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STUDIES OF C-SRC FUNCTION IN DROSOPHILA

by

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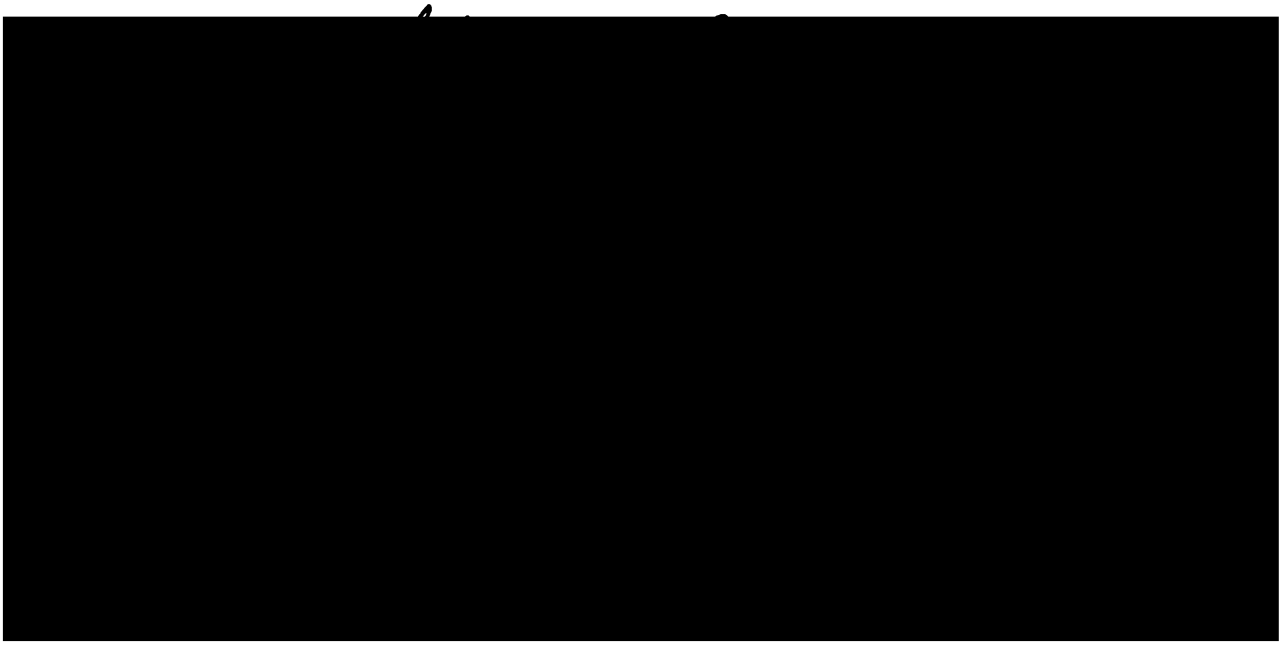

To Martha, MLW, and Leah, WGD

While the work that I have described in this thesis was largely the product of my own hands, the ideas that motivated the experiments are the results of innumerable discussions with a host of collegues. I cannot adequately describe their contributions or thank them sufficiently. However, there are a number of individuals who were so influential in this work that I would be remiss to leave them unmentioned. First, I would like to thank my advisors, Mike Bishop and Tom Kornberg, for their support and advice. They were both crucial in providing both ideas and encouragement. I would also like to thank Harold Varmus for his many ideas during our discussions of my work.

The number of people who have worked in the Bishop and Kornberg labs during my tenure as a graduate student is too large for me to list and thank here (and there are some that I probably would not wish to thank anyway). I would just say that many of the contributed to the ideas and to the work. I have hopefully learned something from each of them (both good and bad). There are two indviduals that I would like to mention explicitly because of their importance to this work and to me personally. These are Josh Kaplan and Alan Wells. I'd like to thank them for their ideas and critical, sometimes too critical, analysis of my work and more importantly for their friendship. My time in graduate school would have probably been less productive, and certainly less enjoyable in their absence.

Studies of c-src function in Drosophila

Michael A. Simon

ABSTRACT

The *Drosophila melanogaster* cellular homolog of the *v-src* gene of Rous sarcoma virus was cloned and characterized. The goal of the analysis was to provide a complete study of *c-src* expression throughout the development of the fly and to generate null mutations in *c-src* that could be used to study the consequences of the lack of *c-src* function.

Sequence analysis of a cDNA clone representing the *c-src* locus suggests that the gene is capable of encoding a 62 kilodalton protein that is very similar to the protein product of the chicken *c-src* gene. Direct biochemical analysis confirmed that the *Drosophila* protein possesses the same enzymatic activity, that of a tyrosine-specific protein kinase, as the chicken protein. This similarity in protein structure and function suggests that study of the *c-src* gene of *Drosophila melanogaster* could provide insight into the function of *c-src* in all metazoans.

Studies on the expression of *c-src* mRNA demonstrated that the locus is expressed as three RNA species each of which is regulated independently during development. *Drosophila c-src* RNA is abundant in embryos and pupae but rare in adults and larvae. *In situ* hybridization revealed that after the first 8 hours of development,

c-src RNA accumulates almost exclusively in neural tissues such as the brain, ventral nerve chord, and eye-antennal discs, and in differentiating smooth muscle. Parallel studies of the localization of *c-src* protein during development have confirmed the RNA hybridization results and further shown that the bulk of *c-src* protein is localized in the processes of neurons while the neurons are differentiating. Subsequently, the *c-src* protein levels diminish as the cells mature. These results suggest that *c-src* plays a role in the development but not in the mature function of neurons.

The *c-src* locus was mapped to polytene chromosome position 64B by *in situ* hybridization. Flies carrying one chromosome that bears a small deletion for the *c-src* region were generated and used in an attempt to isolate mutations that abolish *c-src* function. A number of mutations that map to the *c-src* region were isolated and mapped into 4 complementation groups. These groups were tested for defects in *c-src* function in several ways, but no changes were found. However, none of the groups has been positively eliminated from being *c-src* mutations.

Approved:

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Thesis Committee Chairman

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CHAPTER ONE

INTRODUCTION

1.1 Oncogenes, growth, and differentiation

The development of multicellular organisms is a complex process that requires precise control of both the growth and the differentiation of each cell of the organism. Each cell type must be produced at the proper time and place and in the correct quantity. Individual cells must therefore possess mechanisms for communicating with other cells and deciding on the proper course of action. Cells must then be able to faithfully execute the chosen program. One possible approach to understanding the mechanisms by which cells choose and then execute the correct developmental program is to identify and study genes that can be mutated to cause cells to grow and divide at a time when they should either differentiate or become quiescent. Cancer is a collection of diseases that are characterized by abnormal and excessive growth of cells. Neoplastically transformed cells do not respond properly to signals from other cells that would normally limit their growth or induce terminal differentiation. Therefore, genetic lesions that cause cancers may identify genes that are involved in controlling normal growth and development.

