogenes is associated with increased tyrosine kinase activity and often substantially increased levels of total cellular phosphotyrosine (Cooper et al. 1983). Activated PTK oncogenes need not be expressed at levels higher than their cellular counterparts, but instead bear lesions which increase their kinase activity (Jakobovits et al. 1984; Coussens et al. 1985). A specific inhibitor of tyrosine phosphatases, sodium orthovanadate, is capable of inducing a state that mimics several parameters of transformation (Klarlund, 1985). Obviously the kinase activity of the PTKs must be tightly regulated by the cell. Oncogenic activation is not generally due to loss of transcriptional control as PTKs are widely expressed, so activation must involve altered post-transcriptional regulation.

Examination of PTK regulation is facilitated by dividing them into receptor and non-receptor classes. Each class includes both transforming genes and genes with no known transforming potential. The receptor class contains a number of growth factor receptors (Insulin, EGF, platelet derived growth factor (PDGF), fibroblast growth factor (FGF)) and genes identified by their transforming activity or by homology to tyrosine kinases, all of which contain a single membrane-spanning domain. Ligands have been identified for

only a few of these, but it seems likely that the activity of the others is also controlled by ligand binding. All receptor PTKs appear to have very low basal activity, with their kinase activity turned on by ligand binding. Such a superficial explanation fails to address how kinase activity is squelched, how ligand binding transduces a signal across the membrane or how that signal relieves kinase repression.

Control of non-receptor tyrosine kinase activity is less well understood. Structural motifs have been identified that affect their kinase activity including the SH2 domain (Pawson, 1988) and some progress made towards identifying regulators of their activity, particularly for *src*. The membrane localization of most non-receptor PTKs led to the hypothesis that like receptor PTKs they too might be controlled by signals from outside the cell, and both the *lck* and *fyn* proteins have been shown to associate with cell surface molecules.

In addition to the prevalent PTKs there are two oncogenic serine/threonine kinases: *raf*, and *mos*. C-Raf appears to be a target of a variety of PTKs (Morrison et al. 1988). C-Mos was recently identified as cytostatic factor, which maintains maturation promoting factor in an active state (Sagata et al. 1989).

Oncogenes and Intercellular Communication

An examination of the roles of proto-oncogene products in normal cells leads to the conclusion that many are involved in intercellular signalling. Nothing demonstrates this link more clearly than the identity of the proto-oncogene precursors for *erb*B

(the EGF receptor), v-*fms* (the CSF-1 receptor) and *sis* (PDGF), but many other proto-oncogenes can also be assigned roles in signalling. Development and maintenance of a multicellular organism requires the elaboration of a complex series of cell division and differentiation events whose timing and spatial arrangement are dictated primarily by interactions among cells. Whether mediated by direct cell-cell contact or by diffusible agents each signal must traverse the plasma membrane and induce alterations in intracellular molecules leading eventually to effects within the nucleus.

The need to endow signalling pathways with the capacity to profoundly alter cell fate presumably makes them vulnerable to misuse. The need to avoid unchecked cell growth may limit the number of gene products not directly involved in signalling that are capable of oncogenic activation. Among the kinase oncogenes the receptor class plays an obvious role and the examples of *lck* and *fyn* suggest that at least some non-receptor PTKs are also involved in signalling. Raf associates with or is modified in response to a number of PTKs (Morrison 1988, 1989). Expression of the nuclear oncogenes c-*myc*, c-*fos* and c-*jun* is induced in response to stimulation by several growth factors. Ras proteins are assigned roles in signalling by virtue of both their similarity to G-proteins and the ability of anti-Ras antibodies to block affects of serum stimulation (Mulcahy et al. 1985) and tyrosine kinase oncogenes (Smith et al. 1986).

Accumulating evidence suggests that the signalling pathways along which their proto-oncogene products are so suggestively placed are critical to transformation by oncogenes. Oncogenes might

transform cells either by changing their quantitative activity or by acquiring the capacity to participate in novel interactions. The strongest evidence that the former is true comes from the ability of the unmutated forms of the EGF and CSF-1 receptors to cause transformation in the presence of their ligands (Roussel et al. 1987; Di Fione et al. 1987), but several findings indicate that this may be a general phenomenon. Transformation by *ras* genes requires an intact effector binding domain but activation can be achieved by mutation of codon 12 or 61 to almost any other amino acid (Seeburg et al. 1984; Der et al. 1986). Overxpression of several nuclear oncogenes, whose induction is normally associated with cell proliferation, is sufficient to cause transformation without any alteration of the coding sequences. All these findings support the notion that oncogene products require no novel interactions to induce transformation.

An understanding of the molecular mechanisms of transformation would seem to be quite similar to an understanding of growth factor signal transduction. Ideally a description would include both structural and biochemical descriptions of basal repression of signalling, binding of ligand, transmembrane signal transduction, activation of signalling mechanisms and interactions of substrates throughout each pathway, from plasma membrane to nucleus and back. The current picture falls several steps short of this ideal but a remarkable number of the components of signal transduction pathways have been recently identified. The process as currently understood can usefully be broken down into the three steps of transmembrane

signalling, second messengers and downstream events.

Transmembrane Signalling

Signalling molecules unable to traverse the plasma membrane must bind cell surface receptors and cause them to generate intracellular changes. Two major questions involve the manner of transmitting an alteration to the cytoplasmic receptor domain and how this change produces a signal. Three basic types of receptor molecules have been recognized: those with multiple membrane-spanning domains, those with short cytoplasmic domains and the tyrosine kinase receptors. All three types are involved in the determination of cell fate in various contexts, and significantly each seems to generate signals that affect the others.

The first two classes of receptors are sparsely represented among oncogenes. The *mas* oncogene (Young et al. 1986) contains several membrane spanning domains, as does the 5HT1c receptor which can cause transformation of fibroblasts in the presense of ligand. Such molecules are thought to function either as ion channels whose opening is regulated by ligand binding (Stroud and Finer-Moore, 1985) or by interaction with G proteins (Gilman, 1987). Little is known of the manner in which ligand binding alters receptor state to induce activation. Receptors with short cytoplasmic domains include molecules involved in endocytosis (Goldstein et al. 1985), receptors which interact with cytoskeletal elements (Hynes, 1987) and a large class of unknown signalling function. Endocytic receptors are induced to cluster by ligand binding, which may be a sufficient

signal for internalization. Receptors such as the integrin receptor which interact with the cytoskeleton are poorly understood in terms of the manner of signal transduction and the nature of the induced cytoplasmic events. Some receptors are known to interact with cytoplasmic tyrosine kinases (CD4 with *lck*, the T cell receptor with *fyn*, *crk* and membrane Ig with unknown kinases) and it is thought that many receptors may function by interaction with either cytoplasmic kinases or *ras* proteins.

Most of the characterized growth factor receptors contain an intracellular tyrosine kinase domain. Ligand binding transmits a signal which activates this kinase. An accumulation of evidence from studies with PDGF, EGF, CSF-1 and insulin receptors and the *neu* oncogene indicates that kinase activation results from dimeric interactions between receptors (Schlessinger, 1986). Binding of EGF and PDGF to their receptors has been shown to induce dimeric interactions (Heldin et al 1989; Yarden and Schlessinger, 1987; Cochet et al. 1988). Dimerization of receptor kinases by antibody (Roussel et al. 1990; Hoffetz and Zick, 1986) or by mutations that promote dimerization (Weiner et al. 1989) is capable of activating kinase activity and receptor tyrosine phosphorylation occurs via an interchain mechanism (Ohtsuka et al. 1990; Honegger et al. 1990; Ballotti et al. 1989). Activation of these receptors may stimulate several pathways, but always through their kinase activity (Chou et al. 1987; Chen et al. 1987; Williams, 1989; Downing et al. 1989).

Second Messengers

Many events are triggered rapidly within the cell by binding to cell surface receptors. The list includes activation of protein kinases, activation of G proteins, increased levels of cAMP, cGMP, phosphoinositide and arachidonic acid metabolites and alterations in cytoplasmic pH (Bourne and DeFranco. 1989). In some cases a kinase, G protein or ion-channel is directly coupled to the receptor but many of these effects are not direct. All growth factor receptors seem to affect multiple second messenger pathways (Gill, 1989). Stimulation of the PDGF receptor, for instance, induces tyrosine phosphorylation (Nishimura et al. 1982), phosphoinositide turnover, pH changes, Ca^{++} influx (Escobedo et al. 1988), PI kinase activation (Kaplan et al. 1987) and increased Ser/Thr kinase activity (Morrison et al. 1988). None of these effects save PTK activity has been attributed directly to the PDGF receptor and so must be mediated either by its direct substrates or effectors further down pathways set in motion by PDGF binding.

Several clues have recently emerged concerning activation of various second messenger pathways by protein kinases. In most cases, it has not been possible to firmly establish the manner in which growth factor receptors generate all the observed second messengers. Examination of individual pathways reveals some likely control points. Several growth factor receptors stimulate C kinase, which requires diacyl glycerol and Ca^{++} (Berridge, 1987). Action of phospholipase C (PL C) on phosphoinositides produces both DAG and 1,4,5 inositol triphosphate, which can in turn increase intracellular Ca^{++}. Both the EGF and PDGF receptors are able to phosphorylate PL C

gamma, presumably increasing its activity (Margolis et al. 1989; Meisenhelder et al. 1989; Wahl et al. 1988). There may be other mechanisms by which PTKs can affect C kinase as well. A novel PI kinase, PI 3-kinase, is also associated with and phosphorylated by a number of PTKs (Kaplan et al. 1987; Fukui et al. 1989), and although no role has been established for it in PI metabolism it seems likely to be important. Other PTKs may activate C kinase via a G protein pathway (Imamura et al. 1990; Imamura and Kufe, 1988). C kinase in turn can modulate cytoplasmic pH in addition to its direct effects.

Arachidonic acid metabolites are produced by phospholipase A_2 or the combined action of phospholipase C and diacylglycerol lipase. Obviously regulators of PL C may affect their levels. Control of PL A_2 may be mediated in part by G proteins. cAMP concentration is controlled by adenyl cyclase and cyclic nucleotide phosphodiesterases. Adenyl cyclase is regulated largely by G proteins, while the breakdown process is under complex control. The modulatory role of cAMP is mediated through cAMP-dependent protein kinases. In addition to its role as a regulator of C kinase, Ca^{++} also regulates a variety of Ca^{++}/calmodulin dependent kinases (Edelman et al. 1987).

Many of these second messenger systems are tied to G proteins and/or *ras* proteins. With the exception of *mas*, there are no obvious direct links between growth factor receptor oncogenes and G proteins, nor have molecular mechanisms that link them been established. Ras proteins have been implicated as second messengers by the finding that GAP binds a variety of PTKs and anti-Ras antibodies preclude

serum stimulation of cell growth (McCormick, 1989).

The vast array of both second messenger systems and links among them poses a daunting challenge, without even mentioning the ability of PTKs to transmodulate the activity of other PTKs (Davis, 1988; Gould and Hunter, 1988). Detailed dissection of these interlocked pathways may yield interesting biological insights and clinical possibilities, but within the context of cellular growth control it is tempting to concentrate on the nuclear events that result from receptor signalling.

Most prominent of the downstream consequences of growth factor signalling are the induction of *fos*, *myc* and *jun* expression. Not only are all induced in response to a wide variety of growth signals but experiments with antisense RNA indicate that *fos* and *myc* expression is critical to cell proliferation (Heikkila et al. 1987; Nishikura and Murray, 1987). Although the regulation of *fos*, *myc* and *jun* expression has been very widely studied, the precise route by which growth factor stimulation induces their expression is unknown. *Fos* expression is the most rapidly induced and thoroughly examined. It can be transcriptionally activated by multiple pathways, including both C kinase dependent and independent routes (Gilman, 1987). Activation by direct action of kinases is a favored model not only because of their ubiquitous involvement but because multiple kinases are capable of stimulating *fos* expression, a major transcriptional regulator of *fos* (the serum response factor, SRF) is affected by phosphorylation (Prywes et al. 1986, 1988) and a kinase capable of phosphorylating SRF (casein kinase II) is regulated by growth

factors. Fos and Jun through their transcriptional activity may regulate the expression of a variety of genes (Vogt and Bos, 1989).

The Oncogene fms

Biology of the *fms* Gene

Fms was originally isolated as the oncogene of the feline sarcoma virus SM-FeSV (McDonough et al. 1971; Reynolds et al. 1981; Frankel et al. 1979). A second independent and slightly different transduction of *fms* has also been reported in another feline virus (Besmer et al. 1986). Deletions of the human *fms* locus at 5q33.3-4 (Groffen et al. 1983) have been associated with refractory anemia and acute myelogenous leukemia (Nienhuis et al. 1985; LeBeau et al. 1986). Insertional mutagenesis of the murine *fms* gene has also been found in Friend MuLV induced myeloblastic leukemias (Gisselbrecht et al. 1987). Autocrine activation of c-*fms* has been suggested in some acute myeloblastic leukemias (Rambaldi et al. 1988), and point mutations capable of contributing to the oncogenic activity of *fms* have been found in patients with myelodysplasia and acute myeloblastic leukemia (Ridge et al. 1990).

Major interest in *fms* was aroused by the discovery of its identity with the CSF-1 receptor (Sherr et al. 1985). CSF-1 is a polypeptide growth factor required for survival, proliferation, and the elaboration of differentiated functions by relatively mature monocytes and macrophages (Stanley et al. 1976; Stanley, 1979). The CSF-1 receptor is normally found on only a tiny subset of cells in the mature animal (Byrne et al. 1981), but has also been found on

extraembryonic tissues during development (Regenstreif and Rossant, 1989; Muller et al. 1983) and choriocarcinoma cell lines (Retenmier et al. 1986), though no role has been defined for it in these tissues.

Extensive studies have been made of c- and v-*fms* function in blood cells. In long term bone marrow culture v-*fms* infection is capable of sustaining the growth of murine pre-B cells (Borzillo and Sherr, 1989). Bone marrow cells infected with v-*fms* and transplanted into irradiated mice lead to the development of splenomegaly with a dominant clonal population of cells in a portion of animals. No neoplasias associated with v-*fms* expression were observed in primary recipients, but clonal B-cell lymphoma and erythroleukemia arose in some recipients of secondary transplants (Heard et al. 1987). Chicken bone marrow cells infected with v-*fms* produce erythroid colonies with a high rate of spontaneous differentiation but no myeloid colonies, although v-*fms* is capable of sustaining the viability of macrophages in the absence of chicken macrophage growth factor (Fuhrmann et al. 1989). Expression of v-*fms* in a CSF-1 dependent SV-40 transformed murine macrophage line abrogates their requirement for CSF-1 and enables them to form tumors (Wheeler et al. 1986). In the immature myeloid line FDC-P1, which normally requires IL-3 or GM-CSF for growth, v-*fms* abrogates growth factor requirements but requires extended passage to acquire the capacity to form tumors (Wheeler et al. 1987; Dibb et al. 1990). The CSF-1 receptor, on the other hand, allows FDC-P1 cells to survive in the presence of CSF-1 as the only growth factor, but these cells were not tumorigenic (Dibb

et al. 1990; Rohrschneider and Metcalf, 1989; Kato et al. 1989).

In general the biological effects of v-*fms* and ligand-stimulated c-*fms* are very similar to those observed with other tyrosine kinases. Almost all cause predominantly fibrosarcomas in animals. All transform fibroblasts in culture, leading to anchorage independent growth, higher saturation density, reduced growth factor requirements, altered morphology and the capacity for tumor formation. Perhaps surprisingly, v-*fms* has effects in hematopoetic cells very similar to those seen with other PTK oncogenes (Pierce, 1989). Among the limited differences observed are that it abrogates the exogenous growth factor requirement of v-*myb* transformed chicken myeloblasts in a non-autocrine manner (Fuhrmann et al. 1989) and that CSF-1 treatment of CSF-1 receptor expressing FDC-P1 cells may nudge them slightly towards macrophage differentiation (Rohschneider and Metcalf, 1989). These findings suggest that FMS interacts with a somewhat different set of substrates than other PTKs, including some which may be specific to macrophages.

Biochemical Activities of FMS

Activation of the *fms* protein by either ligand stimulation or mutagenesis as in the case of v-*fms* leads to a number of biochemical changes in the cell. The first of these is probably activation of its PTK activity. Addition of CSF-1 to cells bearing the CSF-1 receptor or to purified CSF-1 receptor leads to receptor dimerization, and tyrosine phosphorylation of the receptor and other proteins (Downing et al. 1988; Yeung et al. 1987; Ohtsuka et al.

1990). Dimerization of receptors seems to be the critical step in kinase activation as crosslinking of a CD2-FMS chimera by anti-CD2 antisera is also capable of stimulating FMS activity (Roussel et al. 1990). The *v-fms* protein is active as a kinase *in vitro* but causes little elevation of total cellular phosphotyrosine (Reynolds et al. 1981; Barbacid and Lauver, 1981). Reexamination of v-*fms* transformed fibroblasts showed that the v-*fms* protein is constitutively phosphorylated on tyrosine (Tamura et al. 1986), unlike the c-*fms* protein and non-transforming hybrids of v- and c-*fms* (Morrison et al. 1988; Downing et al. 1988). Immunoblotting of v-*fms* transformed and CSF-1 stimulated cells indicated tyrosine phosphorylation of a limited set of cellular proteins is elevated by active protein (Morrison et al. 1988; Downing et al. 1988), though the pattern is different than that of PDGF-stimulated cells and much more limited than that seen in v-*src* transformed cells (Morrison et al. 1988). Although point mutations that abolish FMS kinase activity coordinately eliminate transforming activity (Downing et al. 1989) very little is known of its substrates. A plethora of *in vivo* substrates have been identified for Src (Kamps and Sefton, 1988), and the very closely related PDGF receptor is known to phosphorylate PL C gamma, GAP, Raf and PI 3 kinase (see Ullrich and Schlessinger, 1990). Only Raf among this set is phosphorylated in response to FMS activity (Morrison et al. 1988). While PI 3 kinase associates with active FMS in a manner dependent on FMS tyrosine phosphorylation, tyrosine phosphorylation of the PI 3 kinase-associated 85kD band is little altered by active FMS (Shurtleff et al. 1990), and FMS neither

associates with nor phosphorylates GAP or PL C (Downing et al. 1989).

The early effects of CSF-1 stimulation include stimulated hexose uptake (Hamilton et al. 1988), membrane ruffling (Tushinski et al. 1982), pH elevation (Vairo and Hamilton, 1988) and induction of c-*fos* (Orlofsky and Stanley, 1987) and C kinase activation (Imamura et al. 1990). In addition to Raf activation and PI 3 kinase association, one potential pathway by which these events are mediated involves a pertussis-toxin sensitive step (Imamura et al. 1988). Although CSF-1 does not stimulate PI turnover (Whetton et al. 1986), it does stimulate PC degradation via a pertussis toxin sensitive event (presumably involving a G protein; Imamura and Kufe, 1990). As related earlier, C kinase activation might mediate many of the known effects of activated FMS. Since TPA does not mimic all effects of FMS some additional pathways must also be affected. Possibly one or more G proteins mediate these activities, or on the other hand FMS may interact with as yet uncharacterized pathways.

An additional observation that must be accounted for is the ability of microinjected anti-Ras antisera to block v-*fms* transformation (Smith et al. 1986). This is true for transformation by a number of PTK oncogenes, but not by *raf*, and would tend to indicate either that *raf* is not directly phosphorylated by these PTKs or that such phosphorylations are insufficient to produce full Raf activation. In either case some connection between FMS and its downstream effects must involve Ras.

Regulation of FMS activity may exist at several levels, the most obvious being expression of the receptor and availability of

ligand. Generally speaking most of the machinery required to transduce FMS signals must be present in a wide range of cells as FMS can transform or promote the proliferation of many cell types in which it is not normally found. Some regulation of substrates (whether direct or indirect) must exist, however, as some but not all immature myeloid cells are induced to differentiate by FMS (Pierce et al. 1990). Regulation of CSF-1 receptor expression is largely accounted for by mRNA abundance (Rettenmier et al. 1985; Muller et al. 1983; Regenstreit and Roussant 1989), but regulation is complex and involves post-transcriptional events. Initiation of c-*fms* transcription occurs from distinct promoters in placental trophoblasts and monocytic cells (Visvader and Verma, 1989). Message levels gradually increase during monocytic differentiation (Sariban et al. 1985) in a process that appears to involve mRNA stabilization (Weber et al. 1989). Cell surface expression of a functional molecule requires appropriate glycosylation (Nichols et al. 1985; Hadwiger et al. 1986).

CSF-1 is generally required for CSF-1 receptor activity. An odd exception to this rule involves the bovine papillomavirus protein E5 which can somehow cooperate with either the EGF or CSF-1 receptor to cause transformation (Martin et al. 1989). Levels of CSF-1 in serum are generally quite low; it is apparently produced by fibroblasts, monocytes and endothelial cells and it is suspected that its production is induced in sites of local inflammation. It is also found at high levels during pregnancy, produced by the uterine glandular epithelia (Whetton and Dexter, 1989).

