IDENTIFICATION AND CHARACTERIZATION OF HUMAN
AND MURINE c-fes PROTEINS

By

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We accept this thesis as conforming
to the required standard

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The \textit{c-fes} and \textit{c-fps} genes are, respectively, the mammalian and avian proto-oncogenes which were ancestral to the retroviral \textit{fps/fes} oncogenes. While the proteins encoded by \textit{c-fps/fes} proto-oncogenes had been identified in a number of species the homologous proteins had not been isolated or characterized in human or murine cells. This study was undertaken in an attempt to identify \textit{c-fps/fes} proteins in human and murine cells.

Using a cross-reactive rat anti-\textit{v-fps} serum two immunologically cross-reactive proteins of \textit{M} \textsubscript{r} 92,000 (p92) and 94,000 (p94) were immunoprecipitated from a variety of human and murine cells. Functional studies indicated that both of these proteins possessed protein kinase activity and were able to autophosphorylate and to phosphorylate exogenous substrates at tyrosine residues. Structural comparisons suggested that p92 was very closely related in primary sequence to the \textit{v-fes} protein P85\textsuperscript{-} while p94 was more distantly related. On the basis of immunological cross-reactivity, intrinsic protein-tyrosine kinase activity, and structural relatedness it was concluded that p92 represents the human and murine \textit{c-fes} gene product; p94, while possessing some similarities, is probably not a \textit{c-fes} protein but is likely a related protein-tyrosine kinase.
Examination of human and murine cells and cell lines revealed that the expression of c-fes was confined to those of hemopoietic origin and especially to those cells belonging to myeloid lineages. The expression of c-fes was investigated in human myeloid leukemia cell lines during exposure to a chemical inducer of differentiation; complex patterns of expression were observed and these are discussed. The expression of c-fes appears to be differentially regulated in responsive and unresponsive cells and it is possible that it plays a role in commitment to myeloid lineages.
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This thesis is dedicated to Diane, to Gwynneth, and to Keith; may it serve as a symbol of my love, of my dedication, of my commitment.
CHAPTER 1

1.0 INTRODUCTION

1.1 Proto-oncogenes

Oncogenes are so named because they were originally discovered to be the genetic sequences carried by some oncogenic retroviruses which conferred upon those viruses the property of acute tumorigenicity. These genetic sequences have, in most cases, replaced certain retroviral structural or replicative genes, and thus, in addition to rendering these viruses oncogenic, concomitantly render them replication-deficient; these unique genes are called viral oncogenes (v-onc's). Subsequently it was discovered that some non-viral tumors contain homologous DNA sequences which can transform NIH 3T3 cells; these transforming genes are called cellular oncogenes, or activated oncogenes, in order to distinguish them from v-onc's.

Examination of the various species from which the oncogenic retroviruses were originally isolated reveals that genes homologous with the v-onc's are present in the normal genome. These normal sequences are called proto-oncogenes (c-onc's) and it is widely believed that acutely oncogenic retroviruses transduced c-onc's in the course of viral replication, giving rise to v-onc's. In support of recombination events giving rise to v-onc's is the
observation that short stretches of identical nucleotides are found in both viral structural genes and in corresponding c-onc's at the borders of putative recombination junctions (Coussens, et al., 1986). Also, integration of certain non-onc retroviruses next to c-myc or c-erbB sometimes results in the emergence of novel, rapidly transforming retroviruses which have transduced the adjacent c-onc into the viral genome (Miles, et al., 1985; Nusse, 1986). Presumably, cellular genes are transduced randomly from the host genome and the readily detectable neoplastic phenotype conferred by the presence of a v-onc results in their isolation; the finding that different strains of oncogenic retroviruses, isolated independently from virally-induced tumors, carry the same v-onc is consistent with the random transduction of c-onc's.

However, more than mere transduction of a c-onc is required to activate its transforming potential and all v-onc's show mutations relative to the original c-onc. Activated oncogenes isolated from non-viral tumors also inevitably carry structural alterations, point mutations, or are expressed inappropriately relative to the corresponding c-onc; thus, most normal c-onc's are unable, in the absence of mutation or aberrant expression, to transform normal cells or induce tumors (Land, et al., 1983; Foster, et al., 1985; Bishop, 1987).

1.2 Isolation and Classification of Proto-oncogenes
Proto-oncogenes have been isolated by one of four methods (Varmus, 1984): 1.) Probing cellular DNA with v-onc probes in order to identify related cellular sequences. The majority of c-onc's have been identified on the basis of being the normal cellular counterparts of the retroviral transforming genes. 2.) Probing DNA from viral tumors with unique retroviral sequences, such as long terminal repeats (LTR's), in order to identify genes which have been activated by viral integration. The viral LTR's serve as markers and allow identification of adjacent virally-activated c-onc's; a number of genes which have no known equivalent v-onc have been isolated using viral sequences as molecular markers. 3.) Transfection of a suitable cell line with tumor DNA causes transformation if an activated, dominantly-acting c-onc is present. This technique has proved useful in isolating oncogenes from a variety of spontaneous tumors as well as chemical or radiation-induced neoplasms. 4.) Chromosomal translocations are seen frequently in neoplastic cells and specific translocations which disrupt c-onc expression exist in a number of tumors; cloning of sequences adjacent to chromosomal translocation breakpoints has allowed the isolation of several novel oncogenes.

Proto-oncogenes may be classified according to structure, cellular location, or activity. Since precise functions have not been ascribed to many c-onc's their classification has been made on the basis of cellular
location or structural similarity to more extensively characterized c-onc's. The majority of currently identified c-onc's are categorized in Table 1.1 according to the above criteria.

1.3 Functions of Proto-oncogene Products

The exact functions of the majority of c-onc gene products are not well understood but their importance to normal cellular function is underscored by the extreme evolutionary conservation which is observed for many of these genes. For example, ras genes have been identified in the cells of yeasts (DeFeo-Jones, et al., 1983), slime moulds (Reymond, et al., 1984), insects (Neuman-Silberberg, et al., 1984), and mammals (DeFeo, et al., 1981), and the sequence of the highly conserved amino terminal domain is at least 84% homologous between any two species (Barbacid, 1987). Functional integrity is conserved also: mammalian ras genes are able to complement yeast ras<sup>1</sup>-ras<sup>2</sup>- mutants while normal yeast ras genes, if mutated, are able to transform NIH 3T3 cells (Kataoka, et al., 1985; DeFeo-Jones, et al., 1985).

The observation that a single viral oncogene, oblivious to normal cellular regulatory constraints, is able to induce the rapid, uncontrolled proliferation of cells characteristic of malignant neoplasms leads to speculation that the corresponding c-onc's play pivotal roles in the processes of cell division and differentiation. This
### TABLE 1.1

**Classification of Proto-oncogenes**

<table>
<thead>
<tr>
<th>Proto-oncogene</th>
<th>Activity or location of gene product</th>
</tr>
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<tbody>
<tr>
<td>c-src</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-abl</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-fps/fes (EGF receptor)</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-erbB(CSF-1 receptor)</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-ros</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-fgr</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-yeg</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>neu</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>trk</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>lck</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>kit</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>met</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>ret</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>sea</td>
<td>protein-tyrosine kinase</td>
</tr>
<tr>
<td>c-mos</td>
<td>serine/threonine kinase</td>
</tr>
<tr>
<td>c-mil/raf</td>
<td>serine/threonine kinase</td>
</tr>
<tr>
<td>pim-l</td>
<td>serine/threonine kinase</td>
</tr>
<tr>
<td>c-Ha-ras</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>c-Ki-ras</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>N-ras</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>c-sig(PDGF B-chain)</td>
<td>growth factor</td>
</tr>
<tr>
<td>int-2</td>
<td>potential growth factor</td>
</tr>
<tr>
<td>hst</td>
<td>potential growth factor</td>
</tr>
<tr>
<td>c-erbA(thyroid hormone receptor)</td>
<td>transcriptional activator</td>
</tr>
<tr>
<td>c-jun(p39)</td>
<td>transcriptional activator</td>
</tr>
<tr>
<td>c-myc</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>c-myc</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>c-fos</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>c-rel</td>
<td>nuclear protein</td>
</tr>
<tr>
<td>c-ski</td>
<td>nuclear protein</td>
</tr>
<tr>
<td>int-1</td>
<td>controls segmentation (in Drosophila melanogaster)</td>
</tr>
</tbody>
</table>
hypothesis is advanced by the finding that activated cellular oncogenes can also induce transformation and tumorigenicity (Varmus, 1984). What, then, are the normal functions of c-onc's when expressed at appropriate levels, at appropriate times, in appropriate cells? A number of activities have been described for various c-onc gene products, and while precise mechanisms of action in the normal cell are not well understood it is clear that perturbation of these activities may contribute to the neoplastic phenotype. The normal functions of c-onc proteins may be categorized as follows: i.) Growth factors; ii.) Growth factor receptors; iii.) Members of intracellular signal transducing pathways; iv.) Modulators of transcriptional activity.

1.) Growth factors

While a number of c-onc's encode proteins which function as growth factor receptors only one c-onc has been shown unambiguously to encode a known growth factor. Amino acid sequence analysis of platelet derived growth factor (PDGF) revealed homology with the predicted v-sis protein, with the exception of three conservative substitutions (Waterfield, et al., 1983). Subsequently it was shown that cells transformed by v-sis synthesize and secrete a molecule which is functionally and immunologically indistinguishable from PDGF (Owen, et al., 1984; Johnsson, et al., 1985); nucleotide sequence analysis of the human c-sis
gene confirmed that this locus encodes the PDGF B-chain (Chiu, *et al.*, 1984). PDGF is a heterodimeric molecule consisting of an A-chain and a B-chain linked by disulphide bonds, and the A-chain, which is approximately 60% identical to the B-chain, is encoded at a locus distinct from c-sis; the A-chain, unlike the B-chain, is markedly less efficient in transforming fibroblasts (Betsholtz, *et al.*, 1986; Tong *et al.*, 1987; Beckmann, *et al.*, 1988). While individual PDGF A or B chains are functionally inactive, A-A and B-B homodimers are synthesized and secreted by a variety of tumor cell lines and have PDGF agonist activity, raising the possibility of an autocrine mechanism in these cells (Heldin, *et al.*, 1986; De Larco, *et al.*, 1978).

A connection exists between the potential protein product of the gene *int-2* and acidic (aFGF) and basic (bFGF) fibroblast growth factors. The FGF's are members of a family of heparin-binding protein mitogens, while *int-2* is a potential oncogene implicated in the pathogenesis of murine mammary carcinoma by virtue of the frequent proviral integration of mouse mammary tumor virus adjacent to the *int-2* locus (Peters, *et al.*, 1984). Comparison of the predicted primary sequence of *int-2* with that of aFGF and bFGF reveals homology with both of these growth factors, suggesting the *int-2* product may also function as a growth factor (Dickson, *et al.*, 1987). While the *int-2* gene product awaits characterization, a novel oncogene isolated from a Kaposi's sarcoma, and possessing sequence homology
with \( \text{int-2}, \) aFGF, and bFGF, has been shown to encode a protein which is secreted and can stimulate mitogenic activity (Delli Bovi, \textit{et al.}, 1987a; Delli Bovi, \textit{et al.}, 1987b). Thus, it appears that the Kaposi's sarcoma oncogene, \( \text{int-2}, \) \( \text{hat}, \) and the FGF's are members of a gene family which encode a number of secreted growth factors; the normal cellular targets of these factors, the existence of single or multiple receptors, and the mechanisms of action of these receptors all remain to be elucidated.

11.) \textit{Growth factor receptors}

Those \( \text{c-onc}'s \) which function as growth factor or hormone receptors comprise the largest functional group of proto-oncogenes at the present time. The sequences and ligand-binding specificities have been determined for \( \text{c-erbA}, \) \( \text{c-erbB}, \) and \( \text{c-fms}, \) while a number of other genes including \( \text{neu}, \) \( \text{c-ros}, \) \( \text{trk}, \) and \( \text{c-kit} \) are believed to encode receptor molecules by virtue of sequence similarity with known receptors.

The \( \text{c-erbA} \) gene is the normal cellular counterpart of the transforming gene, \( \text{v-erbA}, \) of avian erythroblastosis virus (AEV), a virus known to cause erythroleukemias in chickens. The finding that regions of \( \text{v-erbA} \) show homology to the DNA binding and hormone binding domains of steroid hormone receptors suggested that \( \text{c-erbA}, \) the progenitor of \( \text{v-erbA}, \) might also specify a receptor protein (Weinberger, \textit{et al.}, 1985; Krust \textit{et al.}, 1986). The chicken and human
c-erbA genes were sequenced, revealing structural similarity to human glucocorticoid and estrogen receptors, and ligand binding studies with purified hormones showed that c-erbA protein specifically binds thyroid hormones, an effect which is abrogated by anti-erbA antibodies; steroid hormones which bind related receptors show no affinity for c-erbA protein (Sap, et al., 1986; Weinberger, et al., 1986). The homologous viral protein, p75v-erbA, does not bind thyroid hormones and shows a cytoplasmic distribution in contrast to the exclusively nuclear location of c-erbA protein; these changes in v-erbA ligand binding and subcellular distribution may reflect disordered regulation of thyroid hormone-sensitive genes (Sap, et al., 1986; Boucher, et al., 1988). The retinoic acid receptor gene possesses domain and sequence similarity with c-erbA, and recently a novel retinoic acid receptor gene has been identified at the hepatitis B virus integration site in a hepatocellular carcinoma (Dejean, et al., 1986; Petkovich, et al., 1987; Giguere, et al., 1987; Benbrook, et al., 1988). Thus, c-erbA is a member of a large gene family which encodes a number of nuclear hormone receptors displaying sequence homology in the highly conserved DNA binding and ligand binding domains. While c-erbA is a nuclear hormone receptor and has been shown to bind DNA the majority of proposed receptors encoded by proto-oncogenes function at the cytoplasmic membrane and are protein-tyrosine kinases; the
two best characterized c-onc's encoding receptors are c-fms and c-erbB.

The human c-fms gene encodes a 150 kD glycoprotein, gp150=ms, which is located at the cell surface and possesses protein-tyrosine kinase activity (Woolford, et al., 1985; Rettenmier, et al., 1986). Examination of human and feline tissues for expression of c-fms protein reveals that expression is maximal in spleen; curiously, human placenta and choriocarcinoma cell lines express high levels of c-fms mRNA and protein (Muller, et al., 1983; Sherr, et al., 1985; Rettenmier, et al., 1986). Fractionation of splenocytes indicates that c-fms expression is limited to a fraction composed of macrophages, granulocytes, and blast cells, and flow cytometry reveals that cells possessing c-fms epitopes are almost entirely macrophages (Sherr, et al., 1985). This relatively restricted expression of c-fms protein and its relatedness to other receptor proteins suggested a possible hemopoietic growth factor-receptor function for c-fms, and a number of observations support the assignment of c-fms as the gene encoding the receptor for the macrophage growth factor colony stimulating factor-1 (CSF-1): tyrosine-specific phosphorylation of c-fms protein is increased in the presence of CSF-1, anti-fms antibodies precipitate the CSF-1 receptor, and cloned c-fms confers upon fibroblasts the ability to bind CSF-1 (Sherr, et al., 1985; Roussel, et al., 1987).
The retroviral gene v-erbB, like v-erbA, is also carried by AEV but these two v-onc's are not structurally related. The primary sequence of tryptic peptides obtained from purified human EGF receptor was identical to regions of the deduced v-erbB protein, and sequencing of human EGF receptor cDNA revealed 95% identity with v-erbB in a region encompassing the highly conserved tyrosine kinase domain (Downward, et al., 1984a; Ullrich, et al., 1984). Thus, avian c-erbB, the progenitor of v-erbB, was almost certainly the avian EGF receptor; direct evidence of this identity was established following sequencing of the avian EGF receptor (Lax, et al., 1988). The human EGF receptor is a mol wt 175kD glycoprotein which has the ability to autophosphorylate and to phosphorylate exogenous substrates, and while this kinase reaction occurs in the absence of ligand, binding of EGF by the receptor results in enhanced substrate phosphorylation both in vivo and in vitro (Erneux, et al., 1983; Pike, et al., 1984; Downward, et al., 1984b; Downward, et al., 1985). Like the CSF-1 receptor the EGF receptor consists of an extracellular ligand binding domain, a hydrophobic membrane spanning segment, a conserved intracytoplasmic tyrosine kinase domain, and a regulatory domain at the carboxyl terminus; this typical domain structure is also seen in the receptors for PDGF, insulin, IGF-1, as well as in the potential receptors neu, c-kit, and c-ros (Martin, 1986; Carpenter, 1987). While the ligand binding specificities have been determined for the products
of c-fms and c-erbB, the identity and function of critical in vivo substrates has remained elusive, and the possibility exists that mechanisms other than protein phosphorylation may also be operative in mediating receptor function; until other members of the EGF and CSF-1 signal-transducing pathways are identified the manner in which the specific receptor transmits the mitogenic signal remains speculative.

iii.) Members of intracellular signal transducing pathways

A number of c-onc products exist which are thought to function as intracellular transducers of the growth and differentiation signals initiated when a variety of ligands interact with their receptors. These proposed intracellular messenger proteins utilize diverse mechanisms in order to relay this information from the cell surface and yet little is known of either the afferent or efferent effectors which interact with these proteins.

The ras family of highly conserved genes consists of c-Ha-ras, c-Ki-ras, and N-ras which encode related proteins of 21kD that are localized on the inner surface of the plasma membrane (Taparowsky, et al., 1983; Fujiyama, et al., 1986). The ras proteins bind guanosine diphosphate (GDP) and guanosine triphosphate (GTP) and possess a GTPase activity which hydrolyzes GTP to GDP (Scolnick, et al., 1979; Tamanoi, et al., 1984; McGrath, et al., 1984). The biochemical properties of the ras proteins are very similar
to those of the G proteins, involved in transmembrane signal transduction, and ras proteins show sequence homology with G proteins; these similarities suggest that ras gene products may also be members of signal transducing pathways (Gilman, 1984). The currently favoured model of ras function proposes that ras proteins, following stimulation by an upstream effector molecule, rapidly exchange GDP for GTP, inducing a conformational change in the ras protein; this modified ras protein is then able to interact with downstream molecules resulting in transmission of the relevant signal (Barbacid, 1987). In this scheme ras effector function is abrogated by its intrinsic GTPase action which hydrolyzes GTP to GDP with attendant conversion to the inactive conformation; the observation that oncogenic ras proteins are deficient in GTPase activity is consistent with this model in that mutant proteins, unable to hydrolyze GTP, would remain in a constitutively activated state (McGrath, et al., 1984; Sweet, et al., 1984). The identities of the hypothesized upstream and downstream effector molecules remain cryptic, although some evidence suggests possible interaction with the phosphatidylinositol pathway (Fleischman, et al., 1986; Wakelam, et al., 1986).

The genes c-raf/mil and c-mos encode soluble cytoplasmic proteins which, while displaying homology with protein-tyrosine kinases, function as serine/threonine protein kinases; the raf/mil and mos proteins are able to autophosphorylate and phosphorylate exogenous substrates at
serine and threonine residues (Papkoff, et al., 1983; Hunter, et al., 1986). The normal roles of c-raf/mil and c-mos are unknown but by analogy with protein kinase C, a serine/threonine kinase, they may function as intracellular signal transducers.

The protein-tyrosine kinase pp60<sup>src</sup> is the product of the c-src gene and is the prototypical intracellular protein believed to modify function through tyrosine phosphorylation of target substrates (Takeya, et al., 1983). This 60kD enzyme is structurally related to cAMP-dependent protein kinase and consists of a non-catalytic domain occupying the amino half of the molecule and a catalytic domain located in the carboxyl half; all v-onc, c-onc, and growth factor receptor tyrosine kinases possess domains which are homologous with the kinase domain of c-src (Hunter, et al., 1985). There are 16 amino acid residues which are invariant among all protein-tyrosine kinases and these are centred around the ATP-binding site near the N-terminus of the kinase domain and around the catalytic site near the C-terminus of the kinase domain; there is little, if any, sequence conservation in the non-catalytic amino terminal domains and these may be important in specifying the unique characteristics of each individual kinase (Hunter, et al., 1986). pp60<sup>src</sup> is not an integral membrane protein, lacking both signal peptide and membrane insertion sequences, but is located on the inner surface of the plasma membrane and attached to intracellular membranes.
(Courtneidge, et al., 1980); pp60c-src has the ability to autophosphorylate and to phosphorylate exogenous substrates at tyrosine (Hunter, et al., 1980). The intracellular location of pp60c-src and its protein-tyrosine kinase activity are consistent with a role as a member of a signal-transducing pathway, perhaps coupling a transmembrane receptor to another constituent of the same pathway; however, this conjecture must be balanced with the observation that pp60c-src expression is highest in neurons and platelets, both post-mitotic cells and not known to respond to growth factors (Cotton, et al., 1983; Golden, et al., 1986). While the foregoing observations apply specifically to pp60c-src, most also apply to the large family of intracellular protein-tyrosine kinases, such as c-fgr, c-yes, c-abl, c-fps/fes, which display significant structural and functional homology with c-src and do not encode any known receptor function (Hunter, et al., 1986).

iv.) **Modulators of transcriptional activity**

A group of c-onc's including c-myc, c-myb, c-fos, and c-jun encode proteins which are located in the nucleus of the cell and which bind DNA in vitro (Klempnauer, et al., 1984; Cole, 1986; Verma, et al., 1987; Varmus, 1987). A role for these proteins in transcriptional control has long been hypothesized and evidence exists that both c-fos and c-myc can participate in transcriptional regulation, but the most compelling support for this notion has come with
characterization of the newest member of this group, c-jun (Maki, et al., 1987; Bohmann, et al., 1987). The deduced primary sequence of c-jun specifies a protein of Mₚ 37,000 whose carboxyl-terminal region is homologous with the DNA-binding domain of the yeast transcription factor GCN4, and this region of jun can functionally replace homologous GCN4 sequences in a hybrid molecule (Vogt, et al., 1987; Struhl, 1987; Angel, et al., 1988). Antisera raised against synthetic jun peptides precipitate the mammalian transcription factor AP-1, and both AP-1 and jun proteins protect the same sites in DNAase I protection assays, leading to speculation that c-jun encodes AP-1 or a very similar factor (Bohmann, et al., 1987). However, recent tryptic peptide mapping data show that p39, a protein found complexed with p55α-β in cells, is identical with the product of c-jun; thus, c-jun is a member of a family of evolutionarily conserved genes encoding transcriptional regulatory proteins, and the known association of p39α-β with c-fos proteins in nuclei provokes speculation that several c-onc products may act synergistically in cascades or complexes which modulate transcriptional activity (Curran, et al., 1982; Rauscher, et al., 1988).

1.4 Mechanisms of Proto-oncogene Activation in Neoplasia

While individual v-onc's are capable independently of inducing tumors it is often unclear to what extent the etiology of non-viral tumors can be ascribed to activated
c-onc's, and no single type of non-viral tumor, with the exception of a few carcinogen induced cancers, has been shown to possess a consistently reproducible c-onc mutation (Balmain, et al., 1983; Wiseman, et al., 1986; Bishop, 1987). However, the frequent detection of activated or inappropriately expressed c-onc's in a variety of tumors suggests that they may subserve etiologic roles which correspond to discrete steps in the development of malignant neoplasms.