Such cancer genes, called proto-oncogenes, were first identified as the cellular homologs of the viral oncogenes of acutely transforming retroviruses (reviewed in Bishop, 1983). These viruses are capable of rapidly inducing tumors in animals and directly altering the growth properties of cells in culture. Each

virus contains genetic loci, called viral oncogenes, that are solely responsible for neoplastic transformation and are not required for viral replication (Martin, 1970; Bishop, 1983). These retroviral oncogenes (with two exceptions (Linemeyer *et al.*,1982; Gallo, 1984)) are not originally viral genes at all, but are instead derived from normal cellular genes (proto-oncogenes) by transduction and subsequent mutation (Stehelin *et al.*, 1976; Bishop, 1983). Proto-oncogenes have also been identified in two other settings. Retroviruses that do not themselves have an oncogene can often cause tumors by enhancing expression of genes near the point of proviral insertion into the host cell chromosome (Neel *et al.*, 1981; Payne *et al.*, 1981; Fung *et al.*, 1981; Bishop, 1983). Direct transfer of DNA from tumors and transformed cell lines to normal cells and the subsequent transformation of those cells has also been used to isolate new oncogenes (Shih *et al.*, 1979; Bishop, 1983). The total number of known proto-oncogenes has risen precipitously in recent years to approximately forty while the rate of new discoveries of oncogenes has not substantially declined (Bishop, 1983).

The physiological roles of most of the proto-oncogenes are not presently understood. Thus, it is difficult to evaluate whether the normal functions of proto-oncogenes generally is in the control and execution of growth and differentiation decisions. However, three examples exist in which this is clearly the case. *v-sis*, the oncogene of simian sarcoma virus, is derived from the gene that

encodes the β chain of platelet-derived growth factor (Waterfield *et al.*, 1983; Doolittle *et al.*, 1983; Devare *et al.*, 1983). *v-erbB*, the oncogene of avian erythroblastosis virus, is derived from the gene that encodes the epidermal growth factor receptor (Downward *et al.*, 1984). Finally, *v-fms*, the oncogene of the McDonough strain of feline sarcoma virus, is derived from the gene that encodes the receptor for macrophage colony-stimulating factor (CSF-1) (Sherr *et al.*, 1985).

1.2 The viral src gene

The original and most extensively studied of retroviral the oncogenes is the *v-src* gene of Rous sarcoma virus. RSV is an avian retrovirus that rapidly induces sarcomas in birds. RSV, and hence *v-src*, is capable of transforming a number of different cell types in culture including fibroblasts, chondroblasts, retinal melanoblasts, and myoblasts (Kaighn *et al.*,1966; Fiszman and Fuchs, 1975; Pacifici *et al.*, 1977; Boettiger *et al.*, 1977). Among the changes that are engendered by *v-src* in susceptible cells are (Hanafusa, 1977):

1) more rounded morphology
2) increased hexose uptake
3) collapse of cytoplasmic actin cables
4) reduced serum dependence

5) loss of contact-inhibition by neighboring cells (focus formation)
6) anchorage independent growth
7) reduced serum dependence
8) increased tumorigenicity.

In addition to these effects, *v-src* also acts to suppress the expression of differentiation-specific markers in appropriate cell types. Thus, infected myoblasts fail to fuse, while infected retinal melanoblasts and infected chondroblasts cease to synthesize pigment and type-II collagen, respectively (Fiszman and Fuchs, 1975; Boettiger *et al.*, 1977; Pacifici *et al.*, 1977). An interesting exception to this rule is the ability of *v-src* to induce the differentiation of PC12 pheochromocytoma cells into sympathetic neurons (discussed further in Section 1.4) (Alema *et al.*, 1985).

1.2.1 The enzymatic activity of the product of *v-src*

The protein product of *v-src* was first described by Brugge and Erikson (1977) as a transformation-specific antigen induced in chicken embryo fibroblasts (CEFs) after infection with RSV. Antisera were raised by injecting newborn rabbits with RSV. Some of the rabbits formed tumors and produced high titer antisera that would immunoprecipitate a 60 kilodalton phosphoprotein ($pp60^{v\text{-}src}$)

that was present in RSV infected CEFs, but not in CEFs infected with non-transforming retroviruses. Subsequently, it was shown that when immunoprecipitates containing $pp60^{v\text{-}src}$ are incubated with ATP, the heavy chain of the IgG molecules are phosphorylated by the $pp60^{v\text{-}src}$ molecule to which they are bound (Collett and Erikson, 1978; Levinson *et al.*, 1978). This protein kinase activity is not uniquely directed to immunoglobulins. Purified $pp60^{v\text{-}src}$ is capable of phosphorylating a number of proteins and synthetic peptides on tyrosine *in vitro* (Erikson *et al.*, 1979; Levinson *et al.*,1980; Wong and Goldberg, 1983; Hunter and Cooper, 1985).

When the protein kinase activity of $pp60^{v\text{-}src}$ was originally discovered, $pp60^{v\text{-}src}$ was thought to be a threonine-specific kinase (Collett and Erikson, 1978; Levinson *et al.*, 1978). Phosphorylation of proteins on tyrosine had never been described. Subsequently, tyrosine phosphorylation was detected in immunoprecipitates of the polyoma middle T antigen (though this activity was later shown to be a property of $pp60^{c\text{-}src}$ that is bound to middle T, see Section 1.3.2)(Eckhart *et al.*, 1979). Reevaluation of the activity of $pp60^{v\text{-}src}$ proved that its target residue is also tyrosine and that *v-src*-transformed cells contain elevated levels of phosphotyrosine (Hunter and Sefton, 1980). During the last few years tyrosine-specific protein kinase activity has been attributed to a number of proteins (reviewed by Hunter and Cooper, 1985). These proteins define a large family that share both structural and

sequence homology. Included in this family are a number of oncogene products and growth factor receptors.

1.2.2 $pp60^{v\text{-}src}$ structure

$pp60^{v\text{-}src}$ is synthesized on soluble polyribosomes, but rapidly associates with cellular membranes(Lee *et al.*, 1979; Purchio *et al.*, 1980; Courtneidge *et al.*, 1980; Courtneidge and Bishop, 1982; Kreuger *et al.*, 1983). Detergent solubilization is required to remove $pp60^{v\text{-}src}$ from membranes. This suggests that some portion of the protein directly interacts with the lipid bilayer of the membrane. Proteolytic cleavage studies first suggested that all of the information required for membrane localization resides at the amino-terminal end of the molecule (Levinson *et al.*, 1981). Treatment of membrane preparations of RSV transformed cells with trypsin generates an 8 kilodalton amino-terminal fragment that remains attached to membranes. This region contains no extended hydrophobic region that might be expected to insert into lipid bilayers. The mechanism of membrane attachment remained completely mysterious until the discovery that the fatty acid myristate is post-translationally added to $pp60^{v\text{-}src}$ (Sefton *et al.*, 1982; Buss and Sefton, 1985; Schultz *et al.*, 1985). Subsequent to the removal of the initiating methionine, myristate is covalently linked to the amino group of glycine-2. The myristate is thought to insert directly into the lipid bilayer and anchor $pp60^{v\text{-}src}$ to the

membrane. There is, however, no direct evidence that this is the actual mechanism of attachment. Membrane attachment is not the inevitable fate of all myristylated proteins. The best evidence that myristylation is crucial for membrane localization of $pp60^{v\text{-}src}$ is that mutations that block myristylation also block membrane localization (Cross *et al.*, 1984; Pellman *et al.*, 1985; Kamps *et al.*, 1985).

The kinase catalytic domain has been localized to the carboxy-terminal half of the molecule by proteolytic cleavage (Levinson *et al.*, 1981). Mild trypsin cleavage generates a carboxy-terminal fragment of 30-kilodaltons that has elevated kinase activity relative to the intact protein. This region is also the site of extensive amino acid homology with all other known protein kinases (Hunter and Copper, 1985). The observation that the trypsin fragment has increased activity suggests that a regulatory domain that normally inhibits activity lies somewhere in the amino-terminal half of the molecule, presumably between the membrane binding region and the kinase region (Levinson *et al.*, 1981).