The availability of CSF-1 receptor is subject to several types of downregulation. Ligand mediated degradation is a primary regulator (Tushinski et al. 1982; Rettenmier et al. 1987; Gilbert and Stanley, 1986). In response to ligand the receptor clusters and in then internalized and degraded. This downmodulation requires receptor tyrosine phosphorylation (Downing et al. 1989). Alternatively CSF-1 receptors can also be downregulated by TPA or other inducers of C kinase (Chen et al. 1983; Guilbert et al. 1983). This occurs by a mechanism different from that observed with ligand, and does not require receptor tyrosine phosphorylation (Downing et al. 1989). Downmodulation can also occur in response to stimulation of receptors for other hematopoeitic growth factors (Walker et al. 1985), perhaps via activation of C kinase.

Structure Function Relationships in FMS

The major thrust of this thesis is an examination of the critical structural elements of FMS and how they affect its activity. A powerful tool in beginning this task with several other proteins has been linker insertion mutagenesis. An examination of v-*fms* by this technique (Lyman and Rohrschneider, 1987) confirmed that cell surface expression of a kinase active molecule is required for transformation. A single mutation, creating a four amino acid insertion just N-terminal of the second cysteine in the third Ig domain, generated a properly expressed but non-transforming mutant.

The first step in the approach taken here was to isolate a clone of the cellular homolog of v-*fms*. This allowed structural

comparisons to be made and provided a starting point for examining how structural alterations affect its activity. Next the contribution of various domains to control of *fms* activity was examined by site-directed mutagenesis. An attempt was made to generate multiple transforming alleles of *fms* both as a further means of identifying domains involved in regulation of *fms* activity and as a model for how ligand activation of the CSF-1 receptor might occur. In the last two chapters the role of autophosphorylation in *fms* regulation was investigated by first identifying the sites at which it occurs and then examining the effect that mutation of these sites has on the biological and biochemical activities of *fms*.

A difficulty in writing this thesis was caused by the great strides that have been made in our understanding of *fms* during the course of my work. I have chosen to write each chapter from the perspective of the time at which the work contained was done. Where later findings are particularly relevant, they have been included in the discussion sections of individual chapters.

CHAPTER II:

CLONING AND SEQUENCE ANALYSIS OF c-*fms*

ABSTRACT

To examine the oncogenic activation of v-*fms* I have obtained its normal human homolog. In principle activation could result from ectopic expression or structural alteration of the c-*fms* gene product. All tyrosine kinase oncogenes isolated to date bear mutations, which are generally necessary to activate transforming potential. I have isolated a cDNA clone for the human homolog of v-*fms* and determined the sequence of its protein-coding region, which contains a number of interesting features. Comparison with the sequence of v-*fms* reveals numerous substitutions and a prominent C-terminal truncation. The c-*fms* gene introduced into 3T3 cells in a retroviral vector is not sufficient to cause cell transformation.

RESULTS

Structure of c-FMS

A human placenta cDNA library was screened with a probe containing the 3′ Pst fragment of v-*fms* (Heisterkamp et al. 1983 (Fig.1) and sixteen plaques picked. Following plaque purification, lambda DNA was isolated and restriction mapped to determine overlap. The longest clone (II-5) and an overlapping clone containing additional sequence (II-16) were subcloned into pUC and extensively restriction mapped, then fragments were cloned into M13 for sequencing (Fig. 2). Sequence was determined by the dideoxy chain

Figure II-1. Structure of v-*fms* and probe SM3. The four principal domains of the initial translation product of the v-*fms* gene are indicated. As shown, pSM3 contains the entire kinase domain sequence of v-*fms* and a small portion of the *env* gene which lies immediately 3' of *fms* in the SM-FeSV genome.

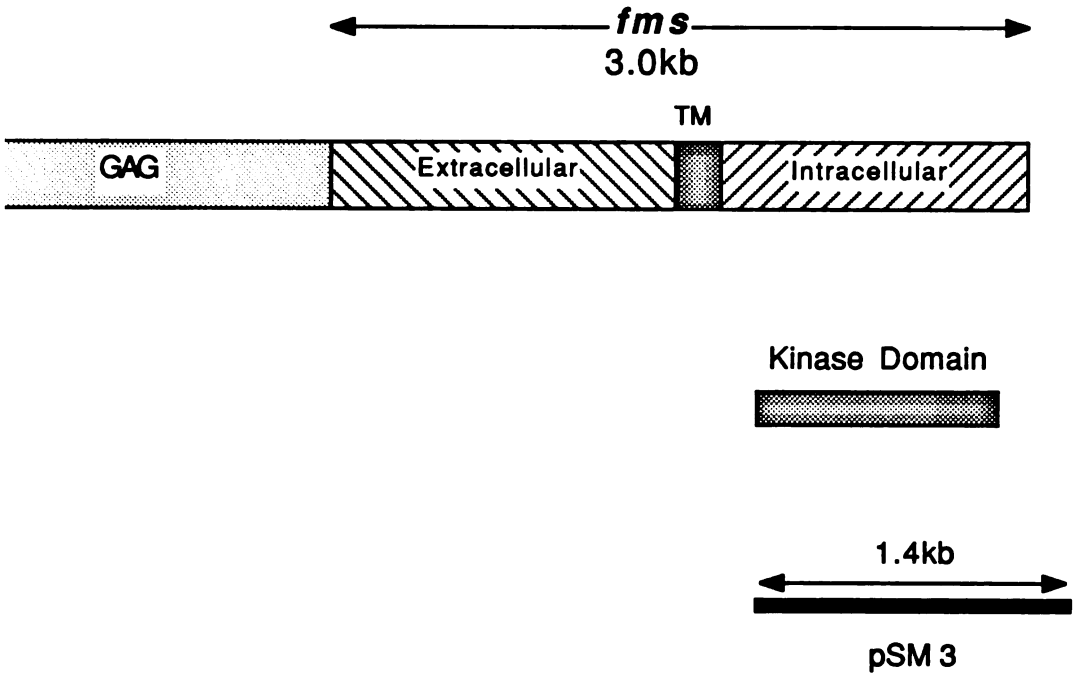
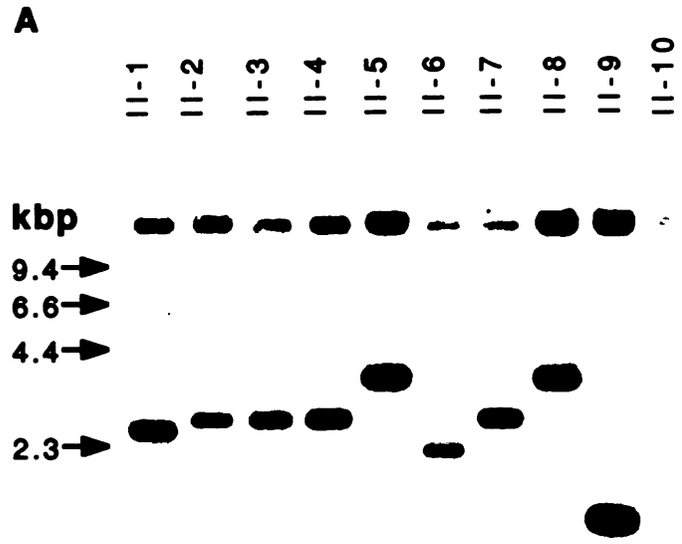
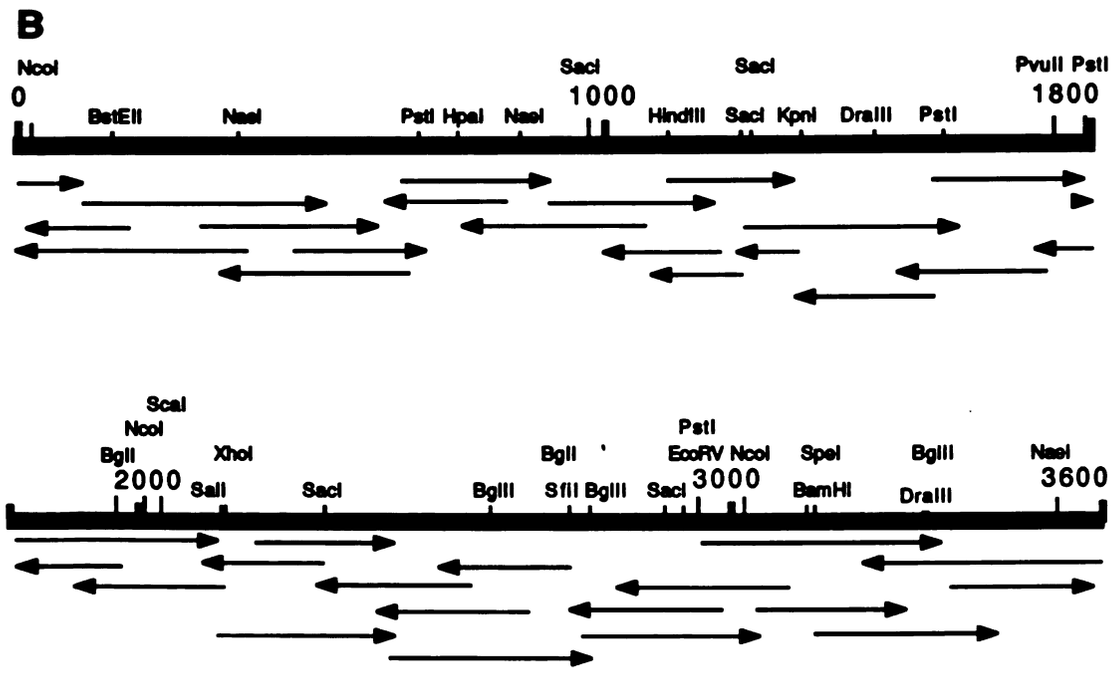

fms
3.0kb
TM
GAG
Extracellular
Intracellular
Kinase Domain
1.4kb
pSM 3

Figure II-2. Structure and sequencing strategy of c-*fms* cDNA. (A) Southern blot of 10 lambda gt-10 clones; the inserts have been cut out with EcoRI and the smaller band represents the cDNA insert. (B) Restriction map of c-*fms* cDNA; the arrows underneath represent sequenced fragments.


A
II-1
II-2
II-3
II-4
II-5
II-6
II-7
II-8
II-9
II-10
kbp
9.4
6.6
4.4
2.3


B
NcoI
0
BstEII
NaeI
SacI
1000
PstI HpaI
NaeI
SacI
HindIII
SacI
KpnI
DraIII
PstI
PvuII PstI
1800
ScaI
NcoI
BglI
2000
XhoI
SalI
SacI
BglII
BglII
SfiI BglII
PstI
EcoRV NcoI
SacI
3000
SpeI
BamHI
BglII
DraIII
NaeI
3600

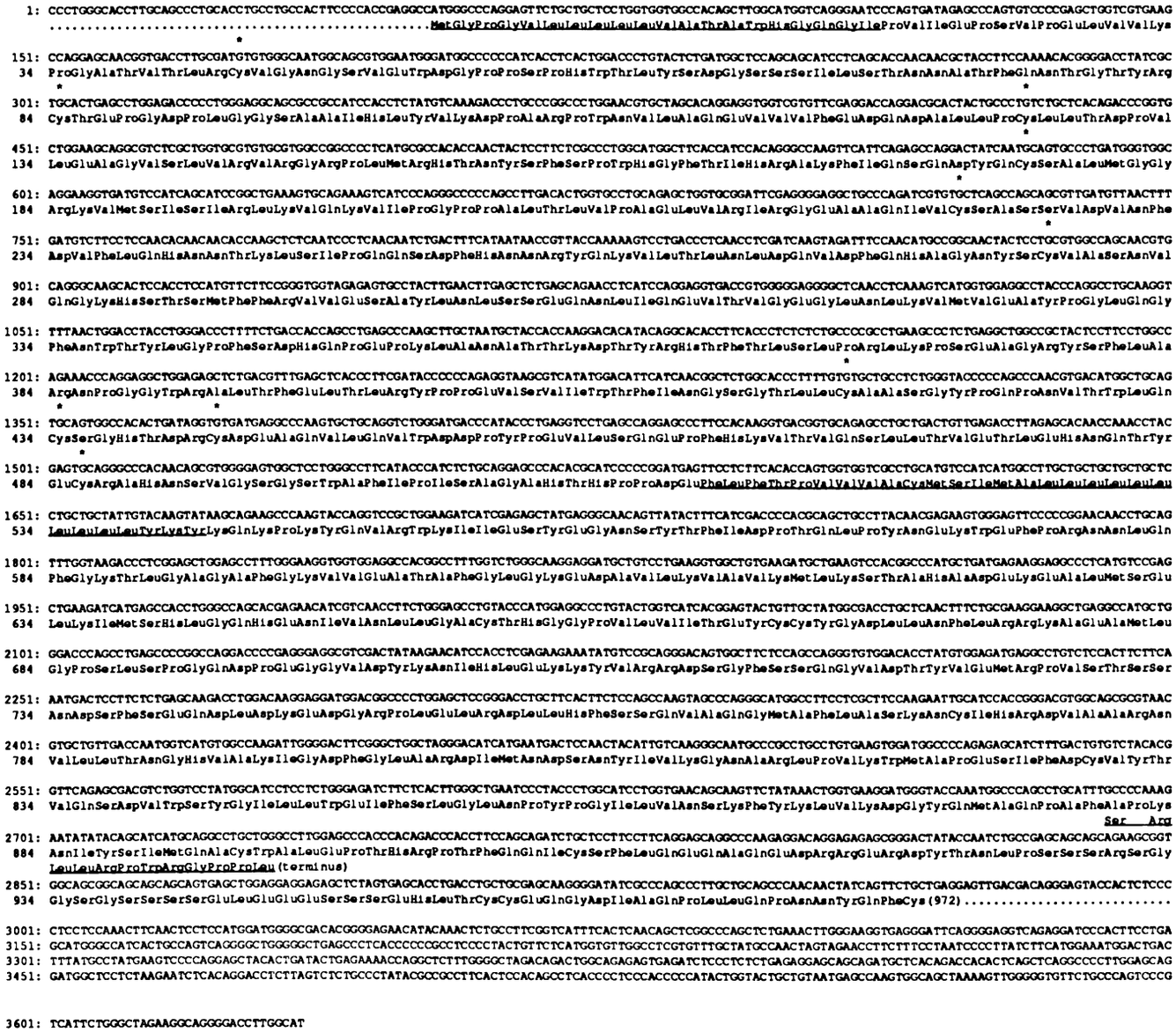
termination method of Sanger et al. (1977). The sequenced fragments are shown below the schematic (Fig. 2); all coding sequence was determined on both strands.

Coding sequence was defined as beginning with the first methionine in frame with the longest open reading frame (Fig.3). This assignment was not conclusive as the total sequence is somewhat shorter (3664bp) than the mRNA length determined by Northern (4.3kb) and no in-frame termination codons were found in the 5' non-coding region. However, the putative initiator lies within a favored translational initiation context (GGCCATGG; Kozak, 1986) and is followed by a stretch of neutral and hydrophobic amino acids likely to function as a signal sequence. In addition, an expression vector containing this ORF and lacking any other potential initiators produces a protein identical in size to authentic c-fms (data not shown). The open reading frame is capable of encoding a protein of 972 amino acids. Overall homology between v-*fms* and human c-*fms* is approximately 85%. The most striking difference between the genes is a substitution of the 40 C-terminal amino acids of c-*fms* by 11 unrelated amino acids. A lengthy stretch of hydrophobic amino acids at amino acids 513-537 is bounded on its C-terminus by several charged amino acids, constituting a likely membrane-spanning domain (Blobel, 1980). This is the only hydrophobic domain in c-*fms* of sufficient length to span a membrane bilayer.

Antibodies

Antisera specific for the *fms* protein were necessary both to

Figure II-3. Sequence of human c-*fms* cDNA. Transmembrane domain and signal sequence are underlined; Cys residues in the extracellular domain are marked with *. There are four differences between my sequence and that of the Verma group: (1) their Leu-10 (CGT) is Val (GTG), (2) their Ala-54 (GCA) is Pro (CCA)-this is shown as Pro in their sequence, (3) their Thr-242 (ACT) is (ACC), and (4) their Ala-245 (GCA) is Ser (TCA). The region of C-terminal divergence between v- and c-*fms* is overlined, and the amino acid sequence of the corresponding region of v-*fms* is shown above.



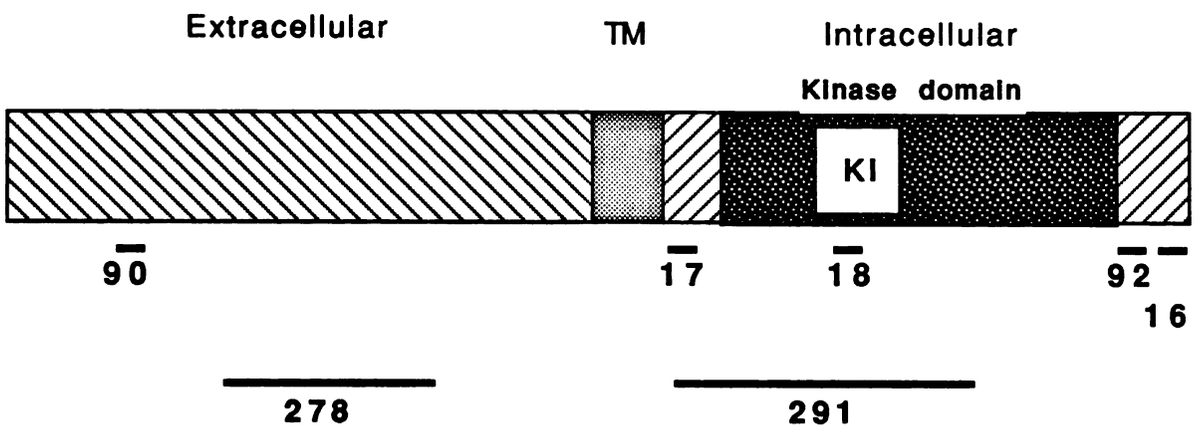
40

monitor protein expression and isolate the *fms* protein. To produce adequate sera two strategies were employed, expression of recombinant fusion protein in bacteria and chemical synthesis of peptides. Both types of antigens were then injected into rabbits and polyclonal sera screened for anti-*fms* activity by Western blotting and immunoprecipitation.

Two fusion proteins were constructed in *lac* fusion vectors (pUR278 and pUR 291;Ruther and Muller-Hill, 1983). One contained amino acids 239-349 from the external domain expressed in pUR278, and the other contained amino acids 600-713, from the cytoplasmic domain, expressed in pUR291 (Fig.4). Following expression in *lon-* e coli, whole-cell lysates were made from bacteria containing either a fusion expression vector or the parental vector. Fusion proteins were identified by Coomassie staining of SDS gels. Bands containing fusion proteins were then excised from preparative SDS gels and protein isolated by electroelution. Both fusion proteins were injected into a series of rabbits but the sera obtained, while reacting well with the bacterial fusion protein, failed to recognize authentic *fms* protein. Possible explanations for this failure include that the *lac* portion of the fusion protein constitutes a dominant epitope, resulting in only a minor immune response to the *fms*-encoded portion of the fusion protein, and that folding or modification of eucaryotic *fms* protein prevents its recognition by sera raised to these bacterially produced proteins.

Peptide antigens resulted in more useful sera. Five synthetic peptides were used, varying in length between 13 and 15 amino acids,

Figure II-4. Antigens used to prepare anti-FMS antisera. (A) The regions of FMS from which the five peptides and two fusion proteins were derived are shown relative to a schematic drawing of the *fms* protein. (B) The sequences of the five peptide antigens.