The mechanisms of c-onc activation are manifold but a useful generalization has been formulated based upon the subcellular locations of the c-onc gene products: nuclear proteins are activated by transcriptional deregulation while cytoplasmic proteins are activated by altered structure or function (Weinberg, 1985). This generalization may be extended further in observing that nuclear and cytoplasmic c-onc proteins, when activated, have different, yet complementary, effects which contribute to carcinogenesis: nuclear proteins generally result in immortalization of primary cells such that they will replicate indefinitely in vitro, while activated cytoplasmic proteins result in the appearance of a transformed phenotype (Ruley, 1983; Land, et al., 1983; Mougneau, et al., 1984; Alema, et al., 1985). In most cases the expression of a pair of c-onc's, one cytoplasmic and one nuclear, is required for the efficient transformation of primary cells; the obvious exceptions are
the retroviral v-onc's which carry full transforming potential in a single gene.

The activating mechanisms which result in loss of transcriptional control or in synthesis of altered gene products may be classified as follows: i.) Insertional mutagenesis by viruses; ii.) Chromosomal translocation; iii.) Gene amplification; iv.) Point mutation; v.) Deletion; vi.) Autocrine stimulation.

i.) Insertional mutagenesis by viruses

Any virus which integrates randomly within host cell DNA to form a provirus has the potential to disrupt normal regulation of c-onc transcription if it integrates within c-onc regulatory sequences; this mode of c-onc activation is known as insertional mutagenesis and is observed when a non-transforming retrovirus integrates in proximity to a c-onc. The provirus can alter transcription by integrating into upstream regulatory sequences, thus bringing the c-onc under control of a viral promoter, or viral integration may occur either upstream or downstream of the gene and increase transcription by virtue of the enhancers carried in the viral LTR's; either of these mechanisms may result in the enhanced transcription of c-myc seen in avian leukosis virus(ALV)-induced B-cell lymphomas (Hayward, et al., 1981; Payne, et al., 1982). DNA viruses may also be able to induce similar aberrations and the integration of human papillomavirus in some cervical carcinoma cell lines has
been shown to occur adjacent to c-myc and is associated with increased steady-state levels of c-myc mRNA (Durst, et al., 1987). Another form of insertional mutagenesis by ALV in murine erythroleukemia involves insertion into and disruption of the coding region of c-erbB such that a truncated EGF-receptor lacking an external domain is produced; interestingly, in this case, integration of ALV results in the production of a mutated cytoplasmic protein while ALV integration into c-myc results in elevated levels of normal myc protein (Nilsen, et al., 1985). Thus, ALV integration serves as a paradigm illustrating some of the unique features distinguishing activation of cytoplasmic and nuclear c-onc proteins.

ii.) Chromosomal translocation

Karyotypic abnormalities are a frequent finding in cancer cells and chromosomal translocations are consistently associated with certain human tumors; cells of Burkitt's lymphoma, a B-cell neoplasm, demonstrate translocations involving chromosome 8, close to the c-myc locus, and chromosomes carrying the immunoglobulin heavy or light chain loci; most exchanges occur with the IgH locus on chromosome 14 (Leder, et al., 1983). As a consequence of translocation the c-myc locus loses normal upstream controls or comes under the influence of powerful immunoglobulin enhancers, or both, and persistently elevated levels of c-myc mRNA, relative to quiescent cells, are detected; it is not
resolved to what extent elevated levels of c-myc mRNA versus loss of negative transcriptional control constitute activation of c-myc (Taub, et al., 1984; Keath, et al., 1984). Recently a translocation involving c-myc in a plasma cell myeloma was described which resulted in production of a chimeric c-myc transcript in which translocated cellular sequences replaced the downstream regulatory region of c-myc; this juxtaposition resulted in greatly enhanced c-myc transcript stability, perhaps achieving the same net effect as increasing transcriptional activity (Hollis, et al., 1988).

The c-abl gene encodes a cytoplasmic protein-tyrosine kinase and its structure is altered in chronic myelogenous leukemia (CML) by the translocation which produces the Philadelphia chromosome (Ph1), found in 90-95% of CML patients. The Ph1 is generated by a reciprocal translocation between chromosomes 9 and 22 and results in fusion of c-abl sequences on chromosome 9 with those of a gene known as bcr on chromosome 22; transcription is initiated within bcr and splices into c-abl exon 2, producing a chimeric mRNA (Shtivelman, et al., 1985). This hybrid mRNA is translated into a novel protein, P210<sup>bcr-abl</sup>, in CML cells and P210<sup>bcr-abl</sup> possesses greatly enhanced tyrosine kinase activity both in vivo and in vitro relative to the normal c-abl product, p145<sup>abl</sup> (Konopka, et al., 1984). While activation of kinase activity is apparent in P210<sup>bcr-abl</sup> the contribution it makes to leukemogenesis is
less clear and the hybrid \textit{bcr/abl} protein, even when highly expressed, does not transform fibroblasts (Daley, et al., 1987). A subset of acute lymphocytic leukemia (ALL) patients also demonstrate a Ph1, but in these patients a smaller transcript distinct from that found in CML is produced and encodes a unique protein, P185$^{\text{ALL-ab1}}$ (Clark, et al., 1988). Hence, chromosomal translocation can activate c-onc's by altering protein function, as illustrated here by two disparate hematologic malignancies, or activation can occur by altering steady-state transcript levels, as seen in the case of a number of c-onc's encoding nuclear proteins such as c-myc.

\textit{iii.}) Gene amplification

Gene amplification is not normally observed in human cells but is seen frequently in cancerous cells and is often heralded by the occurrence of two karyotypic abnormalities, double minute chromosomes and homogeneously staining regions, which disrupt the banding patterns of normal chromosomes. Amplification of specific c-onc's occurs in a wide variety of tumors and may involve genes coding for either cytoplasmic or nuclear proteins, although amplification is more common in genes encoding nuclear proteins; conceptually, it is relatively easy to understand how elevated levels of c-onc product due to gene amplification could assist in the development or maintenance of the neoplastic state. Amplifications involving c-myc,
c-myb, c-erbA, neu, c-erbB, c-Ha-ras, and N-myc have been observed in diverse tumors, including many of hemopoietic origin (Lee, et al., 1984; Pelicci, et al., 1984; Yukota et al., 1986; van de Vijver, et al., 1987; Wong, et al., 1987); however, despite the frequent occurrence of amplified c-myc in solid tumors the elevated level of c-myc mRNA seen in many leukemias is not usually due to amplification or obvious rearrangement at the c-myc locus, implying some other etiology of transcriptional deregulation (Westin, et al., 1982; Rothberg, et al., 1984).

iv.) Point mutation

Oncogenic activation by point mutation occurs in some c-onc's encoding cytoplasmic proteins and is best characterized in transforming alleles of the ras genes. Activated ras genes are the most commonly identified oncogenes isolated from human tumors of all types and point mutation seems to be the dominant mechanism resulting in oncogenic activation. Single nucleotide transition resulting in mutation at any of codons 12, 13, 59, or 61 is sufficient to render a given ras protein transforming, and all the mutations are located in the highly conserved amino terminal effector domain and result in marked reduction in intrinsic GTPase activity (McGrath, et al., 1984; Sweet, et al., 1984; Barbadic, 1987). Mutations at codon 13 of N-ras occur relatively frequently in acute myeloid leukemia and mutation at codon 13 of Ki-ras or of N-ras has been
demonstrated in preleukemia patients many months prior to development of frank myeloid leukemia (Bos, et al., 1985; Hirai, et al., 1987; Liu, et al., 1987). This latter observation, in concert with the isolation of activated Ki-ras alleles from colonic adenomas prior to their malignant conversion, suggests that ras mutation occurs relatively early in the neoplastic process and is consistent with an etiologic role for the ras oncogenes (Forrester, et al., 1987; Bos, et al., 1987). The neu oncogene can also be activated, in addition to gene amplification, by point mutation; in neu the altered codon is situated in the transmembrane spanning segment and is hypothesized to exert its effect by providing a constitutive stimulatory signal, in the absence of bound ligand, to the effector kinase domain (Bargmann, et al., 1986).

v.) Deletion

Deletion of regulatory domains from proteins encoded by v-onc's is a well recognized mode of activation and results in unrestrained activity of the viral protein. Both v-src and v-fms have deleted those carboxyl terminal sequences which exert a negative regulatory influence upon tyrosine kinase activity, and v-erbB has deleted most of the EGF-binding domain in addition to carboxyl terminal residues (Ullrich, et al., 1984; Coussens, et al., 1986; Hunter, 1987a). In a sense the chromosomal translocation which generates P210bcr-abl causes a deletion of amino terminal
abl sequences and results in enhanced tyrosine kinase activity comparable to that seen with P160v-"abl", which also has a deleted amino terminal (Davis, et al., 1985). Some authors believe that truncation of c-"onc"s, due to retroviral transduction or as a result of the frequent chromosomal translocations which are observed in malignant cells, is the dominant mode of c-"onc" activation (Duesberg, 1987). This hypothesis proposes that truncation deletes regulatory regions from the c-"onc" gene and brings it under the influence of regulatory elements contained within the gene with which it recombines; this deletion and recombination mechanism is advanced as being more important than either point mutation or overexpression in the oncogenic activation of c-"onc"s. Most v-"onc"s are indeed truncated versions of c-"onc"s controlled by retroviral transcriptional regulatory elements; the human trk oncogene may also represent an example of this process: a protein-tyrosine kinase gene has been truncated and is fused with an upstream tropomyosin gene, resulting in the production of a novel fusion protein (Martin-Zanca, et al., 1986). While elements of this hypothesis are very attractive they do not completely explain the frequent, and often reproducible association of activated c-"onc"s with a variety of tumors. However, the plausibility of much of this hypothesis suggests that cloning of sequences adjacent to chromosomal translocations may be a very fruitful approach to identifying novel oncogenes.
vi.) **Autocrine stimulation**

Autocrine stimulation of cells refers to the inappropriate elaboration of growth factors by cells which possess receptors for the same factor; the unregulated expression of growth factor results in persistent, unconstrained stimulation of the cell through the cognate receptor (De Larco, et al., 1978). The genes encoding factors which function in an autocrine manner may themselves be c-onc's or, alternatively, the activation of a non-factor-encoding c-onc may result secondarily in the stimulation of growth factor genes. A number of human tumors produce peptides with PDGF-agonist activity and, where characterized, these peptides are usually composed of PDGF A-A chain dimers (Heldin, et al., 1986; Bronzert, et al., 1987). While some human tumor cells express c-sis mRNA and secrete PDGF-like molecules, evidence implicating such factors in an autocrine network comes largely from simian sarcoma virus (v-sis) infected cells, which secrete PDGF-like factors, and where intracellular autocrine stimulation of PDGF-receptors is observed and transformation and proliferation are prevented by anti-PDGF sera (Johnsson, et al., 1985a; Keating, et al., 1988). In the case of simian sarcoma virus transformed cells, the sis gene is introduced by the virus; in spontaneous human tumors the nature of the genetic disruption which results in uncontrolled expression of endogenous c-sis genes has not been elucidated. Cells infected with other viruses may participate in a second type
of autocrine mechanism in which polypeptide growth factors are released as a consequence of infection; the secreted factors are encoded by cellular genes which are expressed following infection but the nature of the genetic alteration which results in their expression is unknown (Twardzik, et al., 1982; Langer-Safer, et al., 1985; Weinberg, 1985). These liberated factors exert a variety of effects and may influence the producing cell itself or surrounding cells. The expression of c-sis by some human leukemic cells may be an example of the latter effect since hemopoietic cells lack PDGF-receptors and are therefore unlikely targets for the PDGF-related molecules they synthesize; whether this c-sis expression is dependent upon activation of another c-onc is unresolved, but any such activation is unlikely to be due to viral infection since the majority of human leukemias are believed to be non-viral (Westin, et al., 1982; Williams, 1986; Sunami, et al., 1987).

1.5 Protein-tyrosine Kinases

The protein-tyrosine kinases constitute the largest functional group of c-onc proteins. In addition to those protein-tyrosine kinases specified by c-onc's and implicated in neoplasia there are a number of cellular protein-tyrosine kinases which bear close structural and functional homology with c-onc-encoded kinases but which have not yet been found to be activated in spontaneous tumors nor carried by any known retrovirus. While these c-onc-related kinases have
not been isolated directly from tumors it is clear that they do possess latent oncogenicity: the kinase domain of slk can provide transforming activity when used to replace the equivalent domain of v-fgr, and pp56^{x} becomes transforming when the influence of an inhibitory phosphotyrosine residue is ablated (Kawakami, et al., 1986; Marth, et al., 1988a).

The protein-tyrosine kinases can be subdivided into two classes, those acting as receptors for various growth factors, and those which are believed to function intracellularly in signal transduction. The majority of known protein-tyrosine kinases are categorized in Table 1.2, according to function and oncogenicity, although this compilation is not exhaustive.

The EGF receptor, encoded by the c-erbB gene, is the best characterized member of the growth factor receptor protein-tyrosine kinases. The EGF receptor is synthesized as a precursor protein, and a membrane signal peptide is cleaved cotranslationally during insertion of the protein into intracellular membranes yielding a mature molecule of 1186 residues (Ullrich, et al., 1984; Hunter, et al., 1985). The amino terminal 621 residues constitute an extracellular ligand binding domain and the carboxyl terminal 542 amino acids comprise an intracellular protein-tyrosine kinase domain; the two domains are separated by a 22 residue hydrophobic membrane spanning segment which is believed to anchor the receptor in the cell membrane. The external
TABLE 1.2

Functional Classification of Protein-tyrosine Kinases

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<thead>
<tr>
<th>Growth Factor Receptors</th>
<th>Intracellular Protein-tyrosine Kinases</th>
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<tr>
<td><strong>Known Oncogenic Potential</strong></td>
<td><strong>Known Oncogenic Potential</strong></td>
</tr>
<tr>
<td>c-fms (CSF-1 receptor)</td>
<td>c-src</td>
</tr>
<tr>
<td>c-erbB (EGF receptor)</td>
<td>c-fgr</td>
</tr>
<tr>
<td>c-erbB-2 (neu)</td>
<td>c-yes</td>
</tr>
<tr>
<td>c-ros</td>
<td>c-fps/fes</td>
</tr>
<tr>
<td>c-kit</td>
<td>c-abl</td>
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<tr>
<td>trk</td>
<td></td>
</tr>
<tr>
<td>met</td>
<td></td>
</tr>
<tr>
<td><strong>Oncogenicity Not Demonstrated</strong></td>
<td><strong>Oncogenicity Not Demonstrated</strong></td>
</tr>
<tr>
<td>PDGF receptor</td>
<td>NCP94</td>
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<tr>
<td>Insulin receptor</td>
<td>hck</td>
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<td>IGF-1 receptor</td>
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domain is extensively glycosylated and possesses multiple cysteine residues which participate in intrachain disulfide bridges; these features help confer upon the receptor its ligand binding characteristics (Mayes, et al., 1984; Soderquist, et al., 1984). The kinase domain displays features typical of all the protein-tyrosine kinases including the canonical sequences present at the ATP binding site and the catalytic site; the carboxyl terminal 15-20kD is not related in sequence to any known protein-tyrosine kinases and probably fulfills a regulatory role since three of four autophosphorylation sites are located in this domain and deletion of the major phosphate acceptor, tyrosine-1173, from v-erbB molecules is associated with reduced in vitro kinase activity (Downward, et al., 1984b; Carpenter, 1987). The EGF receptor possesses constitutive protein-tyrosine kinase activity toward itself and exogenous substrates, but tyrosine phosphorylation is enhanced both in vitro and in vivo following binding of EGF to the receptor (Erneux, et al., 1983; Downward, et al., 1984b). While EGF binding enhances kinase activity, the action of protein kinase C results in conversion of high affinity EGF binding sites to low affinity and in decreased kinase activity (Friedman, et al., 1984; Downward, et al., 1985). Protein kinase C, a serine/threonine kinase, selectively phosphorylates threonine-654, which is located in proximity to the membrane spanning region, and this is postulated to induce conformational changes, resulting in decreased activity
(Hunter, et al., 1984). Despite knowledge of ligand-induced changes in enzymatic activity and its modulation by the action of protein kinase C the relevant physiological substrates have been difficult to isolate; several proteins which are phosphorylated \textit{in vivo} in an EGF-dependent manner have been isolated but their functional significance is unknown (Hunter, \textit{et al.}, 1981; Pepinsky, \textit{et al.}, 1986). EGF receptors are expressed on most cells and binding of EGF results in stimulation of proliferation, especially of epithelial cells. Hemopoietic cells, however, do not express EGF receptors but are able to respond to EGF stimulation if a c-\textit{erbB} gene is introduced, implying either that distal components of the EGF receptor pathway are present in these cells or that the EGF receptor interacts with a common pathway shared by a number of receptors (Pierce, \textit{et al.}, 1988).

The protein \textit{pp60\textsubscript{src}} is the best characterized of all the protein-tyrosine kinases; many of the structural and functional features of \textit{pp60\textsubscript{src}} are typical of all protein-tyrosine kinases but especially of the non-receptor, intracellular c-\textit{onc} kinases. These intracellular proteins lack discernable ligand-binding domains and transmembrane segments, although one gene, \textit{ltk}, encodes an apparent hydrophobic transmembrane region but lacks an extracellular domain (Ben-Neriah, \textit{et al.}, 1988). Much of the current knowledge of the c-\textit{src} protein has come from study of the
viral protein, pp60<sup>v-a,KO</sup>, and while many similarities exist between the two molecules the differences have been instrumental in deciphering the oncogenic activation of not only pp60<sup>v-a,KO</sup> but other protein-tyrosine kinases (Collett, et al., 1978b). pp60<sup>v-a,KO</sup> is synthesized on soluble ribosomes but is tightly membrane associated due to myristylation of glycine-2 following cleavage of the initiating methionine (Buss, et al., 1985; Iba, et al., 1985). pp60<sup>v-a,KO</sup> is bound to internal cell membranes, especially to the cytoplasmic side of the plasma membrane, and myristylation may be critical in orienting the protein in relation to its normal substrates at the cell membrane (Willingham, et al., 1979; Courtneidge, et al., 1980). pp60<sup>v-a,KO</sup> displays a domain structure which serves as a model for other non-receptor protein-tyrosine kinases: an amino terminal domain consisting of residues 1-269 while a carboxyl terminal domain is comprised of residues 270-533. Mild proteolysis of pp60<sup>v-a,KO</sup> generates a 30kD C-terminal fragment which retains full kinase activity but the specific activity of this fragment is actually increased relative to the intact molecule, suggesting that the N-terminal domain subserves some regulatory role (Levinson, et al., 1981). Thus, the N-terminal domain is important for proper subcellular localization and probably for regulation of the intrinsic kinase activity; several serine residues are included within this domain and, while not critical for kinase activity, do seem to enhance activity when
phosphorylated. The amino domain shows little homology with known proteins but the kinase domain shares a number of features with most protein kinases. All protein-tyrosine kinases have the sequence X-Ala-X-Lys and about 20 residues upstream a conserved sequence of Gly-X-Gly-X-X-Gly (Hunter, et al., 1986). These sequence motifs comprise part of the ATP-binding site and the conserved lysine is absolutely required: mutagenesis of lysine-295 in pp60c-src completely abolishes kinase activity and transforming ability (Snyder, et al., 1985). Interestingly, this point mutation also prevents serine phosphorylation of the kinase; this could mean that binding of ATP by the catalytic domain induces a permissive conformation for phosphorylation of serine residues. Toward the carboxyl end of pp60c-src are other highly conserved sequences including residues 385-387(Arg-Asp-Leu), residues 404-406(Asp-Phe-Gly), and residues 430-432(Ala-Pro-Glu); mutagenesis of individual residues within these conserved sequences results in complete ablation of kinase activity (Hunter, et al., 1986). Tyrosine-416 is the major autophosphorylation site in pp60c-src and the equivalent residue is conserved among all tyrosine-protein kinases, although it is not phosphorylated in c-erbB (Downward, et al., 1984b). Despite being the major site of autophosphorylation mutagenesis of tyrosine-416 has minor effects on the kinase activity of pp60c-src, while loss of tyrosine-527 dramatically increases activity; the current evidence suggests that hyperphosphorylation of tyrosine-416
and hypophosphorylation or loss of tyrosine-527 may be critical to the oncogenic activation of pp60c-src (Courtneidge, 1985; Kmiecik, et al., 1987; Piwnica-Worms, et al., 1987). The cell membrane location of pp60c-src and the dramatic effect it exerts upon cells in its activated form suggest that this protein may act as an intracellular transducer of growth control signals, perhaps acting in concert with specific phosphatases to dynamically alter the activity of substrates via levels of tyrosine phosphorylation. However, specific substrates and the exact roles they fulfill have been difficult to define. Much of the limited knowledge available on pp60c-src substrates has come from study of the viral protein, and even overexpressing the normal protein does not result in elevation of whole cell phosphotyrosine or of specific phosphoproteins to the same extent (Coussens, et al., 1985). A number of these phosphoproteins are also elevated in cells transformed by other v-onc-encoded kinases, including v-fps, implying common effector pathways or, alternatively, promiscuous, non-specific phosphorylation of cellular proteins (Cooper, et al., 1981). A number of cytoskeletal proteins, including p36, p81, and vinculin, and glycolytic enzymes contain elevated levels of phosphotyrosine in transformed cells, and yet it remains unclear whether any of these is a legitimate substrate of pp60c-src or if alteration of any of these proteins is directly involved in transformation (Cooper, et al., 1983a; Rohrschneider, et
A recently described 81-85kD protein may well represent an authentic substrate of pp60<sup>c-src</sup> and preliminary indications are that this protein is a phosphatidylinositol kinase (Courtneidge, et al., 1987; Kaplan, et al., 1987). This protein is intriguing for two reasons: it could explain the phospholipid kinase activity which has been associated with pp60<sup>c-src</sup> but, more importantly, it would link c-src proteins with a known signal transducing pathway (Sugimoto, et al., 1984). If this hypothesized connection is verified it still leaves open the question of which afferent molecules stimulate pp60<sup>c-src</sup>, something about which nothing is known. These upstream regulators of pp60<sup>c-src</sup> activity may be members of mitogen signalling pathways but in this regard it is interesting to note that highest levels of pp60<sup>c-src</sup> are found in platelets and neurons, both post-mitotic cell types. However, pp60c-src may serve different functions in different cells, thus not excluding participation in a mitogenic role in other cell types.

1.6 The fps/fes Oncogenes

c-fes is the cellular homologue of the viral gene v-fes, carried by the Snyder-Theilen (ST-FeSV) and Gardner-Arnestein (GA-FeSV) strains of feline sarcoma virus, while c-fps is the cellular counterpart of the v-fps gene, carried by the Fujinami avian sarcoma virus and a number of other avian viruses (Bishop, 1982). Comparison of the sequences
of v-fps and v-fes genes indicates remarkable similarity, suggesting that these viral genes were derived from, respectively, the avian and feline homologues of the same c-onc; v-fes or v-fps probes anneal with identical restriction fragments of human genomic DNA, supporting this view (Hampe, et al., 1982; Groffen, et al., 1983). Thus, with respect to the human gene, either fps or fes can be used when referring to the common locus although c-fes will be employed in this dissertation purely because of greater evolutionary relatedness.