Phosphorylation of $pp60^{v\text{-}src}$ occurs at several residues. Two sites are the most prominent, serine-17 and tyrosine-416. Serine-17 is a site of phosphorylation *in vitro* by cAMP-dependent protein kinase (Collet *et al.*, 1979a; Cross and Hanafusa, 1983). In certain RSV transformed cell lines, the extent of phosphorylation of serine-17 can be stimulated *in vivo* by the treatment with substances that raise intracellular cAMP levels (Roth *et al.*,

1983). However, in most lines the effects of cAMP cannot be detected, perhaps because the basal extent of phosphorylation is very high (60%) (Sefton *et al.*, 1982). Another possibility is that another protein kinase accounts for the bulk of serine-17 phosphorylation *in vivo*. Several other protein kinases have similar target requirements to that of cAMP-dependent protein kinase. The consequences of serine-17 phosphorylation are obscure. Small deletions that remove serine-17 do not markedly affect the biological properties of *v-src* (Cross and Hanafusa, 1983). Perhaps serine-17 phosphorylation is important for some aspect of *c-src* function that is not essential for transformation.

Tyrosine-416 is the major site of tyrosine phosphorylation on $pp60^{v\text{-}src}$ (Smart *et al.*, 1981). The effects of the modification are not marked. Substitution of tyrosine-416 with phenylalanine does not greatly change the specific activity or the biological properties of the protein in mouse cells, but does affect the ability of RSV to induce tumors in chickens (Snyder *et al.*, 1983). Direct comparisons of purified preparations of $pp60^{v\text{-}src}$ that are either phosphorylated or unphosphorylated suggests that phosphorylation may increase specific activity 2-3 fold (Graziani *et al.*, 1983). The identity of the kinase that phosphorylates tyrosine-416 is controversial. Purified preparations produced by some workers autophosphorylate on this site while others' preparations do not (Erikson *et al.*, 1979; Levinson *et al.*, 1980). $pp60^{v\text{-}src}$ is capable *in vitro* of phosphorylating a synthetic

peptide corresponding to the region around tyrosine-416 (Hunter and Cooper, 1985). However, tyrosine-specific kinases generally show little specificity *in vitro*. Mutations that destroy kinase activity or make it thermolabile affect tyrosine-416 phosphorylation accordingly, thus supporting the notion of autophosphorylation (Oppermann *et al.*, 1981; Snyder *et al.*, 1985). The mutations may, however, have affected the protein structure in such a way as to make this residue unavailable to exogenous kinases.

Several other sites of phosphorylation of $pp60^{v\text{-}src}$ have been described. Treatment of *v-src* transformed cells with phorbol esters stimulates phosphorylation of serine-12 (Gould *et al.*, 1985). This is presumably the action of C-kinase. The modification, however, has no discernible effect on either the biology of the cells or the specific activity of $pp60^{v\text{-}src}$. Amino-terminal tyrosine phosphorylations are detected after treating cells with sodium vanadate, an inhibitor of phosphotyrosine phosphatases (Collett *et al.*, 1984). $pp60^{v\text{-}src}$ immunoprecipitated from these cells has an increased specific activity. Incubation of purified $pp60^{v\text{-}src}$ with high levels of ATP can also lead to amino-terminal phosphorylations, apparently at the same sites, and an increase in the *in vitro* kinase activity (Collett *et al.*, 1983; Purchio *et al.*, 1983).

1.2.3 Targets for pp60$^{v\text{-}src}$ phosphorylation

Tyrosine-specific protein kinases show little specificity for substrates *in vitro*. The biochemical search for possible substrates has therefore been conducted by comparing tyrosine phosphorylated proteins from *v-src* transformed cells to those of untransformed cells (reviewed by Cooper and Hunter, 1984). Two experimental approaches have been utilized. In the first, the cells were labelled with [^{32}P] and the cellular proteins were visualized by autoradiography after separation on 2-dimensional gels. The second approach was to directly examine suspected substrates, such as cytoskeletal proteins, for phosphotyrosine content by immunoprecipitation after labelling with [^{32}P]. Such studies have revealed eight proteins that are specifically phosphorylated on tyrosine after transformation by *v-src* (reviewed by Hunter and Cooper, 1986).

All of the available data suggests that none of these identified proteins is an important target of the kinase activity of pp60$^{v\text{-}src}$. This evidence comes from several sources. Analysis of protein phosphorylation in cells that are fully transformed by minimal levels of pp60$^{v\text{-}src}$ suggests that detectable levels of the phosphorylation of certain targets, such as pp36, are not required for transformation (Jacobovits *et al.*, 1984). Examination of phosphorylation patterns induced by dissociated phenotype mutants of *v-src* has also suggested that many of the targets are not

essential for transformation (Weber, 1984). These are mutants that induce some, but not all, of the characteristics of *v-src* transformed cells. These mutants can show virtually any combination of the traits of the transformed phenotype. Comparison of the substrate phosphorylation and transformation parameters induced by different mutants has has shown that in no case does the modification of any known substrate correlate with the induction of any one parameter of transformation. This suggests that either none of the identified substrates is important or the interaction of various phosphorylation events is very complicated. Finally, mutations that block the myristylation of $pp60^{v\text{-}src}$ by changing glycine-2 to alanine block the transformation of cells even though all of the known targets are still phosphorylated (Kamps *et al.*, 1986).

1.2.4 The mechanism of *v-src* action

Since none of the known targets of the kinase activity of $pp60^{v\text{-}src}$ have been demonstrated to be important in maintaining or establishing the transformed phenotype, the question arises as to whether *v-src* acts through its protein kinase activity. Several reports have suggested that $pp60^{v\text{-}src}$ is a phosphatidolinositol kinase (Sugimoto *et al.*, 1984), but this appears to be a contaminating activity (Sugano and Hanafusa, 1985). The best evidence that protein kinase activity is important is that no *v-src* mutation has ever been isolated which can transform cells but does not

possess protein kinase activity. Furthermore, mutations that destroy the ATP binding site of the protein encode pp60$^{v\text{-}src}$ molecules that are defective for both kinase activity and transformation (Snyder *et al.*, 1985; Kamps and Sefton, 1986). This is, however, a somewhat ambiguous result. Some of these mutations cause the protein to be hypophosphorylated at both serine-17 and tyrosine-416 and therefore may radically alter the structure of the protein. Even if kinase activity is essential, until a crucial exogenous substrate is identified the possibility remains that the activity is strictly self-directed and serves to regulate some other activity of the protein.

1.3 The cellular src gene

Molecular hybridization provided the first evidence that *v-src* is derived from a normal cellular gene. Stehelin *et al.* (1976) demonstrated that *src*-specific probes hybridized to sequences present in the genome of uninfected chickens. Susequent cloning and characterization of these sequences demonstrated that this gene, called *c-src*(chicken), is a fully normal cellular gene that is highly conserved during evolution (Parker *et al.*, 1981; Shalloway *et al.*, 1981; Takeya and Hanafusa, 1983; Anderson *et al.*, 1985). The protein encoded by *c-src*, pp60$^{c\text{-}src}$, is located on the inner surface of the plasma membrane and also possesses tyrosine-specific protein kinase activity (Collett *et al.*, 1978; Oppermann

et al., 1979; Courtneidge *et al.*, 1980). However, the specific kinase activity of pp60$^{c\text{-}src}$ is 2-10 fold lower than that of pp60$^{v\text{-}src}$ depending on the assay used (Coussens *et al.*, 1985).