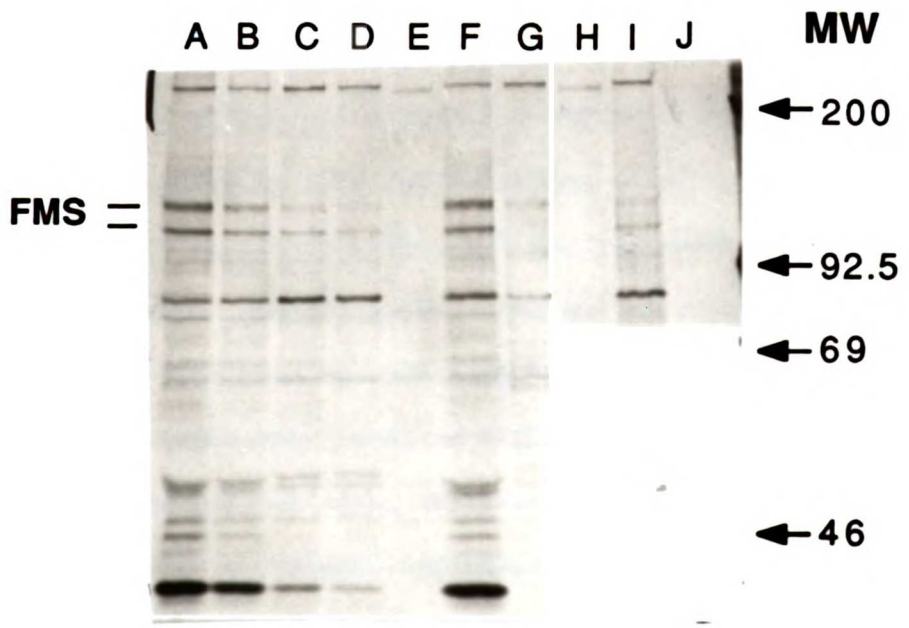
Peptide Antigens

16	952- TCCEZFDIAQPLLQP	-966
17	545- KTQVRWKIIESYEGN	-559
18	690- GQDPEGGVDYKNIHL	-704
92	922- RDYTNLPSSSRSG	-934
90	101- VKDPARPWNVLAQE	-114

derived from various portions of the *fms* protein (Fig.4). Each peptide was coupled to a carrier protein (tuberculin PPD) prior to immunization and at least two rabbits were injected with each antigen. When tested by immunoprecipitation two peptides generated sera capable of recognizing the *fms* protein (Fig.5). Peptide 18, derived from the kinase insert domain, quite consistently resulted in useful titers, while peptide 16, from the C-terminal tail, generated a lower-affinity response. Neither the extracellular domain peptide nor that from the juxtamembrane domain produced useful sera. Since all five peptides are fairly similar in hydrophobicity, sera raised to peptides 16 and 18 may function due to the exposure of their epitopes on the surface of the folded protein. This hypothesis for 18 is consistent with the finding by Taylor et al. (1989) that sera raised to a bacterially expressed protein comprising practically the entire *fms* cytoplasmic domain recognize almost exclusively epitopes within the kinase insert region.

In immunoprecipitation experiments using ^{35}S labelled extracts from either BeWo cells (a human choriocarcinoma cell line) or fibroblasts expressing an introduced c-*fms* gene, both sera recognize a protein of 150kD (Fig.5). This protein is phosphorylated on tyrosine and is associated with elevated levels of poly Glu-Tyr phosphorylation in immune complex kinase assays, consistent with its identity as the *fms* protein. None of the sera work particularly well in Western blotting, although they do identify a band consistent in M_r and intensity with that seen by immunoprecipitation.

Figure II-5. Immune precipitation of *fms* protein with antipeptide antisera. Samples are TPA-induced ML-1 cells (lanes A-G, I) or uninduced ML-1 (H, J). Lanes A-D and F were precipitated with sera 18-1 (titration); E with preimmune serum; (G, H) with LR sera; (I, J) with sera 18-2.


A B C D E F G H I J
MW
FMS
200
92.5
69
46

C-fms Expression

Recombinant c-*fms* was inserted into a eucaryotic expression vector in order to examine its biological activity in fibroblasts. Three retroviral vectors were tested, pMV7 (containing MSV LTRs; Kirschmeier et al. 1988), pLJ (Piwnica-Worms et al. 1987) and pMX 1112 (Brown and Scott, 1987), both of which have MLV LTRs; all contain a neomycin resistance gene as a selectable marker. The *fms* coding region was inserted into each as a Bam fragment containing 60bp of 5′ and 170bp of 3′ untranslated sequence. The resultant constructs were called pLJ-c, pMX-c and pMV-c. After transfection into a Psi-2 packaging line and selection of either pools or single clones of G-418 resistant cells, culture supernatants were harvested and used to infect NIH 3T3 cells. The pLJ virus produced an extremely low titer and was not pursued. When pools of G-418 resistant 3T3 cells were labelled with ^{35}S-Met and precipitated with anti-peptide polyclonal sera, substantially higher expression of c-*fms* was observed in the pMX1112-infected cells relative to the almost negligible expression in pMV7 infectants. pMX1112 was thus chosen for the expression of all other *fms* alleles.

Biological Activity of c-FMS

The ability of c-*fms* to cause morphological alteration, focus formation and anchorage independent growth in fibroblasts was examined. As a positive control the v-*fms* gene was recloned into a homologous expression system. A Bam linker was inserted at the Nhe site approximately 70bp upstream from the authentic *fms* (not *gag-fms*)

initiation site of SM-FeSV. A fragment running from this new Bam site to a Bam site approximately 500bp 3' of the UAG termination codon was then inserted into pMX1112 to make pMX-v, and transfected into Psi-2 cells to produce a virus stock.

Virus stocks from Psi-2 cells producing either pMX-c or pMX-v were introduced into 3T3 cells and the resulting infectants scored for neo^R titer and focus formation. The morphology of G-418 resistant pooled cells was examined and their ability to grow in 0.7% soft agarose tested. While the viral titer of the two stocks was similar as judged by neo^R, pMX-v produced approximately 1/3 as many foci as neo^R colonies, while the pMX-c infectants were essentially as flat as the parental cell line and failed to show anchorage independent growth (Fig.6).

DISCUSSION

Structure of the Extracellular Domain

The external domain of the c-*fms* gene forms a ligand binding structure for the macrophage colony stimulating factor (CSF-1; Sherr et al. 1985). The extracellular domain of human c-*fms* is approximately 75% homologous to v-fms, with only three amino acid insertions and most of the changes conservative (see Fig. 7). The relatively low homology between v-*fms* and c-*fms* may be related in part to the transforming capacity of v-*fms*, but most probably also reflects species divergence. Human CSF-1 does bind to v-*fms* (Lyman et al. 1988), so much of the sequence variation must reflect

Figure II-6. Morphology of fibroblasts expressing v-*fms* or c-*fms*. (A) NIH 3T3 cells infected with pMX-c. (B) NIH 3T3 cells infected with pMX-v. Both are pools of neo resistant cells.

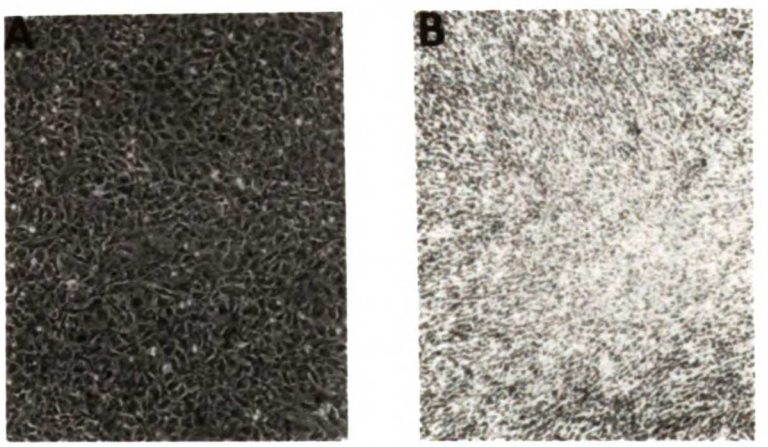
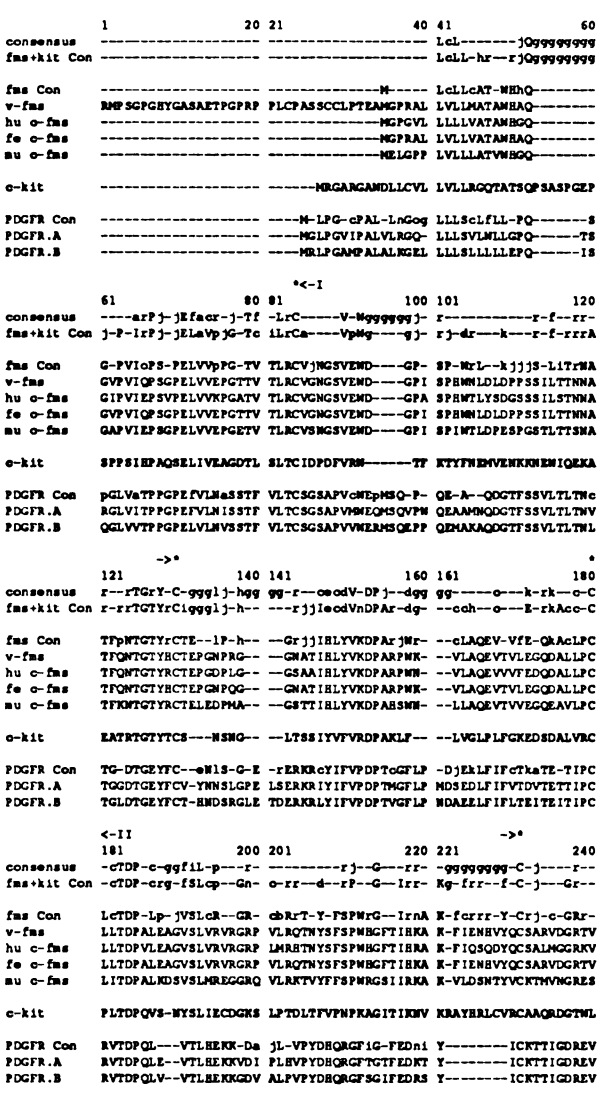
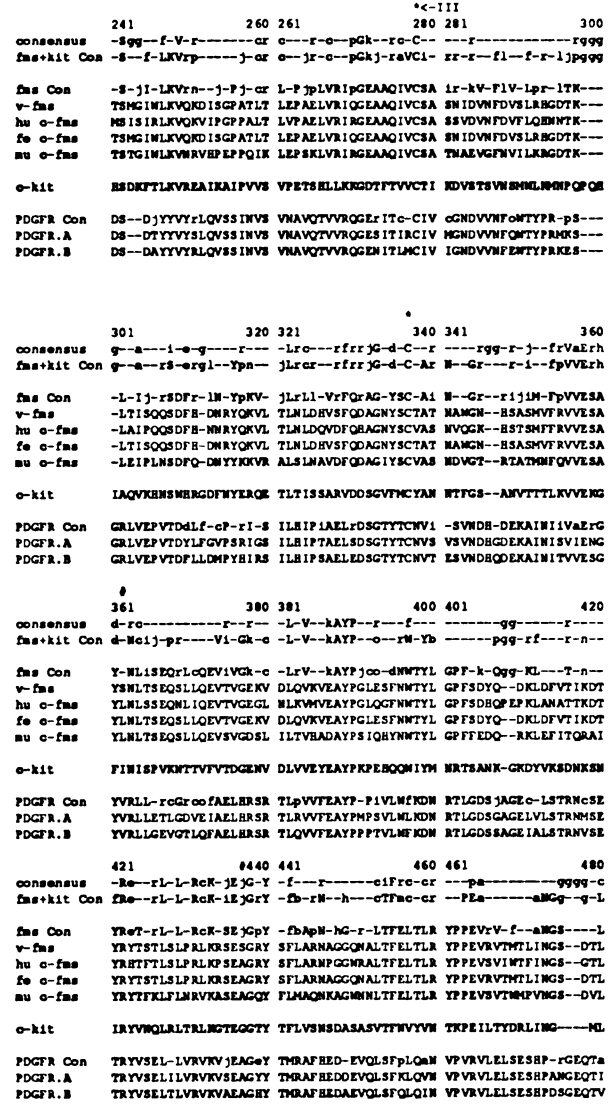
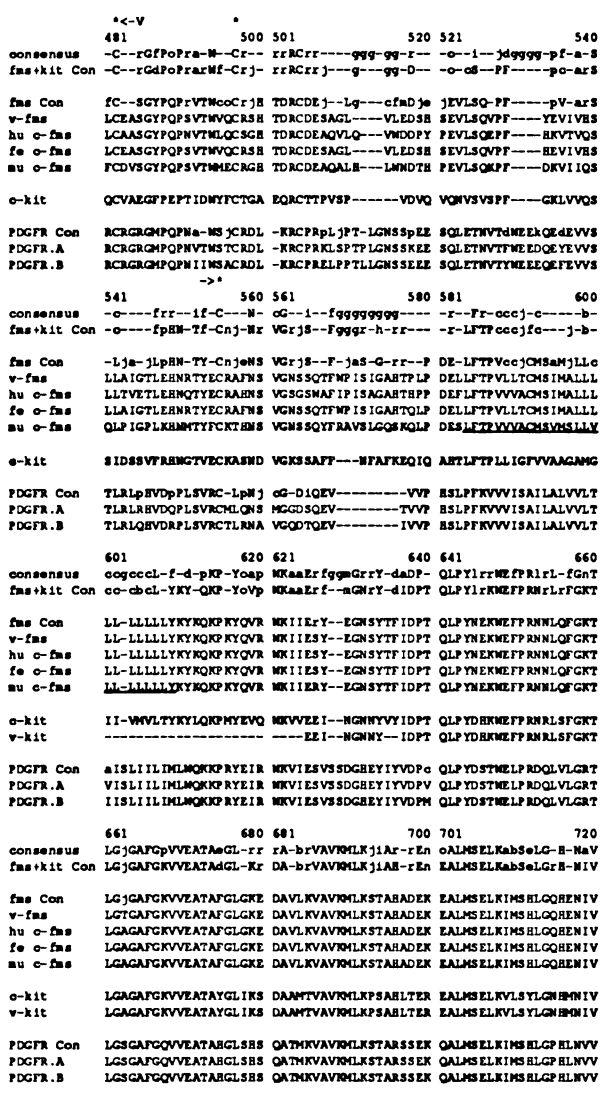
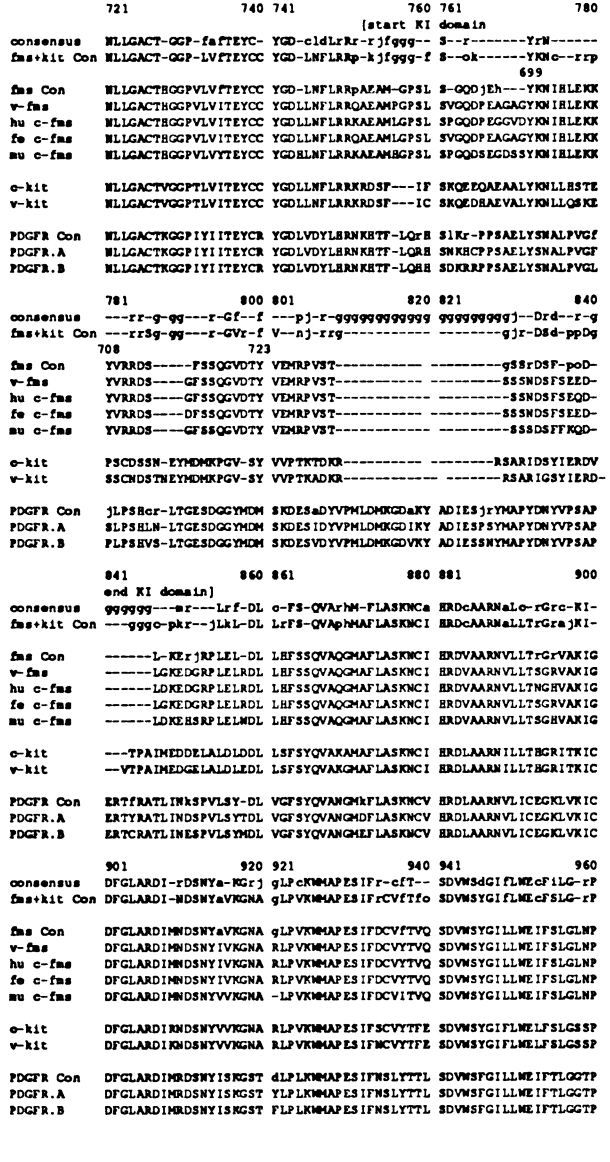

A
B

Figure II-7. Alignment of the PDGF receptor subfamily sequences. Four consensus sequences are shown: the overall subfamily consensus, the consensus for all *fms* genes, the consensus for *fms* and *kit*, and the PDGF receptor consensus. Conserved cystines in the external domain are marked with * and the Ig domains they define are shown by <-domain number->. The transmembrane region of the *fms* proteins is underlined. The boundaries of the kinase insert domain and the C-terminus of the kinase domain are also indicated.









```
                                                   [end of kinase domain]>
               961              980 981             1000 1001            1020
consensus      YP-c-clrpFYr-aKrGdpM -rP-eAj-mcYraMp-CW-- r---RPjFrQof-fcop---
fms+kit Con    YPGc-VlrKFYKbaKkGdpM -rP-eAP-mcYraMpjCW-- kP-rRPTFpQaf-fcopQ-r

fms Con        YPGI-VNrKFYKLVKDGYQM AQP-FAPKNIYSIMQjCW-L EPTrRPTFQQIC-fLQpQAp
v-fms          YPGILVNSKFYKLVKDGYQM AQPAFAPKNIYSIMQACWAL EPTRRPTFQQICSLLQKQAQ
hu c-fms       YPGILVNSKFYKLVKDGYQM AQPAFAPKNIYSIMQACWAL EPTHRPTFQQICSFLQEQAQ
fe c-fms       YPGILVNSKFYKLVKDGYQM AQPAFAPKNIYSIMQACWAL EPTRRPTFQQICSLLQKQAQ
mu c-fms       YPGIHVNNKFYKLVKDGYQM AQPVFAPKNIYSIMQSCWDL EPTRRPTFQQICFLLQEQAR

c-kit          YPGMPVDSKFYKMIKEGFRM VSPEHAPAEMYDVMKTCWDA DPLKRPTFKQVVQLIEKQIS
v-kit          YPGMPVDSKFYKMIKEGFRM LSPEHAPAEMYDIMKTCWDA DPLKRPTFKQIVQLIEKQKN

PDGFR Con      YPELPMNkQFYNAIKRGYRM AQPAHASDEIYEIMQKCWEE KFE-RPPFSQLVLLLERLLG
PDGFR.A        YPELPMNDQFYNAIKRGYRM AQPAHASDEIYEIMQKCWEE KFETRPPFSQLVLLLERLLG
PDGFR.B        YPELPMNEQFYNAIKRGYRM AQPAHASDEIYEIMQKCWEE KFEIRPPFSQLVLLLERLLG

               1021            1040 1041            1060 1061            1080
consensus      gk-----Y-rc-r---r
fms+kit Con    gkrr---YjNLjr---r

fms Con        -kRR--lYjNLPSSj-S
v-fms          EDRRVPNYTNLPSSSSSRLL RPMRGPPL
hu c-fms       EDRRERDYTNLPSSS----- -----RSGGSGSSSSELEEE SSSEHLTCCEQGDIAQPLLQ
fe c-fms       EDRRVPNYTNLPSSSSSSSS SSSSCRTGSGGGSSSEPEEE SSSEHLACCEQGDIAQPLLQ
mu c-fms       LERRDQDYANLPSSGGSSGS DS---GGGSSGGSSSEPEEE SSSEHLACCEPGDIAQPLLQ

c-kit          DSTKHIYSNLANCNPNPENP VVVDHSVRVNSVGSSASSTQ PLLVHEDA
v-kit          KNE

PDGFR Con      EGYKKKYQQVDEEFLRSDHP AILRSQARfPGfHjLRSPLD TSSVLYTAVQPNEjDNDYII
PDGFR.A        EGYKKKYQQVDEEFLRSDHP AILRSQARFPGIHSLRSPLD TSSVLYTAVQPNESDNDYII
PDGFR.B        EGYKKKYQQVDEEFLRSDHP AILRSQARLPGFHGLRSPLD TSSVLYTAVQPNEGDNDYII

               1081            1100 1101            1120 1121            1140
hu c-fms       PNNYQFC
fe c-fms       PNNYQFC
mu c-fms       PNNYQFC

PDGFR Con      PLPDPKPkVADEG--EGSPS LASSTLNEVNTSSTISCDSP LE-QkEPogggggggQ-EPE
PDGFR.A        PLPDPKPDVADEGLPEGSPS LASSTLNEVNTSSTISCDSP LELQEEPQ-------QAEPE
PDGFR.B        PLPDPKPEVADEGPLEGSPS LASSTLNEVNTSSTISCDSP LEPQDEPEPEPQLELQVEPE

               1141            1160 1161
PDGFR Con      joLEQ--DSGCPhP-AEAED SFL
PDGFR.A        AQLEQPQDSGCPGPLAEAED SFL
PDGFR.B        PELEQLPDSGCPAPRAEAED SFL
```

flexibility in those portions of the protein not directly involved in ligand recognition. The extracellular domain contains several potential sites of N-linked glycosylation, but no cysteine-rich stretch similar to those noted in the extracellular domains of the EGF, Insulin, LDL and other receptors is found in c-*fms*. Instead, the extracellular domain can be divided into five domains of feeble homology which are discernibly related to Ig repeats (Williams and Barclay, 1988). Similar motifs have since been found in the PDGF receptors, *kit* and the FGF receptors, as well as the receptor for interleukin 1 (see Ullrich and Schlessinger, 1990). The manner in which these domains create a ligand binding site is unclear. The v-*fms* protein is produced as a *gag-fms* fusion (Barbacid et al. 1980; Ruscetti et al. 1980). Approximately 30 amino acids lie between the end of *gag* homology and the proposed start of c-*fms*. These appear to be derived from the 5' untranslated region of c-*fms* as they are homologous to the 5' untranslated region of the sequenced cDNA.