Human c-fes sequences are located on chromosome 15; they are non-contiguous and, by comparison with the chicken c-fps gene, consist of 18 exons over a span of 11 kilobases (kb) of genomic DNA (Franchini, et al., 1982; Heisterkamp, et al., 1982; Huang, et al., 1985; Roebroek, et al., 1985). The topography of the c-fes gene more closely resembles that of the c-src gene family rather than that of receptor-encoding genes, such as c-erbB, and c-fes lacks transmembrane and extracellular sequences suggesting it is probably not, by itself, a cell surface receptor (Roebroek, et al., 1985). The fps/fes genes encode proteins which display homology with other protein-tyrosine kinases including a putative ATP-binding site and a tyrosine residue equivalent to the major site of autophosphorylation in src proteins. The functional properties of v-fes and v-fps proteins were demonstrated prior to the nucleotide sequence data which related these genes to v-src, and normal cells
from the presumed species of origin of v-fps or v-fes express homologous cellular proteins which are functionally and structurally related to their viral counterparts (Barbacid, et al., 1980; Mathey-Prevot, et al., 1982). The v-fps protein, P130"-fps", autophosphorylates at tyrosine-1073, which is equivalent to tyrosine-416 in pp60"-v-src", but unlike pp60"-v-src" the loss of the major autophosphorylation site from P130"-fps" adversely affects its transforming and kinase activity (Patschinsky, et al., 1982; Weinmaster, et al., 1984). The homologous avian c-fps product, NCP98, phosphorylates this same tyrosine in vitro but, unlike the viral protein, is phosphorylated only at serine in vivo; furthermore it differs from P130"-fps" in its subcellular distribution, being found largely in the soluble cytoplasmic fraction rather than membrane-associated (Mathey-Prevot, et al., 1982; Young, et al., 1984). These distinctive functional features may be among those necessary to result in oncogenic activation of NCP98.

Expression of c-fps transcripts in normal avian tissues is highest in bone marrow cells, and fractionation studies of spleen, bone marrow, and bursa, the avian hemopoietic organs, indicates that NCP98 kinase activity is associated with granulocytes and macrophages (Shibuya, et al., 1982; Samarut, et al., 1985). c-fes transcripts are not detectable in whole mouse fetuses of varying gestation but are detectable in a variety of human malignancies, mainly hemopoietic tumors (Slamon, et al., 1984a; Slamon, et al.,
c-fes proteins were identified in cell lines from a few mammalian species, but no c-fes proteins from human or mouse cells had been identified or characterized (Barbacid, et al., 1980).

1.7 Hemopoiesis

Hemopoiesis, or hematopoiesis, is the lifelong process which ensures that sufficient numbers and varieties of all blood cells are produced throughout life in order to meet the ongoing needs of the organism. Hemopoiesis must not only resupply those blood cells lost normally as a result of finite lifespans, but must also be poised to respond flexibly and rapidly to emergencies such as bleeding or infection. The most basic building block of the hemopoietic system is the pluripotent stem cell, cells which are known functionally by their ability to repopulate any of the hemopoietic lineages. Because of their relatively low frequency in blood forming organs and their lack of unique antigenic or morphologic features the functional characterization of these cells has relied on their ability to form mixed colonies in semisolid media and to repopulate the hemopoietic systems of lethally irradiated mice (Till, et al., 1961; Bradley, et al., 1966; Dexter, et al., 1977). Pluripotent stem cells are unique in that they simultaneously possess the capacity for extensive self renewal and the ability to differentiate into committed
precursors, which then develop along specific lineages (Lord, 1983). The majority of pluripotent stem cells are not actively involved in either division or differentiation but are in a resting Go state from which they can be aroused by various physiological and non-physiological stimuli; the mechanisms by which these stressors stimulate entry into the active cell cycle are unknown.

The pluripotent stem cells give rise to at least three other types of stem cells, namely those stem cells which are committed to myelopoiesis or to T or B lymphopoiesis. These committed stem cells also possess the capacity for extensive self-renewal but their progeny can only differentiate along the respective myeloid or lymphoid pathways (Abramson, et al., 1977; Dexter et al., 1987). Once committed to a particular myeloid lineage the developing cells acquire progressively the functional and morphologic attributes of that lineage concomitant with a decreasing capacity for self renewal. However, cells of the T and B lymphoid lineages are different in that they are able to undergo clonal, self-renewing divisions at later stages of development where significant differentiation, such as immunoglobulin gene rearrangement, has already taken place (McCulloch, 1983); a brief outline of normal hemopoiesis is diagrammed schematically in Figure 1.1. The CFC-Mix, or CFC-GEMM, is the committed progenitor cell of myelopoiesis and is able to differentiate along erythroid, granulocyte/macrophage, and megakarocytic lineages; under some circumstances this cell
Figure 1.1. The human hemopoietic system. The pluripotent stem cell compartment possesses the capacity for extensive self renewal. With increasing differentiation, cells become committed to individual lineages and gradually lose their proliferative ability. (CFC-Mix = mixed colony forming cell; CFU-GEMM = granulocyte/erythrocyte/monocyte/macrophage colony forming unit; Pre-T = pre-T lymphocyte; Eos-CFC = eosinophil colony forming cell; BFU-E = erythrocyte burst forming unit; GM-CFC = granulocyte/macrophage colony forming cell; Bas-CFC = basophil colony forming cell; Meg-CFC = megakaryocyte colony forming cell; Pre-B = pre-B lymphocyte.)
may also generate lymphocytes (Messner, et al., 1981; Fauser, et al., 1985), but it lacks the extensive self renewal capacity characteristic of true pluripotent stem cells and is therefore classified as a multipotent progenitor cell (Messner, 1986; Griffin et al., 1986). The GM-CFC progenitor cell, derived from the CFC-GEMM, is able to produce both macrophages and granulocytes depending on the nature of the microenvironment and colony stimulating factors (CSFs) to which it is exposed; subsequent differentiation of GM-CFC's to mature macrophages or granulocytes is a morphologically well characterized, multistage process and will not be reiterated here.

The microenvironment in which a stem cell or committed progenitor cell finds itself plays an important role in determining the nature of subsequent differentiation events. The microenvironment is composed of stromal cells, extracellular matrix, and both short and long range soluble factors; all of these elements play a part in determining the types of cells which develop. Stem cells which seed in the spleen produce predominantly erythrocytes while granulocytes are the predominant cell type in bone marrow (Wolf et al., 1968); investigations in vitro have shown that a feeder layer established from explanted stromal cells is vital to the maintenance and growth of long term bone marrow cultures (Dexter, et al., 1977). The extracellular matrix is a glycoprotein mesh secreted by stromal cells and probably serves to allow physical attachment of stem cells
at sites of hemopoiesis; recently, a novel adhesion protein, haemonectin, has been isolated which may help account for the granulocyte predominance of bone marrow as it specifically binds cells of granulocytic origin and is located only in the bone marrow (Campbell, et al., 1987). Soluble factors, produced locally by stromal cells and acting at short range, or synthesized distantly by cells at sites of infection or inflammation are important contributors to the microenvironment; the best characterized soluble factors which stimulate granulocyte and macrophage myelopoiesis are the granulocyte-macrophage CSFs.

CSFs are glycoproteins which are synthesized ubiquitously throughout the body, and myeloid progenitor cells, at least in vitro, display an absolute requirement for these factors: the CSFs are necessary in order to maintain proliferation of progenitor cells, orderly differentiation to more mature cells, and functional activity of the mature end-stage cell (Metcalf, 1985). This requirement for CSFs is presumably mirrored in vivo, and the diversity of cell types which synthesize and secrete these factors suggests that the CSFs act as integral components of the body's natural defences by functionally activating granulocytes and macrophages at sites of infection and by stimulating the production of greater numbers of these cells by the bone marrow and spleen. There are four major granulocyte/macrophage CSFs which have been identified and shown to have stimulatory effects on myelopoiesis: 1.)
granulocyte-macrophage CSF (GM-CSF); 2.) granulocyte CSF (G-CSF); 3.) macrophage CSF (M-CSF); 4.) multi-CSF (IL-3). There appears to be a hierarchy of CSF actions in that M-CSF and G-CSF stimulate colonies of, respectively, macrophages or granulocytes while GM-CSF results in mixed granulocyte/macrophage colonies and IL-3 produces colonies in which all hemopoietic lineages except lymphocytes are represented (Metcalf, 1985). The CSFs are unique glycoproteins possessing little sequence homology and appear to be species specific: in general, murine CSFs do not stimulate proliferation of human granulocyte/macrophage precursors (Sachs, 1987). While there is no sequence homology between the CSFs and known oncogene proteins or tumor growth factors, the important niche which the CSFs occupy in the control of myelopoiesis suggests that their dysregulation might be involved in the genesis of myeloid dyscrasias. There are currently no data to indicate that the pathogenesis of human myeloid leukemias involves the unregulated production of CSFs acting in some form of autocrine loop, and the expression of the GM-CSF gene in transgenic mice does not lead to the development of leukemia (Lang, et al., 1987). However, the unregulated expression of a hemopoietic growth factor cDNA in a factor dependent cell line results in conversion to factor-independence and tumorigenicity, suggesting that an autostimulatory loop is certainly a potential mechanism in leukemogenesis (Lang, et al., 1985).
While CSFs are not known to be encoded by c-onc's the product of at least one c-onc functions as a cell surface receptor for a CSF: the c-fms gene encodes a protein, gp150=−fms, which is the receptor for CSF-1 (M-CSF). CSF-1 is absolutely required in vitro for both the activation and maintenance of macrophages and for the proliferation and differentiation of precursor cells to mature macrophages, and this factor is selectively destroyed by the same cells which it stimulates (Tushinski, et al., 1982). Examination of a number of human cells and cell lines revealed that c-fms transcripts and kinase activity are only detectable in monocyte/macrophages or in cells undergoing induced macrophage differentiation, with the exception of cells derived from the placental trophoblast (Sariban, et al., 1985; Woolford, et al., 1985; Rettenmier, et al., 1986). The elevated expression of c-fms in placenta probably reflects some non-hemopoietic developmental role in this tissue as the pregnant uterus secretes high levels of CSF-1 in response to the hormones estradiol and progesterone (Pollard, et al., 1987). Binding of murine CSF-1 to its receptor on macrophages results in enhanced tyrosine kinase activity of the c-fms protein, and anti-fms antisera are able to immunoprecipitate 125I-CSF bound to its receptor, suggesting that gp150=−fms and the CSF-1 receptor are identical (Sherr, et al., 1985); formal proof that c-fms encodes the CSF-1 receptor came with the demonstration that a transfected c-fms gene confers upon fibroblasts the
ability to bind and to be transformed by CSF-1 (Roussel, et al., 1987). Thus, at least one CSF is bound by a c-onc-encoded receptor although the mechanisms by which the receptor mediates the myriad of CSF-1-induced responses are completely unknown; the effects are likely to be the result of protein-tyrosine phosphorylation but substrates for such proposed modification have not yet been identified. It is possible, if not likely, that other c-onc's will encode hemopoietic growth factor receptors since ligands have not yet been characterized for a number of protein-tyrosine kinases which display the topology typical of transmembrane receptors.

1.8 Myelogenous Leukemias

Leukemias are hemopoietic malignancies which result as a consequence of arrested maturation of a committed cell lineage leading to overgrowth of immature, nonfunctional clonal populations. Normal hemopoietic cells and their precursors are crowded out of their usual microenvironmental niches in blood-forming organs by relentless production of the undifferentiated clone, eventually leading to clinical symptoms associated with deficiencies of the various normal cells. This overproduction of immature progeny is not the result of a reduced cell cycle time, in fact leukemic cells often have a prolonged cell cycle time compared with their normal counterparts (Baserga, 1981), but is due to
proliferation becoming uncoupled from normal regulatory constraints.

The myelogenous leukemias are classified as either acute (AML) or chronic (CML) according to clinical presentation and the predominant cell type identified in smears of peripheral blood and bone marrow, the cells in CML being mainly defective granulocytes while those in AML are primarily blast cells or partially differentiated granulocyte/macrophage precursors. Both AML and CML are clonal hemopathies, that is, the leukemic cells are believed to all be the progeny of a single malignant ancestral cell; evidence of clonality has come from a number of sources including analysis of isoenzymes, karyotypic abnormalities, and analysis of DNA polymorphisms (Fialkow, et al., 1977; Fialkow, et al., 1981a; Fearon, et al., 1986).

In CML the malignant clone is believed to originate in a pluripotent stem cell since the Philadelphia chromosome (Ph1), when present, can be detected in cells of multiple lineages (Kalousek, et al., 1984), and the clonal nature of this disease has been confirmed by examining isoenzymes of glucose-6-phosphate dehydrogenase (G6PD) in CML cells (Fialkow, 1984). Despite evidence that the defect in CML has its roots in a pluripotent stem cell and that all lineages are derived from this abnormal precursor, normal differentiation programs are followed by these other lineages; this raises the possibility that the genetic
derangement in the stem cell manifests as a selective growth advantage unique to the abnormal granulocytic cells. It has been suggested that such a growth advantage might be due to the elevated protein-tyrosine kinase activity of P210 \* in granulocytic cells (Fialkow, 1984), but this must be balanced with the observation that not all CML's are Phi-positive and some cases of Phi-positive acute lymphocytic leukemia (ALL) also display increased \textit{abl}-associated tyrosine kinase activity (Kurzrock, \textit{et al}., 1987; Chan, \textit{et al}., 1987). However, the very frequent association of the Phi with CML strongly suggests that this translocation is involved in the pathogenesis of this disease, although it may not be the initiating event since clonality can be demonstrated in CML cells prior to the emergence of the Phi (Fialkow, \textit{et al}., 1981b). Interestingly, those cases of ALL which are Phi-positive, like CML, appear to originate in a pluripotent stem cell while Phi-negative ALL's show no evidence of involvement of myeloid cells, suggesting in these cases that the disease arises in stem cells committed to lymphopoiesis (Tachibana, \textit{et al}., 1987).

The AML's comprise a more heterogenous group of diseases than CML, as assessed by morphology of the malignant cells and the presumed cell of origin. The clonal nature of AML has been demonstrated in studies of G6PD isoenzymes and of DNA restriction-fragment polymorphisms, but the cell of origin remains obscure. While it is clear that all three lineages of myelopoiesis may derive from a
malignant progenitor, implying involvement of a multipotent stem cell, it is not resolved whether lymphopoiesis, suggesting a lesion at the level of a true pluripotent stem cell, is also affected (Fialkow, et al., 1981a; Griffin, et al., 1986). Studies of cell surface markers and other indicators of cell lineage have indicated the existence of lineage infidelity in AML, that is, the co-expression of several distinctive lineage-specific characteristics in the same cell (Smith, et al., 1983). It is not clear whether the co-existence of markers of both myeloid and lymphoid lineages in AML blasts represents unique, highly abnormal pathways of differentiation or whether AML is a clonal expansion of transient precursor cells which exist during normal hemopoiesis and which normally co-express several markers (McCulloch, 1983; Greaves et al., 1986). A multistep pathogenesis for AML has been suggested in which genetic damage results in the generation of abnormal clones of preleukemic cells and subsequent events allow the emergence of a frankly malignant clone (Fialkow, 1984). Support for this proposal has come from the findings that clonal expansion is already present in the preleukemic syndromes which are frequent forebears of AML (Jacobson, et al., 1982), and that activating mutations are present in the ras genes of some preleukemic patients long before progression to acute leukemia (Hirai, et al., 1987; Liu, et al., 1987). In this model, the acquisition of chromosomal abnormalities or other genetic damage occurs later and
confers a selective advantage upon the leukemic clone; the existence of abnormal karyotypes, which are frequently identified in AML's, may herald such unrestrained growth, although consistent involvement of c-onc's in the chromosomal alterations is not an invariant feature (Yunis, 1983). The leukemic cells in AML are phenotypically less mature than those of CML and the leukemic AML cells appear to be blocked from differentiating beyond identifiable precursor forms to mature granulocytes or monocytes. However, this blockage is not absolute and apparently normal granulocytes derived from the malignant clone have been identified in AML patients in remission (Fearon, et al., 1986), and some leukemic cells are able to differentiate normally, both in vivo and in vitro, when stimulated with CSF's (Dexter, et al., 1987; Sachs, 1987). Also, characterizing the profiles of myeloid surface markers on leukemic cells suggests that some differentiation, albeit aberrant, does occur in AML patients in vivo (Griffin, et al., 1986); however, this differentiation is clearly ineffective in diverting the majority of blast cells into normal developmental programs. The fact that differentiation can take place at all belies the assumption that all leukemic blasts are equal in their proliferative capacity, and it has been shown that the vast bulk of leukemic cells are proliferatively inert (Griffin, et al., 1986); the cells responsible for the relentless production of blasts are clonogenic AML "stem cells", morphologically
indistinguishable from other blasts but possessing extensive self-renewal capacity (Buick, et al., 1979; Chang, et al., 1980). Thus, in populations of blast cells a hierarchy of diminishing replicative capacity exists, but, unlike normal myelopoiesis, it is not accompanied by a concomitant commitment to differentiation. Much effort has been expended investigating the induction of differentiation in AML's using both physiologic and non-physiologic inducers in the hope that both therapeutic and mechanistic insights might be realized (Pegoraro, et al., 1980; Koeffler, et al., 1984). Much useful knowledge has come from the examination of fresh cells but more information has resulted from the systematic study of the effects of inducers upon myeloid leukemia cell lines (Koeffler, 1983), and the use of leukemic cell lines has allowed characterization of changes in expression of c-onc's which accompany differentiation, thereby allowing glimpses of their normal functions.

1.9 Purpose and Experimental Approach

While well characterized at the DNA level (Groffen, et al., 1982; Shibuya, et al., 1982), details of the expression of c-fps and c-fes proto-oncogenes in human and murine cells have remained largely undeciphered. Previous studies have characterized the c-fps/fes gene products from a number of avian and mammalian sources but did not identify or characterize either the human or murine proteins (Barbacid, et al., 1980; Mathey-Prevot, et al., 1982). Detectable
levels of c-fes transcripts are present in human hematologic malignancies but in few other tumors (Slamon, et al., 1984b), and thus I employed immunoprecipitation techniques and a variety of functional and structural assays in order to try and identify the human and murine c-fps/fes homologous proteins in a variety of hemopoietic cells.

Differentiation to a mature phenotype is an intrinsic property of normal hemopoietic cells and is a phenomenon which can be studied to some extent in vitro by employing cell lines and exogenous inducers of differentiation. Evidence indicates that levels of protein-tyrosine kinases may vary with the differentiative state of hemopoietic cells (Barnekow, et al., 1986; Golden, et al., 1986; Quintrell, et al., 1987), and may play a supporting role in leukemogenesis (Dexter, et al., 1984). Therefore, I have investigated human myeloid leukemia cell lines during exposure to a chemical inducer of macrophage differentiation in order to determine if similar changes in expression of c-fes occur: I evaluated the expression of both c-fes mRNA and p92a-e kinase activity before and after exposure of cell lines to the inducer tetradecanoyl phorbol acetate (TPA).
CHAPTER 2

2.0 Materials and Methods

2.1 Cells, Cell Lines, and Tissues

The following human cell lines were used: HL-60, derived from a patient with promyelocytic leukemia (Collins, et al., 1977); HL-525, a subline derived from HL-60, was obtained by subculturing HL-60 cells 102 times at 5-8 day intervals in increasing concentrations of TPA (Mitchell, et al., 1986; Homma, et al., 1986); KG-1, derived from a patient with acute myelogenous leukemia (Koeffler, et al., 1978); KG-1a, an undifferentiated subline of KG-1 which has lost the capacity to differentiate (Koeffler, et al., 1980); K562, a cell line showing erythroid characteristics but originally obtained from a patient with chronic myelogenous leukemia (CML; Lozzio, et al., 1975); HEL, an erythroleukemia cell line (Martin, et al., 1982); MOLT-4, an immature T-lymphocyte derived cell line (Minowada, et al., 1972); SU-DHL-4, an immunoglobulin-positive histiocytic B-lymphoma cell line (Epstein et al., 1978); WAY-1, an Epstein-Barr virus-transformed B-lymphocyte cell line (D. Howard, personal communication); NCI-H82, a small-cell lung carcinoma cell line (Little, et al., 1983); U-937, a monocyte-like cell line obtained from a patient with generalized histiocytic lymphoma (Sundstrom, et al., 1976).
The following murine cell lines were used: P388AD-4, an adherent macrophage-like cell line (Cohen, et al., 1981); WEHI-3B, a myelomonocytic leukemia derived cell line (Warner, et al., 1969); B6SUTA, an IL-3-dependent, non-tumorigenic cell line with some properties of a multipotential progenitor cell (Greenberger, et al., 1983); EL-4, a thymoma cell line (Farrar, et al., 1980); NS-1, a non-secreting myeloma cell line (Kohler, et al., 1976); P815, a mastocytoma cell line (Dunn, et al., 1957); MEL, a Friend virus-induced erythroleukemia cell line (Friend, et al., 1971); P19 and O1A1, embryonal carcinoma cell lines (McBurney, et al., 1982); Y1, an adrenocortical tumor cell line (Yasamura, et al., 1966); and NIH 3T3, a fibroblast cell line (Barbacid, et al., 1981).

A number of virus-transformed cell lines were used: E26 virus-transformed avian myeloblasts were grown as previously described (Beug, et al., 1984); FSV-transformed Rat II cells were grown according to published methods (Weinmaster, et al., 1983); and ST-FeSV-transformed NIH 3T3 fibroblasts were grown as previously described (Barbacid et al., 1981). No infectious viruses were produced by any of these cell lines as all the viruses were replication defective retroviruses.

Adherent cell lines were maintained in 100 mm Falcon dishes while suspension cultures were grown in 250 ml tissue culture flasks. Cells were grown in RPMI-1640 medium (Gibco) supplemented with HEPES, 10% fetal bovine serum
(FBS), penicillin and streptomycin at 37°C in a 5% CO₂ atmosphere, or in Dulbecco's modified Eagle's medium receiving the same supplements. When required, individual cell lines were supplemented with 3% horse serum (cell line Y1) or 2% chicken serum (avian cell lines). All cell lines were tested periodically for contamination by Mycoplasma species using a commercially available cytotoxicity test kit (Mycotect; Bethesda Research Laboratories), and all experiments were performed on Mycoplasma-negative cells.

Normal mouse bone marrow cells were obtained from the femurs and tibias of DBA/2 mice by flushing the marrow cavities with cold PBS and pelleting the cells, while early erythroblasts were obtained from the spleens of acetylphenylhydrazine-treated mice as described previously (MacDonald, et al., 1985). Normal mouse spleens, hearts, livers, and kidneys were obtained from DBA/2 mice, the tissues homogenized, and lysates prepared for use in in vitro kinase reactions. Peripheral blood leukocytes and bone marrow cells from healthy donors or from patients with CML or AML were separated by buoyant density centrifugation over Ficoll-Hypaque and contaminating erythrocytes were removed by lysis with TRIS/NH₄Cl, as described (Territo, et al., 1977).

Exposure of HL-60 or HL-525 cultures to TPA was done as follows: healthy cells, in logarithmic growth phase, were split with fresh media 24 hr prior to addition of TPA. On
the day of induction the cells were counted and cell density was adjusted to $8 \times 10^6$ / ml with fresh medium. Cells were placed in 750 ml tissue culture flasks and TPA (10 ug / ml in 95% ethanol; Sigma) was added directly to the culture and gently mixed; final concentration of TPA was $1.6 \times 10^{-6}$ M and ethanol 0.1%. No morphologic alterations were ever seen in control cultures receiving only ethanol. Little, if any, adherence was seen in cultures of less than 8 hr exposure to TPA and hence these early cultures were harvested by simply pouring the cells into centrifuge tubes and pelleting them. In cultures induced for longer than 8 hr, the flasks were first scraped gently with a plastic cell scraper (Costar) to dislodge any adherent cells prior to pelleting.