1.3.1 Structure, modification and regulation of pp60$^{c\text{-}src}$

The basic structure of pp60$^{c\text{-}src}$ is very similar to that of its viral counterpart. DNA sequence analysis of the viral and chicken cellular genes indicates that only a few differences exist between pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ in primary sequence (Takeya and Hanafusa, 1983; Takeya *et al.*, 1982). The major difference is at the carboxy-terminus. During the transduction and propagation of RSV the last 19 amino acids of *c-src* were replaced by 12 amino acids encoded by DNA sequences that normally reside 900 baspairs downstream of the *c-src* coding region. In addition, there are a number of amino acid substitutions between *v-src* and *c-src* scattered through the genes (Takeya and Hanafusa, 1983).

Several aspects of the modification of pp60$^{c\text{-}src}$ are identical to that of the viral protein. pp60$^{c\text{-}src}$ is also myristylated and associated with the plasma membrane (Courtneidge *et al.*, 1980; Buss and Sefton, 1985). Serine-17 is phosphorylated as in pp60$^{v\text{-}src}$ (Collett *et al.*, 1979b; Karess and Hanafusa, 1981). However, the pattern of tyrosine phosphorylation is different. The major site of tyrosine phosphorylation is tyrosine-527 which lies within the carboxy-terminal region of the protein where pp60$^{c\text{-}src}$

and pp60$^{v\text{-}src}$ are unrelated (Cooper *et al.*, 1986). In addition to these modifications, there are several modifications that are seen only in neuronal cells and which will be discussed in Section 1.3.5

1.3.2 Transformation: *v-src* versus *c-src*

Some aspect of *c-src* structure, regulation, or expression must prevent *c-src* from transforming cells as *v-src* does. Expression of *c-src* in normal fibroblasts is considerably lower than that of *v-src* in transformed fibroblasts. Several groups have examined whether dose is the sole reason that *c-src* does not normally transform cells by introducing *c-src* genes that are transcribed from strong heterologous promoters into fibroblast lines. *c-src* expression at levels greater than those required by *v-src* for full transformation do not transform (Parker *et al.*, 1984; Iba *et al.*, 1984; Shalloway *et al.*, 1984). However, extremely high levels of *c-src* can induce slight morphological changes and induce weak focus formation (Johnson *et al.*, 1984). This implies that there is some qualitative difference between *v-src* and *c-src*. The nature of this difference is not firmly established. However, the observation that *c-src* over-producing cells do not show a large increase in total cellular phosphotyrosine suggests that the qualitative difference may be simply a quantitative difference in kinase activity due to negative regulation of the activity pp60$^{c\text{-}src}$ rather than a difference in target specificity (Coussens *et al.*,

1985; Iba *et al.*, 1985).

c-src does appear to be capable of contributing to the transformation of cells in at least one setting. Polyoma virus transforms rodent cells largely through the action of a single viral gene that encodes a protein called middle T antigen (Smith and Ely, 1983). Tyrosine phosphorylation of proteins was first discovered in immunoprecpitates containing middle T antigen (Eckhart *et al.*, 1979). This activity is not an intrinsic property of middle T antigen itself, but is instead at least partially due to $pp60^{c\text{-}src}$ that is complexed with middle T antigen (Courtneidge and Smith, 1983). The specific activity of the middle T-bound $pp60^{c\text{-}src}$ is increased relative to uncomplexed $pp60^{c\text{-}src}$ (Bolen *et al.*, 1984; Courtneidge, 1985). There is no direct evidence that the activation of $pp60^{c\text{-}src}$ is the mechanism of transformation by polyoma virus, but several mutants of middle T that do not bind $pp60^{c\text{-}src}$ cannot transform cells (Bolen *et al.*, 1984).

The construction of hybrid genes in which portions of *v-src* were substituted for *c-src* has shown that *v-src* contains several amino acid changes that are each individually capable of causing transformation when substituted into *c-src* (Iba *et al.*, 1984; Kato *et al.*, 1986; Yacuik and Shalloway, 1986). These activating mutations lie within the kinase domain, in the amino-terminal region of the protein, and in the carboxy-terminal tail of the protein where the viral and cellular genes diverge. In each case the *in vitro* kinase activity of the resultant protein is elevated

relative to that of pp60$^{c\text{-}src}$. This suggests that the kinase activity of pp60$^{c\text{-}src}$ may normally be regulated by domains that lie in both the amino-terminal portion of the protein (near amino acid 95) and in the carboxy terminal tail of the protein. Similar regulation of the kinase activity of pp60$^{v\text{-}src}$ was also seen in protease digestion studies (Levinson *et al.*, 1981). The nature of the regulation in the amino-terminus is not understood

The nature of the carboxy-terminal change is clearly the removal of an inhibiting element present in *c-src* rather than a positive effect of *v-src*. This has been demonstrated by either replacing the carboxy-terminus of *c-src* with random seqences or by truncating the gene (Yacuik and Shalloway, 1986). In either case, the resulting protein is competent for transformation. This inhibitory effect appears to be the result of tyrosine phosphorylation at residue 527. Two lines of evidence support this conclusion. pp60$^{c\text{-}src}$ from cells treated with the phosphotyrosine phosphatase inhibitor sodium vanadate has reduced kinase specific activity (Courtneidge, 1985). Since tyrosine-527 is the major site of tyrosine phosphorylation, this effect is most likely due to the increased phosphorylation of tyrosine-527. Direct biochemical evidence for this conclusion comes from the observation that phosphatase treatment of immunoprecipitates of pp60$^{c\text{-}src}$ increases the kinase activity of the protein (Cooper and King, 1986).

1.3.4 *c-src* expression in normal cells.

The role of *c-src* in normal cells cannot be directly inferred either from the actions of *v-src* or mutant *c-src* alleles because these are dominant mutations that may have acquired functions distinct from those of normal *c-src*. Another approach to understanding the normal role of *c-src* has been to study the temporal and spatial pattern of *c-src* expression in order to determine when and in what tissues *c-src* might be functioning. *c-src* expression has been studied in several organisms including chickens, rats and humans. The most comprehensive study has been of *c-src* expression in the fruit fly, *Drosophila melanogaster*, and is described in Chapters 3 and 4 of this thesis.

The expression of $pp60^{c\text{-}src}$ has been studied in chickens using antibodies generated against $pp60^{v\text{-}src}$ that also recognize $pp60^{c\text{-}src}$. Cotton and Brugge (1983) quantitated the levels of $pp60^{c\text{-}src}$ in extracts from various tissues of staged chick embryos. Neural tissues such as brain, retina, and spinal ganglia showed 8-10 fold higher levels of $pp60^{c\text{-}src}$ activity and protein than those found either in limb bud tissue or in cultured chick embryo fibroblasts. The level of neural $pp60^{c\text{-}src}$ was highest between 8 and 12 days of embryonic development and subsequently declined as hatching approached. Limb bud tissue showed low levels throughout embryogenesis.

Maness and her collegues have extended these observations by

examining the precise timing and location of pp60$^{c\text{-}src}$ expression in specific neural tissues of the developing chicken embryo. They have concentrated on the retina (Sorge *et al.*, 1984), cerebellum (Fults *et al.*, 1985), and neural plate (Maness *et al.*, 1986) because each of these tissues has an ordered structure which makes the identification of cell types relatively easy. The technique that they employed was immunoperoxidase staining of sectioned material. Retinal expression was detected in each layer of the retina that contains neurons and glial cells (Sorge *et al.*, 1984). The staining was always first apparent at the time when neurons in the region cease proliferating and begin to differentiate. The levels of pp60$^{c\text{-}src}$ subsequently decline until hatching at which time the levels are similar to those in a fibroblast. Cerebellar expression of pp60$^{c\text{-}src}$ was very similar to that of the retina (Fults *et al.*, 1985). Expression was detected in each layer at approximately the time of the onset of neuronal differentiation and declined as hatching approached. These results have been used to argue that *c-src* is involved in the process of cellular, particularly neuronal, differentiation rather than in cellular proliferation. However, the association is strictly correlative and therefore not conclusive.