Structure of Cytoplasmic Domain

The cytoplasmic domain of c-*fms* is approximately 95% homologous to v-*fms*, save for an unrelated C-terminus, and contains a tyrosine kinase domain. Extensive homology among the family of protein tyrosine kinases has been defined, most completely by Hanks et al. (1988). By their alignment, the *fms* kinase domain is separated from the inner face of the plasma membrane by a fifty amino acid juxtamembrane domain, spans the following 322 amino acids, and is followed by a 62aa tail. The kinase domain has all the hallmarks of

a tyrosine kinase, including AAR at positions 780-782, KWMAPE at positions 820-825 and a tyrosine analogous to the major autophosphorylation site of *src* (Tyr-416) and other PTKs, at position 809. A distinctive feature is the presence of a stretch of approximately 71aa between the domains Hanks et al. call V and VI. Termed a 'kinase insert' (KI) domain, this helps to distinguish a subclass of tyrosine kinases, as it is found only in the PDGF receptors, CSF-1 receptor and the *kit* gene. Much smaller inserts are seen in the fibroblast growth factor receptors (Ruta et al. 1989; Pasquale and Singer, 1989). The KI domain may mediate substrate interaction or perform some regulatory function. There are no other particularly remarkable features of the *fms* kinase domain, though it should be noted that unlike most PTKs, the *fms* analog of $srcY_{416}$ is not adjacent on its amino side to an acidic amino acid. The much tighter conservation between v- and c-*fms* of cytoplasmic than extracellular sequence may result from a requirement for the cytoplasmic domain to interact with a wide variety of substrates.

Juxtamembrane sequences have been shown to play a regulatory role in some receptor PTKs. Specifically, Thr-654 of the EGF receptor is known to become phosphorylated (presumably by C-kinase) in response to PDGF binding to the cell and this phosphorylation causes reduction in binding and kinase activities (Davis, 1988). No analogous consensus C kinase sites exist in *fms* and although C kinase does downregulate Fms this appears to occur by a proteolytic mechanism (Downing et al. 1989). The juxtamembrane domain is conserved among members of the PDGF receptor subfamily, suggesting

some important function.

The C-terminal tail of c-*fms* differs substantially from that of v-*fms*. The final forty amino acids of c-*fms* have been replaced by 11 unrelated amino acids in v-*fms* and within the deleted stretch is a Tyr at position 969. The v-*fms* C-terminal deletion might be linked to its transforming activity, or alternatively may be an incidental consequence of retroviral transduction. Deletion of actual or potential sites of tyrosine phosphorylation in their C-termini is an almost universal feature of tyrosine kinase oncogenes (Hunter, 1987). Such deletions are capable of activating the transforming capacity of *src* family oncogenes and affect the activity of the insulin and EGF receptors (McClain et al. 1987; Khazaie et al. 1988; Bertics et al. 1988). This region of c-*fms* is unrelated to the C-termini of even other members of the PDGF receptor subfamily, and distinguished by being extremely hydrophilic, containing many charged amino acids and stretches of serine residues. Comparison of the human, cat and mouse c-*fms* sequences shows substantial divergence in a serine-rich stretch that bisects the tail domain. This is followed by 34 amino acids which are almost absolutely conserved, suggesting that the serine-rich region may serve as a variable linker to a functionally significant terminus.

The feline c-*fms* cDNA was obtained and sequenced after this work (Woolford et al. 1988). Nine substitutions distinguish v-*fms* from its progenitor (see Fig. 7). Though the feline gene provides a much better indication of potential activating lesions than does the human c-*fms* gene, there were advantages to isolating the human gene.

A major one is the much more extensive knowledge existing concerning human growth factors, both in terms of identity and physiology. A second advantage was the availability of a good cDNA library, derived from a source known to express c-*fms*. Prior to the finding that c-*fms* was the gene for the CSF-1 receptor, or at least that it was expressed in monocytes, the only known source for a feline c-*fms* cDNA would have been a cat placenta library, construction of which posed practical obstacles.

Sequence alignment of the four characterized *fms* genes and their relatives *kit* and the two PDGF receptors reveals limited conservation of the extracellular region, amounting to little more than the fundamental Ig superfamily motifs. Interestingly the fourth Ig repeat is missing both cysteines in all these genes; either it was lost in some ancestral receptor gene or the fourth domain may play a distinctive role in ligand binding or transmembrane signal transduction in all these receptors. Cytoplasmic sequences are much more tightly conserved within this subfamily, with the exception of the KI domain and the C-terminal tail. Again the extensive conservation may reflect constraints imposed by substrate or regulatory interactions that distinguish this subfamily, or it may simply reflect evolutionary history. The latter possibility seems unlikely in view of the high degree of divergence of the KI and C-terminal domains, which vary dramatically. Little similarity can be seen in the C-termini, as even the two PDGF receptors and three CSF-1 receptors show divergence. The KI domains are conserved within CSF-1 receptor and PDGF receptor families but not between them. Some

conservation can also be discerned about CSF-1 receptor Tyr-699 and possibly Tyr-723.

Structure and Biological Activity

Clearly some structural differences between v-*fms* and human c-*fms* are critical for transforming activity. As has been pointed out the v-*fms* gene bears an obvious C-terminal truncation analogous to C-terminal mutations of *src* and *fps* that are capable of inducing transformation (Cooper, 1989). Although this is an obvious candidate for an activating lesion, it should be noted that a similar truncation does not inevitably confer ligand-independent transforming capacity on the EGF receptor gene (Velu et al. 1989).

A variety of other differences exists between v-*fms* and human c-*fms*, and while many of them are probably attributable to the evolutionary distance between human and cat proto-oncogenes, some may contribute to oncogenic activation. In this respect the choice of human c-*fms* as the proto-oncogene to study imposes limitations an our analysis, but it has attendant advantages in the availability of its ligand and some knowledge of the function of the human CSF-1 receptor.

CHAPTER III:

ACTIVATION OF THE TRANSFORMING ACTIVITY OF *c-fms*

ABSTRACT

The v-*fms* gene is an activated oncogene derived from the gene for the receptor for CSF-1. By determining the primary structure basis for activation of c-*fms* transforming potential it should be possible to learn something of how c-*fms* activity is regulated and how v-*fms* induces transformation. There are approximately 100 amino acid changes distinguishing v-*fms* of feline origin from the human c-*fms* gene, making it difficult to assign a role in activation to any particular substitution. I have employed two strategies in an attempt to activate c-*fms*. First, I have created both N- and C-terminal deletions and examined the transforming potential of these truncated genes. None of the alleles constructed was oncogenic. Second, I have taken advantage of the error-prone nature of retroviral replication to randomly mutate c-*fms*, and select for spontaneous transforming variants. None was isolated by this scheme and potential explanations are discussed.

INTRODUCTION

Analysis of the molecular basis of oncogene activation has provided insights into both the regulation of proto-oncogene products and the mechanism of transformation by their activated derivatives. For example, activation of c-*src* enhances its kinase activity and frequently involves loss of an *in vivo* tyrosine phosphorylation site, phosphorylation of which decreases Src kinase activity (see Cooper, 1989).

The mechanism of activation of receptor tyrosine kinases is less well understood than that of the SRC family. The v-*erb*B, *kit* and *fms* proteins all show truncations of their normal C-termini (Downward et al. 1984a; Besmer et al. 1986; Qui et al. 1988; Hampe et al. 1984; Coussens et al. 1986; see ch.II of this work). In the case of v-*erb*B this truncation removes the major sites of EGF receptor tyrosine phosphorylation (Downward et al. 1984b). Truncation of the EGF receptor C-terminus is not, however, necessary (Nilsen et al. 1985) nor does it necessarily contribute to the transforming activity of the activated EGF receptor in all contexts (Velu et al. 1989). Many receptor PTK oncogenes do not have C-terminal alterations, and in fact other types of activating lesions appear quite frequently. The *neu* oncogene has a single point mutation in its transmembrane domain (Schechter et al. 1985; Bargmann et al. 1986a,b), while several others have truncated N-termini, including *trk*, *met*, *kit*, *ros*, and v-*erb*B (Martin-Zanca et al. 1986,1989; Park et al. 1987; Qui et al. 1988; Neckameyer et al. 1986; Downward et al. 1984a).

An understanding of the mechanism of PTK receptor activation should cast light on several diverse questions. First of these concerns the relationship of transforming function to normal proto-oncogene function. Does *fms* activation simply provide constitutive signals identical to those of its normal progenitor, or does it interact with novel substrates? Such a question is difficult to address with non-receptor PTKs as there is no good method for stimulating the normal activity of these proteins. Ligand stimulation of a receptor allows the changes in the overall pattern

of tyrosine phosphorylation and the phosphorylation state of individual potential substrates that occur as a primary result of PTK stimulation to be identified, and compared in turn with the changes that occur in the presence of an activated oncogene. Identification of FMS substrates involved in transformation should be facilitated by the limited changes in total cellular phosphotyrosine induced by *fms* transformation (Barbacid and Lauver, 1981; Morrison et al. 1988). This is in sharp contrast to *src*, which causes a dramatic increase in total phosphotyrosine (Sefton et al. 1980) and increases the tyrosine phosphorylation of a large number of different proteins (Cooper and Hunter, 1983).

Another question of great interest concerns the mechanism by which binding of ligand to the extracellular domain of a receptor produces an effect within the cytoplasm. To the extent to which oncogenic forms of *fms* mimic ligand-activated CSF-1 receptor, activating mutations should identify structures involved in transmembrane signalling by the CSF-1 receptor and provide a model for biochemical and structural studies of its mechanism.

To study the primary structure basis of oncogenic activation of *fms*, deletion mutations were constructed *in vitro*. Since many activated PTKs have C-terminal truncations, including v-*fms*, such a mutation was made in c-*fms*. N-terminal truncation is another commonly observed lesion in receptor PTK oncogenes, so two internal deletions of the extracellular domain were constructed and their transforming activity examined either alone or in concert with C-terminal truncation. An attempt was also make to generate additional

activated alleles of the *fms* gene by random mutagenesis.

RESULTS

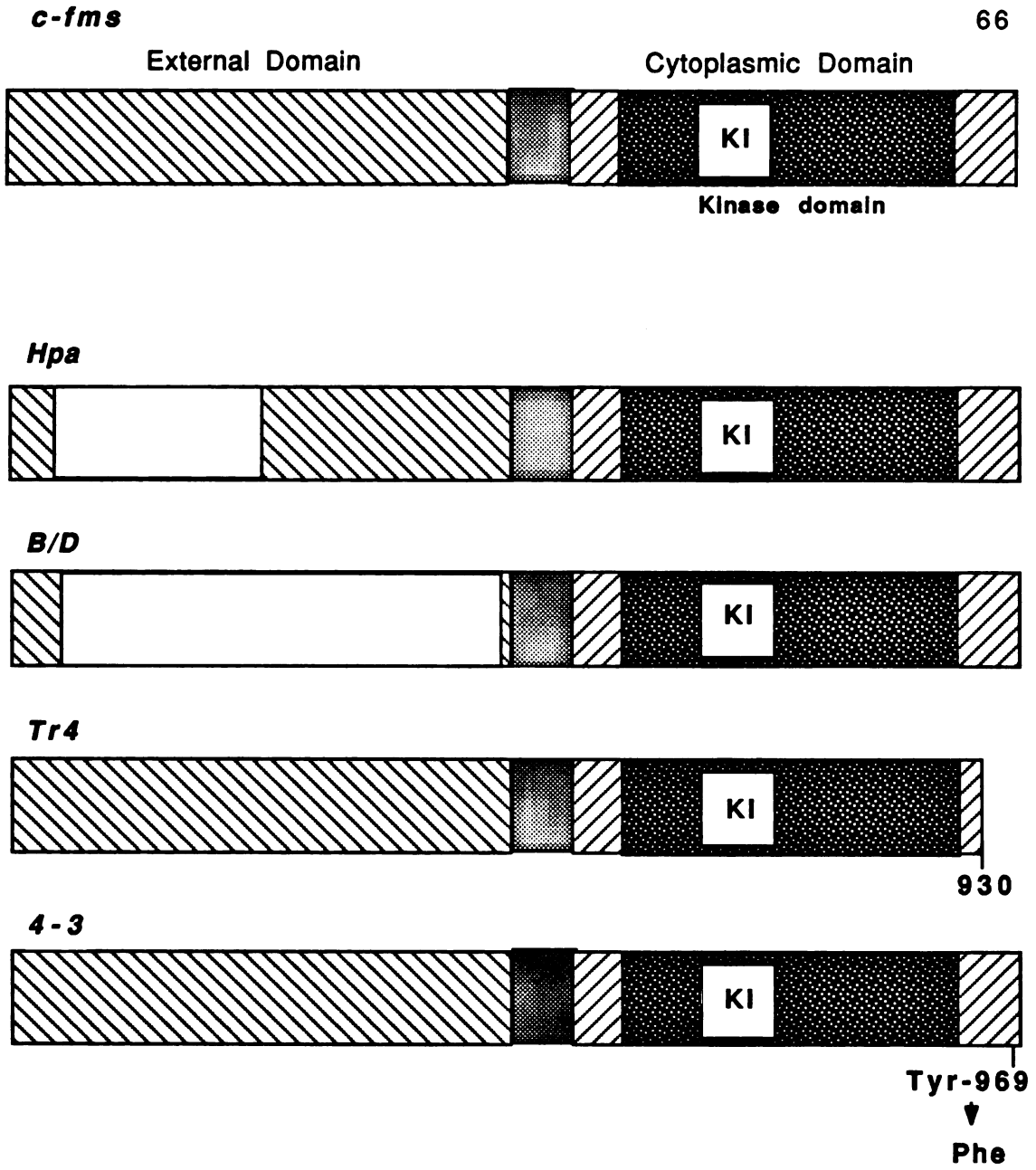
Construction of Deletion Mutants

Deletions of the extracellular domain of c-*fms* were constructed that lacked either amino acids 38-231 or 42-464 (see Fig. 1). Mutant *Hpa* was constructed by digesting c-*fms* with BstEII and removing the 5' extension with Mung Bean exonuclease, then digesting with XhoI. A HpaI-XhoI fragment was then inserted into this fragment to create a deletion of amino acids 38-231. Mutant *B/D* was constructed by digesting a c-*fms* clone with BstEII and DraIII, then using an adaptor to join the two ends, creating a deletion of amino acids 42-464. Examination of Ig domains in the c-*fms* extracellular domain indicates that *Hpa* removes the Cys residues of the first two domains and one from the third domain. Mutant *B/D* removes all but the final Cys of the fifth domain.

Mutations affecting the C-terminus of c-*fms* were constructed by oligonucleotide site-directed mutagenesis. Mutant *Tr4* introduced a UGA termination codon in place of Arg-931. This is the point at which v- and c-*fms* diverge. Mutant *4-3* replaces Tyr-969 with Phe. Tyr-969 is the only potential site of tyrosine phosphorylation that lies within the region of C-terminal divergence between v- and c-*fms*.

A total of four double mutants were constructed with both C- and N-terminal alterations. *B/DTr4* and *B/D4-3* contain both the B/D deletion and either a truncated or mutated C-terminus. *HpaTr4* and *Hpa4-3* contain similar pairs of mutations.

Figure III-1. Structures of N- and C-terminal mutants. The locations of the external, transmembrane, cytoplasmic, kinase and kinase insert ('KI') domains are shown schematically. Only the transmembrane domain is not shown to scale. Locations of deletions in *Hpa* and *B/D* are shown by blank boxes. The lesions in *Tr4* (a truncation at 930) and *4-3* (a Tyr-969 to Phe substitution) are indicated.


c-fms
External Domain
Cytoplasmic Domain
KI
Kinase domain
Hpa
KI
B/D
KI
Tr4
KI
930
4-3
KI
Tyr-969
Phe

Expression of c-fms Mutants

All eight mutants were cloned into both an SP6 vector for *in vitro* translation and into pMX1112neo for expression in mammalian cells. SP6 constructs were transcribed and translated *in vitro* and the [^{35}S]-Met-labelled translation products separated by SDS-PAGE. The products (Fig.2) of the various mutants were of the expected molecular weights, confirming both the identities of the clones and that no dramatic structural alterations had occurred during their manipulation. Junction fragments and the region containing the Tyr-969 codon were sequenced as additional confirmation.

Viral stocks were produced by transfection of pMX1112neo constructs into Psi-2 cells followed by drug selection and harvesting of tissue culture supernatants. 3T3 cells were infected with Psi-2 virus and split for neomycin resistance and focus formation assays. None of the mutants showed transforming activity.

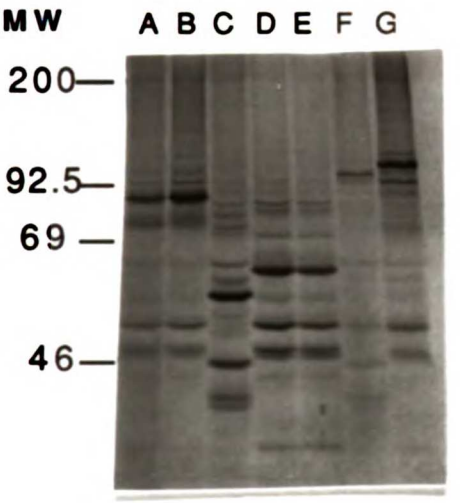
Expression of mutant constructs in mammalian cells was tested by immunoprecipitation. Neomycin resistant pools of cells were lysed and immunoprecipitated with FMS-specific antisera. Immune-complex kinase assays were performed, and the products displayed by SDS-PAGE and autoradiography. Mutants containing the *Hpa* deletion produced no active *fms* protein. All other mutants demonstrated total cellular kinase activity very similar to *wt* FMS.

Random Mutagenesis

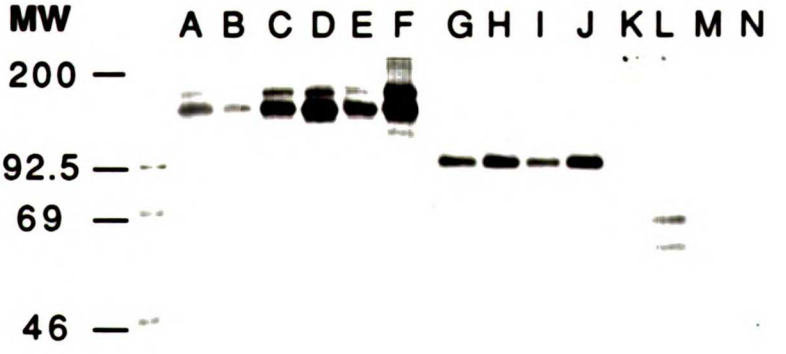
In an attempt to isolate novel activated alleles of *fms* a random mutagenesis scheme was employed. Replication of retroviruses

Figure III-2. Protein products of *fms* mutants. (A) Autoradiograph of [^{35}S] Met-labelled proteins produced by *in vitro* transcription and translation of mutant clones. Identities of clones are indicated above each lane. A is *B/D*; B is *B/D4-3*; C is *HpaTr4*; D is *Hpa4-3*; E is *Hpa*; F is *Tr4*; G is *4-3*. (B) Immune complex kinase autophosphorylation products of mutants. Clonal cell lines expressing mutants were lysed, immunoprecipitated with anti-FMS sera, autophosphorylated and separated by SDS PAGE prior to autoradiography. Lanes (A-F) are *4-3*; lanes (G-H) are *B/D*, and lanes (K-N) are *Hpa*.

A



B



is an error prone process, with a mutation frequency estimated at 10^{-3} to 10^{-5} per site per generation (Gopinathan et al. 1979; Coffin et al. 1980; Dougherty and Temin, 1988; Leider et al. 1988). To take advantage of this process as a mutagen, cells expressing either c-*fms* or *Tr4* were cocultivated with an MLV producer line to produce a replication competent stock of *fms* virus. $6x10^5$ cells containing a 5 to 1 ratio of *fms*-expressing to MLV producer cells were plated in 100mm dishes and grown 2 days, until just subconfluent. Cell culture supernatants were then harvested and used to infect NIH 3T3 cells.

Cells infected with replication-competent *fms* viral stocks were subject to either focus assay or neomycin selection. Drug resistant cells were used as a source for additional *fms* virus, either by harvesting cell culture supernatants or by cocultivation with fresh 3T3 cells. In either case cells infected with these new stocks were assayed for focus formation.