2.2 Preparation of Immune Reagents

Anti-fps serum (J6) was obtained from T. Pawson and was originally prepared by injecting 4-week-old Fischer X Wistar rats with $5 \times 10^6$ FSV strain L5-transformed Rat-1 cells as previously described (Ingman-Baker, et al., 1984). Non-immune serum was obtained from the same rats prior to inoculation with FSV-transformed cells. Anti-pEX-2-abl rabbit antiserum (Konopka, et al., 1984) was obtained from O. Witte and goat antiserum to ST-FeSV P87$^{---}$ (anti-STaut) was obtained from M. Barbacid (Barbacid, et al., 1980).

Staphylococcus aureus strain Cowan I (IgGsorb; The Enzyme Center) was prepared by incubation at 4°C with rabbit
anti-rat gamma globulin (RARIG; Cappel-Worthington) for 2 hr. The RARIG-coated *Staph. aureus* was washed three times with PBS and resuspended at a final concentration of 10% in either lysis buffer or kinase lysis buffer prior to use in immunoprecipitation.

2.3 Radiolabelling of Cells

Healthy cells in logarithmic growth phase were counted and 1 X 10⁷ cells were pelleted and washed with PBS prior to resuspension in 1.0 ml of RPMI-1640 medium lacking methionine and supplemented with 3-5% FBS. After a period of pre-incubation of 10-30 min in a 35 mm well (2x3 well plate; Linbro), (35S)methionine (100uCi/ml, 1000 Ci/mmol; Amersham Corp.) was added to a final concentration of 250 uCi/ml and the cells were incubated at 37°C for periods of time ranging from 20 min to 8 hr. Adequate incorporation of label was determined to have taken place after a 30 min period of incubation. Following the labelling period the cells were pelleted and washed twice with 2.0 ml ice cold PBS and then lysed on ice in 0.5 ml of lysis buffer (1% Nonidet P-40; 0.5% sodium deoxycholate; 10 mM Tris-HCl(pH7.5); 100 mM NaCl; 1 mM EDTA; 2 mM ATP; 10 ug/ml aprotinin; 1mM phenylmethylsulfonylfluoride (PMSF); 50 ug/ml leupeptin). The crude lysates were cleared by centrifugation at 27,000 X g at 4°C for 30 min and the cleared supernatants were transferred to fresh 1.5 ml
Eppendorf tubes and held at 4°C until immunoprecipitated; the pellets were discarded.

For \textit{in vivo} phosphate labelling, medium was switched from RPMI-1640 to DMEM several days prior to radiolabelling; cells tolerated changes in media well with no discernable alteration in growth kinetics or morphology. $5 \times 10^6$ cells were pelleted, washed once with PBS, and suspended in 1.0 ml of phosphate-free DMEM supplemented with 3-5% FBS, following which $^{32}$P-orthophosphate (2.0 mCi/ml, carrier free; ICN Pharmaceuticals Inc.) was added to a final concentration of 1.0 mCi/ml. Cells were labelled for 12 hr at 37°C following which they were harvested and lysates prepared exactly as described for methionine labelling.

To determine if p92 was phosphorylated \textit{in vivo} in a labile manner which was susceptible to the action of phosphotyrosine phosphatases, cells were labelled with $^{32}$P-orthophosphate as described above except that sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases, was added to the culture medium to a final concentration of 100 uM. Sodium orthovanadate (100 uM) was also included in the lysis buffer and all the wash buffers used during the immunoprecipitation of proteins from these cells.

2.4 \textbf{Immunoprecipitation}

Cleared cell lysates were incubated with either immune or non-immune serum for 45 min at which time 10 volumes,
relative to the volume of serum, of a 10% suspension of RARIG-coated *Staph. aureus* was added and incubated for an additional 45 min. The immune complexes were pelleted in a microfuge and washed successively with 1 M NaCl - 10 mM Tris-HCl (pH 8.0) - 0.1% NP-40; with 100 mM NaCl - 1 mM EDTA - 10 mM Tris-HCl (pH 8.0) - 0.1% NP-40 - 0.1% SDS; and with 10 mM Tris-HCl (pH 8.0) - 0.1% NP-40; all washes were performed at 4°C. Immune complexes were prepared for SDS-polyacrylamide gel electrophoresis as described in section 2.5.

For TPA-kinetic studies the same immunoprecipitation procedure as above was followed except that the volumes of \((^{35}S)\)methionine-labelled lysates from all time points were adjusted so that equivalent amounts of total TCA-precipitable radioactivity were being precipitated. Volumes of serum and of RARIG-coated *Staph. aureus* were adjusted so that equimolar concentrations of each reagent were maintained for all immunoprecipitations.

2.5 Immune Complex Kinase Reaction

Samples of 5 X 10^7 to 1 X 10^8 cells were harvested, washed, lysed in kinase lysis buffer (1.0% NP-40 - 20 mM Tris-HCl (pH 7.5) - 150 mM NaCl - 1 mM EDTA - 0.5% sodium deoxycholate), and lysates were cleared at 27,000 xg at 4°C for 30 min. Samples of cleared lysate were immunoprecipitated as described above and the resulting immune complexes were washed once in kinase lysis buffer and
twice in kinase reaction buffer (20 mM Tris-HCl(pH 7.5) - 10 mM MnCl₂), the immune complexes were then incubated with 2-20 uCi of (gamma-³²P)ATP (3,000 Ci/mmol; 10 uCi/ul; Amersham Corp.) in 35 ul of kinase reaction buffer at 20°C for 15 min. Kinase reactions were terminated by the addition of 0.5 ml of kinase lysis buffer and the immune complexes were washed three times with kinase lysis buffer, on ice. Following this, the immune complexes were suspended in 50 ul of SDS-electrophoresis sample buffer (10% glycerol - 5% 2-mercaptoethanol - 2.3% SDS - 0.0625 Tris) and heated at 37°C for 10 min in order to disrupt the immune complexes. The RARIG-coated Staph. aureus was pelleted by centifuging at full speed in a microfuge for 3 min, and the supernatants, containing the radiolabelled proteins, were prepared for immediate SDS-polyacrylamide gel electrophoresis or were stored overnight at -20°C.

Phosphorylation of exogenous substrates was assayed by adding 5 ug of acetic acid-denatured rabbit muscle enolase (Cooper, et al., 1984) to the kinase reaction mixture, prior to the addition of (gamma-³²P)ATP, and then incubating at 30°C for 15 min. The reaction was terminated by addition of an equal volume of 2X SDS-electrophoresis sample buffer to the reaction mixture followed by heating at 37°C for 10 min to disrupt the immune complex; supernatants were prepared as above and were subjected to electrophoresis or stored overnight at -20°C.
In order to compare *in vitro* kinase activity from one time point to the next in the TPA-kinetic studies, the protein content of each lysate was determined by a Coomassie blue-binding assay (Sedmak, *et al.*, 1977) and the volume of individual lysates adjusted to ensure that equivalent amounts of total protein were subjected to immunoprecipitation; adjustments were made, where necessary, to maintain equimolar concentrations of the immune reagents.

2.6 SDS-Polyacrylamide Gel Electrophoresis

Radiolabelled proteins in SDS-electrophoresis sample buffer were heated at 100°C for 3 min before being loaded onto the SDS-polyacrylamide gel system as originally described by Laemmli (1970). A 15 cm vertical slab gel apparatus was used (Protean II; Bio-Rad Laboratories) and the stacking gel contained 4.5% polyacrylamide while the separating gel contained 7.5% polyacrylamide; in the case of V8 protease analysis a 12.5% separating gel was employed. The proteins were separated electrophoretically at a constant power of 2 watts, which was increased to 3 watts once the tracking dye in the sample buffer had entered the separating gel. A buffer system of 0.025 M Tris - 0.192 M glycine - 0.1% SDS - pH 8.3 was utilized and electrophoresis was continued until the tracking dye reached the bottom of the gel, at which time the current was stopped. After electrophoresis, gels containing (\(^{32}\)P)-labelled proteins were soaked overnight in 0.04% Coomassie brilliant blue-R -
7.5% acetic acid - 50% methanol in order to fix proteins and stain molecular weight standards of known size. Stained gels were destained for one to three hours in 7.5% acetic acid - 50% methanol and for 10 min in distilled water before being dried onto filter paper (3MM; Whatman) using a Hoefer slab gel dryer. Labelled proteins were detected according to the method of Laskey and Mills (1977) by exposing the dried gels to Kodak XAR-5 film at -80°C in the presence of an intensifying screen (Lightning Plus; DuPont). Gels containing (35S)methionine-labelled proteins were treated identically except that following destaining gels were impregnated with fluor (En3Hance; New England Nuclear Corp.) for one hour before drying and autoradiography. In the case of gels in which the 32P-labelled proteins were subjected to further analysis, for example tryptic peptide mapping, the gels were removed from the electrophoresis apparatus, wrapped in Saran wrap and exposed wet to XAR-5 film at 4°C in order to locate bands of interest; these bands were then excised from the wet gel.

All films were developed using a Kodak MII film processor, and molecular weights of relevant proteins were calculated from a linear regression plot of the log of molecular weight versus the relative mobility for standard proteins of known molecular weight which were run on the same gel and were detected by Coomassie blue staining. In those immune complex kinase reactions which included enolase, the location of phosphorylated enolase in SDS-
polyacrylamide gels was confirmed by the parallel electrophoresis of unlabelled enolase and detection by staining with Coomassie blue.

2.7 Phosphoamino Acid Analysis

Analysis of phosphoamino acid composition of in vivo or in vitro phosphorylated proteins or phosphopeptides was carried out essentially according to published methods (Beemon, et al., 1978; Cooper, et al., 1983b), with only minor modifications. \(^{32}\)P-labelled proteins were recovered by overlaying wet or dried gels with the corresponding autoradiogram and identifying and excising the piece of gel containing the protein of interest. The proteins were eluted from the gel slices overnight in 5 ml of PAA elution buffer (50 mM \(\text{NH}_4\text{HCO}_3\) - 0.1% SDS - 5% 2-mercaptoethanol) at 37°C. The gel fragments were recovered by centrifugation at 500 xg for 10 min and a second elution was performed on these, utilizing similar conditions, for 4 hr. The two eluates were combined and centrifuged at 27,000 xg for 10 min in order to remove any residual gel fragments. To the combined eluates was added 25-30 \(\mu\)g of bovine gamma-globulin, as a carrier, and proteins were precipitated overnight at 4°C by the addition of 100% TCA to a final concentration of 20%. The precipitated proteins were pelleted by centrifugation at 25,000 xg for 20 min at 4°C and washed twice with 95% ethanol prior to drying under vacuum. The dried pellets were resuspended in 0.2 ml of 6 N
HCl and boiled for 2 min before being sealed in a glass tube and hydrolyzed at 110°C for 90 min; the hydrolyzed proteins were transferred to microfuge tubes and the liquid was removed by lyophilization. The dried pellets were resuspended in 3.0 ul of pH 1.9 buffer (88% formic acid - acetic acid - water; 50:156:1794 by volume) and then mixed with 3.0 ul of a solution of non-radioactive authentic phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine; all at 0.1 mg/ml) prior to loading onto a 20 cm X 20 cm thin-layer cellulose (TLC) plate (0.1 mm; E. Merck Lab). Following sample application the plate was dampened with pH 1.9 buffer and subjected to electrophoresis at 1,000 volts for 180 min towards the anode. The plate was then dried, re-wetted with pH 3.5 buffer (pyridine - acetic acid - water; 10:100:1890 by volume), and electrophoresed towards the anode at 1,000 volts for 80 min in a direction which was perpendicular to the original electrophoresis. Subsequently, the plate was dried and exposed to film, with an intensifying screen, at -70°C; following autoradiography the non-radioactive phosphoamino acids were located by spraying the plate with a ninhydrin stain (0.1 gm ninhydrin - 70 ml ethanol - 21 ml acetic acid - 2.9 ml 2, 4, 6-collidine) and developing the colour by heating gently over a hot plate for 10 min.

The procedure followed in order to determine phosphoamino acid composition of tryptic phosphopeptides was similar except that areas of the TLC plates containing
phosphopeptides were scraped off into microfuge tubes and the peptides eluted by incubation overnight at 37°C in 0.5 ml pH 2.1 buffer. The cellulose fragments were removed by centrifugation and the eluted phosphopeptides were dried by lyophilization before being subjected to hydrolysis and electrophoresis as described above.

2.8 Tryptic Peptide Analysis

Labelled proteins were identified and extracted from gels, precipitated with TCA, washed, and dried exactly as described above for phosphoamino acid analysis. Proteins were dissolved in 100 ul formic acid and to this was added 25 ul methanol and 40 ul performic acid (prepared fresh by combining 0.9 ml formic acid with 0.1 ml 30% hydrogen peroxide for 1 hr at 20°C). The dissolved proteins were incubated at -5°C for 2 hr following which 3.0 ml of water was added, the solution quick frozen in a dry ice-ethanol bath, and lyophilized to dryness. The oxidized proteins were dissolved in 0.5 ml of 50 mM NH₄CO₃ and digested with 5 µg of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (TPCK-trypsin; Worthington) for 6 hr at 37°C. Following digestion 2.5 ml water was added, the solution quick frozen, and lyophilized; the lyophilization was repeated once and the peptides were then dissolved in 1.0 ml of pH 2.1 electrophoresis buffer (water - formic acid - acetic acid; 90:2:8 by volume), transferred to a 1.5 ml microfuge tube, quick frozen and lyophilized to dryness.
The lyophilized sample was resuspended in 5 ul of pH 2.1 buffer and applied to a 20 cm X 20 cm TLC plate, the plate was dampened with pH 2.1 buffer and electrophoresed towards the cathode for 1 hr at 1,000 volts. After electrophoresis the plate was air dried and subjected to ascending chromatography in N-butanol - acetic acid - water - pyridine (75:15:60:50, by volume) in a direction perpendicular to that of electrophoresis. \(^{32}\text{P}\)-labelled phosphopeptides were detected by exposing the TLC plate to XAR-5 film in the presence of an intensifying screen at -80°C. In some cases, in order to investigate relatedness of proteins, two different proteins were isolated and digested with trypsin separately before being applied together to the same TLC plate; conditions were otherwise identical with those used for a single protein.

2.9 Partial Proteolytic Cleavage with V8 Protease

Limited proteolysis with V8 protease was performed essentially as described previously (Cleveland, et al., 1977); proteins labelled in in vitro kinase reactions were separated by electrophoresis and the relevent proteins were identified by autoradiography and excised from the gels. The gel slices containing the labelled proteins were soaked in V8 protease buffer (0.125 M Tris-HCl(pH 6.8) - 1 mM EDTA - 0.1% SDS) for 30 min at 20°C and were placed in the bottoms of wells of a fresh SDS-polyacrylamide gel system with a 5 cm-long stacking gel and a 12.5% polyacrylamide
separating gel. The samples were overlaid with the same V8 buffer but containing 20% glycerol; the usual SDS-electrophoresis buffer was then added to the apparatus. To each well was added 20 ul of a solution consisting of: 10% glycerol, 0.0001% bromphenol blue, and various concentrations of *Staphylococcal aureus* V8 protease, dissolved in V8 protease buffer. Electrophoresis was performed at a constant power of 2W until the bromphenol blue tracking dye was 0.5 cm from the separating gel, at which point the power and cooling system were turned off for 30 min; electrophoresis was then resumed at 3W until the dye front had reached the end of the separating gel. Gels were fixed, stained, dried and exposed to film exactly as described in section 2.6.

2.10 Isolation and Analysis of Total Cellular RNA

Total cellular RNA was isolated according to published methods (Cathala, et al., 1983). Briefly, 1 X 10⁶ cells were pelleted in a benchtop centrifuge for 3 min at 800 xg in a 50 ml plastic tubes, washed once with 10 ml of PBS at 4°C, and re-pelleted for 3 min at 800 xg in pre-weighed 30 ml Corex tubes. Cell cultures which had been induced with TPA and which showed any cell adherence were scraped with a cell scraper prior to harvesting and washing the cells. The cell pellet was weighed after the second centrifugation and 7.0 ml/gm(of wet cells) of guanidine lysis buffer (5 M guanidine monothiocyanate - 10 mM EDTA - 50 mM Tris-HCl(pH
7.5) - 8%(v/v) 2-mercaptoethanol) was added to each tube and the cells were homogenized by vortexing three times for 10 seconds each. The cell lysate was transferred onto ice and total cellular RNA was precipitated by the addition of 7 volumes of 4 M LiCl per volume of homogenate, and incubated 15-20 hr at 4°C. The RNA's were pelleted at 11,000 xg for 60 min at 4°C, and the supernatants, containing mainly DNA and denatured proteins, were discarded. To remove DNA and protein contaminants the pellet was resuspended in 3 M LiCl (volume identical with that of 4 M LiCl); the pellet was disrupted by forcing it successively through 18, 22, and 25 gauge needles using a sterile syringe. The RNA's were re-pelleted at 11,000 xg for 60 min at 4°C, the supernatant discarded, and the pellet dissolved in RNA solubilization buffer (10 mM Tris-HCl(pH 7.5) - 1 mM EDTA - 0.1% SDS). The solubilized RNA's were extracted according to accepted methods (Maniatis, et al., 1982) with phenol, phenol:chloroform, and chloroform. After the final chloroform extraction the RNA's were precipitated from the aqueous phase by adding 0.05 volumes of 3 M sodium acetate (pH 5.1) and 2 volumes of 95% ethanol and incubating 12 hr at -20°C. The RNA's were pelleted by centrifugation at 12,000 xg for 10 min at 4°C, washed once with 10 ml of 70% ethanol, re-pelleted at 12,000 xg for 10 min and dried under vacuum. RNA pellets were dissolved in 100 ul of water and the concentration of RNA was determined
spectrophotometrically by measuring the OD_260 of duplicate 1:100 dilutions of RNA.

Total RNA was fractionated according to the method of Lehrach et al. (1977) as follows: a 0.8-1.0% agarose-formaldehyde gel was poured into a 9.5 X 14.5 cm horizontal electrophoresis tray (Dedicated Design Line; Hoefer Scientific Instruments), a 15 tooth comb was inserted, and the gel was allowed to set at room temperature. RNA samples were prepared for loading as follows: a volume of RNA solution calculated to contain 10 ug of total cellular RNA was placed in a 1.5 ml microfuge tube and to it was added 12.0 ul deionized formamide, 4.0 ul 37% formalin, 5.0 ul 5X gel running buffer (0.2 M morpholinopropanesulfonic acid - 50 mM sodium acetate - 5 mM EDTA), and a volume of water sufficient to bring the total volume to 25 ul. After mixing, these samples were incubated at 70°C for 10 min and then 2.5 ul of RNA loading buffer (50% glycerol - 1 mM EDTA - 0.4% bromphenol blue - 0.4% xylene cyanol) was added to each tube and mixed; a 3 ug aliquot of authentic RNA molecular size standards (RNA ladder; Bethesda Research Laboratories) was prepared in exactly the same manner as the RNA samples. The agarose gel was submerged in 1,100 ml of 1X gel running buffer, the comb removed, RNA samples and size standards loaded into wells, and subjected to electrophoresis at 70 volts for 3 hr. The gel was stained in 500 ml of acridine orange (15 ug/ml in water) for 30 min and was then destained twice in 500 ml of water for a total
of 60 min; the stained gel was exposed to ultraviolet light in order to measure the locations of the size standards and to ascertain that there was no degradation of the 28s or 18s rRNA. Any gel showing evidence of degradation of either 28s or 18s rRNA was assumed to have been subject to the action of ribonucleases and was discarded.

RNA was blot-transferred from the gel to a nylon membrane (HYBOND-N; Amersham Corp.) according to the manufacturer's instructions, which are essentially modifications of commonly employed protocols (Southern, 1975; Thomas, 1980). Following blot transfer for 12-16 hr the membrane was removed, rinsed in 2X SSC, wrapped in Saran wrap and exposed to ultraviolet light for 5 min in order to bind the RNA to the membrane.

The membrane was placed in a heat-sealable bag and pre-hybridization fluid (0.6 M NaCl - 0.18 M Na₂HPO₄ - 3 mM EDTA - 1% N-lauroyl sarcosine - 2.5% dextran sulfate - 100 ug/ml heat-denatured herring testes DNA) was added at a ratio of 0.1 ml/cm² area of nylon membrane. Air bubbles were forced out, the bag sealed, and incubated at 65°C for 5 hr. After pre-hybridization the bag was cut open and the fluid emptied out; this was replaced by hybridization fluid at 0.05 ml/cm² area of membrane, the bag was re-sealed, and the membrane allowed to hybridize at 65°C for 16-20 hr; the hybridization fluid had exactly the same composition as the pre-hybridization mix except that it contained the labelled
probe, described in section 2.12, at a concentration of 0.5-1.0 \times 10^7 \text{ CPM/ ml of fluid.}

After hybridization the membrane was removed from the bag and washed twice in 1X SSC (3 M NaCl - 0.3 M trisodium citrate; pH 7.0) - 0.1% SDS - 0.1% sodium pyrophosphate at 20°C for 15 min each and once in 0.1X SSC - 0.1% SDS - 0.1% sodium pyrophosphate at 65°C for 30 min in a shaking water bath. Following the final wash the membrane was air-dried until damp, wrapped in Saran wrap, and exposed to XAR-5 film in the presence of an intensifying screen at -70°C for 1-4 days.

2.11 **Morphologic Markers of Cellular Differentiation**

Morphology of TPA-exposed or control cells growing in culture was recorded with a Wild inverted microscope with photographic attachment. Growth and viability counts were done by preparing dilutions of cells, mixing with an equal volume of 0.5% eosin-Y in PBS, and counting viable and non-viable cells in a hemocytometer chamber on an inverted microscope. Cytocentrifuge-slide preparations of unstained cells were prepared from TPA-exposed and control cultures at appropriate intervals using a Cytospin II apparatus (Shandon Instruments). Slides were stained with Wright-Giemsa stain using an automated processor (Coulter Instruments) or were stained for (alpha)-naphthyl acetate esterase or for chloracetate esterase according to previously published procedures (Yam, et al., 1970). Stained slides were
evaluated for differentiation with a Zeiss Photomicroscope II, and photographed with ASA 400 film on the same microscope.

2.12 Preparation of Radiolabelled Nucleic Acid Probes

The DNA used initially to probe membrane-bound RNA was a 3.5 kbp BglII-XhoI fragment of Gardner-Arnstein FeSV provirus (Even, et al., 1983) which had been cloned into pGem-1 and was obtained from Dr. Peter Greer; use of this probe was discontinued due to high levels of binding not only to c-fes mRNA but also to 28s and 18s rRNA. Subsequently a human cDNA probe, also obtained from Dr. Peter Greer, was used in hybridization; this probe consisted of a 1.8 kbp EcoRI fragment, encoding exon 8 to the 3' end of the human c-fes gene (Roebroek, et al., 1985), which had been cloned into the EcoRI site of pUC18. The actin probe was a 1.3 kb PstI fragment of bovine (beta)-actin cDNA cloned into the PstI site of pBR322 (Degen, et al., 1983). The preparation of the above fragments from the original plasmid DNA was similar in each case and will be outlined only briefly.