Studies by the same group of pp60$^{c\text{-}src}$ expression in the developing chick neural plate weaken the argument (Maness *et al.*, 1986). A peak of *c-src* expression is seen in the neural ectoderm. This expression is transient and disappears by the time of neural

tube closure. The cells that express pp60$^{c\text{-}src}$ at this stage are still mitotic and will remain so for several days. There is no detectable expression during this period in either the mesodermal or the endodermal layers. Maness and her group have argued that their results taken altogether imply that *c-src* has two functions. One is manifested by the early expression and may be involved in determination of neuronal cell character, while the other is important for terminal differentiation of neurons. A more reasonable interpretation (at least to this author) is that *c-src* is involved in neuronal development in a presently unknown manner.

Neuronal cells are not the only cells with high levels of pp60$^{c\text{-}src}$. Platelets contain levels of pp60$^{c\text{-}src}$ that are greater even than those in neuronal cells (Golden *et al.*, 1986). In addition, differentiation of the pre-monocytic cell line, HL-60, into macrophage causes an increase in pp60$^{c\text{-}src}$ kinase activity that has been attributed by one group to increased levels of pp60$^{c\text{-}src}$ (Gee *et al.*, 1986) and by another to increased specific kinase activity of pp60$^{c\text{-}src}$ (Barnekow and Gessler, 1986). The increase in total *c-src* kinase activity is also observed if normal promonocytes are allowed to differentiate *in vitro*. How the role of *c-src* in these cell types relates to the role in cells of neuronal origin is completely obscure.

1.3.5 Tissue specific modification and activation of pp60$^{c\text{-}src}$

While the absence of pp60$^{c\text{-}src}$ from a given cell type is clear evidence that *c-src* has no role in those cells, the presence of the protein in another cell does not necessarily imply that *c-src* has a function there. The possibility exists that pp60$^{c\text{-}src}$ is in many cells but is only activated in certain cells or under certain conditions. Therefore, an understanding of both the nature and the control of modifications or interactions that regulate the kinase activity of pp60$^{c\text{-}src}$ could suggest a biological role for *c-src*.

Activation of pp60$^{c\text{-}src}$ has been described in both neuronal and nonneuronal settings. Addition of platelet-derived growth factor (PDGF) to cultured mouse fibroblasts alters the electrophoretic mobility in SDS-polyacrylamide gels of approximately 10% of the pp60$^{c\text{-}src}$ molecules (Ralston and Bishop, 1985). This alteration occurs within minutes and is the result of tyrosine phosphorylation at a site in the amino-terminal portion of the protein. The consequence of this modification is an approximately 10-fold increase in the specific kinase activity of the protein as measured by either immune complex kinase activity *in vitro* or by phosphorylation of pp36 (a known substrate for pp60$^{c\text{-}src}$) *in vivo*. Whether this activation of pp60$^{c\text{-}src}$ is important for PDGF stimulation of cell growth is unclear. The low stoichiometry of the modification might suggest that it does not.

Brugge *et al.* (1985) have studied the activation of pp60$^{c\text{-}src}$

in primary cultures of either neurons or astrocytes from rat embryos. Both astrocyte and neuronal cultures contain approximately 20-fold higher levels of *c-src* protein than do primary fibroblast cultures. However, the specific activity of the pp60$^{c\text{-}src}$ from the neuronal cultures was 6-to 12-fold higher than that of pp60$^{c\text{-}src}$ from either fibroblasts or astrocytes. In addition, the pp60$^{c\text{-}src}$ expressed in neuronal cultures contained a modification in the amino-terminal portion of the molecules that lowered electrophoretic mobility in SDS-polyacrylamide gels. The nature of this modification is unknown, but it is apparently not tyrosine phosphorylation. A similar modification and activation has also been detected in embryonal carcinoma cells that have differentiated into neurons (Lynch *et al.*, 1986). The combination of elevated expression and activation in neurons strongly suggests that this is an important site of *c-src* function.

Activation of pp60$^{c\text{-}src}$ has also been described in several human neuroblastoma cell lines (Bolen *et al.*, 1985). Neuroblastoma cell lines were found to contain pp60$^{c\text{-}src}$ possessing 20- to 40-fold higher specific activity than that found in either human glioblastoma cells or human fibroblasts. This activation was associated with a change in electrophoretic mobility of approximately 50% of the pp60$^{c\text{-}src}$ molecules. pp60$^{c\text{-}src}$ molecules from the neuroblastoma cells were found to possess at least one site of tyrosine phosphorylation that was not present in pp60$^{c\text{-}src}$ molecules isolated from glioblastoma cells or fibroblasts.

1.4 The function of c-src: New approaches

The studies that have been reviewed in the previous sections provide some evidence about when and in which cells *c-src* may have an important role. However, these studies are inadequate in at least two ways. In no case has a comprehensive study of all cells throughout all of development been achieved. Important sites of expression could easily have been missed. Furthermore, the correlative nature of the studies limits the strength of the conclusions that may be drawn. The mere presence of $pp60^{c\text{-}src}$ in a cell, or even its activation there, does not formally prove that the *c-src* has any role in that cell. The present data only allow a very weak conclusion that $pp60^{c\text{-}src}$ appears to be involved in some aspect(s) of neuronal and platelet function. The timing of *c-src* expression in neuronal cells hints that this role may be related to some aspect of differentiation rather than to cell division.

The action of *v-src* on neuronal cells or platelets could provide clues about the role of *c-src* in these cells. The effect of *v-src* on the pheochromocytoma line, PC12, has been well-studied. PC12 cells treated with nerve growth factor (NGF) shift from a chromaffin cell phenotype to that of sympathetic neurons. The major manifestation of this switch is the growth of long neurites from the cell body. Infection of these cells with Rous sarcoma virus causes this transition to neuronal phenotype in the absence of NGF (Alema *et al.*, 1985). This is in marked contrast to the

effects of *v-src* on other differentiating cells. In every other case, *v-src* either inhibits or does not affect differentiation. That *v-src* can drive the differentiation process of neuronal cells suggests that *c-src* might be an important element in the control of that process in normal cells. This inference is, however, completely dependent on the assumption that *v-src* is merely an activated version of *c-src*. For example, if *v-src* has acquired a different spectrum of targets for its kinase activity, then the effect of *v-src* on PC12 cells may not be relevent to *c-src* function. Since the relevent targets of both *v-src* and *c-src* are unknown, this possibility cannot be discounted.

A potentially powerful approach to understanding the role of *c-src* would be to examine the consequences of the absence of all *c-src* function from a cell or an entire organism. The goal of the work described in this thesis was to establish an experimental system in which this could be accomplished. The fruit fly, *Drosophila melanogaster*, was chosen as the experimental organism because it is the only multi-cellular organism for which genetic techniques exist that allow one to mutate a gene that has been identified solely as a DNA sequence.