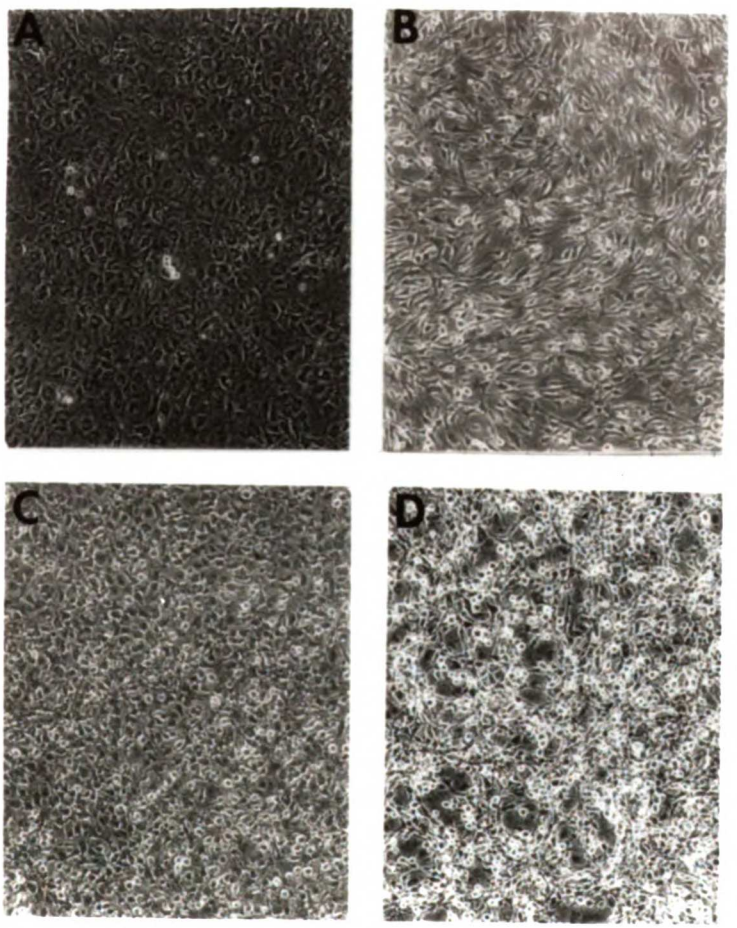
It is difficult to assess the number of viral replications that occurred during the process of passing virus. Infection with culture supernatants produced neo titers in the range of $1.5x10^3$-$4.0x10^4$. This probably underestimates the number of infections in the focus assay as cells not infected by the initial viral inoculum would be subject to infection by virus produced by new producer cells in focus assay, but not in neo selection. The infection of naive cells by co-cultivation with cells producing *fms* virus cannot be easily scored by neo assay due to the large number of neo resistant cells initially present as producers. All cells in cocultivation assays are likely to become infected with either *fms* or helper virus and since the

ratio of these two should be little different than in cell culture supernatants the number of *fms* virus infections obtained by cocultivation should be at least as high as that observed by infection with culture supernatants.

Foci appeared on focus assay plates at a frequency of roughly one focus per four plates. There was no obvious difference in frequency of focus formation between uninfected control cells and cells infected with *fms* viral stocks. Foci were picked from plates in cloning cylinders and expanded under neo selection. Supernatants were harvested from cells with apparently altered morphology (see Fig. 3) and titered for neo resistance and focus formation.

In total, 209 plates were infected with culture supernatant and 86 plates were co-cultivated; each plate was split 10:1 for focus assay. From these plates 51 foci were picked, of which 10 grew under neo selection and showed altered morphology. On infection of naive 3T3 cells with virus harvested from isolated foci, none of these viral stocks gave focus formation activities greater than 0.4% of their neo titer. This frequency of focus formation was only marginally greater than background, indicating one of three possibilities: (1) cells were transformed by mutation of cellular genes; (2) cells were transformed by a mutation of the *fms* gene but produced no virus containing the transforming allele, or (3) cells contained a mixed population of *fms* proviruses, with only a fraction of the neo viruses coding for activated *fms* alleles. The first possibility is difficult to exclude or address. As the initial frequency of focus formation is very close to background, many foci

Figure III-3. Morphology of transformed clones isolated by random c-*fms* activation protocol. (A): fibroblasts infected with a control virus. (B-D): clones shown were picked from focus assay plates, then expanded under neo selection.


A
B
C
D

are likely to fall into this class. If the second possibility is true, it is probably not due to lack of helper virus as prolonged culture in the presence of MLV helper should have ensured nearly complete infection, so any provirus containing an activated *fms* gene must lack some sequence required in *cis* for packaging. In the third case, transforming titer could be enhanced by biological cloning of the initial focus. Biological cloning was performed with all 10 promising primary foci by plating at low density on plastic and picking 12 well separated colonies for expansion. Of these, virus was harvested from subclones showing altered morphology and used to infect 3T3 cells. None showed enhanced focus/neo titer ratios.

DISCUSSION

Site Directed Mutants

Two types of site directed mutants were examined, N-terminal deletions and C-terminal alterations. C-terminal alterations were inspired by the observation that the C-termini of activated alleles of PTK oncogenes are frequently mutated (see Hunter, 1989). Indeed, Browning et al. (1986) had observed that replacement of the C-terminus of v-*fms* with that of c-*fms* diminished transforming activity by 98% when sequences 3′ of the BglI site (codon 894 of c-*fms*) are exchanged. Although this lesion apparently contributes to transforming activity it is clearly not sufficient. No transformation was seen by either truncated c-*fms* or c-*fms* with Tyr-969 replaced by Phe, a mutation that restores full transforming activity to a v-*fms* N-terminus/c-*fms* C-terminus chimera (Roussel et al. 1987).

N-terminal deletions are also frequently observed in activated alleles of receptor PTKs (Hunter, 1989). They are sufficient to activate the erythroblastosis-inducing potential of erbB (Nilsen et al. 1985; Gamett et al. 1986). Although two different deletions were constructed in the extracellular domain of c-*fms*, the failure of one (*Hpa*) to produce kinase-active protein sufficiently explains its failure to transform. The *B/D* allele lacks almost the entire extracellular domain of c-*fms* and although infected cells contain kinase active FMS at levels comparable to those observed in v-*fms* transformants, they are not transformed. Fusions have been constructed linking the extracellular domain of CD2 to the transmembrane and intracellular domains of *fms* (Roussel et al. 1990) and these are also non-transforming, and in fact non-mitogenic in the absence of crosslinking by anti-CD2 antibodies. Though it is possible that some other deletions(s) of the extracellular domain of FMS are transforming there must be significant constraints on activation by this mechanism. The c-*erb*B and *neu* genes, in contrast, can be activated by a wide variety of extracellular domain deletions (see Haley et al. 1989), and *erb*B can even tolerate removal of the entire extracellular domain (Bassiri and Privalsky, 1986). While it is obviously impossible to deduce that any large deletion is sufficient to activate the transforming activity of the EGF receptor family, the wide latitude observed in N-terminal deletions sufficient to activate the EGF receptor family indicates that it probably differs in this respect from the PDGF receptor family.

Further evidence for a distinction between these two families

comes from experiments attempting to induce mitogenic activity with anti-receptor antibodies. Antibodies to the external domain of the EGF receptor are capable of inducing mitogenesis (Yarden and Schlessinger, 1987; Gill et al. 1984). No antibody has been found that is capable of inducing CSF-1 receptor activity (Roussel et al. 1990). The dichotomy of behavior of these two receptors suggests that the mechanism by which ligand induces receptor activation may be quite different for these two receptor classes.

High concentrations of EGF will transform fibroblasts expressing an abundance of EGF receptors, indicating that *erbB* may transform cells by mimicking a constitutively activated receptor (Velu et al. 1987; DiFiore et al. 1987). Extensive study of the EGF receptor has indicated that receptor dimerization triggered by ligand binding is the critical event that activates receptor PTK activity (Yarden and Ullrich, 1988). Dimerization is thought to promote intermolecular receptor phosphorylation and all the other intracellular consequences of receptor activation. There is no evidence that *erbB* protein resembles activated EGF receptor in being constitutively dimerized, but this is the state of the oncogenically activated *neu* protein (Weiner et al. 1989). Dimerization is also thought to be a critical step in transduction of signals across the plasma membrane by the CSF-1 receptor. No direct evidence has been obtained that this is the case, but ligand stimulation induces intermolecular receptor phosphorylation (Ohtsuka et al. 1990) and as cited above antibody crosslinking of receptor-CD2 chimera induces mitogenesis. Studies of the closely related PDGF receptor have

demonstrated ligand-stimulated dimerization (Heldin et al. 1989). EGF receptor dimerization may be actively inhibited by its extracellular domain; ligand binding induces a conformational change (Greenfield et al. 1989) which might eliminate this obstruction. Substantial truncations might therefore activate the EGF receptor by allowing constitutive dimerization and stimulation of pathways normally activated only by ligand binding. Both the ligands and the ligand binding domains of the PDGF receptor family differ radically from those of the EGF receptor family. Significantly, both CSF-1 and PDGF are active as dimers and may therefore bind the two receptors simultaneously to cause dimerization. This distinction between the EGF receptor and PDGF receptor families could account for the failure of a truncation of the extracellular domain of c-*fms* to activate its transforming capacity.

The mutations responsible for v-*fms* transforming activity have been identified. Conclusive determination of the critical mutations required the sequence of the feline c-*fms* gene (Woolford et al. 1988). Mutation of Leu-301 is certainly an essential feature, and a mutation of Ala-374 is also necessary in at least some assay systems (Woolford et al. 1988; Roussel et al. 1988). In both these studies the C-terminal truncation was found to contribute to efficient transformation as expected from the work of Browning et al. (1986) and Roussel et al. (1987). There is no direct evidence that activated FMS exists as a dimer, but constitutive ligand stimulation of normal CSF-1 receptor expressed in 3T3 cells is sufficient to induce a transformed phenotype (Roussel et al. 1987; Rohrschneider et

al. 1989) and thus the simplest model is that oncogenically activated FMS resembles the ligand stimulated CSF-1 receptor and exists as a dimer. CSF-1 receptor dimerization is unlikely to consist simply of proximity resulting from ligand interactions with two receptors simultaneously, since monoclonal antibodies to the extracellular domain of the CSF-1 receptor fail to duplicate the activity of CSF-1. Some conformational change in the extracellular domain of the receptor must occur in the process of ligand binding or ligand induced dimerization. Both amino acids 301 and 374 lie within the fourth Ig domain of FMS, which is also the only domain lacking Cys residues. These two distinctions suggest some special role for the fourth domain, perhaps in mediating intermolecular interactions within a receptor dimer or inducing a conformation of the other domains that stabilizes receptor interactions.

Random Mutagenesis

The random mutagenesis strategy was motivated in large part by the finding of Iba et al. (1984) that non-transforming alleles of *src* are frequently activated in the process of expanding viral stocks. Similar findings of frequent activation of transforming activity have been obtained with *rel* (Miller et al. 1988) and *trk* (Oskam et al. 1988). In all these cases it is difficult to determine precisely the rate of activation. Iba and coworkers transfected non-transforming alleles of *src* in RSV vectors onto CEF cells and harvested virus after nine days of viral spread. Transforming titres from the resultant viral stock were approximately 10^{-4} those obtained with transforming alleles. Transfection of NIH 3T3 cells with non-

transforming alleles of *trk* leads to transformation of 10^{-3}-10^{-4} of transfected cells. Introduction of non-transforming REV-T virus into cells causes transformation with about 2% the efficiency of parental REV-T, and each transformed focus produces high-efficiency transforming virus.

The mechanism of oncogene activation is quite distinct in these three cases. Activated alleles of *src* have sustained one or two point mutations (Levy et al. 1986), as have activated alleles of *fyn* (a member of the *src* family) generated by a similar protocol (Kawakami et al. 1988). Analysis of *trk* oncogenes has inevitably shown alteration (generally deletion) of the extracellular domain of TRK), often accompanied by fusion to unrelated sequences (Coulier et al. 1990; Oskam et al. 1988). The *rel* gene is activated not by mutation of coding sequences but by increased expression of spliced *rel* mRNA, generally resulting from small deletions.

As a result of the variety of activation mechanisms, observations of the rate of *rel*, *trk* and *src* activation cannot all be applied to *fms*. *Rel* transformation is unlike *fms* in resulting from overexpression. *Trk* activation was observed to involve large deletions, frequently with the remaining sequence fused to a portion of another gene, in all but a single instance. This might be result of the transfection protocol employed but in any case distinguishes it from the experiment described here. The *src* and *fyn* results are quite similar in principal, but produced transforming alleles in both cases at a frequency of 10^{-3}-10^{-4}.

In the *fms* activation experiments described here somewhere on

the order of 10^6 events were examined. If *fms* were as easily activated as *src* or *fyn* the expected yield of transforming alleles would be 10^2-10^3, so either *fms* is subject to oncogenic activation by a much more limited spectrum of lesions or the protocol employed here yields far fewer mutations than expected. *Src* has been oncogenically activated by a number of distinct lesions (see Cooper, 1989). In particular, almost any mutation that disrupts phosphorylation of Tyr-527 seems to be sufficient for activation, so nonsense mutations between the end of the kinase domain and amino acid 527 would be expected to be activating, providing a fairly large target for activating mutation. Since only a single activated allele of *fms* has been examined, it is impossible to make any firm estimate of its target size for activating mutation. Analysis of HZ-5 (Besmer et al. 1986), a second activated allele of *fms*, would be interesting in this regard. It is possible that Leu-301 must be mutated in order to activate *fms* transforming activity, which would constitute an exceedingly limited target for mutagenic activation of *fms*.

Even with a target size as small as a single codon one might have expected to obtain an activated allele of *fms* by this procedure. Calculation of the mutation rate of an SNV vector *in vivo* yielded an estimate of 2×10^{-5} per base pair per generation (Dougherty and Temin, 1988), versus 1.4×10^{-4} per nucleotide for an RSV vector (Lerder et al. 1988). These rates are in rough agreement with those determined *in vitro* using purified reverse transcriptase (Riccetti and Buc, 1990). Calculation of mutation rates at a single specific site is complicated by a huge variation in mutation rate dependent on

sequence context, as Ricchetti and Buc found frequencies ranging from 10^{-2}-10^{-6} depending on the test template. They also found substantial differences between polymerases, up to 100 fold between HIV-1 and AMV reverse transcriptase on the same template. The enzyme relevant to *fms* activation, MoMLV RT, was also tested but found to have a K_m for nucleotides two logs greater than the other two enzymes, which raised the limit for detection of mutation by two logs. Taken together this all indicates an expected frequency of mutation at a single site of roughly 10^{-4}-10^{-5}, but possibly ten fold lower or higher.

Though mutations are predicted to occur at a frequency predicted to produce 10 to 30 mutagenic events at codon 301 during the course of these experiments, only those mutations which produced an activated allele would have been detected. Judging from the experiments of Roussel et al. (1990) the set of activating amino acid substitutions might be Ser, Thr, Asp, Glu, Pro which would be produced by only 1 of 9 single base pair mutations at codon 301. Six possible mutants are not transforming, and two others (Val and Trp) seem unlikely to transform on structural grounds. A further limitation of detection is the ability of activated c-*fms* to form a focus. Since an MLV v-*fms* construct forms foci at 25% the frequency of SM-FeSV (Roussel et al. 1987), and alleles activated by changes at 301 and 969 form foci 10-50% as efficiently as v-*fms*, only 2-12% of activated alleles infecting a single cell might be detected in this assay. Thus, given pessimistic assumptions, a survey of 10^6 events might be one to three orders of magnitude below the number of events

required to obtain a single *de novo* activated allele, if mutation of Leu-301 is required.

All that can be clearly deduced from the failure to isolate new activated alleles of *fms* is that the target size for activating mutations cannot be extensive. Additional alleles would still be useful even now that the activating lesions of v-*fms* have been identified. The simplest way of identifying one would be to sequence the *fms* gene of HZ-5. The strategy described here is still an attractive one for the generation of novel alleles. Its main limitations are the limited number of infections that occur per plate (because of the low titer of *fms* virus) and the resulting high ratio of background cellular transforming events to *fms* transformants. A higher viral titer would not only ease the primary focus screen but also dramatically reduce the number of foci resulting from spontaneous transformation due to mutation of cellular genes, relative to those resulting from *fms* mutation, and thus reducing the number of secondary screens required to identify activated alleles of *fms*.

CHAPTER IV:

AUTOPHOSPHORYLATION SITES OF THE c-*fms* PROTEIN

ABSTRACT

A hallmark of protein tyrosine kinases is their ability to autophosphorylate. In many cases this has been shown to affect both the biochemical and biological activities of the protein. The CSF-1 receptor is phosphorylated on tyrosine in response to ligand and its activated homolog is constitutively tyrosine phosphorylated. Tryptic phosphopeptide mapping indicates that receptor autophosphorylation can occur at a number of sites. To examine the regulatory function of CSF-1 receptor autophosphorylation I have identified two major sites, Tyr-699 and Tyr-708. Both of these sites lie within the kinase insert (KI) domain of the protein, a region shown to be essential for the mitogenic activity of the closely related PDGF receptor.

INTRODUCTION

A major regulatory role for tyrosine phosphorylation of PTKs was first indicated by the finding that major sites are frequently absent from the transforming alleles of PTK oncogenes (Hunter, 1987). In various proteins tyrosine phosphorylation has been shown to affect the transforming capacity (Piwinca-Worms et al. 1987; Snyder and Bishop, 1984), kinase activity (Rosen et al. 1983; Piwnica-Worms et al. 1987), substrate association (Kazlauskas and Cooper, 1989) and downmodulation (Ohtsuka et al. 1990) of proteins. Although study of

the regulatory role of autophosphorylation has been complicated by a paucity of identified physiologically significant substrates for most PTKs (see Kamps and Sefton, 1988), knowledge of tyrosine phosphorylation sites offers a window through which important regulators (Okada and Nadagawa, 1989) and substrates (Kazlauskas and Cooper, 1989) can be viewed.

The most extensive studies of the regulatory role of autophosphorylation have been conducted with *src* and the Insulin receptor. Both v- and c-*src* autophosphorylate predominantly on Tyr 416 *in vitro*, while *in vivo* nontransforming alleles are phosphorylated at Tyr-527 and transforming alleles at Tyr-416 (Smart et al. 1981; Patschinsky et al. 1982; Cooper et al. 1986). The phosphorylation on Tyr-416 appears to enhance both the kinase and transforming activities of Src (Kmiecik and Shalloway, 1987; Piwnica-Worms et al. 1987; Snyder and Bishop, 1984), while Tyr-527 phosphorylation reduces them (Kmiecik and Shalloway, 1987; Piwnica-Worms et al. 1987; Courtneidge, 1985; Cooper and King, 1986). Phosphorylation of the two sites appears to be largely exclusive (Kmiecik and Shalloway, 1987), and thus phosphorylation of either site can be seen not only to mediate direct biochemical and biological effects but also to repress the effects of modification at the alternative site.

Autophosphorylation of the insulin receptor also affects its kinase activity (Herrera et al. 1985; Cobb et al. 1989; Flores-Riveros et al. 1989). Phosphorylation of the receptor on several sites is induced by binding of its ligand (Kasuga et al. 1982; White

et al. 1984; Petruzzelli et al. 1984), probably by an intramolecular reaction. The net effect on specific activity is greater than 100 fold (Cobb et al. 1989). Mutation of Tyr-1150 and -1151 results in a loss of insulin-stimulated glycogen synthesis, while Tyr-1146 mutants lose mitogenic activity. Both mutants have reduced insulin-stimulated kinase activity.

A recent intriguing finding concerning a regulatory role for autophosphorylation was made with the PDGF receptor. Here the major site of ligand-stimulated autophosphorylation, Tyr-751, lies in the KI domain and is required for association with GAP and at least three other proteins. At least some of these are normally substrates for the activated PDGF receptor and thought to be critical for its mitogenic activity (Kazlauskas and Cooper, 1989; Kaplan et al. 1990).

The CSF-1 receptor becomes rapidly phosphorylated on tyrosine in response to ligand binding. This event is required for receptor downregulation (Downing et al. 1989). In order to examine this and other functions I have identified two major sites of *in vitro* CSF-1 receptor autophosphorylation.

RESULTS

Isolation of Phosphorylated Receptor

Genes for *wt* and mutant alleles of CSF-1 receptor were introduced into the Mo-MLV vector pMX1112. Viral stocks produced by transfected Psi-2 cells were harvested and used to establish either pools or clonal cell lines expressing the desired gene. To facilitate protein analysis, high expressers of *fms* were selected

either by screening clonal cell lines or FACS sorting of pooled cells.

Following cell lysis, cleared lysates were immunoprecipitated with anti-FMS antibodies and phosphorylated *in vitro* in an immune-complex kinase assay. Labelled proteins were separated on SDS gels and visualized by autoradiography (Fig.1). The CSF-1 phosphorylated receptor was excised from gels and extracted.

Peptide Mapping

Phosphorylated proteins were digested with either TPCK-trypsin or V8 protease, then resolved on cellulose TLC plates by electrophoresis in pH 1.9 buffer followed by ascending chromatography in the opposite dimension. Phosphoamino acid analysis of FMS labelled in immune complex kinase assays detected tyrosine as the only labelled amino acid (data not shown), so all labelled peptides must contain P-Tyr. Tryptic digestion of *wt* FMS produced three consistent major spots in this system (labelled A,B and C; Fig. 2A). Several other minor spots were also observed, some of which varied in intensity between experiments. Digestion with V8 protease produced four major phosphopeptides (D,E,F and G; Fig. 2B).

The relationships among these sites were examined by purifying labelled peptides and redigesting with a second protease (Fig. 3). Tryp A could not be digested with V8 protease, while tryp B and C were both cleaved by V8 to produce spots with identical mobilities. Digestion of V8 spot D with trypsin produced a peptide with the same mobility as tryp spots B and C redigested with V8.