Competent E. coli were transformed with purified plasmid DNA according to standard procedures (Maniatis, et al., 1982) and grown on LB plates containing an appropriate antibiotic. Resistant colonies were identified and grown up overnight in 4-5 ml LB broth tubes supplemented with antibiotics, and plasmid DNA was isolated from the bacterial
pellet using a modified version (Wilimzig, et al., 1985) of
the boiling method for small scale extraction of plasmid DNA
(Holmes, et al., 1981). Following isolation of the
respective plasmids the presence of the appropriately sized
insert was confirmed by restriction enzyme analysis using
standard procedures; an estimate of the concentration of
insert, and hence the plasmid, was made by comparing it with
DNA fragments of known size and concentration using an
ethidium bromide spotting plate. After determination of
plasmid concentration 1 ug of plasmid DNA was digested to
completion with the appropriated restriction enzyme and the
fragments were separated electrophoretically on a 1% low
melting-point agarose gel. The band representing insert DNA
was identified in each case and the band excised from the
gel and stored at -20°C until use. Insert DNA (16-22 ng)
was labelled to high specific activity (1-3 X 10^9 CPM/ug)
using oligo-labelling by hexanucleotide primers, exactly as
described (Feinberg, et al., 1984), in an overnight
reaction. The radiolabelled probe was purified from
unincorporated nucleotides over an ion-exchange mini-column
(NACS Prepac; Bethesda Research Laboratories) entirely
according to the manufacturer's specifications. Aliquots of
the unpurified reaction mix and of the column-purified probe
were assayed by liquid scintillation counting in order to
calculate specific activity and yield for every labelling
reaction. Purified probe DNA was held at 4°C until needed
at which time it was denatured by heating at 95°C for 10 min
followed by rapid cooling and addition to the calculated volume of hybridization fluid.
CHAPTER 3

3.0 Identification and Characterization of Murine and Human c-fes Proteins.

3.1 Introduction

c-fps/fes proteins from a variety of species are highly conserved, based upon nucleotide sequence analysis, immunological cross-reactivity, and functional comparison (Barbacid, et al., 1980; Hampe, et al., 1982; Mathey-Prevot, et al., 1982; Huang, et al., 1985). FSV-transformed or ST-FeSV-transformed rat fibroblasts have been used in syngeneic animals to induce the production of anti-tumor antibodies or have been employed to immunize heterologous species; in either case, antisera are produced which are able to specifically recognize viral and cellular fps/fes proteins (Barbacid, et al., 1980; Ingman-Baker, et al., 1984). Initially, v-fps or v-fes proteins possessing protein-tyrosine kinase activities similar to that of pp60v-fes were identified (Barbacid, et al., 1980; Feldman, et al., 1980); subsequently, immunologically-related normal cellular proteins were shown to have similar protein-tyrosine kinase activity and these cellular proteins were demonstrated to have sequences similar to viral transforming proteins by proteolytic mapping and eventually by nucleotide sequence analysis (Mathey-Prevot, et al., 1982; Huang, et al., 1985; Roebroek, et al., 1985). Despite the identification of c-fes proteins in a number of divergent
species, these proteins were not detected in human or murine cells (Barbacid, et al., 1980); however, this study was limited in the number of cell lines which were examined for the presence of c-fes proteins, and this suggested that it might be possible to identify murine and human c-fes proteins by searching a more extensive array of cells, tissues, and cell lines for expression. Also, none of the murine or human cell lines which were tested were of hemopoietic origin; studies of the avian c-fps protein, NCP98, showed that it is preferentially expressed in granulocytes and macrophages (Samarut, et al., 1985), further suggesting that the previous inability to detect homologous c-fes proteins might be circumvented by scrutinizing hemopoietic cells of various lineages.

3.2 Results

3.2.1 Characterization of Rat anti-fps Sera

In the course of producing monoclonal antibodies directed against v-fps proteins, a number of sera were collected from Fischer or Wistar rats in which sarcomas had been induced by the injection of FSV-transformed fibroblasts, as described previously (Ingman-Baker, et al., 1984). These tumor-bearing rat sera had been shown to possess reactivity towards the v-fps protein Pl40Gag-Fps in in vitro kinase reactions and one serum, designated J6, demonstrated particular ability to immunoprecipitate Pl40Gag-Fps (T. Pawson, personal communication). In order
to determine if this serum might be suitable for immunoprecipitation of c-fes proteins, it was evaluated for its ability to precipitate v-fps (P140<sup>-p</sup>) and v-fes (P85<sup>-p</sup>) proteins in in vitro kinase reactions. J6 serum specifically immunoprecipitated an 85kD protein from ST-FeSV-transformed NIH 3T3 fibroblasts which was phosphorylated in vitro (Figure 3.1, lane 2); comparison with the equivalent protein recognized by caprine anti-v-fes serum (ST<sub>aut</sub>; Barbacid, et al., 1980) suggested that this protein was P85<sup>-p</sup> (Figure 3.1, lane 3). The ability of J6 serum to recognize P140<sup>-p</sup> was confirmed by in vitro kinase activity of immunoprecipitates prepared from lysates of FSV-transformed Rat 2 fibroblasts (Figure 3.1, lane 6). Interestingly, the ST<sub>aut</sub> serum was unable to recognize P140<sup>-p</sup> in this assay system, implying that it was less broadly cross-reactive with fps/fes proteins than J6 (Figure 3.1, lane 4), and that another explanation for the previously documented inability to detect human and murine c-fes proteins (Barbacid, et al., 1980) could be that the proteins were not recognized by the ST<sub>aut</sub> serum, rather than not expressed by the cells.

Enolase is a glycolytic enzyme which is known to be phosphorylated both in vivo and in vitro by a number of viral protein-tyrosine kinases, including P85<sup>-p</sup> (Cooper, et al., 1983a; Cooper, et al., 1984). Enolase is frequently included in in vitro kinase assays in order to assess phosphorylation of exogenous substrates by protein-
Figure 3.1: Identification of v-fes and v-fps proteins by rat anti-fps serum. To assay for in vitro kinase activity, ST-FeSV-transformed NIH 3T3 cells (lanes 1-3) or FSV-transformed Rat II fibroblasts were lysed and immunoprecipitated with J6 anti-fps serum (lanes 2 and 6), or STmut anti-fes serum (lanes 3 and 4), or non-immune rat serum (lanes 1, 5, and 7). Immune complexes were incubated with (gamma-32P)ATP and in vitro-phosphorylated proteins were identified by electrophoretic separation and autoradiography. The locations and sizes (in kD) of molecular weight standards are indicated to the right.
tyrosine kinases and has been shown to be phosphorylated exclusively at tyrosine \textit{in vitro} (Cooper, \textit{et al.}, 1984). J6 serum was tested for its ability to specifically precipitate P85 in an \textit{in vitro} kinase reaction which included denatured rabbit muscle enolase, and this resulted in the autophosphorylation of P85 and the phosphorylation of enolase (Figure 3.2, B); phosphoamino acid analysis indicated that enolase was phosphorylated exclusively at tyrosine (Figure 3.2, C). This result suggests that P85 possesses intrinsic tyrosine kinase activity; while a co-precipitating kinase could conceivably result in the "autophosphorylation" of P85 it is more difficult to invoke this same explanation to account for the phosphorylation of enolase since the amino acids surrounding the site of tyrosine phosphorylation in enolase are much different from those in the \textit{v-fps/fes} transforming proteins (Cooper, \textit{et al.}, 1984). Chicken myeloblasts transformed by the E26 virus produce high levels of NCP98, the normal avian \textit{c-fps} product; J6 serum is also able to precipitate NCP98, which is autophosphorylated in an immune complex kinase assay (Figure 3.2, A).

Thus, the tumor-bearing rat serum employed in this study was able to immunoprecipitate a number of viral and cellular \textit{fps/fes} proteins of the expected molecular weights from cell lines known to express such proteins, and each had an associated protein-tyrosine kinase activity. It appeared that this serum recognized a wider spectrum of \textit{c-fps/fes}
products than the caprine antiserum and this reagent was therefore used in an attempt to identify cross-reacting human and murine c-fes proteins.

3.2.2 Examination of Human and Murine Hemopoietic Cells for Expression of c-fes Proteins

A previous investigation failed to detect cross-reactive c-fes proteins in murine and human cells but this study did not survey any hemopoietic cells; in chickens hemopoietic cells are known to express high levels of NCP98, and thus I examined fresh murine and human hemopoietic cells to determine if an equivalent activity is present. Fresh mouse spleen cells and bone marrow cells were obtained from DBA/2 mice, and human peripheral blood leukocytes and bone marrow cells were obtained from healthy donors; in addition, a murine multipotential hemopoietic progenitor cell line, B6SUTA, was also examined (Greenberger, et al., 1983). Mouse bone marrow and spleen cells were collected and washed prior to preparation of cell lysates and hence represent heterogenous populations of hemopoietic cells. The human peripheral blood samples were collected over Ficoll-Hypaque and were consequently enriched for lymphocytes, monocytes, and their precursors; the human bone marrow samples were also collected over Ficoll-Hypaque but consisted mainly of erythroid and myeloid precursor cells, with few lymphocytes or monocytes. Cell pellets were prepared, lysed, and proteins immunoprecipitated for use in in vitro immune complex
Figure 3.2: Identification of c-fps and v-fes proteins by rat anti-fps serum. E26-transformed avian myeloblasts (lanes 1 and 2) or ST-FeSV-transformed NIH 3T3 cells (lanes 3 and 4) were lysed and immunoprecipitated with J6 anti-fps serum (lanes 2 and 4) or non-immune serum (lanes 1 and 3). Immune complexes were incubated with (gamma-\(^{32}\)P)ATP and, in the case of panel B, with 5 ug of acid-denatured rabbit muscle enolase; In vitro phosphorylated proteins were separated by electrophoresis and identified by autoradiography. The locations and sizes (in kD) of molecular weight standards for panel A are indicated to the left. The location of enolase was confirmed by electrophoresis of unlabelled enolase and detection by Coomassie blue staining. The 85 kD protein identified in panel B was isolated from the gel and subjected to acid hydrolysis, followed by two-dimensional electrophoretic separation of phosphoamino acids, as shown in panel C. Authentic phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) were located by staining with ninhydrin and are indicated.
kinase assays. A 92kD protein (p92) present in lysates of both human peripheral leukocytes and bone marrow cells was specifically phosphorylated in an \textit{in vitro} kinase reaction (Figure 3.3, lanes 1 and 3); a protein of similar size was also present and was phosphorylated in lysates prepared from mouse bone marrow cells (Figure 3.3, lane 5). Unfractionated spleen cells consist mainly of lymphocytes and erythrocytes, and to a lesser degree of monocyte/macrophages; analysis of mouse spleen cells reveals a 92kD phosphoprotein which co-migrates with p92 found in bone marrow cells (Figure 3.3, lane 7). However, lysates of total spleen cells also contain a 94kD protein (p94) which is recognized by the anti-\textit{fps} serum and which is also phosphorylated \textit{in vitro} (Figure 3.3, lane 7). Examination of cells from the multipotential hemopoietic progenitor cell line B6SUtA indicated that they too expressed p92 (Figure 3.4, lane 3); this cell line, while immortalized, displays many of the features of normal bone marrow progenitors. In no case did the non-immune serum precipitate any unique protein: p92 and p94 were specifically immunoprecipitated by the anti-\textit{fps} tumor serum. Fresh human peripheral leukocyte preparations occasionally demonstrated the presence of p94 but this was not a consistent observation and seemed to correlate with the presence of erythrocytes in the cell pellet.

On the basis of immunological cross-reactivity with the viral proteins P140\textit{fps} and P85\textit{fps}, and the c-\textit{fps}
Figure 3.3: Identification of p92 and p94 in human and murine hemopoietic cells. Lysates of normal human peripheral blood leukocytes (lanes 1 and 2), normal human bone marrow cells (lanes 3 and 4), normal mouse bone marrow cells (lanes 5 and 6), and normal mouse spleen cells (lanes 7 and 8) were immunoprecipitated with J6 anti-fps serum (lanes 1, 3, 5, and 7) or non-immune serum (lanes 2, 4, 6, and 8). The immune complexes were incubated with (gamma-\(^{32}\)P)ATP and the in vitro-phosphorylated proteins were separated by electrophoresis and detected by autoradiography. The locations and sizes (in kD) of molecular weight standards are indicated to the left.
Figure 3.4: Identification of p92 in a murine multipotential hemopoietic progenitor cell line. Bone marrow cells from DBA mouse femurs (lanes 1 and 2) or B6SUtA cells were lysed and immunoprecipitated with J6 anti-fps serum (lanes 1 and 3) or non-immune serum (lanes 2 and 4). Immune complexes were incubated with (gamma-32P)ATP and the in vitro phosphorylated proteins were separated by electrophoresis and identified by autoradiography.
protein NCP98, it seems likely that the proteins identified in cell lysates of human and murine hemopoietic cells represent c-fes proteins. This assertion is supported by the finding that the molecular weights of these two proteins, 92kD and 94kD, are similar to the molecular weights of feline NCP92 and of avian NCP98 (Barbacid, et al., 1980; Mathey-Prevot, et al., 1982). Also, a protein-kinase activity is associated with both p92 and p94, and it is not inconceivable that this activity is due to autophosphorylation at tyrosine residues, a property associated with authentic fes proteins. Either or both of p92 or p94 may be c-fes proteins: the differences in molecular weight can be accounted for by a number of potential mechanisms including post-translational protein processing, glycosylation, or production of differentially spliced transcripts.

3.2.3 Examination of Human and Murine Cell lines for Expression of c-fes Proteins

Relatively large amounts of protein are required for more extensive characterization and in order to have a readily available supply of cells expressing c-fes proteins a number of mouse and human cell lines were screened for expression of p92 and p94 using immune complex kinase assays. The cell lines examined reflected a variety of different tissue sources but were predominantly of hemopoietic origin, as these appeared to hold the most
promise of expressing detectable levels of p92 or p94. All of the hemopoietic cell lines screened expressed either p92, p94, or co-expressed both these proteins (Table 3.1); additionally, several non-hemopoietic cell lines expressed p94, but p92 was not observed in any of them. Representative autoradiographs are shown in Figure 3.5, demonstrating the presence of p92 or p94 or the p92/p94 doublet in individual cell lines; also shown are the results of an immune complex kinase assay of HL-60 cells utilizing a different anti-fps serum (Figure 3.5, lanes 9 and 10). This antiserum, Fl-1, was also obtained from tumor-bearing rats but consistently showed lower affinity for both p92 and p94 and thus was not routinely employed. Most of the cells expressing p92 were derived from myeloid leukemias, although two B-lymphoma cell lines and an erythroleukemia cell line also expressed p92 (Table 3.1), consistent with published results which indicate that c-fes transcripts are present in most hematologic malignancies, especially AML and CML, but in few other tumors (Slamon, et al., 1984). This study also found low levels of c-fes transcripts in four out of four lung tumors examined, but the one lung carcinoma cell line tested here for the presence of p92/p94 was negative. Two other groups also reported that c-fes transcripts were detectable only in normal myeloid cells, AML, and CML, with the exception that c-fes mRNA could be detected in megakaryocytes and in immature erythroid precursors (Ferrari, et al., 1985; Emilia, et al., 1986). The presence
<table>
<thead>
<tr>
<th>Species</th>
<th>Cell type or tissue</th>
<th>Expression(^1)</th>
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<tbody>
<tr>
<td>Human</td>
<td></td>
<td>p92</td>
</tr>
<tr>
<td>HL-60</td>
<td>(promyelocytic leukemia)</td>
<td>+</td>
</tr>
<tr>
<td>KG-1</td>
<td>(myeloblastic leukemia)</td>
<td>+</td>
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<tr>
<td>KG-1a</td>
<td>(KG-1 variant cell line)</td>
<td>-</td>
</tr>
<tr>
<td>K562</td>
<td>(erythroleukemia)</td>
<td>-</td>
</tr>
<tr>
<td>HEL</td>
<td>(erythroleukemia)</td>
<td>+</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>(T-cell leukemia)</td>
<td>-</td>
</tr>
<tr>
<td>SU-DHL-4</td>
<td>(B-cell lymphoma)</td>
<td>+</td>
</tr>
<tr>
<td>WAY-1</td>
<td>(B-lymphocyte)</td>
<td>+</td>
</tr>
<tr>
<td>U937</td>
<td>(histiocytic lymphoma)</td>
<td>+</td>
</tr>
<tr>
<td>NCI-H82</td>
<td>(small-cell lung cancer)</td>
<td>-</td>
</tr>
<tr>
<td>Peripheral leukocytes (normal)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Peripheral leukocytes (AML)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Bone marrow cells (normal)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td>p92</td>
</tr>
<tr>
<td>B6SUtA</td>
<td>(progenitor cell)</td>
<td>+</td>
</tr>
<tr>
<td>WEHI-3B</td>
<td>(myeloblast leukemia)</td>
<td>+</td>
</tr>
<tr>
<td>P815</td>
<td>(mastocytoma)</td>
<td>+</td>
</tr>
<tr>
<td>P388AD</td>
<td>(macrophage-like)</td>
<td>+</td>
</tr>
<tr>
<td>EL-4</td>
<td>(thymoma)</td>
<td>-</td>
</tr>
<tr>
<td>MEL</td>
<td>(erythroleukemia)</td>
<td>-</td>
</tr>
<tr>
<td>NS1</td>
<td>(myeloma)</td>
<td>-</td>
</tr>
<tr>
<td>P19, OLAl</td>
<td>(embryonal carcinoma)</td>
<td>-</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>(fibroblast)</td>
<td>+</td>
</tr>
<tr>
<td>Y1</td>
<td>(adrenal cortex)</td>
<td>-</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Spleen (normal)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Spleen (early erythroblasts)</td>
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<tr>
<td>Heart</td>
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<td>Kidneys</td>
<td></td>
<td>-</td>
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</table>

\(^1\) The presence or absence of detectable p92 or p94, as determined by \textit{in vitro} kinase assays, is indicated by a "+" or "-" respectively.
Figure 3.5: Identification of p92 and p94 in human and murine hemopoietic cell lines. Cells from MEL (lanes 1 and 2), HEL (lanes 3 and 4), K562 (lanes 5 and 6), HL-60 (lanes 7, 8, 9, and 10), MOLT-4 (lanes 11 and 12), or KG-1 (lanes 13 and 14) cell lines were lysed and immunoprecipitated with J6 anti-fps serum (lanes 2, 4, 6, 7, 11, and 13), Fl-1 anti-fps serum (lane 9), or non-immune serum (lanes 1, 3, 5, 8, 10, 12, and 14). Immune complexes were incubated with (gamma-32P)ATP and in vitro phosphorylated proteins were identified by electrophoretic separation and autoradiography.
of p94 did not correlate with any particular cell type or lineage, and if p92 and p94 are linked in some precursor/product relationship it is curious that more of the cell lines did not display both p92 and p94 activity.

The human cell line HL-60 was chosen as a source of p92, K562 as a source of p94, and HEL as a source of p92/p94 (Figure 3.5). These cell lines were chosen for further characterization for a variety of reasons, including availability of the cell lines and ease of culture, relatively high levels of expression of their respective proteins, and relatively low levels of non-specific precipitation of unrelated cellular proteins from lysates of these cells. Also, cell lines, while not necessarily clonal, represent far more homogeneous populations of cells than could have been arrived at by using fresh tissues.

3.2.4 Characterization of Human p92 and p94

The sine qua non of protein-tyrosine kinases, whether encoded by viral or by cellular genes, is their ability to phosphorylate substrates at tyrosine residues; the majority of v-onc and c-onc protein-tyrosine kinases have now been shown to autophosphorylate at one or more tyrosine residues within the enzyme itself. p92 and p94 would be expected to contain phosphotyrosine if the incorporation of label seen in the immune complex kinase assay is due to an analogous autophosphorylating activity; accordingly, p92 and p94 proteins, phosphorylated in vitro, were extracted from SDS-
polyacrylamide gels and analyzed for phosphoamino acid content. HL-60 p92 is phosphorylated at tyrosine, although phosphoserine and phosphothreonine are also present (Figure 3.6, A), and K562 p94 is phosphorylated exclusively at tyrosine (Figure 3.6, B). While Figure 3.6, A suggests that phosphoserine and phosphotyrosine are present in equimolar amounts, repeat experiments showed that the majority of phosphate label was incorporated as phosphotyrosine (see Figure 3.11, A). The detection of phosphoserine and phosphothreonine in p92 could be due to an intrinsic serine/threonine kinase activity, such as that described for mos or mil/raf, or might be due to contaminating, co-precipitating cellular kinases which utilize p92 as a substrate. Low levels of phosphoserine or phosphothreonine have also been detected in the autophosphorylated products of well characterized protein-tyrosine kinases such as pp60c-src, pp60v-src, and P140gsp (Collett, et al., 1978a; Hunter, et al., 1980; Feldman, et al., 1980); further characterization was undertaken in order to try and resolve whether the observed phosphorylation of serine and threonine was due to an intrinsic activity of p92 or to some co-precipitating kinase.

A number of glycolytic enzymes, including enolase, are phosphorylated at tyrosine by fps/fixa-encoded protein-tyrosine kinases (Cooper, et al., 1983a). The phosphorylation of enolase in vitro occurs at a single tyrosine residue and this same unique tyrosine is also
Figure 3.6: Phosphoamino acid analysis of p92, p94, and enolase. $^{32}$P-labelled proteins, phosphorylated in in vitro kinase reactions as described, were isolated from SDS-polyacrylamide gels and subjected to acid hydrolysis, followed by two-dimensional electrophoretic separation of phosphoamino acids. The positions of authentic phosphoserine (p-ser), phosphothreonine (p-thr), and phosphotyrosine (p-tyr) were determined by staining with ninhydrin and are indicated. (A) HL-60 p92; (B) K562 p94; (C) enolase phosphorylated in vitro by HL-60 p92; (D) enolase phosphorylated in vitro by K562 p94; (E) enolase phosphorylated in vitro by NCP98.
modified *in vivo* (Cooper, *et al.*, 1984); if p92 and p94 represent authentic c-fes proteins it is likely that they too will phosphorylate enolase at tyrosine. The ability of these proteins to phosphorylate rabbit muscle enolase in an *in vitro* kinase assay was determined; p92 from HL-60 (Figure 3.7; lane 1) and p94 from K562 (Figure 3.7; lane 5) were able to cause the phosphorylation of enolase in the immune complex kinase assay. Under the same reaction conditions NCP98 from E26-transformed cells also resulted in the phosphorylation of enolase (Figure 3.7, lane 3); phosphoamino acid analysis of enolase from each of these reactions demonstrated predominantly phosphotyrosine (Figure 3.6; C, D, and E). Phosphorylated enolase was extracted from SDS-polyacrylamide gels and subjected to digestion with trypsin; trypsin cleaves after lysine and arginine residues and, depending on the content of amino acid residues in the phosphate-labelled cleavage fragments, unique patterns result following electrophoretic and chromatographic separation. Digestion of enolase labelled *in vitro* by p92, p94, or NCP98 showed that a single co-migrating tryptic phosphopeptide was generated, indicating that all three proteins possessed the same substrate specificity for enolase (Figure 3.8; A, B, and C). Phosphoamino acid analysis of this peptide revealed, in each case, exclusively phosphotyrosine (data not shown), establishing that p92 and p94 phosphorylate the same tyrosine residue as known fps/fes proteins. These results show that the kinase activities
Figure 3.7: Phosphorylation of enolase in vitro by p92, p94, and NCP98. HL-60 cells (lanes 1 and 2), E26-transformed avian myeloblasts (lane 3), or K562 cells (lanes 4 and 5) were lysed and immunoprecipitated with J6 anti-fps serum (lanes 1, 3, and 5) or non-immune rat serum (lanes 2 and 4). Each immune complex was precipitated and resuspended in a 30 ul reaction mixture containing 5 ug of acid-denatured rabbit muscle enolase prior to the addition of (gamma-32P)ATP. In vitro phosphorylated proteins were identified by electrophoretic separation and autoradiography. The location of enolase was confirmed by electrophoresis of unlabelled enolase and detection by staining with Coomassie blue.
Figure 3.8: Tryptic phosphopeptide analysis of enolase phosphorylated in vitro by p92, p94, and NCP98. Rabbit muscle enolase, phosphorylated in vitro by p92, p94 or NCP98 (as in Figure 3.7), was eluted from SDS-polyacrylamide gels and digested with trypsin. The resulting digests were analyzed by electrophoresis on TLC plates at pH 2.1, followed by chromatography in the second dimension. The locations of the $^{32}$P-containing phosphopeptides were determined by autoradiography. (A) enolase phosphorylated by p94; (B) enolase phosphorylated by NCP98; (C) enolase phosphorylated by p92.
associated with p92 and p94 phosphorylate enolase at tyrosine contained almost exclusively within a single major tryptic phosphopeptide, implying that these proteins are indeed protein-tyrosine kinases. These data also support the assumption that the phosphoserine and phosphothreonine observed in phosphorylated p92 (Figures 3.6, A; 3.11, A) were due to a co-precipitating kinase since enolase phosphorylated \textit{in vitro} by NCP98, a known tyrosine-specific kinase, likewise contained trace amounts of phosphoserine and phosphothreonine; also, a single tryptic peptide containing the major phosphoacceptor tyrosine residue is completely consistent with previously published results for other \textit{fps/fes} proteins (Cooper, \textit{et al}., 1984).