CHAPTER TWO

ISOLATION AND SEQUENCE ANALYSIS OF DROSOPHILA C-SRC

2.1 Introduction

Shilo and Weinberg (1981) provided the first evidence that *Drosophila* cells might contain tyrosine-specific protein kinases. They reported that the genome of the fruit fly contained DNA sequences that were capable of annealing to *v-src* sequences under conditions of reduced hybridization stringency. The hybridizing sequences were not further characterized in their initial report. The experiments described in this chapter were therefore undertaken to determine whether *Drosophila* cells contain tyrosine-specific protein kinases. The first approach was to show directly that the *Drosophila* cells contain both tyrosine kinase activity and protein-bound phosphotyrosine. The other approach was to isolate and analyze the *Drosophila* DNA sequences that hybridize to the *v-src* gene and show that they are capable of encoding a protein that is homologous to $pp60^{v\text{-}src}$ and other tyrosine kinases.

2.2 Drosophila cells contain tyrosine-specific protein kinases

In order to conclude that *Drosophila* cells contain tyrosine-specific protein kinases, it was necessary to demonstrate both that the enzymatic activity exists in extracts of *Drosophila* cells and that the product of the activity, phosphotyrosine, exists *in vivo* (Simon *et al.*, 1983). The first requirement was tested using antisera raised in newborn rabbits against RSV-induced tumors

(Levinson *et al.*, 1978; Brugge and Erikson, 1977). All of the tumor sera immunoprecipitate pp60$^{v\text{-}src}$, but have varying affinity for avian and mammalian pp60$^{c\text{-}src}$ (Oppermann *et al.*, 1979). When an immune complex cotaining either pp60$^{v\text{-}src}$ or pp60$^{c\text{-}src}$ is incubated with ATP, a tyrosine of the immunoglobulin heavy chain is phosphorylated by the *src* protein (see Section 1.2.1). The results of such an immune complex kinase assay performed with extracts from Rat 2 cells and *Drosophila* K_c cells is shown in Figure 2-1. Tumor serum 2 recovered detectable kinase activity from each of the extracts while normal rabbit serum did not. Five of seven tumor sera tested immunoprecipitated detectable kinase activity in K_c cells (data not shown). Phosphoamino acid analysis of the IgG chains phosphorylated by the K_c cell extract demonstrated that the phosphorylation was on a tyrosine residue (Fig. 2-2).

The abundance of phosphotyrosine *in vivo* was examined by labelling K_c cells with [^{32}P] and assaying the percentage of total phosphoamino acids that was phosphotyrosine. In both avian and mammalian cells, phosphoserine and phosphothreonine account for the vast majority of phosphoamino acids. Phosphotyrosine accounts for only 0.01% of total phosphoamino acids. Figure 2-3 shows the result of an analysis of phosphoamino acids from K_c cells. Phosphotyrosine represented 0.1% of total phosphoamino acids in K_c cells. Thus, *Drosophila* cells contain both phosphotyrosine and a tyrosine-specific protein kinase activity that can be specifically immunoprecipitated by antisera to pp60$^{v\text{-}src}$.

Figure 2-1. ***Drosophila*** **cells possess protein kinase activity that can be immunoprecipitated by serum from rabbits bearing RSV-induced tumors.** Extracts were prepared from Rat 2 and *Drosophila* K_c cells and assayed for tyrosine-specific protein kinase activity as described by Simon *et al.* (1983). The lanes are: 1) Rat 2 cells with tumor serum 2; 2) Rat 2 cells with normal rabbit serum; 3) K_c cells with tumor serum 2; 4) K_c cells with normal rabbit serum. Phosphorylation of the heavy chain of the immunoglobulins is observed with extracts from both cell types with anti-RSV tumor serum but not with normal rabbit serum. The position of the immunoglobulin heavy chain is indicated on the left.

FIGURE 2-1

Figure 2-2. The *Drosophila* protein kinase activity that is immunoprecipitated by serum from rabbits bearing RSV-induced tumors is tyrosine-specific. The immunoglobulin heavy chain band from Figure 2-1 (lane 3) was excised and analyzed for phosphoamino acid content. The positions of phosphoamino acid markers were determined by ninhydrin staining. The phosphorylation of the immunoglobulin heavy chain is predominantly of tyrosine residues.

FIGURE 2-2

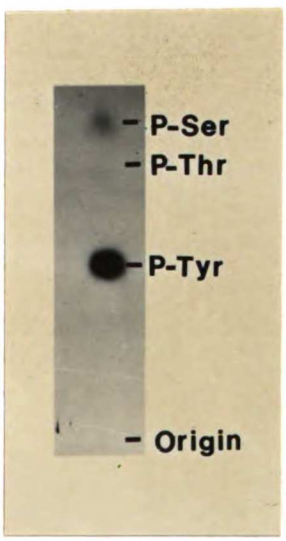
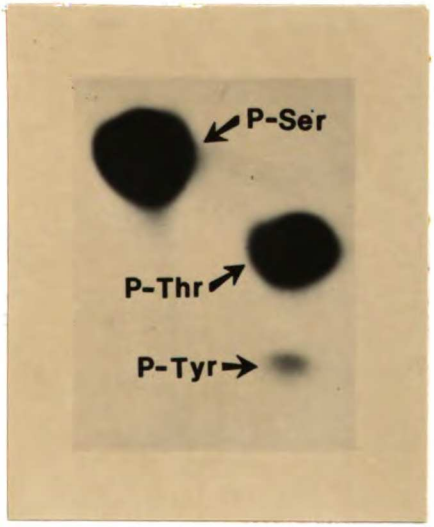


Figure 2-3. *Drosophila* cells contain phosphotyrosine. Approximately 1 x 10^7 K_c cells were labelled for 3 hours with 5 mCi of radioactive orthophosphate and analyzed for phosphoamino acid content. Markers were detected by ninhydrin staining. K_c cells contain phosphotyrosine as approximately 0.1% of total phosphoamino acids.

FIGURE 2-3



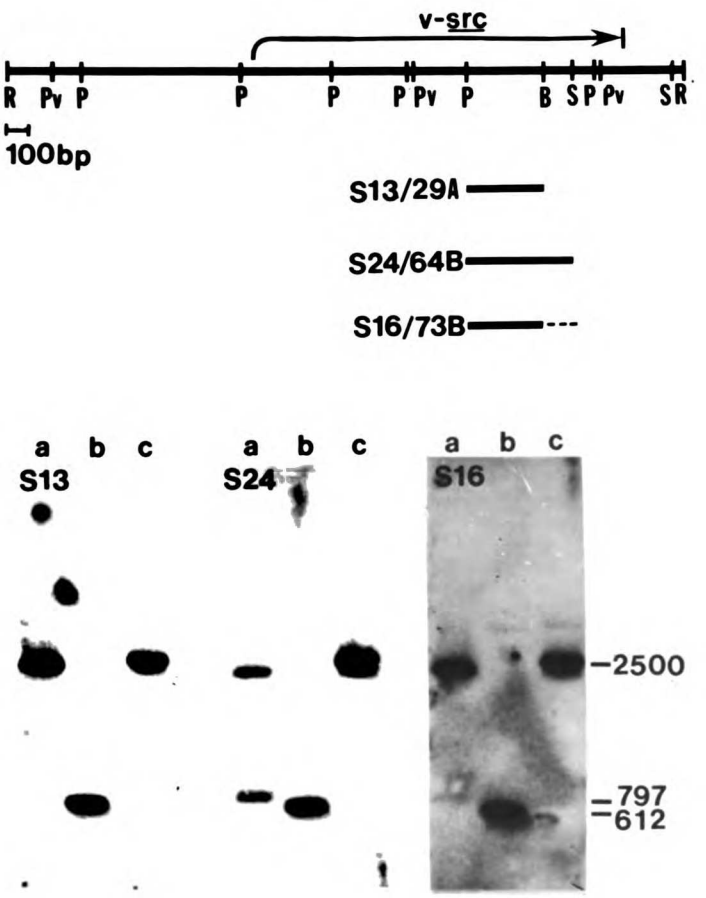
2.3 Isolation of Drosophila sequences that hybridize to v-src

Shilo and Weinberg (1981) reported only that the *Drosophila* genome contained sequences that hybridize with *v-src* sequences. This did not prove that the detected sequences actually encode proteins related to $pp60^{v\text{-}src}$. The possibility remained that the hybridization signals were the result of a short stretch of homology between otherwise unrelated sequences. In order to eliminate this trivial possibility and to isolate *Drosophila* tyrosine kinase genes for further study, the *Drosophila* sequences that hybridize to *v-src* were isolated and analyzed (Simon *et al.*, 1983; Simon *et al.*, 1985).