Figure IV-1. Starting material for determination of FMS autophosphorylation sites. *fms* protein was isolated from high expressing fibroblasts. After precipitation with anti-FMS antisera and labelling with [^{32}P] ATP the protein was isolated on SDS PAGE gels.

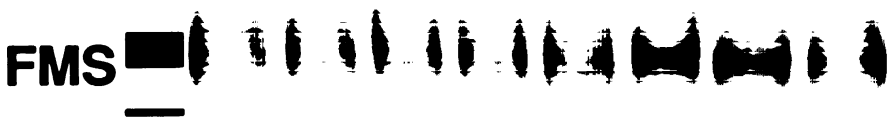
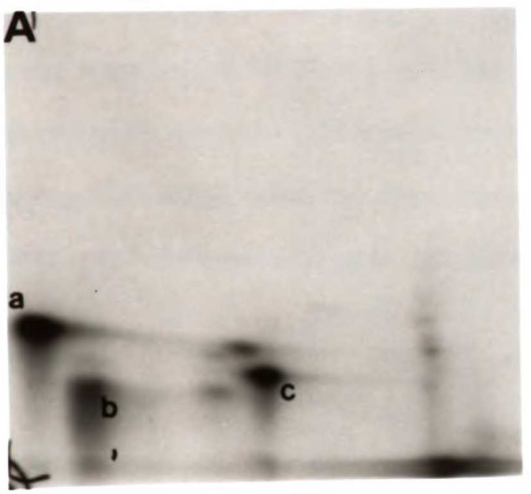

FMS

Figure IV-2. Two-dimensional peptide maps of FMS. Electrophoresis was in the horizontal dimension; chromatography in the vertical dimension. (A) Tryptic peptide map of autophosphorylated FMS. Spots A, B and C are labelled. (B) Staph. V8 protease peptide map. Spots D, E, F and G are marked.


A
a
b
c

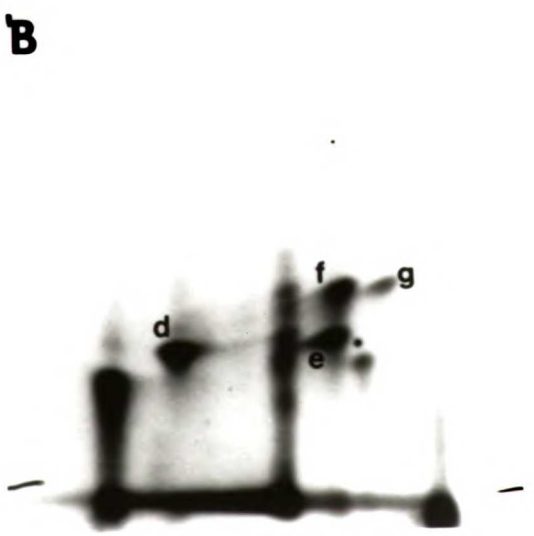
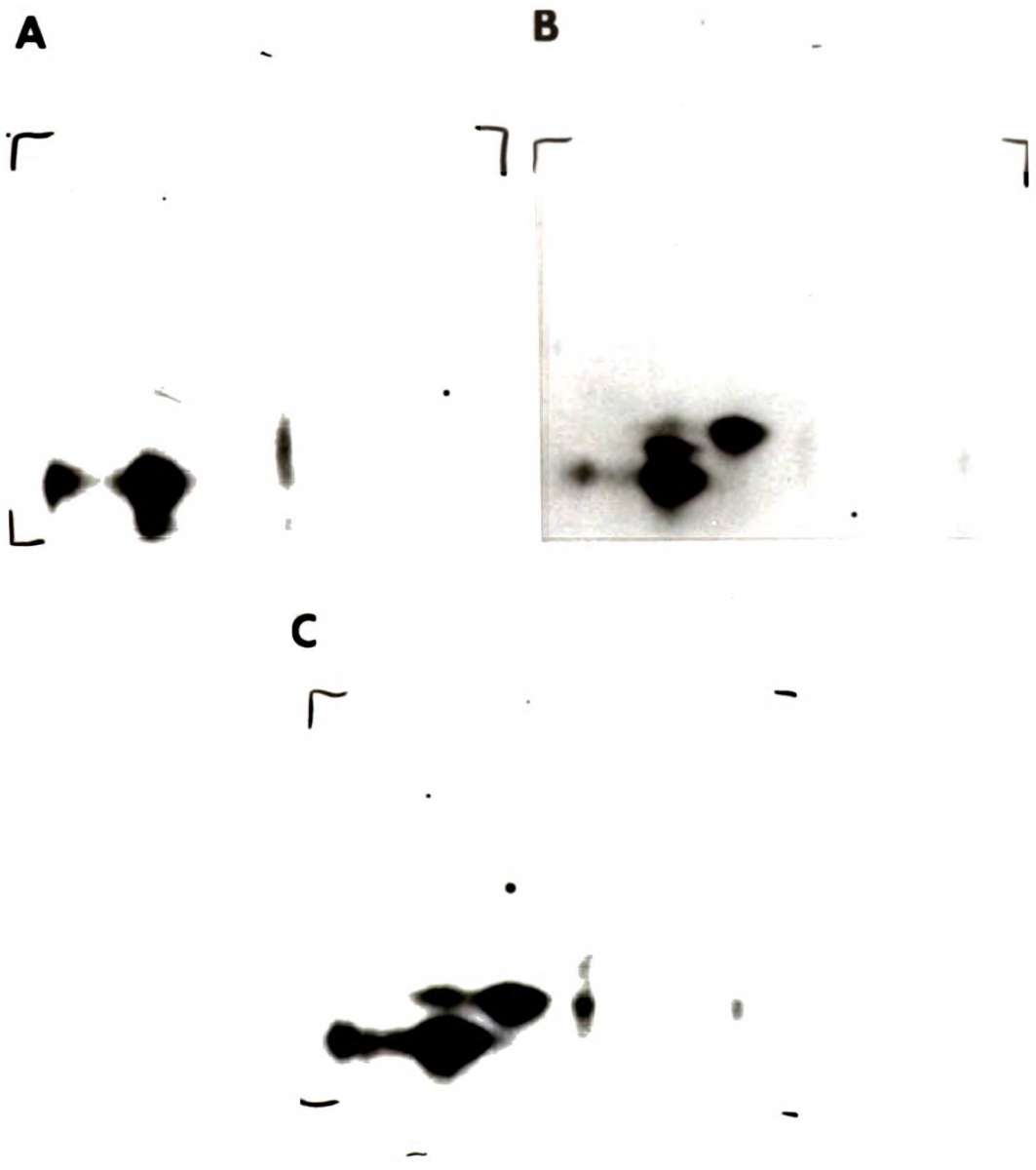

B
d
e
f
g

Figure IV-3 Peptide maps of individual phosphopeptides redigested with a second protease. Phosphopeptides were isolated following two-dimensional separation. V8 peptides were subject to overnight digestion with trypsin, tryptic peptides were digested with V8 protease. Each was then subjected to two dimensional-separation as in Fig. 2. (A) tryptic digest of V8 D. (B) V8 digest of Tryp B. (C) V8 digest of Tryp C.


A
B
C

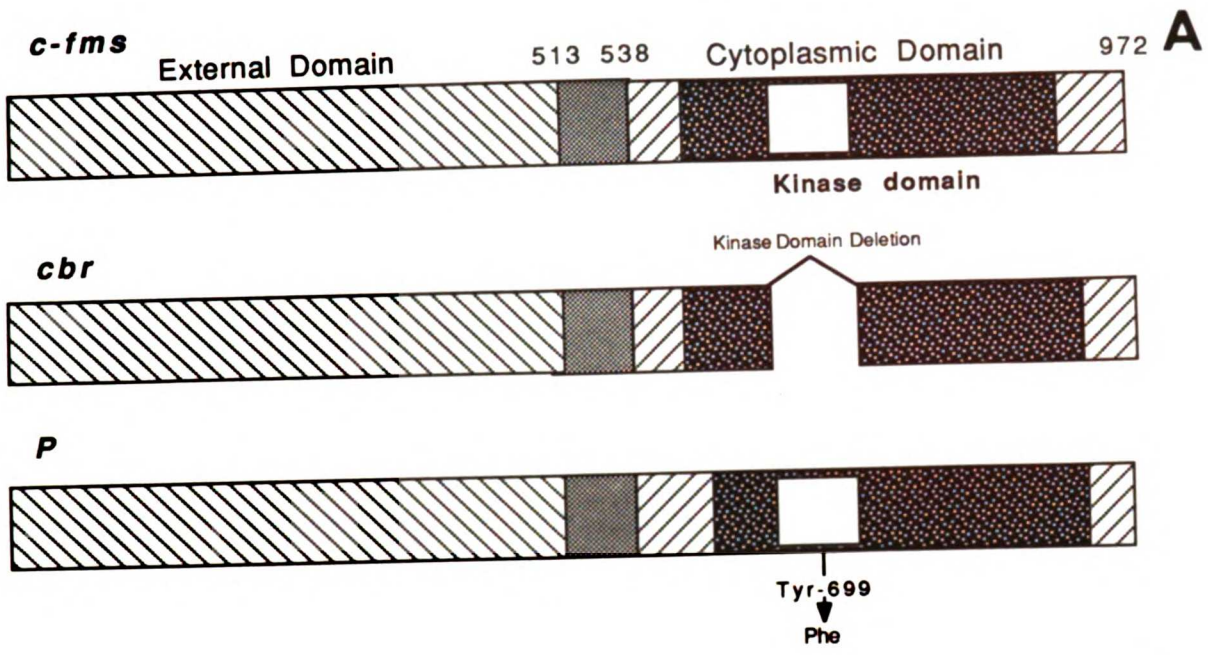
The protein products of mutants *P* (Tyr-699 replaced by Phe) and *cbr* (a deletion of amino acids 680 to 749) were isolated and phosphorylated *in vitro* in the same manner as *wt* FMS. These were chosen because Taylor et al. (1989) had shown that some phosphorylation sites were lost when the KI domain of FMS was deleted, and the major site of phosphorylation of the closely related PDGF receptor lies in its KI domain (Kazlauskas and Cooper, 1989). Tryptic maps of *cbr* lacked all three major phosphopeptides seen in *wt* FMS, along with one consistent minor spot (Fig. 4). Tryptic maps of *P* lacked two major phosphopeptides. Phosphopeptides missing from maps of mutants could have been lost either due to the absence of the actual site in the mutant or because structural alterations prevented phosphorylation of a particular site. Nonetheless the mutant data indicated that all three major phosphopeptides were probably derived from the KI domain. Mutation of Tyr-699 eliminated two major spots (B and C), which redigestion experiments had already suggested were probably related.

Edman Degradation

Tryptic peptides A, B and C and V8 peptides D,E,F and G were isolated from chromatography plates and prepared for sequencing. Since the peptides were not sufficiently pure to allow actual sequence information to be obtained, the samples eluted following each cycle of Edman degradation were collected and counted in a scintillation counter.

Degradation of spot A released the bulk of label after a single cycle (Fig. 5). There are three tyrosines within the cytoplasmic

Figure IV-4. Phosphopeptide maps of *fms* mutants. (A) Schematic diagram of mutants *P* and *cbr*. *P* contains a Tyr-699 to Phe substitution, while *cbr* is a mutant from which the KI domain has been deleted. (B) Tryptic phosphopeptide maps of (1) *wt fms*, (2) mutant *P* and (3) mutant *cbr*. Spots A, B and C are marked on the *wt* map. Arrows mark the origins.


c-fms
External Domain
513 538
Cytoplasmic Domain
972
A
Kinase domain
cbr
Kinase Domain Deletion
P
Tyr-699
Phe

B

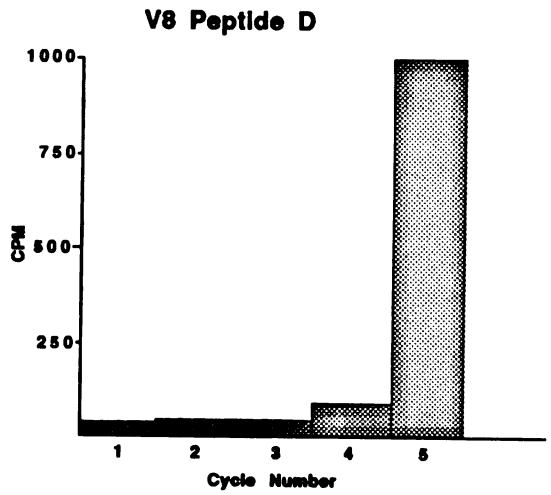
1

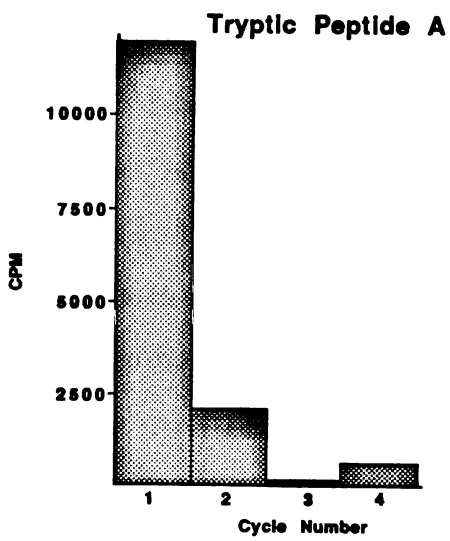
2


a
b
c
a

3

Figure IV-5. Edman degradation of phosphopeptides. Phosphopeptides isolated from two dimensional separations were subjected to sequential Edman degradation. The eluate following each cycle of degradation was collected and Cherenkov counts determined by scintillation counting. The phosphate label released following each cycle is indicated.


V8 Peptide D
1000
750
500
250
CPM
1
2
3
4
5
Cycle Number


Tryptic Peptide A
10000
7500
5000
2500
CPM
1
2
3
4
Cycle Number

domain of FMS that lie just C-terminal to Lys or Arg residues and could thus contain phosphotyrosine as the N-terminal amino acid of a tryptic peptide. Two of these (Tyr-540 and Tyr-546) lie just inside the transmembrane domain and would be unlikely to be affected by deletion of the KI domain. Thus one site of tyrosine phosphorylation must be Tyr-708.

Degradation of spots B and C released no label within the first 10 cycles. Only 9 Tyr residues in the FMS cytoplasmic domain lie greater than 10 amino acids from the N-terminus of a tryptic peptide. Both B and C are related to peptide D. Degradation of peptide D released phosphate label after 5 cycles. Only Tyr-699 lies at the proper distance from a Glu residue to release phosphotyrosine after 5 cycles. Tyr-699 is also greater that 10 cycles away from the closest trypsin cleavage site, which lies in the sequence Arg-Arg-Lys-Ala. Since trypsin does not work well as an exopeptidase, tryptic digestion of FMS labelled at Tyr-699 would be expected to produce two phosphopeptides with either Arg-Lys-Ala or Lys-Ala at their N-termini, accounting for the relationship between spots B and C. As both of these spots are absent from tryptic maps of mutant P, Tyr-699 must be a site of FMS autophosphorylation.

Close examination of tryptic maps of FMS reveals as many as eleven minor spots in addition to those accounted for by Tyr-699 and -708. One of these is lost in cbr mutants and thus is probably Tyr-723, the only other tyrosine residue in the KI domain. *In vitro* autophosphorylation of PTKs has consistently labelled a tyrosine analogous to Src Tyr-416, which in FMS lies at 809 (Cooper, 1989;

Kazlauskas and Cooper, 1989; Tornqvist et al. 1987), so one spot probably represents a peptide containing this residue. The array of additional peptides may include some partial digestion products (which might be expected from peptides containing Tyr-708 and Tyr-723). The identities of these peptides are unknown. Although some may represent authentic sites, others may be artifacts of the labelling method.

DISCUSSION

What is the role of FMS autophosphorylation? Both major sites lie within the KI domain, deletion of which has only limited effects on the transforming activity of *fms* (Taylor et al. 1989). Superficially this might indicate that they are unimportant, but another possibility is that the KI domain inhibits CSF-1 receptor kinase activity. Gill has proposed a model (Bertics and Gill, 1985; Gill et al. 1988) in which tyrosines in the C-terminal tail of the EGF receptor normally occupy a substrate binding site of the kinase and serve to damp residual activity prior to ligand binding. After binding they are phosphorylated, causing the tail to vacate the substrate pocket. All the EGF receptor autophosphorylation sites lie in its C-terminus (Downward at al. 1984; Margolis et al. 1989), whereas no FMS tyrosine phosphorylation has been detected in its C-terminus (van der Geer and Hunter, 1990). The C-terminal regions of FMS and all PDGF receptor family proteins are radically different from those of the EGF receptor and *neu* proteins (see Hanks et al. 1988). It seems possible that a function similar to that of the C-terminus in the EGF receptor family is played in the PDGF receptor

family by the KI domain which characterizes this family. If the KI domain is indeed inhibitory then deletion might remove inhibitory function, relief which is normally mediated by tyrosine phosphorylation in this region.

Another possible role for autophosphorylation is the creation of sites of interaction with other proteins. This seems to be the case for the PDGF receptor (Kazlauskas and Cooper, 1989; Coughlin et al. 1989; Kaplan et al. 1990). A major difference between the CSF-1 and PDGF receptors is that the KI domain of the PDGF receptor appears to be essential for mitogenesis (Escobedo and Williams, 1988; Severinsson et al. 1990). The KI domain of the CSF-1 receptor might mediate interactions with proteins involved specifically in monocyte function, or be partially functionally redundant for some interaction (Shurtleff et al. 1990).

An additional possibility is that autophosphorylation is involved in receptor downmodulation. FMS kinase activity is required for ligand-mediated downmodulation (Downing et al. 1989) but can be supplied in *trans* (Ohtsuka et al. 1990). It is unclear whether tyrosine phosphorylation of the kinase-inactive mutant receptor is the key ingredient provided by active FMS, or whether inactive FMS is downregulated by virtue of dimerization with active FMS. This issue can be addressed using mutants lacking potential phosphorylation sites.

The autophosphorylation sites I have identified are modified in an *in vitro* immune complex kinase assay. The spectrum of sites in these assays is not necessarily identical to those used *in vivo*

(Cooper, 1989; Kazlauskas and Cooper, 1989). Results published following the substantial completion of this work indicate that murine homologs of Tyr-699 and -708 (Tyr-697 and -706) are indeed phosphorylated *in vivo* in response to ligand stimulation of the receptor (Tapley et al. 1990; van der Geer and Hunter, 1990), as is Tyr-809. In chapter V the effects of some of these autophosphorylation sites on kinase and transforming activities are examined.

CHAPTER V:

EFFECTS OF AUTOPHOSPHORYLATION SITES ON THE BIOLOGICAL AND BIOCHEMICAL ACTIVITIES OF THE *fms* PROTEIN

ABSTRACT

Receptor autophosphorylation is an early response of the CSF-1 receptor to binding of its ligand. Three of the sites at which this occurs have been identified. Tyr-699 and Tyr 809 were independently mutated to Phe in both *wt* Hu CSF-1 receptor genes and CSF-1 receptor genes bearing activating mutations. These genes were introduced into 3T3 cells and their effects on the transformation state of the cell were analyzed. Mutation of Tyr-809 to Phe in a *wt* CSF-1 receptor had no effect on the cell, while the same mutation in activated CSF-1 receptor substantially reduced its transforming potential. Mutation of Tyr-699 to Phe slightly increased the transforming efficiency of an oncogenically activated CSF-1 receptor, while in the *wt* CSF-1 receptor gene this mutation produced marginal transformation.

INTRODUCTION

As discussed in chapter IV, autophosphorylation can have several different effects in different proteins. In proteins of the *src* family, *in vivo* tyrosine phosphorylation is found constitutively on a residue near the C-terminus (Cooper, 1989). This inhibits transforming activity and the kinase activity of these proteins. PTK receptors do not generally show constitutive tyrosine phosphorylation, but instead autophosphorylate in response to ligand binding (Yarden and Ullrich, 1988). The first receptor PTK to be sequenced, the EGF receptor, had a previously identified oncogenic

homolog, *erb*B. This pair appeared initially to fit into the paradigm established by *src*-like genes, with a large deletion of the C-terminus being one of the prominent differences between *erb*B and its progenitor (Downward et al. 1984a). Key differences were however soon found that distinguish the two classes. First, autophosphorylation of the EGF receptor occurs at the C-terminus (Downward et al. 1984b), rather than at the analog of Src Tyr-416. Second, deletion of the EGF receptor C-terminus is neither sufficient nor-in many systems-required for oncogenic activation (Nilsen et al. 1985; Haley et al. 1989; Velu et al. 1989). Finally, while autophosphorylation of Src enhances its kinase activity (see Cooper, 1989), EGF receptor autophosphorylation has equivocal effects on EGF kinase activity (Honegger et al. 1988; Bertics and Gill, 1985).