Mapping of tryptic peptides can be used to establish structural relatedness between proteins, and this technique was employed to investigate how closely p92 and p94 might resemble P85\textsuperscript{\textit{fps/fes}}, a known \textit{fes} protein. HL-60 p92, K562 p94, and ST-FeSV/NIH 3T3 P85\textsuperscript{\textit{fps/fes}} were phosphorylated \textit{in vitro} in individual immune complex kinase reactions and the labelled proteins were extracted from SDS-polyacrylamide gels and analyzed by tryptic peptide mapping. Trypsin cleavage of p92 generated two major phosphate containing peptides (Figure 3.9; A, 1 and 2). Cleavage of P85\textsuperscript{\textit{fps/fes}} also generated two predominant phosphopeptides, closely resembling p92 in this respect (Figure 3.9, B). A mixing experiment was performed to establish if the two peptides from p92 co-migrated with those of P85\textsuperscript{\textit{fps/fes}}, which would
Figure 3.9: Tryptic phosphopeptide analysis of HL-60 p92 and ST-FeSV P85*-m. Human p92 and viral P85*-m, phosphorylated in *in vitro* kinase reactions, were eluted from SDS-polyacrylamide gels and digested with trypsin. The resulting digests were analyzed by electrophoresis on TLC plates at pH 2.1, followed by chromatography in the second dimension. The locations of the *32P*-containing phosphopeptides were determined by autoradiography. An equal amount of radioactive label was loaded onto each plate. (A) HL-60 p92; (B) ST-FeSV P85*-m; (C) mixture of equal amounts of p92 and P85*-m. The two major phosphopeptides isolated from HL-60 p92 are numbered 1 and 2; the sample origins are shown with arrows.
imply identity; as shown in Figure 3.9, C, phosphopeptides 1 and 2 from p92, while not separating identically to those from P85=3=2=3, have migrated in a very similar fashion, suggesting a high degree of amino acid sequence similarity. Trypsin digestion of *in vitro* phosphorylated K562 p94 released only one major phosphopeptide (3.10; B, peptide 3), and the appearance of the p94 tryptic peptide map was quite different from that of p92 (Figure 3.10, A); p94 obtained from the murine erythroleukemia cell line MEL resulted in a pattern identical with that of human p94 (Figure 3.10; C). In order to establish definitively that p94 peptide 3 was distinct from either of p92 peptides 1 or 2, phosphorylated p92 and p94 were digested with trypsin and the resulting peptides were separated on the same thin-layer chromatography plate in a mixing experiment (Figure 3.10, D): it is quite clear that p92 and p94 phosphopeptides migrate independently. The human erythroleukemia cell line, HEL, co-expresses p92 and p94, both of which can be phosphorylated *in vitro* and resolved on SDS-polyacrylamide gels as a doublet (see Figure 3.5); tryptic peptide mapping of the p92/p94 doublet generates a pattern identical to that obtained from mixing p92 and p94 (Figure 3.10, E). This evidence confirms that the divergent patterns observed in the individual tryptic peptide maps of p92 and p94 were not a result of obtaining these two proteins from different cell lines or some other experimental artifact: isolating and mapping the same proteins from a single cell line, HEL, gave
Figure 3.10: Comparitive tryptic phosphopeptide analysis of p92 and p94. HL-60 p92, K562 p94, HEL p92/p94, or MEL p94 were phosphorylated in \textit{in vitro} kinase reactions, eluted from SDS-polyacrylamide gels, and digested with trypsin. The resulting digests were analyzed by electrophoresis on TLC plates at pH 2.1, followed by chromatography in the second dimension. The locations of the $^{32}$P-containing phosphopeptides were determined by autoradiography; an equal amount of radioactive label was loaded onto each plate. (A) HL-60 p92; (B) K562 p94; (C) MEL p94; (D) mixture of equal counts per minute of phosphopeptides from HL-60 p92 and K562 p94; (E) HEL p92/p94 doublet. The two phosphopeptides isolated from p92 are numbered 1 and 2 (as in Figure 3.9), and the major phosphopeptide isolated from p94 is numbered 3.
identical results. Peptides 1, 2, and 3 were scraped off of their respective locations on the thin-layer chromatography plates and analyzed for phosphoamino acid content; all three of the peptides, representing the major phosphoacceptor sites for p92 and p94, contained exclusively phosphotyrosine (Figure 3.11; B, C, E). These data further support the proposal that p92 and p94 are tyrosine-specific kinases: p92 and p94 have been shown to phosphorylate the exogenous substrate enolase at the same tyrosine as other protein-tyrosine kinases, and the results of tryptic peptide mapping show that the enzymes themselves are autophosphorylated almost exclusively at tyrosine residues contained within one or two tryptic peptides. The absence of detectable phosphoserine or phosphothreonine in either of peptide 1 or 2 from p92, and the lack of any other major phosphopeptides which might represent sites of serine or threonine phosphorylation support the contention that the phosphoserine and phosphothreonine seen in Figure 3.6 is due to some contaminating kinase; none the less, the observed phosphorylation of p92 at serine or threonine residues may well represent significant regulatory modifications.

The structural comparison of p92 and p94 was extended by performing partial proteolytic digests of the in vitro phosphorylated proteins with Staphylococcus aureus V8 protease and mapping the resulting fragments; V8 protease cleaves after glutamic acid and aspartic acid residues and is well suited to generate peptides for structural
Figure 3.11: Phosphoamino acid analysis of tryptic phosphopeptides from p92 and p94, and of \textit{in vivo} phosphorylated p92. The areas on TLC plates (A) and (B) shown in Figure 3.10 containing, phosphopeptides 1 and 2(p92) and phosphopeptide 3(p94), were scraped off, the phosphopeptides eluted from the cellulose fragments, and subjected to acid hydrolysis. In separate experiments, HL-60 p92 was phosphorylated in an \textit{in vitro} reaction or was labelled \textit{in vivo} with $^{32}$P-orthophosphate; the $^{32}$P-labelled proteins were eluted from SDS-polyacrylamide gels and subjected to acid hydrolysis. The phosphoamino acids were analyzed by two-dimensional electrophoresis and autoradiography; the locations of authentic phosphoserine (p-S), phosphothreonine (p-T), and phosphotyrosine (p-T) were determined by staining with ninhydrin. (A) HL-60 p92, labelled \textit{in vitro}; (B) HL-60 p92, peptide 1; (C) HL-60 p92, peptide 2; (D) HL-60 p92, labelled \textit{in vivo}; (E) K562 p94, peptide 3.
comparison of proteins (Cleveland, et al., 1977). p92, p94, and P85gag-fes were phosphorylated in individual immune complex kinase reactions and subjected to V8 protease mapping; in parallel with the results of tryptic peptide mapping, the pattern of proteolytic fragments generated from p92 (Figure 3.12; lanes 3, 4) closely resembled that of P85gag-fes (Figure 3.12; lanes 1, 2), and a majority of P85gag-fes phosphopeptides co-migrated with equivalent peptides from p92, as indicated. A number of peptides unique to p92 were also observed and these presumably represent divergent primary structure relative to P85gag-fes, resulting in a somewhat different mosaic of peptides. Partial proteolytic digestion of p94 yielded a pattern distinct from that of either p92 or P85gag-fes and did not appear to share any of the seven identified co-migrating fragments (Figure 3.12; lanes 5, 6). Thus, these data extend and support the results of tryptic peptide mapping which suggest that p92 is structurally very closely related to the fes protein P85gag-fes, and that p94, while demonstrating functional similarities, is more distantly related at the level of primary sequence.

The feline (NCP92) and avian (NCP98) fps/fes homologues are synthesized de novo in the same cells in which they are detected by immune complex kinase assays (Barbacid, et al., 1980; Mathey-Prevot, et al., 1982). HL-60 cells were metabolically labelled and radioactive proteins were immunoprecipitated and separated electrophoretically; p92
Figure 3.12: V8 protease digestion of p92, p94, and P85*. P85*, p92, K562 p94, and ST-FeSV P85* were phosphorylated in vitro and the labelled proteins were identified by electrophoresis and autoradiography. Gel fragments containing the ^32P-labelled proteins were excised and applied to the wells of a new 12.5% SDS-polyacrylamide gel, the proteins were digested in situ with V8 protease, and the resulting cleavage products identified by electrophoresis and autoradiography. The proteins digested and the amounts of V8 protease used were: (lane 1) P85*, 50 ng; (lane 2) P85*, 200 ng; (lane 3) p92, 50 ng; (lane 4) p92, 200 ng; (lane 5) p94, 50 ng; (lane 6) p94, 200 ng. Comigrating fragments are identified by arrows to the left, while molecular weight standards (in kD) are on the right.
was specifically precipitated by the J6 anti-fps serum from lysates of HL-60 cells labelled with either \(^{35}\text{S}\text{Methionine}\) (Figure 3.13, lane 1) or with \(^{32}\text{P}\text{-orthophosphate}\) (Figure 3.13, lane 5), indicating that it is synthesized in these cells. \textit{In vivo} labelled p92 co-migrates with p92 labelled in \textit{in vitro} kinase reactions (Figure 3.13; lane 3). p92 was also detectable in the cell line KG-1 (Figure 3.14; lane 1), while p94 could not be immunoprecipitated from lysates of K562 cells labelled with \(^{35}\text{S}\text{Methionine}\) (Figure 3.14; lane 3) or with \(^{32}\text{P}\text{-orthophosphate}\) (data not shown). These data are consistent with the conclusion that p92 is immunologically related to known fps/fes proteins; the inability to precipitate labelled p94 could mean that it is not synthesized by these cells or that it is labelled but not detected. The structural comparisons presented here show that p94 is more distantly related than p92 to P85\(^{\text{p21-ras}}\)-\(^{\text{c-src}}\), suggesting, as a consequence, that fewer cross-reactive epitopes may be present and hence low levels of labelled p94 remain undetected by J6 anti-fps serum. Alternatively, p94 might be bound by the antiserum with the same affinity as p92 but not detected due to a significantly lower rate of synthesis or smaller steady-state pools of protein; this explanation is probably not the correct one since repeated experiments utilizing longer labelling periods and greater volumes of lysate all failed to demonstrate synthesis of p94 (data not shown).
Figure 3.13: Synthesis of p92 in HL-60 cells. HL-60 cells were metabolically labelled in vivo with \((^{35}\text{S})\text{methionine} \text{ (lanes 1 and 2)}\) or with \(^{32}\text{P}\)-orthophosphate \text{ (lanes 5 and 6)}\) and lysates were prepared, or lysates were prepared from unlabelled HL-60 cells \text{ (lanes 3 and 4)}\). Lysates were immunoprecipitated with J6 anti-fps serum \text{ (lanes 1, 3, and 5)}\) or with non-immune serum \text{ (lanes 2, 4, and 6)}\); the immune complex from the unlabelled lysate was labelled in an in vitro kinase reaction. The radiolabelled proteins in each case were then separated by electrophoresis through 7.5% SDS-polyacrylamide gels and identified by autoradiography; the location of p92 is indicated with an arrow.
Figure 3.14: Metabolic labelling of KG-1 and K562 cells. KG-1 cells (lanes 1 and 2) or K562 cells (lanes 3 and 4) were metabolically labelled *in vivo* with \(^{35}\text{S}\)methionine and lysates prepared. These lysates were immunoprecipitated with J6 anti-fps serum (lanes 1 and 3) or with non-immune serum (lanes 2 and 4) and the labelled proteins were identified by electrophoretic separation and autoradiography; the locations of molecular weight markers (in kD) are indicated to the left and the location of p92 is shown with an arrow.
The $^{32}$P-orthophosphate-labelled p92 in Figure 3.13 was extracted from the gel and analyzed for its phosphoamino acid content; in contrast to in vitro labelled p92 (Figure 3.11; A) in which phosphotyrosine is the predominant phosphorylated species, only phosphoserine was detected in p92 from $^{32}$P-orthophosphate-labelled cells (Figure 3.11; D). A similar pattern of phosphorylation has been described for NCP98, in which in vitro autophosphorylation occurs at tyrosine and yet only phosphoserine is detectable when the protein is labelled in vivo (Mathey-Prevot, et al., 1982); however, the equivalent viral transforming protein of PRC II virus, P105<sup>g-fp</sup>:ESJ", is phosphorylated at tyrosine both in vivo and in vitro, suggesting that retroviral transduction may result in unmasking of previously unavailable tyrosine residues (Beemon, 1981). c-fes proteins may also be susceptible to similar modifications, since human c-fes protein is not phosphorylated at tyrosine in vivo while v-fes protein, like v-fps, is phosphorylated at tyrosine both in vivo and in vitro (Barbacid, et al., 1980). Preincubation of $^{32}$P-labelled cells with sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases, and inclusion of this reagent in all buffers did not result in the appearance of discernable phosphotyrosine in p92 (data not shown). This suggests that p92, like NCP98, is not phosphorylated at tyrosine in vivo and that the lack of detectable phosphotyrosine is not due to phosphatase cleavage during isolation; removal of c-fps/fes proteins
from their normal cellular context, either by cell disruption or retroviral expression, facilitates autophosphorylation of tyrosine, possibly by removing these enzymes from the vicinity of inhibitory molecules.

The K562 cell line was derived from a patient with CML and possesses an amplified and translocated c-\textit{abl} gene, resulting in the production of a c-\textit{abl} protein, P210\textsuperscript{c-\textit{abl}}, which is considerably larger than the normal P145\textsuperscript{c-\textit{abl}} (Heisterkamp, \textit{et al.}, 1983); as a result of its aberrant structure, P210\textsuperscript{c-\textit{abl}} possesses considerably greater protein-tyrosine kinase activity than its normal human counterpart (Konopka, \textit{et al.}, 1984). In order to show that J6 anti-\textit{fps} serum did not react promiscuously with other protein-tyrosine kinases, and to show that p94 detected in K562 cells did not represent a processed form of P210\textsuperscript{c-\textit{abl}}, K562 lysates were immunoprecipitated with either J6 anti-\textit{fps} serum or with pEX-2 anti-\textit{abl} serum and used in immune complex kinase assays. J6 serum recognized only p94 (Figure 3.15; lane 3) and pEX-2 serum recognized only P210\textsuperscript{c-\textit{abl}} (Figure 3.15; lane 4); for comparison, HL-60 p92 and HEL p92/p94 are shown in Figure 3.15, lanes 1 and 2. These data imply that J6 serum does not react indiscriminately with any protein-tyrosine kinase and that p94 is not a processed form of P210\textsuperscript{c-\textit{abl}}; furthermore, the results suggest that p94, while being structurally distinct, shares some common antigenic determinants with \textit{fps/fes}-encoded proteins which
Figure 3.15: Identification of p92, p94, and P210<sup>c-abl</sup> in lysates of human hemopoietic cells. Lysates were prepared from HL-60 cells (lane 1), HEL cells (lane 2), or K562 cells (lanes 3 and 4) and were immunoprecipitated with J6 anti-fps serum (lanes 1, 2, and 3) or with pEX-2 anti-abl serum (lane 4). Immune complexes were incubated with (gamma-<sup>32</sup>P)ATP and in vitro phosphorylated proteins were identified by electrophoretic separation and autoradiography. The locations and sizes of molecular weight standards (in kD) are indicated, as are the locations of p92, p94, and P210<sup>c-abl</sup>. 
may not be present in other protein-tyrosine kinases, as exemplified by P210°-b1.

3.3 Discussion

The data presented here extend previously published results, which demonstrated the presence of immunologically cross-reactive \textit{fps/fes} proteins possessing protein-tyrosine kinase activity in cells from a variety of species (Barbacid, \textit{et al.}, 1980; Mathey-Prevot, \textit{et al.}, 1982). One of these previous reports alluded to the expression of NCP98 in hemopoietic cells (Mathey-Prevot, \textit{et al.}, 1982), but the association of maximal kinase activity with granulocytic cells and macrophages was not made until somewhat later (Samarut, \textit{et al.}, 1985). The other early report in this area demonstrated a 92kD protein-kinase in non-hemopoietic cells obtained from several species, but human and murine cell lines, derived from fibroblasts, were negative for an equivalent activity (Barbacid, \textit{et al.}, 1980).

My results demonstrate clearly that proteins possessing immunological determinants in common with \textit{v-fps} and \textit{v-fes} proteins are present in a variety of murine and human cells. These proteins, p92 and p94, are of similar molecular mass as the \textit{c-fps/fes} proteins described previously and also appear to possess intrinsic protein-tyrosine kinase activity. It is not established unequivocally from my results that the associated kinase activity is intrinsic to p92 and p94, as discussed in section 3.2.1.; however,
applying the same burden of proof which other investigators have employed, it is likely that the observed "autophosphorylation" and phosphorylation of enolase is due to an enzymatic activity present within these molecules. Investigators confronted this same conundrum soon after the discovery that pp60\(^{\text{v-src}}\) is associated with a protein kinase activity, and the possibility was raised that a cellular kinase might be activated by v-src, rather than the kinase activity residing in the viral protein (Richert, et al., 1979). Ample evidence consistent with the latter interpretation is available, including the demonstration that pp60\(^{\text{v-src}}\), translated in vitro from virion RNA, results in the phosphorylation of tyrosine (Hunter, et al., 1980), and also that pp60\(^{\text{v-src}}\) is inseparable from its presumed kinase activity despite extensive purification (Purchio, 1982). The vast weight of evidence also supports the conclusion that protein-tyrosine kinase activity is an intrinsic property of fps/fes proteins; this evidence includes sequence homology between fps/fes kinase domains and the src kinase domain (Hampe, et al., 1982; Groffen, et al., 1983), mutant viruses encoding conditional kinase activity (Pawson, et al., 1980), and functional comparisons with other protein-tyrosine kinases (Feldman, et al., 1980; Cooper, et al., 1984). Thus, since p92 and p94 are specifically immunoprecipitated by an anti-fps sera which reacts with known fps/fes proteins, and since they appear to possess an intrinsic protein-tyrosine kinase activity, I
conclude that it is likely that either or both are cellular \textit{fps/fes} proteins. It is apparent that p92 is synthesized in the same cells in which it is identified by \textit{in vitro} kinase activity, but p94 synthesis could not be identified by metabolic labelling cells with either $^{32}$P-orthophosphate or with ($^{35}$S)methionine. While it is possible that the particular cell lines examined did not themselves synthesize p94 it is more likely that the antiserum did not detect low level synthesis due to fewer cross-reactive epitopes being present on p94 than on p92; other investigators, using different anti-\textit{fps} sera, have demonstrated synthesis of p94 in hemopoietic cells, suggesting that the latter possibility is the correct one (Feldman, \textit{et al.}, 1985; Feldman, \textit{et al.}, 1986). Also, the results of mapping proteolytic fragments generated by cleavage of p92 and p94 with trypsin or with V8 protease imply that p92 is structurally more closely related to P85$\approx$-\textit{fes}, a known \textit{fes} protein, than p94; the two major phosphopeptides derived from trypsin digestion of p92 virtually co-migrated with the two major peptides generated from P85$\approx$-\textit{fes}, indicating that, while not identical, the primary sequences of these peptides are closely related. Presumably this conservation of structure is mirrored in the conservation of relevant epitopes, allowing low levels of metabolically labelled p92 to be detected by immunoprecipitation. Digestion of p94 with trypsin yielded only one major phosphopeptide which did not appear to co-migrate with either of the peptides from p92 or
P85$$^\text{PQ}$$; published data indicate that tryptic digestion of NCP98 yields a pattern reminiscent of that seen for either p92 or P85$$^\text{PQ}$$ (Mathey-Prevot, et al., 1982), while phosphopeptide mapping of avian p94 indicates that it is distinct from NCP98 (Feldman, et al., 1986). The observation of Feldman, et al., (1986) that two tryptic phosphopeptides were generated following cleavage of avian p94, as opposed to the single peptide reported here for human p94, suggests that they may be different proteins, rather than homologues, or that different phosphorylation sites are utilized in avian and human p94; while they also identified a cross-reactive 94kD protein-tyrosine kinase in human hemopoietic cells it was not compared with avian p94 by tryptic peptide mapping. Tryptic peptide mapping of ($^{35}$S)methionine labelled p92 and p94 would likely have yielded valuable insights into relatedness of these two proteins but, as shown in Figure 3.14, labelled p94 could not be detected with the available antiserum; also, insufficient ($^{35}$S)methionine-labelled p92 was recovered from gels to allow exploitation of this technique. Structural comparison by mapping V8 protease digests of in vitro phosphorylated p92 and p94 was consistent with the data obtained from tryptic peptide mapping and indicated that p92 and P85$$^\text{PQ}$$ shared several co-migrating fragments while p94 exhibited its own unique pattern; since V8 protease cleaves peptide bonds at sites distinct from trypsin this is further evidence that p92 is related to P85$$^\text{PQ}$$. A
number of V8 protease cleavage fragments of *in vitro* phosphorylated p92, obtained from either HL-60 cells or normal human bone marrow cells, have been reported to co-migrate with those obtained from NCP98 (Feldman, *et al*., 1985), supporting the identification of p92 as a c-*fps/fes* protein; while p94 was also identified in this study no structural comparisons were undertaken. In summary, I have isolated and characterized a 92kD protein, present in both mouse and human cells, which I believe to be the product of the c-*fes* gene, and I designate this protein p92*-e-*. I have also characterized a 94kD protein which, while displaying some structural and functional similarities, appears to be the product of another gene; I designate this protein p94. Essentially identical results to those presented here were obtained independently by other investigators (Feldman, *et al*., 1985; Feldman, *et al*., 1986), and formal proof that human p92*-e-* is the product of the c-*fes* gene has recently been published (Greer, *et al*., 1988; Smithgall, *et al*., 1988). Very new data derived from the cloning and sequencing of an apparently full length cDNA encoding p94 establishes conclusively that p92 and p94 are unique, but related, proteins; the gene encoding p94 has tentatively been assigned the name FER (Hao, *et al*., 1989).