A recombinant DNA library of the *Drosophila* genome cloned into bacteriophage λ was screened by hybridization at reduced stringency with a [^{32}P]-labelled 800 base pair (bp) *Pvu*II fragment of *v-src* (See Fig. 2-4) (Maniatis *et al.*, 1978). Thirty positive clones were isolated and placed into one of three independent groups of overlapping clones on the basis of analysis with restriction endonucleases. The approximate location of the nucleotide sequences within *v-src* was determined by hybridization of Southern blots of digested *v-src* DNA with probes made from a representative of each of the three classes of positive clones. The results are shown in Figure 2-4. The patterns of hybridization are distinct. Clone S13 hybridized only within a 400 bp region defined by *Pst*I and *Bgl*I sites, while clones S16 and S24 each hybridized to the

Figure 2-4. Determination of the location within *v-src* of the sequences that hybridize to *Drosophila* clones S13, S24, and S16. The restriction map of the *Eco*RI-B fragment of the Schmidt-Ruppin A strain of RSV is shown (P=*Pst*I, B=*Bam*HI, S=*Sph*I, Pv=*Pvu*II) (DeLorbe *et al.*, 1980). The *v-src* coding sequences are indicated by the arrow. The plasmid containing the *Eco*RI-B fragment was digested with: a) *Eco*RI and *Bgl*II, b) *Eco*RI and *Pst*I, and c) *Eco*RI and *Sph*I and then blotted onto nitrocellulose and probed with each of the three bacteriophage genomes using reduced stringency conditions. The locations of the homologous sequences are summarized under the map of *v-src*. The dotted line indicates weak homology.

FIGURE 2-4


v-src
R Pv P P P P Pv P B S P Pv SR
100bp
S13/29A
S24/64B
S16/73B
a b c
S13
a b c
S24
a b c
S16
2500
797
612

same region and also to an adjacent 100 bp region defined by *Bgl*I and *Sph*I sites. The intensity of this additional hybridization was not equivalent between the two clones. S24 hybridized more intensely. It is worth noting that the hybridizing regions lie within the portion of *v-src* that encodes the tyrosine kinase domain and is highly conserved among all tyrosine kinases (Hunter and Cooper, 1985).

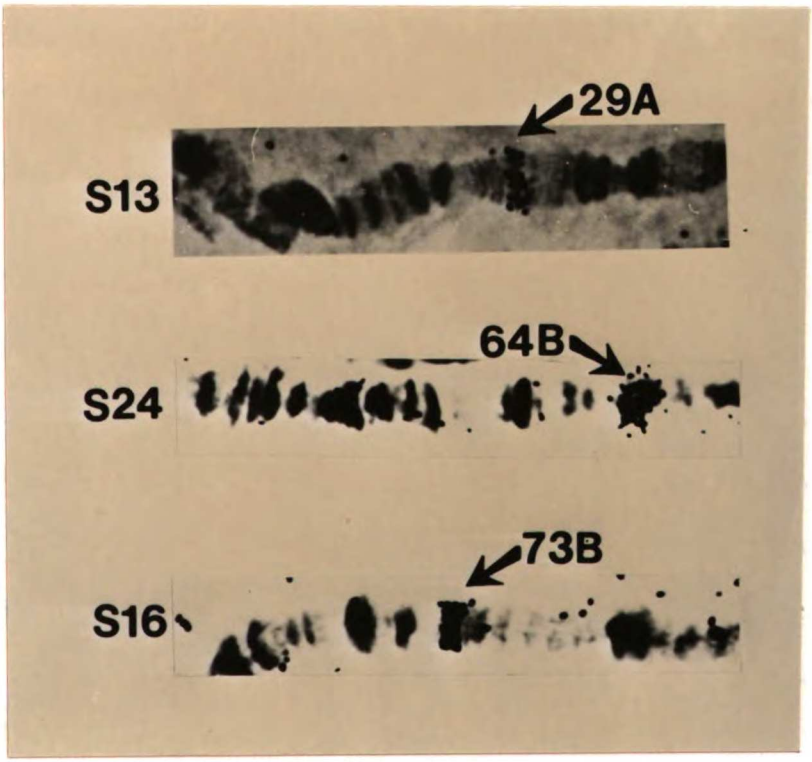
Hybridization experiments with clones S13, S16 and S24 showed little cross-hybridization among the three clones even under conditions of reduced stringency. This permitted the determination of the chromosomal location of each of the clones without interference from related sequences present in the other clones. *In situ* hybridization to *Drosophila* salivary chromosomes indicates that S13 maps to region 29A, S24 to region 64B and S16 to region 73B (Fig. 2-5). The 64B and 73B loci have also been described and mapped by others (Hoffman-Falk *et al.*, 1983; Hoffman *et al.*, 1983).

2.4 Isolation and analysis of a cDNA encoded by the 64B locus

Since the 64B locus appeared to contain the greatest homology to *v-src*, it was named *Drosophila c-src* and became the focus of all of the subsequent studies to be described in this thesis. The first priority was to establish that the 64B locus does encode a protein that is homologous to avian $pp60^{c\text{-}src}$. This was accomplished by isolating and analyzing cDNAs derived from mRNA encoded

Figure 2-5. Determination of the chromosomal locations of clones S13, S24,and S16. Salivary glands from third instar Canton S larvae were squashed and hyridized with [^{3}H]-labelled probe from each of the three clones. The chromosomal location of the hybridization of each of the clones is indicated above the appropriate panel.

FIGURE 2-5



by the 64B locus. A early embryonic library constructed in λgt10 by M. Goldschmidt-Clermont and D. Hogness was screened using an isolated fragment of λ S24 that hybridizes to *v-src* and therefore should include coding sequences. A single λ clone, 64B-A4, containing a 3.2 kilobasepair (kb) insert was chosen for further study. The insert was sequenced and found to contain a single long open reading frame. The nucleotide sequence and predicted amino acid sequence of the open reading frame are shown in Figure 2-6. The protein predicted by the cDNA sequence is a 552 amino acid polypeptide that is approximately 40% homologous with chicken pp60$^{c\text{-}src}$ (see Fig. 2-7 for comparison) (Takeya and Hanafusa, 1983). The site of translational initiation has been tentatively assigned to the AUG codon at position 1 of the sequence, because it is the first AUG of the open reading frame and is immediately followed by sequences that are homologous to the amino-terminal end of chicken pp60$^{c\text{-}src}$. This AUG also shows several features that are common to initiator codons, including a purine at position -3, cytosines at positions -2 and -1, and a guanosine at position +4 (Kozak, 1984).