The insulin receptor is the only other receptor PTK whose biochemical properties have been studied in detail. It undergoes autophosphorylation both at sites analogous to Src Tyr-416 and sites in its unique C-terminal regions (Tornqvist et al. 1987; Flores-Riveros et al. 1989). No activating mutants of the insulin receptor exist. Effects of autophosphorylation are complex, including increased kinase activity (Cobb et al. 1989; White et al. 1988) and various metabolic and growth stimulatory effects (Wilden et al. 1990; McClain et al. 1988; Debant et al. 1988).

The major autophosphorylation site of the PDGF receptor was only recently determined. Although it lies intriguingly in a critical region, the KI domain (Kazlauskas and Cooper, 1989), study of its biological significance has been complicated. At a

biochemical level it is necessary for PDGF-stimulated receptor association with a set of proteins. Since the KI domain is necessary for PDGF-induced mitogenic activity (Escobedo et al. 1988; Severinsson et al. 1990) the suggestion has been made that this association is involved in the mitogenic response to PDGF.

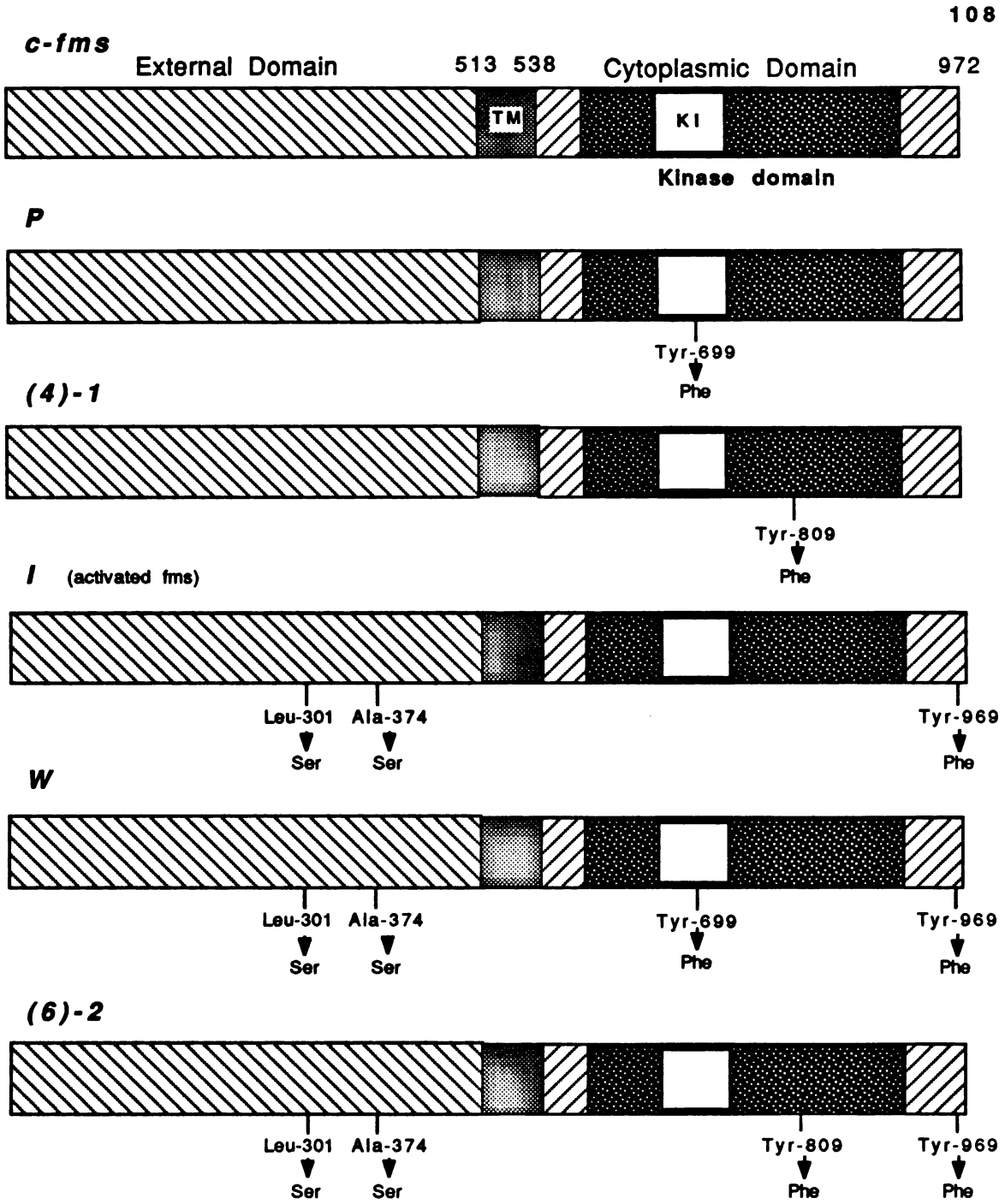
While the KI domain of the CSF-1 receptor can be deleted without loss of transforming activity (Taylor et al. 1989), this deletion does have some effect on the morphology of transformed cells. One model for the function of the KI domain is that it acts as a repressor of kinase activity and that this repression is relieved by tyrosine phosphorylation. To test this model, as well as the function of the Src Tyr-416 analog, I have introduced both *wt* and activated CSF-1 receptor alleles bearing a mutation at Tyr-699 or Tyr-809 into 3T3 cells to test the transforming and kinase activities of these genes.

RESULTS

Mutation and Expression

Site directed mutagenesis was employed to specifically alter Tyr-809 and Tyr-699 in a Pst-SfiI fragment of the CSF-1 receptor. After sequencing of the entire mutant fragment, each was then recloned into either a *wt* CSF-1 receptor backbone, or one which had been activated by a set of three mutations: (1) C-terminal truncation at the point of divergence between v- and c-*fms* (Ser-930), (2) mutation of Leu-301 to Ser, and (3) mutation of Ala-376 to Ser (Fig. 1). All four autophosphorylation site mutants, along with *wt*

Figure V-1. Structures of activating and autophosphorylation site mutants. The lesions that distinguish each mutant from *wt* c-*fms* are indicated below a schematic drawing of c-*fms*


c-fms
External Domain
513 538
Cytoplasmic Domain
972
TM
KI
Kinase domain
P
Tyr-699
Phe
(4)-1
Tyr-809
Phe
I (activated fms)
Leu-301
Ala-374
Ser
Ser
Tyr-969
Phe
W
Leu-301
Ala-374
Ser
Ser
Tyr-699
Phe
Tyr-969
Phe
(6)-2
Leu-301
Ala-374
Ser
Ser
Tyr-809
Phe
Tyr-969
Phe

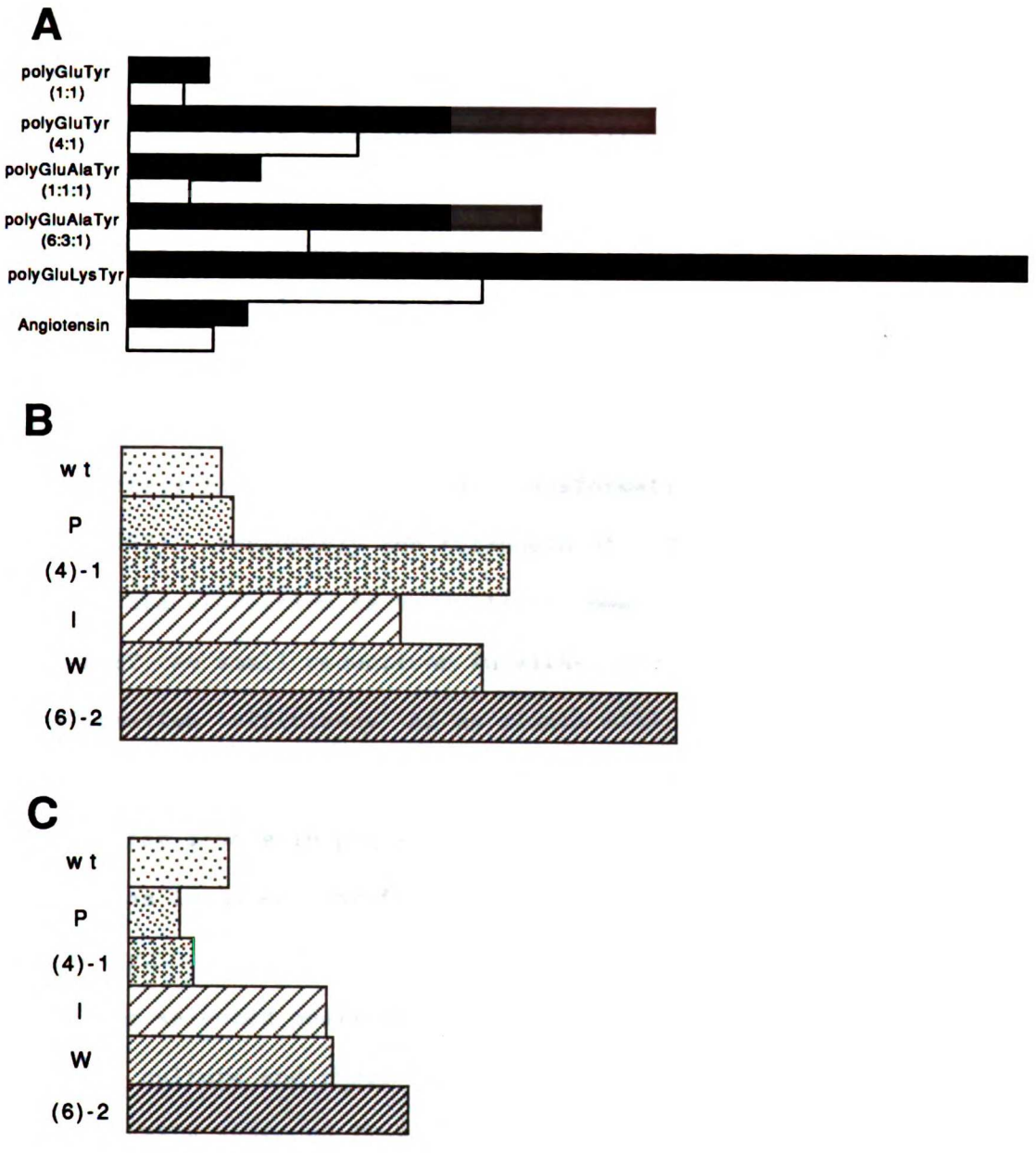
CSF-1 receptor and CSF-1 receptor activated by the three mutations described above, were subcloned into the MuLV vector pMX1112neo (Fig.1). Viral constructs were transfected into Psi-2 cells, selected for neomycin resistance, and cell culture supernatants harvested from pooled, drug-resistant cells. Supernatants were used to infect a cloned NIH 3T3 cell line (C7) and infectants were selected either as pools or individual clones of neo^R cells.

Kinase Activity

To measure the kinase activity of CSF-1 receptor mutants, cells expressing CSF-1 receptor were labelled to steady state (20h) with $[^{35}S]$ Met and CSF-1 receptor protein isolated by immune precipitation. One fourth of each precipitate was separated by SDS PAGE and CSF-1 receptor bands excised and counted. Another fourth was subjected to *in vitro* kinase assay, then separated by SDS PAGE. The remainder was split in half and used for duplicate kinase assays using poly EY (4:1) as an exogenous substrate. Labelled poly EY was isolated on glass fiber filters and counted. A variety of exogenous substrates for CSF-1 receptor have been tested, including poly EY (1:1), poly EAY (1:1:1), poly EAY (6:3:1), poly EKY and angiotensin. Poly EY (4:1) was chosen as the best of these (Fig.2).

In brief, *(4)-1* had a slightly elevated (about 2 fold greater) activity on exogenous substrates. The same mutation in an activated background was perhaps marginally more active than *I*. Activated CSF-1 receptor (*I*) was significantly more active in both exogenous substrate and autophosphorylation assays (3-4 fold) than was *wt* CSF-1

FigureV-2. Kinase activity of FMS and mutant proteins. (A) Comparison of the kinase activity of *wt fms* protein using various polypeptide substrates. White bars are background obtained with immuneprecipitates of cells not expressing *fms*. (B) Comparison of activities of *wt* and mutant *fms* proteins with poly GluTyr (4:1) as a substrate, normalized to amount of *fms* protein. (C) Comparison of autophosphorylation activities of *wt* and mutant *fms* proteins.


A
polyGluTyr (1:1)
polyGluTyr (4:1)
polyGluAlaTyr (1:1:1)
polyGluAlaTyr (6:3:1)
polyGluLysTyr
Angiotensin
B
w t
P
(4)-1
I
W
(6)-2
C
w t
P
(4)-1
I
W
(6)-2

receptor. Mutations of Tyr-699 (*P* and *W*) had slight effects on both autophosphorylation and exogenous substrate kinase activity.

Biological Activity

3T3 (C7) cells were infected with virus expressing various CSF-1 receptor alleles and split to score neo^R and focus forming activity. After two weeks, plates were stained and neo*R* colonies and foci counted (Fig. 3). None of the alleles, including v-*fms*, were particularly potent transforming genes in this assay. Others have observed that transformation by v-*fms* expressed from a MuLV LTR is approximately 1/4 as efficient as transformation by SM-FeSV (Roussel et al. 1987). Presumably the threshold of v-*fms* activity required for cell transformation is very little lower than the level expressed by SM-FeSV, so small reductions in either protein levels or the transformation 'efficiency' of an allele will result in substantially reduced numbers of foci. Since all alleles examined are expressed at very similar levels in pools of infectants (data not shown), differences in numbers of foci probably reflect transformation efficiency.

Drug selected pools and clones of infected cells were grown in monolayer culture and their morphology examined (Fig. 4). *I* and *W* infected cells appeared flagrantly transformed, almost as rounded and overgrown as v-*fms* infected cells. *(6)-2* cells were less rounded in appearance. Unlike cells infected with neo, CSF-1 receptor or *(4)-1* viruses, *P* cells showed some morphological alteration.

Both neo-selected pools and high-expressers of these alleles were grown in soft agarose to test their capacity for anchorage

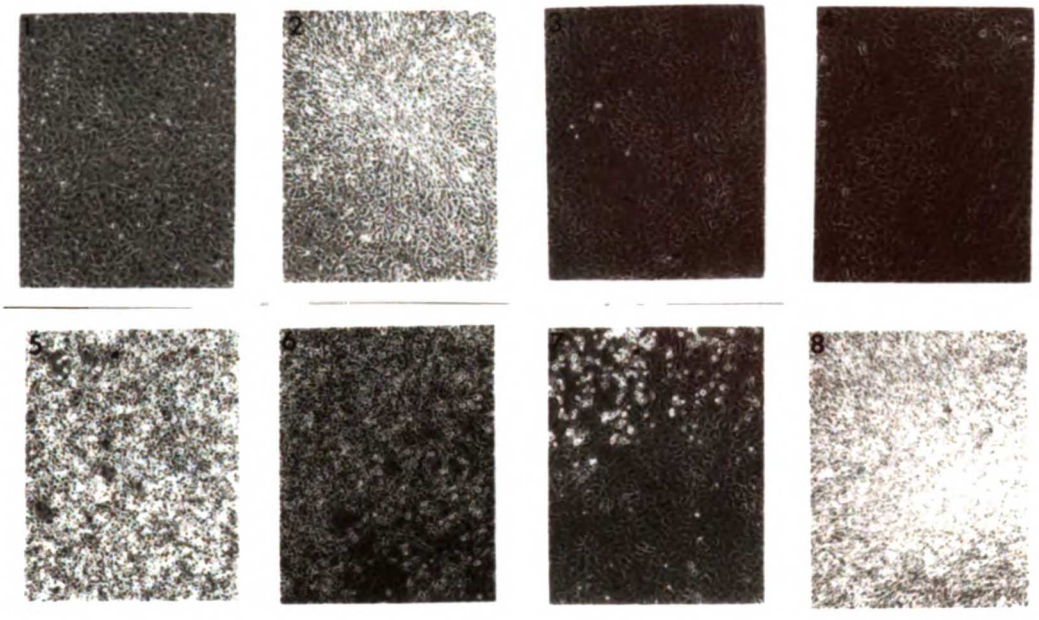
Figure V-3. Relative transforming activities of *fms* alleles. Focus forming activity is expressed as a percentage of neo resistant colonies obtained in parallel assay. Mutant *P* formed small foci with intermediate morphology. Soft agar growth was tested by plating 10^3, 10^4, 10^5 and 10^6 cells in 60mm dishes. Mutant *P* consistently formied small colonies at high densities. Mutant *(6)-2* grew with ~10% efficiency in soft agar (half that of other activated alleles) and appearance of colonies was delayed about one week.

Allele	Focus Formation	Soft Agar Growth
c-fms	<0.1%	-
P	(0.4%)*	(+/-)*
(4)-1	<0.1%	-
I	18%	+++
W	13%	+++
(6)-2	0.8%	(++)*
v-fms	28%	+++

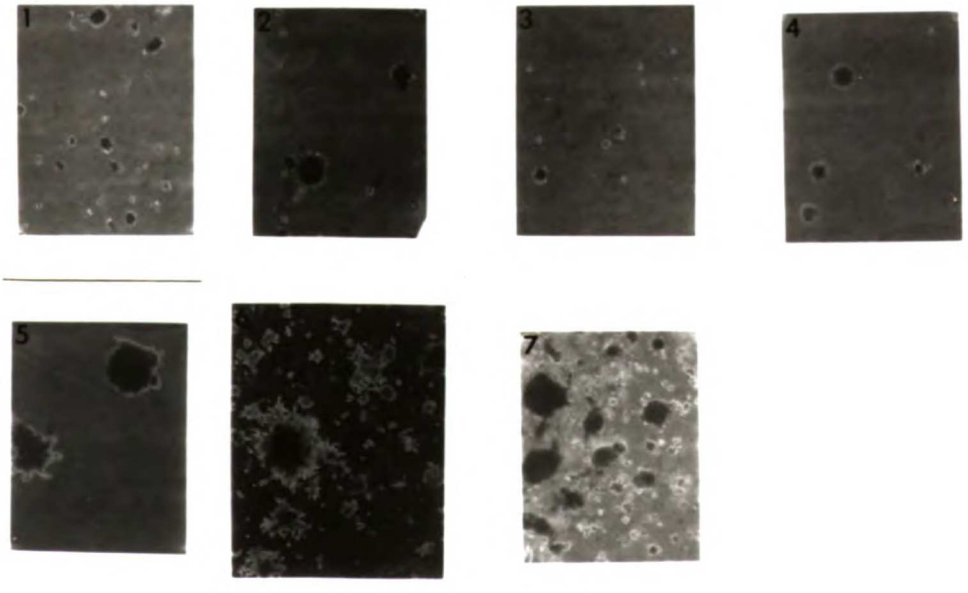
*(see text)

Figure V-4. Morphology of fibroblasts infected with various *fms* alleles. All are pools of neomycin-resistant cells: (A) in monolayer culture; (B) in soft agar. (1) c-*fms*. (2) *P*. (3) *(4)-1*. (4) control (neo-only virus) infected fibroblasts. (5) *I*. (6) *W*. (7) *(6)-2*. (8) v-*fms*.

A



B



independent growth. Consistent with the results of focus assays, *I*,*W* and v-*fms* infected cells grew well in soft agar, while CSF-1 receptor and *(4)-1* infected cells failed to grow. The sharpest contrast between focus and colony forming activity involved cells expressing mutant *(6)-2*. In focus assays this allele transformed approximately 100 fold less efficiently than did *I*, while in SA assays it produced almost as many colonies as activated CSF-1 receptor. Only a delay in the appearance of colonies distinguished *(6)-2* from *I* in this assay.

DISCUSSION

At least one CSF-1 receptor autophosphorylation site seems to be important for the biological activity of the molecule, that at Tyr-809. Substantial precedent for the importance of sites related to Tyr-416 of *src* exists, yet its effects in different systems vary widely. Replacement of *src* Tyr-416 reduces both kinase and biological activities (Piwnica-Worms et al. 1987; Kmiecik et al. 1988; Snyder and Bishop, 1984). Replacement of Tyr-1150 and -1151 in the insulin receptor causes reduced insulin-stimulation of kinase activity but a higher basal activity and retains insulin-stimulated thymidine incorporation (Dabant et al. 1988). Mutation of insulin receptor Tyr-1146 or PDGF receptor Tyr-825, in the other hand, radically limits their phosphorylation of exogenous substrates and ability to stimulate mitogenesis (Fantl et al. 1989; Wilden et al. 1990). The homologous mutation (at Tyr-809) in CSF-1 receptor does not reduce autophosphorylation or exogenous substrate phosphorylation *in vitro* and substantially reduces without eliminating transforming

activity. Phosphorylation of FMS on Tyr-809 must therefore either be necessary for the interaction of FMS with a set of substrates involved in transformation, or affect some aspect of *in vivo* kinase activity not reflected in *in vitro* kinase assays.

In sharp contrast with the effects of Tyr-809 mutation, replacement of Tyr-699 with Phe reduced kinase activity not at all and mutant *W* was if anything slightly more efficient as a transforming allele than was *I*. On the surface this demonstrated that not all receptor autophosphorylations have an upregulatory function. It would also tend to discredit the idea that autophosphorylation within the KI domain is a necessary step in relieving inhibition of CSF-1 receptor kinase activity. It should be borne in mind, however, that there exists another autophosphorylation site in the vicinity of Tyr-699 whose modification may make modification of either site functionally redundant. In either case phosphorylation of Tyr-699 is not essential for formation of any substrate complex essential for transformation in contrast to what has been suggested for Tyr-751 of the PDGF receptor.