Modulation of protein function involving a dynamic balance of phosphorylation and dephosphorylation has been proposed as an important regulatory mechanism in the normal cell; these modifications, catalyzed by protein kinases and
protein phosphatases respectively, are believed to result in activation or deactivation of specific substrate proteins (Ingebritsen, et al., 1983; Hunter, 1987b). Envisaged within this concept are cascades or pathways of proteins, their afferent activation dependent on regulatory kinases and phosphatases, and these proteins in turn acting in an efferent manner to control the activity of other downstream proteins, perhaps again by reversible phosphorylation and dephosphorylation. Examples of the most proximal members of such proposed pathways exist in the form of cell surface receptors, as typified by the EGF receptor. It is known that the tyrosine kinase activity innate to the EGF receptor is influenced positively by the binding of ligand and negatively by phosphorylation of the receptor by protein kinase C; however, the downstream molecule, or molecules, modified by the EGF receptor remain to be identified. Thus, the EGF receptor displays some of the characteristics of members of these hypothetical signalling cascades, namely negative regulation by protein kinase C and ligand-dependent increased phosphorylation of cellular substrates; the contribution of tyrosyl-specific phosphatases to the regulation of EGF receptor activity is less clear but still completely plausible. The association of pp60^src with an 81-85kD protein believed to be a phosphatidylinositol (PI) kinase has been described (Kaplan, et al., 1987; Courtneidge, et al., 1987); this protein may be a legitimate example of a physiologically relevant substrate of
pp60^c-src, and might account for the PI kinase activity reported to be associated with v-src and v-ros (Macara, et al., 1984; Sugimoto, et al., 1984). Direct regulation of this putative PI kinase by c-src-mediated phosphorylation has yet to be demonstrated; however, it is known that the phosphorylation of key tyrosine residues in many protein-tyrosine kinases, including c-src and v-fps, has profound effects upon enzymatic activity (Weinmaster, et al., 1984; Kmiecik, et al., 1987; Piwnica-Worms, et al., 1987). In this respect it is interesting that p92^c-src is phosphorylated at tyrosine in vitro and yet phosphotyrosine is not detected in vivo; p92^c-src might be released from some inhibitory complex during cell lysis, allowing phosphorylation at tyrosine in vitro, or the turnover of phosphate at tyrosine residues in vivo might be at an extremely low level, not allowing detection. However, this second possibility seems less likely since the addition of sodium orthovanadate, an inhibitor of phosphotyrosine-phosphatases, did not result in detectable phosphotyrosine. It is also entirely possible that c-fes functions normally in vivo without significant phosphorylation of tyrosine and it is only in an aberrant context, such as an in vitro reaction or when c-fes is transduced by a retrovirus to yield v-fes, that phosphotyrosine is detected: the phosphoserine detected in p92^c-src in vivo may well represent an important regulatory modification in the normal cell.
Little is known of the \textit{in vivo} substrates upon which \( p_{92} \) acts, or of the proteins which might utilize \( p_{92} \) as a substrate; these molecules have been difficult to elucidate for all protein-tyrosine kinases. While a number of protein-tyrosine kinases act as cell surface receptors it is probable that most, lacking external domains and transmembrane sequences, do not act independently as receptors. However, protein-tyrosine kinases lacking the typical features might still function in receptor complexes by supplying the intracytoplasmic signalling apparatus to a transmembrane receptor protein. A protein-tyrosine kinase operating in this fashion could be envisaged as associating exclusively with a specific ligand-binding protein or, alternatively, acting as a common transducer for a number of different ligand-binding proteins. The latter model is certainly consistent with observations that different growth factors, acting through different receptors, often result in similar biochemical changes within cells (Kruijer, \textit{et al.}, 1984; Muller, \textit{et al.}, 1984; Gee, \textit{et al.}, 1986), while the former model closely parallels the structure of the receptors for insulin and IGF-1. The insulin and IGF-1 receptors consist of an external ligand-binding protein complexed through disulphide bonds with an internally situated protein-tyrosine kinase (Ullrich, \textit{et al.}, 1985; Ullrich, \textit{et al.}, 1986); these receptors differ slightly from the model presented here in that the internal chain supplies the transmembrane segment in addition to the kinase domain.
However, the recently described association of p56\textsuperscript{la} with the T-lymphocyte antigen CD4 supports the first model: p56\textsuperscript{la} is entirely internal while CD4 spans the membrane; however, the ligand which binds CD4 is unknown (Rudd, et al., 1988; Veillette, et al., 1988). The gene ltk, encoding a transmembrane protein-tyrosine kinase devoid of an extracellular domain, has been described; this too may be an example of a c-onc acting as part of a multi-unit receptor where the ligand binding region is on a separate molecule (Ben-Neriah, et al., 1988). It is entirely possible that p92\textsuperscript{-} is also a constituent of a multi-unit receptor, and a recent report suggests that the c-fes protein may be an essential component for responsiveness to GM-CSF (Smithgall, et al., 1988). It is noteworthy that the gene immediately upstream of human c-fes, designated fur, has a predicted protein sequence displaying many features found in known receptors, including a cysteine-rich extracellular domain, a transmembrane segment, and a short intracellular segment (Roebroek, et al., 1986a; Roebroek, et al., 1986b); the short intracytoplasmic region of fur might associate with p92\textsuperscript{-}, or with some other effector molecule, in response to binding of ligand. Both c-fes and fur are in close proximity cytogenetically to the gene encoding gp150, a myeloid membrane antigen expressed in the same types of hemopoietic cells as p92\textsuperscript{-} (Look, et al., 1986); it is possible that all three of these genes are expressed coordinately to form a receptor complex for some hemopoietic
growth factor. However, \textit{fur} is expressed in a much greater variety of tissues than either c-fes or gp150 (Schalken, \textit{et al.}, 1987), suggesting either that its location adjacent to c-fes is only coincidental and they are not expressed together, or that \textit{fur} subserves different functions, perhaps in association with different effector proteins, when expressed in non-hemopoietic tissues; support for the latter hypothesis comes from c-fms, encoding the CSF-1 receptor of macrophages, which is also highly expressed in placenta and plays a part in controlling placental development (Pollard, \textit{et al.}, 1987). While it is tempting to ascribe a hemopoietic growth factor-receptor role to p92\textsuperscript{c-src}, based on expression restricted to hemopoietic cells as reported here and elsewhere (Feldman, \textit{et al.}, 1985; Smithgall, \textit{et al.}, 1988), this conjecture must be balanced by several caveats. Firstly, p92\textsuperscript{c-src} is expressed normally in post-mitotic leukocytes; these cells do not respond to growth factors by engaging in a new round of cell division. A similar pattern of expression has been described for c-src, in which highest levels of pp60\textsuperscript{c-src} are found in platelets and neurons, and it has been proposed that its function is to maintain the differentiated state (Cotton, \textit{et al.}, 1983; Golden, \textit{et al.}, 1986). Secondly, p92\textsuperscript{c-src} activity, while most often detected in myeloid cell lines, is also present in cells of erythroid, eosinophilic, and B-lymphocytic origin; the only known growth factor to affect cells in all these lineages is IL-3 and its receptor appears to be
distinct from \( p92^{\alpha-fes} \) (Palaszynski, et al., 1984; Nicola, et al., 1986). Thirdly, \( p92^{\alpha-fes} \) activity is present in fibroblasts, as reported here for NIH 3T3 and elsewhere (Barbacid, et al., 1980); however, these observations are not inconsistent with \( c-fes \) expression restricted primarily to hemopoietic cells since fibroblasts can be derived from pluripotent hemopoietic stem cells (Lord, 1983). Finally, \( c-fes \) transcripts can be detected in some lung neoplasms (Slamon, et al., 1984b), and low levels of \( p92^{\alpha-fes} \) activity are found in normal mouse lung (Feldman, et al., 1985), although the single lung carcinoma-derived cell line examined here was negative. Whole lung tissue is composed of several different cells, including large numbers of alveolar macrophages, and the precise contribution of any one cell type to the overall \( p92^{\alpha-fes} \) activity is unknown; however, \( fur \) transcripts have also been detected in human lung cancers, suggesting that the concomitant presence of \( c-fes \) transcripts may be more than casual (Schalken, et al., 1987).

While the preceding discussion has focussed on the possibility that \( p92^{\alpha-fes} \) may function as a cell surface receptor, either alone or in concert with other proteins, it is equally likely that it functions as an intracellular member of a signal transducing pathway. Subcellular fractionation studies have not been performed for \( p92^{\alpha-fes} \), but the distribution of NCP98 was found to be largely, but not entirely, cytoplasmic (Young, et al., 1984); this might
imply that c-fps, and by inference c-fes, does not usually function at the cell membrane, although the equivalent viral protein, P130^c-fes, is membrane-associated (Feldman, et al., 1983). p92^c-fes may be a member of an expanding family of intracellular protein-tyrosine kinases, including hck, lck, and ltk, which do not fulfill the criteria of typical transmembrane receptors and yet are believed to play a role in signal transduction (Marth, et al., 1985; Ziegler, et al., 1987; Ben-Neriah, et al., 1988). Several of these genes, including c-fes, are expressed in post-mitotic cells, and detectable hck transcripts increase in myeloid cells following induction of macrophage differentiation (Quintrell, et al., 1987). These genes are similar to c-src in this respect and may serve in maintaining the differentiated phenotype; if this is so their gene products may hold some promise as lineage-specific markers of differentiation.

Many of the considerations and speculations presented above for p92^c-fes also apply to p94; while the data here and elsewhere (Feldman, et al., 1985; Feldman, et al., 1986) establish that p94 is not a c-fes protein, it does appear to be a legitimate member of the protein-tyrosine kinase family. p94 is expressed in a more ubiquitous fashion than p92^c-fes and thus may participate in some process critical to all cells; unfortunately, much less is known about p94 in all respects than the fps/fes proteins, thus effectively limiting speculation on potential functions and regulation
of p94 to that which can be made about protein-tyrosine kinases in general.
4.0 Expression of c-fes in Human Leukemia Cell Lines During Chemically Induced Differentiation.

4.1 Introduction

AML may be conceptualized as a disease of disordered maturation of myeloid cells, due to an unknown defect, resulting in the clonal overgrowth of blast cells which appear blocked from differentiating normally. However, this arrested maturation is not absolute and analysis of X-chromosome-linked DNA polymorphisms has indicated, in some patients, that morphologically normal granulocytes do develop from the malignant clone in vivo (Fearon, et al., 1986). Also, many primary myeloid leukemias will undergo differentiation in vitro following stimulation with physiologic inducers of differentiation, such as purified CSF's or vitamin D₃, or with non-physiologic inducers, such as TPA or DMSO (Pegoraro, et al., 1980; Koeffler, et al., 1984; Sachs, 1987). However, leukemic cell explants, like all primary tissue cultures, have a finite lifespan in vitro, thus precluding systematic studies of differentiation over a prolonged period of time; in order to circumvent this limitation in vitro, investigators have employed myeloid leukemia cell lines. A number of myeloid leukemia cell lines exist, representing developmental blocks at various identifiable stages, and the finding that many of these cell lines differentiate in a fashion similar to primary
leukemias in vitro suggests that they may be valid models for studying leukemic differentiation (Koeffler, et al., 1981; Koeffler, 1983; McCarthy, et al., 1983).

Considerable evidence has emerged linking the expression of c-onc's with the control of cell division and differentiation; studying c-onc expression during differentiation of leukemic cells is valuable because it affords mechanistic insights into normal and leukemic myeloid differentiation, and because the possibility of therapeutic progress exists. c-onc's of all functional classes are expressed in myeloid leukemias but the significance of this expression to the emergence, maintenance, and expansion of the malignant clone is unknown. However, the finding that mutated ras genes are present in preleukemic cells prior to the emergence of acute leukemia is suggestive of an etiologic role, as opposed to some views which propose that point mutations of c-onc's are not a major mechanism in malignant neoplasia (Duesberg, 1987). Furthermore, many v-onc's cause acute leukemia in animals, implying that unregulated or mutant oncogene expression may be an important mechanism in leukemogenesis.

Examining c-fes expression during myeloid differentiation is hampered by the requirement of relatively large numbers of cells in order to obtain sufficient RNA and protein for study; in order to circumvent this problem I elected to examine differentiation of the human myeloid
leukemia cell line HL-60 following exposure to the inducer TPA. Additionally, HL-60 was chosen because a TPA-resistant subline, designated HL-525, became available, allowing evaluation of differences in c-fes expression between a differentiation-responsive cell line and its unresponsive counterpart.

4.2 Results

4.2.1 Examination of HL-525 Cells for Expression of c-fes

HL-60 cells have been shown to synthesize active p92* but whether or not HL-525 possessed a similar activity was unknown. Results published for KG-1 and its differentiation-resistant subline, KG-1a, indicated that KG-1 possesses p92* activity while KG-1a does not (Feldman, et al., 1985). This raised the intriguing possibility that expression of p92* protein-tyrosine kinase activity in myeloid leukemias correlates with ability to respond to inducers, while absence of this same activity correlates with resistance; accordingly, HL-525 cells were tested for the presence of functional p92* in an in vitro kinase assay. The results indicate clearly that p92* can be immunoprecipitated from HL-525 cells and that it is capable of autophosphorylation and phosphorylation of the exogenous substrate enolase (Figure 4.1, lane 3), similar to that seen in parental HL-60 cells (Figure 4.1, lane 1); these results would seem to be quite different from those obtained for KG-1 and its subline.
Figure 4.1: Identification of p92\textsuperscript{e} in HL-525 cells. To assay for p92\textsuperscript{e} activity, lysates of HL-60 cells (lanes 1 and 2) and HL-525 cells (lanes 3 and 4) were immunoprecipitated with J6 anti-\textit{fps} serum (lanes 1 and 3) or with non-immune rat serum (lanes 2 and 4) and the immune complexes were incubated with (\textit{gamma}-\textsuperscript{32}P)ATP in reaction mixtures which contained 5 ug of acid-denatured rabbit muscle enolase. The \textit{in vitro}-phosphorylated proteins were analyzed by electrophoretic separation and autoradiography; the location of enolase was confirmed by electrophoresis of unlabelled enolase. The locations of p92 and enolase are indicated by arrows.
KG-1a. However, other investigators subsequently determined that \( p92^- \) is detectable in KG-1a cells when cultures of low passage-number, relative to the original isolate, were tested as opposed to high passage-number cultures; thus, the loss of \( p92^- \) from KG-1a cells appears to be an artifact related to length of time in culture (T. Pawson, personal communication). Therefore, there does not seem to be a correlation between the ability to detect \( p92^- \) kinase activity and the ability to differentiate \textit{in vitro} in response to inducers.

4.2.2 Examination of \textit{c-fes} Expression During Exposure of HL-60 and HL-525 Cells to the Inducer TPA.

HL-60 cells, growing in suspension culture, have the ability to differentiate to more mature cells in response to a variety of physiologic and non-physiologic inducers. Cells resembling granulocytes, monocytes, macrophages, and eosinophils can all be derived from HL-60 cell cultures following addition of the appropriate inducer; however, it should be emphasized that all of these "differentiated" cells are deficient in some feature possessed by their normal counterparts (Collins, 1987). The phorbol ester TPA, a tumor promoter, exerts a somewhat paradoxical effect on HL-60 cells in that it stimulates them to undergo morphologic transformation to cells resembling mature macrophages and displaying some of the same functional attributes of these cells (Huberman, \textit{et al.}, 1979; Rovera,
et al., 1979a; Rovera, et al., 1979b; Koeffler, et al., 1981). The TPA-resistant HL-60 subline, HL-525, is refractory to the macrophage-inducing effects of TPA, and numerous morphological and biochemical differences between HL-60 and HL-525 following exposure to TPA have been documented (Solanki, et al., 1981; Huberman, et al., 1982; Fisher, et al., 1984; Anderson, et al., 1985).

1.) Morphology

In order to ensure that the expected morphologic differences between HL-60 and HL-525 developed following addition of TPA, cultures were observed microscopically for overall morphology and samples were withdrawn at timed intervals for preparation of cytocentrifuge slides and diagnostic staining. HL-60 and HL-525 cells in culture are indistinguishable prior to the addition of TPA (Figure 4.2, A and C); following induction with TPA for 48 hr cultures of HL-60 showed marked phenotypic change, including development of pseudopodia, enlarged macrophage-like cells, marked adherence to the plastic flasks, and clumping of cells (Figure 4.2, B). In contrast, HL-525 cells exposed to TPA for an equivalent period of time showed no discernable morphologic changes, and the most striking feature of these cultures was extreme crowding of cells, indicative of ongoing mitosis (Figure 4.2, D). Sequential cell counts performed on induced and uninduced cultures of HL-60 and HL-525 cells confirmed that there was cessation of cell
Figure 4.2: Morphologic characteristics of HL-60 and HL-525 cells in suspension culture following exposure to TPA. HL-60 cells (A) and HL-525 cells (B) were grown in tissue culture flasks in suspension culture. TPA was added to each culture to reach a final concentration of 10 ng/ml; HL-60 cells (C) and HL-525 cells (D) were photographed in situ 48 hr following addition of TPA to the culture flasks. Magnification, x40.
division in HL-60 after addition of TPA while the growth kinetics of HL-525 were unaffected; these observations were completely consistent with published results for these cell lines (Huberman, et al., 1979; Huberman, et al., 1982). Wright-Giemsa staining of slides prepared from uninduced HL-60 cells showed uniform blast-like cells (Figure 4.3, A), while HL-60 cells induced for 48 hr showed the presence of large macrophage-like cells, monocyte-like cells, and the persistence of some blast cells similar to those seen in the uninduced culture (Figure 4.3, B); these observations are similar to published reports (Rovera, et al., 1979a; Rovera, et al., 1979b). HL-525 cells prior to treatment with TPA appeared very similar to uninduced HL-60 cells (Figure 4.3, C); following 48 hr of induction a few morphologic changes were apparent, but these were quite different from those observed for HL-60 and consisted mainly of variation in cell size, some mild cell enlargement, and vacuolization. Some monocytic cells but no macrophage-like cells were identified, and similar changes have been noted by other investigators (E. Huberman, personal communication; Homma, et al., 1986). Diagnostic staining for chloracetate esterase, a marker of granulocytes, and (alpha)-naphthyl esterase, a marker of monocyte/macrophages, confirmed that induced HL-60 cells displayed the staining characteristics of monocyte/macrophages while HL-525 did not (Table 4.1); these data are also consistent with published observations (Huberman, et al., 1982; Mitchell, et al., 1986). Thus, by
Figure 4.3: Morphologic differentiation of HL-60 and HL-525 cells. HL-60 cells (A) and HL-525 cells (C) growing in suspension culture were cytocentrifuged onto glass slides and stained with Wright-Giemsa stain. HL-60 cells (B) and HL-525 cells (D) exposed to 10 ng/ml TPA for 48 hr were cytocentrifuged onto glass slides and stained with Wright-Giemsa; adherent HL-60 cells were dislodged gently with a cell scraper before cytocentrifugation. Magnification, x400.
<table>
<thead>
<tr>
<th>Stain</th>
<th>Cell type</th>
<th>% of cells staining positive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>nil TPA</th>
<th>48 hr TPA</th>
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<tbody>
<tr>
<td>(alpha)-naphthyl acetate esterase</td>
<td>HL-60</td>
<td>0%</td>
<td>80%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HL-525</td>
<td>5%</td>
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</tr>
<tr>
<td>Chloracetate esterase</td>
<td>HL-60</td>
<td>100%</td>
<td>20%</td>
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<tr>
<td></td>
<td>HL-525</td>
<td>90%</td>
<td>80%</td>
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<sup>a</sup> Cytocentrifuge slide mounts of control cells or of cells exposed to TPA for 48 hr were prepared and stained for (alpha)-naphthyl acetate esterase and chloracetate esterase according to the methods of Yam, et al. (1970); cells were evaluated microscopically in order to determine numbers of cells displaying positive staining characteristics.
a number of morphologic criteria, HL-60 cells responded to TPA by assuming a macrophage-like morphology while the TPA-resistant subline HL-525 did not.