One of the most interesting aspects of Tyr-699 is that mutation to Phe seems to provoke partial activation. Focus formation by allele *P* was only slightly above background levels and soft agar growth was also marginal, but together with the observed morphological alterations these suggest some biological activity in the absence of ligand. Perhaps Tyr-699 and -709 function coordinately to inhibit transforming activity, yet such a simplistic model does not account for the finding that mutants which lack the

fms kinase insert domain (see ch. 4) are not transforming in the absence of additional activating mutations. A more complex role for the KI domain in general and by extension for those autophosphorylation sites that lie within it is suggested by the finding (Shurtleff et al. 1990) that KI deletion mutants show impaired but not completely deficient association with PI 3 kinase. A role for this domain in both protein association and regulation makes functional dissection difficult.

Two potential roles for Tyr-699 autophosphorylation that were not examined are as a signal for downregulation and as a mediator of monocyte specific interactions were not examined in these experiments. Mutant *P* transforms fibroblasts in the presence of added CSF-1 with approximately the same efficiency as does CSF-1 receptor indicating that Tyr-699 autophosphorylation is not strictly necessary for ligand-induced mitogenesis. Since fibroblasts expressing CSF-1 receptor are relatively efficiently transformed by addition of CSF-1 this is not a particularly telling assay, but the ability of *P* to mediate transformation in the presence of limiting quantities of CSF-1 might be indicative of a role for autophosphorylation at Tyr-699 in receptor downregulation. Further examination of the function of autophosphorylation should probably be conducted in concert with Tyr-708 mutants. I constructed such a mutant and found it to be inactive in kinase and transformation assays. When both Tapley et al. (1990) and van der Geer and Hunter (1990) found a similar mutant to be kinase active, my construct was resequenced and found to have acquired an additional Ala to Val

mutation at 911. Although this amino acid is not well conserved among tyrosine kinases (Hanks et al. 1988), it appears to be required for CSF-1 receptor activity.

None of the mutants studied causes any dramatic reduction in CSF-1 receptor kinase activity. This is particularly surprising for Tyr-809 mutants as analogous mutations in *src*, the PDGF and insulin receptors do alter kinase activity, and *(6)-2* has clearly altered biological activity. This could reflect the fact that CSF-1 receptor is a weak kinase both *in vivo* and *in vitro* for the substrates that have been tested, implying that none are decent models for interaction of the CSF-1 receptor kinase with physiological substrates. Mutation of Tyr-809 might significantly reduce CSF-1 receptor activity towards one or a small number of critical substrates. Anti-phosphotyrosine blotting of cell extracts from cells infected with the *(6)-2* virus shows no detectable differences in the pattern of tyrosine phosphorylated proteins relative to *I* expressing cells (data not shown).

The precise role of CSF-1 receptor autophosphorylation remains to be elucidated. Since deletion mutants that lack the KI domain are still transforming and the only major autophosphorylation site outside that domain is also dispensable for transformation, none of the major sites appears to be strictly required. At least Tyr-809 serves to enhance activity and that may also be true of the others; mutants lacking all three major sites have yet to be examined. The fact that both KI deletions and Tyr-809 mutations affect transformation without eliminating it also lends support to the idea

that transformation by *fms* may occur via multiple pathways, no one of which is sufficient to account fully for the activity of the *wt* gene. The ability to tamper with individual regulatory sites offers a potential opportunity to identify and dissect the contributions of each.

CHAPTER VI: CONCLUSIONS AND PROSPECTS

The burgeoning understanding of cell growth control pathways that has appeared in the last ten years is a culmination of forty or more years of work in wildly diverse fields of biology. A sufficient number of participants in various control mechanisms has been identified that common themes are beginning to emerge and interconnections become clearer. It is now possible to imagine describing the precise biochemical events triggered within a cell by interaction with a growth factor, and the contribution that each process makes towards altering the fate of that cell. As such an understanding approaches the idea that some single distinctive pathway--or even a pair of pathways--serves to transduce growth signals from the cell membrane to the nucleus fades away. This idea is replaced with the realization that a very complex matrix of regulatory interactions governs cell fate, and exposure of a cell to a stimulus modifies that matrix in several ways.

Progress towards an understanding of the interactions that make up this web depends on an examination of details. Quite possibly there exist master integrators of multiple signalling pathways, but the ripples eventually producing a binary decision start far away and travel through circuitous channels. As usual, lambda provides an illustrative example. The lysis/lysogeny decision may ultimately depend on the balance of lambda repressor and cro, but a multitude of influences affect that balance and the alternative which those two proteins eventually determine.

Study of the *fms* protein--the CSF-1 receptor--is very exciting

within this context. Several intriguing features of the control and propagation of its signalling potential have been touched in the preceding chapters, and further exploration of these features promises to be rewarding. To start at the beginning, there is the control of c-*fms* expression, which is observed in two very distinct cell types. Following translation of the protein, its appearance at the cell surface requires mature glycosylation, an observation that could be of interest in the study of protein sorting and membrane traffic. Similarly a point mutation in v-*fms* results in delayed and inefficient transport of the v-*fms* protein to the cell surface. There are other intriguing examples of unusual regulatory mechanisms that affect the activity of FMS, both positively and negatively. In macrophage cells, stimulation of other growth factor receptors causes a loss of responsiveness to CSF-1. Treatment of cells with TPA causes a loss of *fms* protein via a proteolytic mechanism. The polyomavirus E5 protein, which causes fibroblast transformation by c-*fms* in the absence of added ligand, is thought to affect the downregulation of FMS. All of these observations point to the existence of mechanisms that modulate receptivity to signalling by peptide hormones that are not only significant to *fms*, but poorly understood and perhaps of general importance.

Another level of regulation of FMS signalling potential involves downregulation in response to ligand. FMS is very rapidly degraded in response to ligand stimulation, but the same is not true of kinase inactive mutants. Downregulation must therefore involve some aspect of FMS kinase activity, whether tyrosine phosphorylation

or the assumption of a conformation that depends on binding of ATP. The ability of active FMS to induce ligand-stimulated downregulation *in trans* is in contrast to findings reported with the EGF receptor, indicating diversity rife with regulatory potential.

My studies have concentrated on the regulation of the signalling function of *fms* protein expressed at the cell surface. The focus has been further narrowed to two basic questions: (1) what is the structural difference between active and inactive FMS, and (2) what role does autophosphorylation--an immediate consequence of activation--play in mediating signalling by FMS. Each of these has been addressed primarily from the direction of genetics, ie the correlation between mutation and function. Although a number of obvious, potentially fruitful genetic approaches to *fms* function remain, an important future direction for research will be towards a more biochemical understanding of *fms*.

The oncogenically activated homolog of a PTK receptor provides a model of ligand activation. By isolating c-*fms* it was possible to identify the structural basis of this activation, at the level of amino acid sequence, and produce reagents for study of the *c-fms* protein. Several of the initial goals of this project were overtaken by the findings of other groups. The Sherr/Stanley alliance, employed biochemical characterization of the CSF-1 receptor and antisera to the v-*fms* protein (crossreactive towards the c-*fms* protein) to demonstrate the identity of the c-*fms* protein and the CSF-1 receptor. Soon afterwards, it was shown that ligand activation of the CSF-1 receptor, expressed in fibroblasts, could cause

transformation. This answered the longstanding and critical question of whether oncogenic activation of PTKs required their endowment with some unique capacity to interact with novel substrates or simply release from normal constraints on their kinase activity. Although now taken for granted, that is truly a profound finding and was initially a central goal of my work.

A second primary goal of the isolation of c-*fms* was to identify the mutations that provide the basis for its oncogenic activation in v-*fms*. An important and interesting question first posed long ago concerns the mechanism by which a hormone unable to traverse the plasma membrane is capable of inducing changes in a cell. This question can be divided into an examination of the changes which hormone binding induces in the extracellular domain of a receptor, to bring about activation of intracellular signalling, and the structural and biochemical changes produced in the intracellular domain, which constitute activation. As an important class of mediators for peptide hormones, tyrosine kinase receptors are a particularly important arena in which to address these questions.

Identification of activating lesions provides insights into structures involved in the control of *fms* activity. Mutations may either induce a conformation mimicking that normally caused by ligand binding, or they may relieve inhibitory interactions. An obvious candidate to be the activating lesion of *fms* was the C-terminal alteration. Though this does contribute to transforming activity in the presence of other mutations, it is not sufficient. This contribution to transforming activity is both puzzling, and

interesting in light of the failure of FMS to show tyrosine phosphorylation of this region. Future experiments that might shed light on the function of this region include construction of chimeric *fms* genes, containing C-termini from the PDGF receptor, *kit* or the EGF receptor; mutagenesis of sites in this region other than Tyr-969; and biochemical approaches to identifying either intra- or intermolecular interactions.

The great divergence between v- and human c-*fms* made identification of activating lesions difficult, a problem I sought to overcome by producing novel oncogenic alleles of *fms*. Deletion of the extracellular domain failed to activate *fms*. In light of emergent knowledge of the subfamily of receptors into which *fms* falls, the mechanism of EGF receptor activation, and the activating lesions of v-*fms*, a more extensive study is in order. First, a larger set of deletion mutants should be constructed. Second, chimeric receptors should be constructed between the EGF receptor, PDGF receptor and *fms*, to examine the sufficiency of their extracellular domains to induce transmembrane signalling. If EGF receptor/*fms* chimera prove responsive to EGF, then the question of the ability of deletions of the EGF receptor N-terminus to activate chimeric receptors could address the difference in transmembrane signal transduction mechanisms between these two receptor families.

My random mutagenesis strategy was a deep well which came up dry. All that can be extracted from it is the knowledge that activation of *fms* transforming ability must require either specific or compound mutations. For all that, the singular nature of the v-

fms activating lesion--a point mutation in the extracellular domain--makes a second try worth the effort (for some brave gambler). Downregulation makes study of the structure of ligand-activated receptors incredibly challenging; biochemical and structural analysis of a mutant receptor whose extracellular domain assumes a conformation analogous to that induced by ligand binding is a potential gusher. Isolation of independent activated alleles of *fms* would serve either to focus attention on the unique role of 301, or to define regions involved in producing active conformations of the receptor. Preliminary to any concerted effort to isolate new alleles, a greater biochemical definition of the states (are they dimers?) of v-*fms* and ligand-activated CSF-1 receptor is in order.

The last phase of this work addressed a well defined modification, associated with both oncogenic activation and ligand stimulation of *fms*. In order to examine function it was first necessary to establish location. As mentioned in earlier discussions, I had determined two sites when other groups published the identities of one and three sites.

Both sites whose functions were probed by mutation gave intriguing results. Mutation of Tyr-809 substantially reduced one measure of transforming activity, and in this respect is similar to Tyr-416 of *src*. In sharp contrast, however, Tyr-809 mutation has no detectable inhibitory effect of FMS kinase activity. Such a mutant reinforces one of the initially attractive aspects of studying transformation by *fms*: that the restricted set of associated tyrosine phosphorylations should facilitate identification of

physiologically significant substrates. Although an initial survey of tyrosine phosphorylated proteins in *wt* and Tyr-809 mutant cells revealed no obvious differences, a thorough study of cell lysates with anti-phosphotyrosine antibodies, and of coprecipitating proteins, might be revealing. The hint of oncogenic activation by Tyr-699 mutation is also tantalizing. Among the tempting experiments to follow up this finding are combining Tyr-699 and Tyr-969 mutations, in an attempt to enhance the oncogenic activity of Tyr-699, and a search for proteins that interact with Tyr-699 (by probing Western blots and expression libraries with both tyrosine phosphorylated and unphosphorylated peptides derived from this region). Two sets of experiments that should yield interesting results are examination of Tyr-708 mutants, both alone and in combination with Tyr-699 mutations, and study of the functions of these mutants in macrophage cell lines.

The different potentials of *fms* in fibroblasts and macrophages addresses a final regulatory issue, the determination of cell response to stimulation by the cell-type specific set of regulatory molecules functioning downstream of the receptor. It seems quite possible that the highly-variable KI domain mediates interactions with substrates peculiar to those cell types in which c-*fms* is normally found, macrophages and placental trophoblasts. Examination of the function of autophosphorylation site mutants in macrophages might provide additional insight into their function.

Two other, more speculative and labor intensive approaches to the question of how cell-type specific response is achieved, involve

transfection and transgenotes. v-*fms* does not transform myeloid cells without the cooperation of a nuclear oncogene, and transfection of fibroblast DNA into v-*fms* expressing macrophages might lead to the isolation of a gene responsible for this difference in susceptibility. Transgenic mice could be employed to study a variety of questions about the developmental role of *fms*.

The study of *fms* has in the past provided, and promises in the future to continue providing, valuable insight into questions of PTK signalling. Though many of the initial goals of my study of *fms* were reached by other labs, these experiments have provided independent confirmation, and some interesting results concerning the involvement of certain structural elements of *fms* with control of its activity.

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MATERIALS AND METHODS

Materials. Cloned SM-FeSV v-*fms* was from M. Roussel. NIH 3T3 (C7) cells were a flat subclone isolated by E. Liu. Antisera LR was a kind gift of L. Rohrschneider. Placental cDNA library was from Y. Ebina. MLV producer cells were from J. Kaplan.

Reagents. All chemicals were obtained from Fischer, Sigma or Pierce. Staph V8 protease was from Boehringer, TPCK trypsin from Cooper. Radiochemicals from ICN and NEN. Immunological reagents were from Pharmacia and Cappel. Enzymes were from NEB and Boehringer.

Cell culture, transfection and infection. 3T3 cells were grown in DME H-21+10%FCS. Psi-2 producer lines were produced by transfection, then harvested and used to infect fibroblasts as described (Cepko et al. 1984). MLV producer cells were a clone of MLV infected fibroblasts, and were grown as uninfected cells.

Soft agar and focus assays. Base layers of 5ml 0.7% low-melting agarose containing 1X medium were poured in 60ml dishes. Cells were suspended in a total of 8ml, 0.35% SA+1X medium, and layered on bases. A top layer of 5ml 0.7% SA+1X medium was poured on top and cells incubated for three weeks at 37. For focus assay cells were grown 2-3 weeks and scored visually.

Labelling. Cells were labelled for 4-20h. in Met^- DME+5% serum with 150μCi/ml [^{35}S] Met. 20h. labellings contained 10% normal DME.

Cell lysis. Cells were washed 3X with cold PBS, then lysed on plates for 1h. at 4^0 with shaking. Monolayers were scraped, then spun 30' @ 20,000g and the pellet discarded. Lysis buffer contained 50mM Tris(7.4), 150mM NaCl, 20mM EDTA, 1% cholate, 30% glycerol,

25μg/ml STI, 5μg/ml leupeptin and 5μg/ml aprotinin. Aliquots were stored up to 3 months at -80°.

Immunoprecipitation and immune complex kinase assays. Performed as described by Woolford et al. (1985), with the following modifications. Lysates were washed 5X in the RIPA buffer described above, then 2X in 10mM Tris (7.4). Kinase precipitates were then resuspended in 30λ (20mM $MnCl_2$, 50mM Hepes (7.4) 170μCi [^{32}P] ATP) and incubated 10' at 30°, then washed once in RIPA. Precipitates were washed once in 1M NaCl, 10mM Tris (7.4), then boiled in Laemmli buffer for 5'. Exogenous substrates were added at 1mg/ml, and supernatants spotted onto Whatmann P91 paper, then washed 1X30' in 30% HAc, 5X>30' in 10% HAc, 1X10' in 95% EtOH, dried and counted.

Oligonucleotide site directed mutagenesis. Performed by manufacturers protocol (Bio-Rad Mutagen kit).

Cloning. Performed by standard methods.

Sequencing. According to manufacturers protocol (USB Sequenase kit).

Sequence analysis. Used the *pima* algorithm (Smith and Smith, 1990).

In vitro transcription and translation. Performed according to Promega protocol.

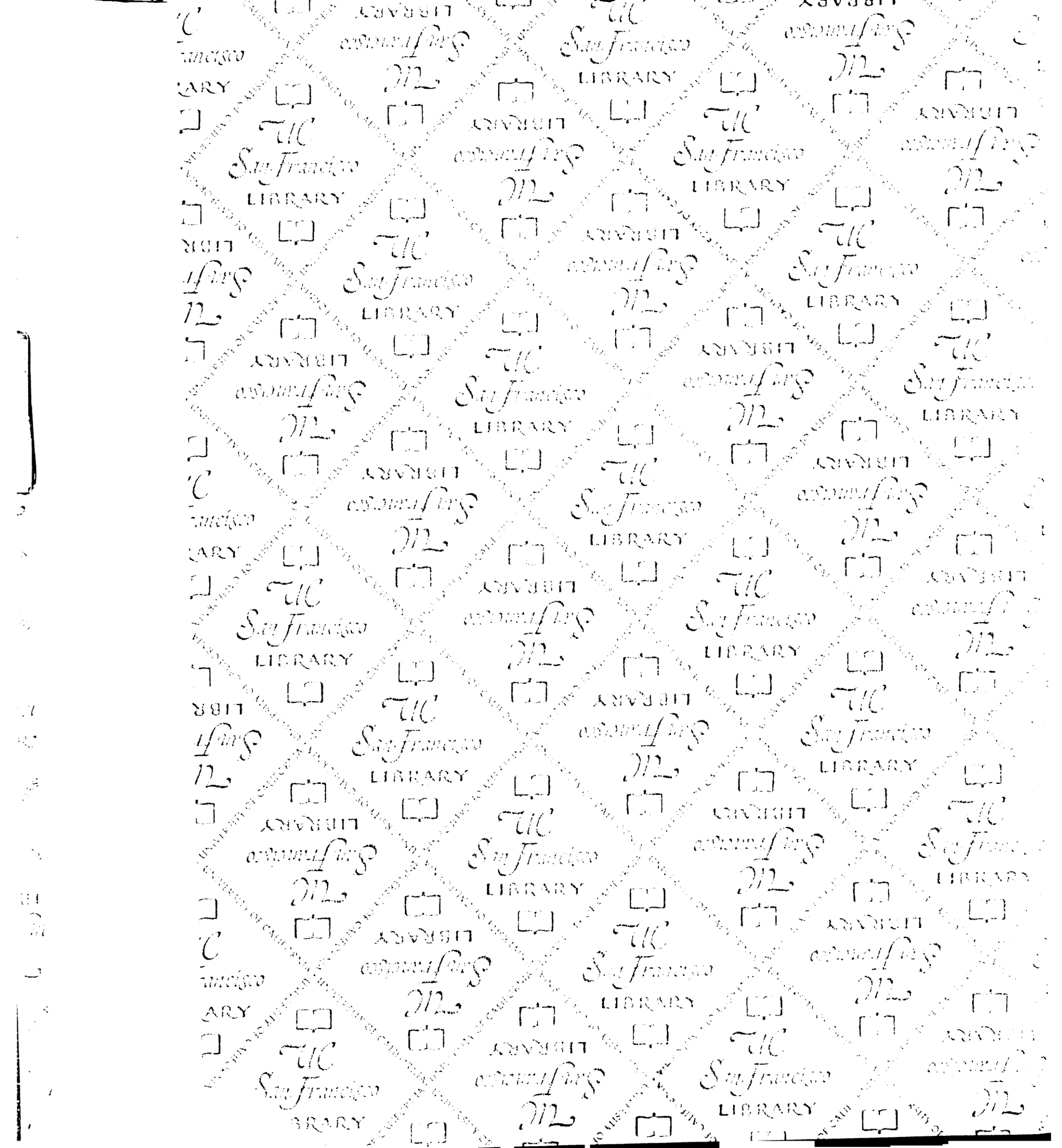
Antisera production. Peptides were coupled to tuberculin PPD with glutaraldehyde, acetone precipitated and resuspended in PBS. Injections were with 200μg peptide at 2wk. intervals; first with Freunds complete adjuvant, then with incomplete Freunds. Bleeds were performed one week after injection. Fusion protein antigens were

produced as described (Ruther and Muller-Hill, 1983), then electroeluted and injected as above, without coupling.

Peptide mapping. Proteins isolated form SDS gels were performate oxidized and digested overnight, then again for 5h. with 10μg trypsin in 50mM ammonium bicarb (8.0), or 10μg of V8 in AmBicarb (7.9). Samples were washed twice by lyopholization, then resuspended in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid in H_2O) and spotted onto cellulose TLC plates (EM Scientific, Art5577). Electrophoresis was for 20' to 40' at 1000V. Chromatography was is 15ml n-butanol, 50ml pyridine, 15ml acetic acid, 60ml H_2O.

Peptide extraction. Plates were scraped and peptide eluted in pH 1.9 buffer, then lyopholized 3X.

Edman degradation. Performed as described in Wettenhall et al. (1990), but using Sequelon membranes (MilliGen) as a support (coupling by manufacturers protocol).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