**ii.) c-fes transcripts**

Published data indicate that the expression of some c-onc's, including protein-tyrosine kinases, is altered when differentiation is induced in HL-60 cells with TPA (Mitchell, et al., 1985; Barnekow, et al., 1986; Gee, et al., 1986). Although reports indicate that c-fes transcripts and *in vitro* kinase activity increase in HL-60 cells after stimulation of granulocyte differentiation by dimethyl sulfoxide, TPA-induced differentiation was not examined (Ferrari, et al., 1985; Smithgall, et al., 1988). In order to determine whether or not changes in the expression of c-fes accompany the morphologic alterations of TPA-induced differentiation, levels of steady-state c-fes mRNA in uninduced and induced HL-60 cells were examined. Uninduced HL-60 cells synthesize a 2.5 kb RNA which hybridizes to a human c-fes probe in Northern blots (Figure 4.4, A; 0 hr); a transcript of identical size was detected when RNA blots were probed instead with a 3.5 kbp BglII-XhoI fragment encoding Gardner-Arnstein FeSV v-fes sequences (data not shown). The c-fes transcript identified in Figure 4.4 was calculated to be 2.5 kb in length using authentic RNA molecular size standards as references, and this size is in good agreement with a 2.6 kb c-fes transcript which was
Figure 4.4: Detection of c-fes and actin mRNA in uninduced and TPA-induced HL-60 cells. Total RNA was extracted from uninduced (0 hr) cells and from cells induced with TPA for 1, 2, 4, 8, or 24 hr. 10 μg of total RNA from each sample was electrophoresed, subjected to blot hybridization with a human c-fes probe, and transcripts were detected by autoradiography (A). After autoradiography, membranes were stripped of c-fes probe and hybridized to a bovine actin probe utilizing conditions identical to those for c-fes (B). Sizes of authentic RNA molecular size standards are given to the left, in kb.
identified in mRNA isolated from human hemopoietic malignancies (Slamon, et al., 1984b). Although the concentration of c-fes transcripts appears to be decreasing slowly with length of induction, a trivial explanation for the observed decline might be that the amount of RNA loaded in each lane was unequal, accounting for this perceived decline. In order to control for this possibility the membrane was stripped of c-fes probe and re-probed for (beta)-actin mRNA, which has been shown to remain constant in TPA-induced HL-60 cells (Sariban, et al., 1985). c-fes transcripts were therefore normalized to (beta)-actin transcripts by densitometric scanning of the autoradiograms and establishing a ratio of c-fes expression to that of (beta)-actin for each lane. These ratios were calculated as a percentage, where the value obtained for uninduced cells was defined as 100%, and plotted as a function of time following induction with TPA (Figure 4.6, HL-60); When disparities in loading are accounted for in this manner it is clear that levels of c-fes mRNA decrease rapidly following exposure of HL-60 cells to TPA: the number of c-fes transcripts dropped to approximately 50% of pre-induction levels in the first 1 hr and then declined more slowly to a level approximately 10% of pre-induction by 24 hr (Figure 4.6, HL-60). Although this particular induction was terminated at 24 hr, continuing the induction to 48 hr in parallel experiments (data not shown) did not result in any significant change in levels of c-fes.
transcripts, which remained constant at approximately 10% of pre-induction values, as shown in Figure 4.6. Cultures of HL-525 cells were also exposed to TPA and Northern blots prepared in order to compare the response of this differentiation-resistant subline with that of the parental line. A c-fes transcript of identical size to that of HL-60 was detected in uninduced HL-525 cells (Figure 4.5, A; 0 hr), but the response of HL-525 cells to TPA-stimulation was more complex: the addition of TPA resulted in a rapid decline in c-fes mRNA during the first 8 hr, which essentially paralleled the decrease observed in HL-60 cells (Figure 4.6, HL-525). But, between 8 and 24 hr post-induction, c-fes mRNA levels began to increase so that by 24 hr the number of c-fes transcripts had been restored to 100% of uninduced levels; over the remaining 24 hr transcript levels declined somewhat to about 80% of pre-induction (Figure 4.6, HL-525).

iii.) Synthesis of p92=--

Northern blot experiments indicated that levels of steady-state c-fes mRNA declined rapidly following addition of TPA, suggesting some form of transcriptional regulation. In order to ascertain whether de novo synthesis of p92=-- was altered as well, radiolabelled p92=-- was isolated from uninduced and induced HL-60 cells which had been metabolically labelled with (35S)-methionine (Figure 4.7). Exposure to TPA resulted in a decrease in p92=-- synthesis
Figure 4.5: Detection of c-fes and actin mRNA in HL-525 cells and HL-525 cells exposed to TPA. Total RNA was extracted from uninduced (0 hr) cells and from cells induced with TPA for 2, 8, 24, or 48 hr. 10 ug of total RNA from each sample was electrophoresed, subjected to blot hybridization with a human c-fes probe, and transcripts were detected by autoradiography (A). After autoradiography, membranes were stripped of c-fes probe and hybridized to a bovine actin probe utilizing conditions identical to those for c-fes (B). Sizes of authentic RNA molecular size standards are given to the left, in kb.
Figure 4.6: Quantitation of c-fes mRNA in HL-60 cells and HL-525 cells exposed to TPA. The autoradiograms in Figures 4.4 and 4.5 were scanned using soft laser densitometry and a ratio of intensity of c-fes versus intensity of actin was calculated for each time point. The ratios are plotted as percentages, where the ratios obtained for 0 hr in each cell line were assigned a value of 100%.
within 2 hr following induction, and production of p92\textsuperscript{c-fes} continued to decrease over the next 2 hr, at which point synthesis stabilized at this reduced level. The decline in p92\textsuperscript{c-fes} synthesis lagged behind the decline observed in c-fes mRNA since repeat experiments, concentrating on the initial period following induction, showed that no detectable drop in synthesis occurred until approximately 90 min post-induction (Figure 4.8). By 4 hr the synthesis of p92\textsuperscript{c-fes} stabilized at the new, reduced equilibrium level and synthesis remained detectable at 24 hr after induction with TPA (Figure 4.7). Repeat experiments showed that p92\textsuperscript{c-fes} synthesis was maintained at a low, but constant, level from 8 hr to 50 hr post-induction (data not shown). At no point following TPA-induction did \textit{de novo} synthesis of p92\textsuperscript{c-fes} ever become undetectable, a low level of persistent synthesis always being present, consistent with the results of RNA analysis which indicated that despite a reduction in the size of the transcript pool c-fes mRNA remained detectable from 8 hr to 48 hr post-induction (Figures 4.4, 4.6).

It was not possible to investigate the synthesis of p92\textsuperscript{c-fes} in radiolabelled HL-525 cells following exposure to TPA due to lack of sufficient J6 anti-fps serum. In an attempt to secure additional antisera, tumors were induced in Fischer X Wistar rats by injecting v-fps transformed fibroblasts and sera were tested exactly as described for the production of J6 antiserum (Ingman-Baker, et al., 1984);
Figure 4.7: Synthesis of p92=-e- in uninduced and TPA-induced HL-60 cells. HL-60 cells were induced with TPA for 0, 2, 4, 8, and 24 hr, radiolabelled with (35S)methionine for 30 min and cell lysates were prepared and immunoprecipitated. Radiolabelled proteins were separated electrophoretically and identified by autoradiography. The times indicated show the total length of exposure to TPA and include the labelling period. Lysates from each time point were immunoprecipitated with immune (I) or non-immune serum (N), as indicated. The locations and sizes (in kD) of molecular weight standards are indicated to the left.
Figure 4.8: Synthesis of p92°-e in uninduced and TPA-induced HL-60 cells. HL-60 cells were induced with TPA for 0, 45, 90, and 180 min, radiolabelled with (35S)methionine for 30 min and cell lysates were prepared and immunoprecipitated. Radiolabelled proteins were separated electrophoretically and identified by autoradiography. The times indicated show the total length of exposure to TPA and include the labelling period. Lysates from each time point were immunoprecipitated with immune (I) or non-immune (N) serum, as indicated. The position of p92°-e is indicated with an arrow.
however, no antiserum able to immunoprecipitate p92<sup>-c-fes</sup> as efficiently as J6 antiserum was identified (data not shown). As an alternative approach, I attempted to raise monoclonal antibodies by injecting mice with a recombinant TRP-E/v-fps protein (Sadowski, et al., 1986), but the presence of anti-fps antibodies could not be demonstrated by immune complex kinase assay and thus hybridomas were not produced (data not shown).

iv. p92<sup>-c-fes</sup> in vitro kinase activity

p92<sup>-c-fes</sup> is the human c-fes protein and possesses protein-tyrosine kinase activity; in order to determine if this enzymatic activity was altered during TPA-induced differentiation, p92<sup>-c-fes</sup> from uninduced and induced HL-60 cells was tested for its ability to autophosphorylate and to phosphorylate enolase in an in vitro kinase reaction. In contrast to the data which indicated a decrease in steady-state c-fes mRNA and reduced p92<sup>-c-fes</sup> synthesis, the in vitro kinase activity of p92<sup>-c-fes</sup> in HL-60 cell lysates remained invariant up to 48 hr post-induction (Figure 4.9). No detectable decrease in enzymatic activity, as assessed by either autophosphorylation or enolase phosphorylation, was seen between 8 hr and 24 hr post-induction despite very low levels of both c-fes transcripts and p92<sup>-c-fes</sup> synthesis during the corresponding period of time; both enolase and p92<sup>-c-fes</sup> were phosphorylated to a similar extent from one time point to the next (Figure 4.9). The in vitro kinase
Figure 4.9:  p92 in vitro kinase activity in uninduced and TPA-induced HL-60 cells. HL-60 cells were induced with TPA for the indicated lengths of time, cell lysates were prepared, and immunoprecipitated with immune (I) or non-immune (N) serum. Each immune complex was resuspended in a 30 ul reaction mixture containing 5 ug of acid-denatured rabbit muscle enolase. Phosphorylated proteins were analyzed by electrophoresis and detected by autoradiography. The location of enolase was confirmed by electrophoresis of unlabelled enolase and detection by Coomassie blue staining.
activity of p92\textsuperscript{c-fes} from HL-525 cells exposed to TPA was also determined (Figure 4.10). The data for HL-525 cells is very similar to that obtained for HL-60 cells, showing neither a decrease nor an increase in \textit{in vitro} kinase activity. In contrast to HL-60 cells, where full kinase activity is maintained despite reduced mRNA and p92\textsuperscript{c-fes} synthesis, there is no evidence to indicate that p92\textsuperscript{c-fes} kinase activity is increased in HL-525 as a result of relatively higher levels of c-fes transcripts; data regarding synthesis of new p92\textsuperscript{c-fes} might have been helpful in this regard but could not be obtained, as mentioned, due to lack of immune reagents. The data presented in Figure 4.10 suggests an increase in autophosphorylation and enolase phosphorylation with length of exposure to TPA, but this increase can be accounted for by overloading of protein onto gels since duplicate experiments, where more equal loading was obtained, failed to demonstrate a similar increase (data not shown).

In order to obtain additional quantitative information on steady-state levels of p92\textsuperscript{c-fes} protein, as opposed to its kinase activity, I attempted to identify p92\textsuperscript{c-fes} in fresh lysates prepared from uninduced and induced HL-60 and HL-525 cells using a rabbit anti-v-fps serum (Sadowski, et al., 1986) in a Western blotting procedure. Despite its ability to identify HL-60 p92\textsuperscript{c-fes} in \textit{in vitro} kinase assays (Greer, et al., 1988), no unique 92 kD protein could be identified in Western blots of HL-60 or HL-525 lysates using
Figure 4.10: p92\textsuperscript{\textgamma} in vitro kinase activity in HL-525 cells and HL-525 cells exposed to TPA. HL-525 cells were exposed to TPA for the indicated lengths of time, cell lysates were prepared, and immunoprecipitated with immune (I) or non-immune (N) serum. Each immune complex was resuspended in a 30 ul kinase reaction mixture containing 5 ug of acid-denatured rabbit muscle enolase. Phosphorylated proteins were analyzed by electrophoresis and detected by autoradiography. The location of enolase was confirmed by electrophoresis of unlabelled enolase and detection by Coomassie blue staining.
this antiserum (data not shown). Similar inability to recognize protein-tyrosine kinases in an immunoblot procedure, despite demonstrated ability to immunoprecipitate the same proteins from cell lysates, has been reported for other rabbit antisera (Feldman, et al., 1986).

4.3 Discussion

The data presented here establish that c-fes expression in HL-60 cells is altered during the course of TPA-induced differentiation to macrophage-like cells. Steady-state levels of c-fes mRNA decrease very rapidly after induction with TPA, followed closely by a decline in p92c-fes synthesis; this suggests that the production of new c-fes protein is transcriptionally regulated, although evidence exists that translational regulation may also play an important role in controlling synthesis of protein-tyrosine kinases (Marth, et al., 1988); the data presented here do not address whether c-fes expression might also be regulated at the translational level. Only a single 2.5 kb species of c-fes transcript is identified, suggesting that the reduction in steady-state c-fes mRNA is not due to a shift in the balance of subpopulations of mRNA's to one which is more unstable; presumably, if subpopulations of c-fes mRNA of differing stabilities exist, these would be manifested as transcripts of different sizes as described, for example, for c-myc (Swartwout, et al., 1987). The observed decline in c-fes mRNA might conceivably be due to enhanced
ribonuclease activity accompanying macrophage differentiation, but this seems unlikely since there is no decrease in the overall population of polyadenylated mRNA associated with TPA-induced differentiation of HL-60 (Harley, 1987), and since RNA obtained from induced cells showed no more evidence of degradation than did RNA from uninduced cells. Thus, it is most likely that the reduced amount of steady-state mRNA is due to reduced transcription from the c-fes gene. Likewise, the decreased synthesis of p92\(^{-e-}\) is probably secondary to a smaller pool of c-fes transcripts being available for translation; increased proteolytic degradation of newly synthesized p92\(^{-e-}\) seems unlikely to account for the decrease in labelled protein because there is no detectable reduction at any time in steady-state p92\(^{-e-}\), as assessed by in vitro kinase activity. In fact, the unaltered p92\(^{-e-}\) protein-tyrosine kinase activity seen during differentiation suggests that the normal degradative pathways responsible for p92\(^{-e-}\) turnover may be acting at a reduced level. Neither c-fes transcripts nor synthesis of new c-fes protein ever disappear completely, implying that there is an ongoing need for p92\(^{-e-}\) synthesis during differentiation, albeit in reduced amounts; this low level of synthesis might be enough to resupply the steady-state pool of p92\(^{-e-}\), which would be degraded at a reduced rate. An alternative explanation for the apparently constant nature of the p92\(^{-e-}\) kinase activity might be that as synthesis of new protein is
decreasing there is a concomitant increase in the specific activity of the pre-existing pool of p92⁰, perhaps as a result of a change in phosphorylation status. Evidence exists that the specific activity of c-src can be modulated by phosphorylation of specific tyrosine residues (Piwnica-Worms, et al., 1987; Kmiecik, et al., 1987; Cartwright, et al., 1987), and the phosphorylation state of c-src in myeloid cells induced to differentiate with TPA has been reported to change (Gee, et al., 1986). However, it would seem unlikely, in the face of decreased synthesis and normally functioning degradative pathways, that the pre-existing pool of p92⁰ could continuously increase its specific activity sufficiently without demonstrable reduction in activity at some time. Clearly, much of the above is speculative; however, observations on the expression c-src during TPA-induced differentiation of HL-60 cells generate many of the same questions. Following exposure to TPA the in vitro kinase activity of pp60⁰ increases and yet it is not clear if this increase is due to alterations in specific activity or due to translation of new protein (Gee, et al., 1986; Barnekow, et al., 1986). In fact, regulation at both levels may contribute to the observed increase in kinase activity, and although pp60⁰ activity increases, as opposed to p92⁰ which decreases, it is somewhat analogous in that augmented pp60⁰ activity appears not to require any increase in transcription from c-src just as p92⁰ activity appears
to remain constant despite decreased transcription of c-fes. However, it should be emphasized that the relationship between in vitro kinase activity and normal in vivo kinase activity has not been established for any c-onc-encoded protein-tyrosine kinase; therefore, the apparently constant nature of p92 activity in vitro may belie significantly altered function in vivo. For example, p92 initial rates might be significantly reduced in vivo and yet this would not be identified in the standard in vitro kinase assay, employed here, which allows phosphorylation reactions to proceed to completion; however, since the in vitro kinase reactions allow complete substrate phosphorylation they do at least act as a good indicator of c-fes steady-state levels. It is interesting to note that pp60 activity increases during the differentiation to mitotically inert, macrophage-like cells, consistent with results indicating that it is highly expressed in a number of terminally differentiated, post-mitotic tissues. A number of other genes encoding protein-tyrosine kinases which are expressed predominantly or exclusively in hemopoietic cells have been described, including lck, hck, and ltk, and the expression of at least one of these, hck, also increases during TPA-induced differentiation of HL-60 (Quintrell, et al., 1987); thus, the expression of c-fes may be regulated somewhat differently from other protein-tyrosine kinases during TPA-induced differentiation of HL-60 cells.
What insights, then, can be gleaned about the function of p92°=e from alterations in its expression during induced differentiation of a myeloid leukemia cell line? The most likely role for p92°=e, based on structural and functional comparisons with other protein-tyrosine kinases, is as a member of some intracellular signal-transducing pathway. If such a pathway were rendered unnecessary by differentiation to a more mature cell then this could explain the decrease in c-fes transcripts and p92°=e synthesis, but the continued presence of high levels of functional protein mitigates against such an interpretation. But, the presence of p92°=e in myeloid cells of all stages of maturity and mitotic capacity, as well as in HL-60 prior to and after differentiation, suggests that the expression of c-fes may be necessary to maintain commitment to the myeloid lineage. While c-fes mRNA can be detected in some non-myeloid neoplasms (Slamon, et al., 1984b) and some normal erythroid precursors (Emilia, et al., 1986), these may be examples of normally regulated c-fes expression which might occur transiently early in hematopoiesis and disappear as these cells become committed to their respective lineages; the persistence of c-fes kinase activity in some B-cell and erythroid neoplasms may therefore represent a form of lineage infidelity. Compatible with this proposal of a lineage determination role are the multiple observations that c-fps/fes proteins in vivo are expressed almost exclusively in hemopoietic tissues (Mathey-Prevot, et
al., 1982; MacDonald et al., 1985; Feldman et al., 1985), and that within hemopoietic tissues c-fps/fes expression is confined, in mature cells, to those of myeloid lineages (Samarut, et al., 1985; Feldman, et al., 1985; MacDonald et al., 1985; Emilia, et al., 1986); the few non-hemopoietic tissues which express significant levels of c-fps/fes proteins are known to be rich in fixed tissue macrophages and this may account partially or completely for the ability to detect these proteins.

If p92° functions as one of the subunits of a multimeric receptor complex, as hypothesized in Chapter 3, do the observed changes in the expression of c-fes during induced differentiation support such a hypothesis? While at first glance the change in HL-60 from an immature, proliferative cell to a more differentiated, non-mitotic cell without any accompanying change in p92° activity implies that it does not function as a growth factor receptor, it is still possible to reconcile the observed results with the hypothesis. In this regard the CSF's serve as a useful paradigm, their presence being required by dependent cells at all stages of maturity for division, differentiation, and proper effector function (Sachs, 1987); by analogy, p92° could fulfill disparate roles, like the CSF's, by binding the same ligand at different stages of differentiation with vastly different consequences. Thus, in undifferentiated cells this hypothetical receptor complex may be necessary for stimulation of mitosis while in
differentiated cells it might function to maintain viability or promote an effector function; a precedent for this proposal exists within the family of protein-tyrosine kinases in c-fms, the CSF-1-receptor, which mediates the multiple functions of CSF-1, including stimulation of division and commitment to the macrophage lineage among precursor cells, maintenance of cell viability, and stimulation of macrophage effector functions. It is known that the CSF-1-receptor binds CSF-1 with high affinity and the myriad effects mediated by this interaction may involve selective substrate utilization in a differentiation-dependent manner; a similar scheme could be envisaged for p92, thus explaining the persistence of the enzyme. Also, if p92 does function as a receptor then it would not be likely to bind TPA, which functions through its own specific receptor; in the absence of ligand-mediated receptor turnover the observed kinase activity might not be unexpected. It will be interesting to examine the expression of the fur and gpl50 genes during induced differentiation to see if their regulation resembles that of c-fes, as might be expected if their gene products are associated in a receptor complex.

The changes in expression of c-fes in HL-525 cells add at least a little clarification to some of the preceding conjecture. HL-525 cells, while remaining morphologically immature, possessed in vitro kinase activities which, like HL-60, remained invariant during exposure to TPA. This is
certainly consistent with a myeloid determination role for p92-α, although it in no way excludes participation in a receptor complex. Interestingly, the change in c-fes transcripts seen in HL-525 exposed to TPA was not uniform, initially declining almost identically to HL-60, but quite rapidly returning to pre-exposure levels. The divergent regulation of c-fes implies that the mRNA changes seen in HL-60 cells are differentiation related and are not the result of some unique direct action of TPA upon c-fes regulation; utilization of different inducers which also result in macrophage-like differentiation would likely resolve this issue in a more direct manner. The nature of the differences between HL-60 and HL-525 which result in altered regulation of c-fes mRNA are unknown. The changes seen in the c-fes transcript pool could be explained by induction of some enzyme system which deactivates TPA, thus allowing c-fes transcript levels to increase; however, human cells are not known to possess the esterase which results in the hydrolysis of TPA in rodent cells, and HL-525 cells have not been shown to degrade or modify TPA (E. Huberman, personal communication). The nature of the defect which renders HL-525 unresponsive to TPA may be of considerable interest as the same defect might be responsible for differentiation-resistant AML cells which are isolated from patients (Pegoraro, et al., 1980). Unfortunately, due to technical constraints, I was unable to determine if de novo synthesis of p92-α exhibited kinetics similar to those of
the mRNA; however, if synthesis is transcriptionally regulated, as seems to be the case in HL-60 cells, then return to pre-induction levels of $p92^{-\kappa}$ synthesis would be expected. Since neither an increase nor a decrease in $p92^{-\kappa}$ in vitro kinase activity is observed, it would be reasonable to conclude that the degradative pathways are also functioning at normal capacity, maintaining a dynamic balance in steady-state levels of $p92^{-\kappa}$.

Many, if not all, of the effects of TPA on cells are believed to be the result of the direct stimulation of protein kinase C, a membrane-associated serine/threonine kinase, and activation of protein kinase C results in both stimulatory and inhibitory effects upon various substrate proteins (Nishizuka, 1988). TPA exposure results in reduced phosphorylation of cellular proteins in HL-525 cells compared with HL-60 (Anderson, et al., 1985), although this is not believed to be due to a reduction in protein kinase C levels or to decreased binding of TPA by protein kinase C (Solanki, et al., 1981). The observed differences between HL-60 and HL-525 cells could be due to disruption in the complex regulation of protein kinase C: in addition to modifying the $c-fes$ transcript pool it could also conceivably inactivate the breakdown of $p92^{-\kappa}$, possibly by phosphorylation of relevant regulatory proteins. However, $p92^{-\kappa}$ itself is not known to be a substrate for protein kinase C, and there is no evidence that TPA, through protein kinase C, alters the specific activity of $p92^{-\kappa}$,
either in HL-60 or HL-525 cells. But, protein kinase C is known to modulate the activity of other protein-tyrosine kinases by specific serine and threonine phosphorylation (Carpenter, 1987; Livneh, et al., 1988), and subtle distinctions, which are not detected here, may exist in the activities of HL-60 and HL-525 p92<sup>++</sup> following exposure to TPA. Results have shown that protein kinase C translocates from the soluble to the membrane fraction of HL-60 cells within minutes of exposure to TPA; this same subcellular translocation does not occur in HL-525 cells and may be one of the crucial determinants of TPA-unresponsiveness (Homma, et al., 1986). A translocation mechanism might be used by the cell to expand the repertoire of available substrates for protein kinase C or for other kinases, potentially including p92<sup>++</sup>; the failure of such a proposed translocation in HL-525 cells would have major functional ramifications, and yet no indication would be expected from the in vitro kinase assay, which does not distinguish between membrane-associated and soluble forms of protein-tyrosine kinases. Subcellular protein localization is clearly an important correlate of correct function, and aberrant localization, such as exists when comparing NCP98 with its transforming counterpart P130<sup>++</sup>, may be an important determinant of oncogenicity (Young, et al., 1984); differences in intracellular distribution of p92<sup>++</sup> may contribute to the malignant phenotype, yet these alterations would not be detected in the assay system used here.
CHAPTER 5

5.0 SUMMARY

These investigations were undertaken in order to identify and characterize the human and murine c-fes proteins. The identification of these proteins was important because some data from experimental animals indicated that homologous c-fps/fes proteins were confined to hemoietic cells, and data on c-fes transcripts in human tumors tended to support this observation. Thus, the possibility existed that differences in the expression of these proteins contribute significantly to the neoplastic phenotype and might be detectable.

Using a variety of immunoprecipitation techniques two proteins of Mr 92,000 and 94,000 were identified and shown to be protein-tyrosine kinases. Structural and functional comparisons suggested that the 92 kD protein is very closely related to known c-fps/fes proteins and hence was named p92α. On the other hand, the 94 kD protein appeared to be a legitimate protein-tyrosine kinase, but further characterization resulted in the conclusion that it does not represent a c-fps/fes protein. The results of other investigators showing that c-fps/fes proteins are present primarily in hemopoietic tissues was confirmed for human and murine cells; however no obvious qualitative or quantitative differences in p92α were delineated between normal and leukemic sources.
Changes in the expression of c-fes in a differentiation-responsive and unresponsive myeloid leukemia cell line were investigated following exposure to the inducer TPA. The most dramatic changes occurred in the levels of c-fes transcripts, and each cell line displayed its own distinctive response. However, despite remarkable alterations in mRNA levels, p92=c-fes in vitro kinase activity remained constant following exposure to TPA. Although changes in specific activity could not be ruled out, the unwavering ability to detect full levels of c-fes protein led to the conclusion that this protein is fulfilling some important role in the myeloid leukemia cells examined. The normal role of p92=c-fes, like most protein-tyrosine kinases, remains enigmatic. However, the tight association between the expression of this enzyme and hemopoietic cells, especially of the myeloid lineages, alludes to some important, regulatory or lineage determining role.
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