The amino-terminal 10 kd of the predicted *Drosophila c-src* protein, pp62$^{c\text{-}src}$, show little homology to the chicken protein. The only substantial homology lies within the first 11 amino acids. These sequences are probably important for myristylation of pp62$^{c\text{-}src}$ (see Section 4.5) since studies of pp60$^{v\text{-}src}$ myristylation have demonstrated that essential sequences are located within

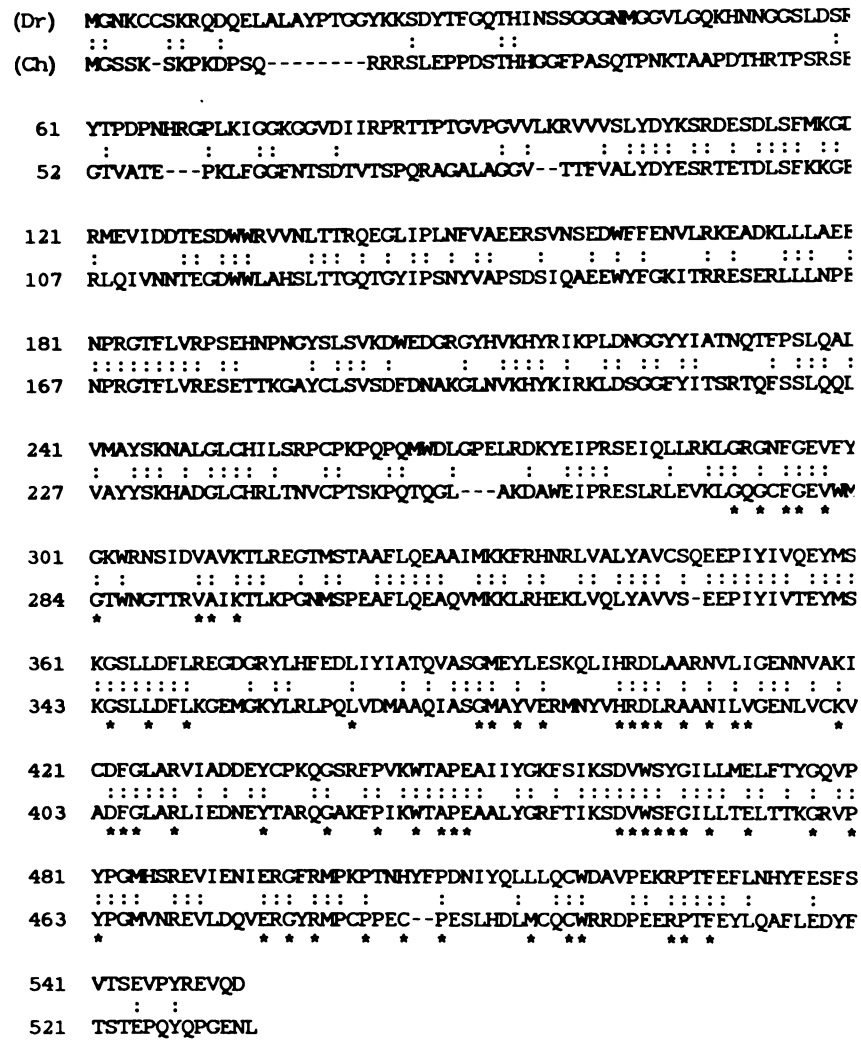
Figure 2-6. Sequence of the open reading frame in a cDNA clone of *Drosophila c-src*. The direction of transcription (5′-3′) is from left to right. The sequences of both strands were obtained except for nucleotides -6 to 21. Translation of the sequence is shown above the DNA sequence. The AUG shown at position 1 lies approximately 900 bp from the 5′ end of the cDNA clone.

FIGURE 2-6


MetGlyAsnLysCysCysSerLysArgGlnAspGlnGluLeuAlaLeuAlaTyrProThrGlyGlyTyrLysLysSerAspTyr
-6 TAAGCCATGGGCAACAAATGCTGCAGCAAGCGACAGGATCAGGAACTGGCACTGGCCTATCCCACTGGGGGCTACAAGAAATCCGACTAC
ThrPheGlyGlnThrHisIleAsnSerSerGlyGlyGlyAsnMetGlyGlyValLeuGlyGlnLysHisAsnAsnGlyGlySerLeuAsp
85 ACCTTTGGCCAGACGCACATCAACAGCAGCGGCGGCGGCAACATGGGCGGGGTTCTTGGCCAGAAGCATAACAACGGTGGCTCGCTGGAC
SerArgTyrThrProAspProAsnHisArgGlyProLeuLysIleGlyGlyLysGlyGlyValAspIleIleArgProArgThrThrPro
175 TCGCGCTACACGCCCGATCCCAATCATCGGGGTCCGTTGAAAATCGGCGGAAAGGGCGGCGTTGACATCATCAGACCACGCACCACACCA
ThrGlyValProGlyValValLeuLysArgValValValSerLeuTyrAspTyrLysSerArgAspGluSerAspLeuSerPheMetLys
265 ACCGGTGTTCCTGGTGTCGTGCTCAAGCGGGTTGTCGTGTCCCTGTACGACTATAAATCCGCGATGAATCCGATCTGAGCTTCATGAAG
GlyAspArgMetGluValIleAspAspThrGluSerAspTrpTrpArgValValAsnLeuThrThrArgGlnGluGlyLeuIleProLeu
355 GGTGACCGCATGGAGGTCATCGACGACACCGAGTCCGATTGGTGGCGCGTCGTGAACCTAACCACCCGCCAGGAGGGCCTCATCCCGCTC
AsnPheValAlaGluGluArgSerValAsnSerGluAspTrpPhePheGluAsnValLeuArgLysGluAlaAspLysLeuLeuAla
445 AATTTCGTGGCCGAGGAGCGCAGCGTGAACAGCGAAGACTGGTTCTTTGAGAATGTGCTACGAAAGGAGGCTGACAAGTTGCTGCTGGCC
GluGluAsnProArgGlyThrPheLeuValArgProSerGluHisAsnProAsnGlyTyrSerLeuSerValLysAspTrpGluAspGly
535 GAGGAGAATCCGCGTGGCACTTTCCTCGTGCGACCTTCAGAGCACAATCCCAATGGATACTCGCTGTCGGTGAAGGATTGGGAGGATGGA
ArgGlyTyrHisValLysHisTyrArgIleLysProLeuAspAsnGlyGlyTyrTyrIleAlaThrAsnGlnThrProSerLeuGln
625 CGTGGATACCATGTGAAGCACTACCGCATCAAGCCACTGGATAATGGCGGCTACTACATAGCCACCAATCAGACTTTCCCCTCGCTTCAG
AlaLeuValMetAlaTyrSerLysAsnAlaLeuGlyLeuCysHisIleLeuSerArgProCysProLysProGlnProGlnMetTrpAsp
715 GCCTTGGTCATGGCATACAGCAAAAACGCTCTTGGCCTGTGTCACATATTGTCGCGTCCCTGCCCCAAACCGCAGCCCCAGATGTGGGAC
LeuGlyProGluLeuArgAspLysTyrGluIleProArgSerGluIleGlnLeuLeuArgLysLeuGlyArgGlyAspPheGlyGluVal
805 TTGGGTCCGGAGCTGCGCGATAAGTACGAGATTCCGCGCTCGGAGATTCAGCTGCTGCGCAAACTGGGACGCGGCGACTTTGGCGAGGTC
PheTyrGlyLysTrpArgAsnSerIleAspValAlaValLysThrLeuArgGluGlyThrMetSerThrAlaAlaPheLeuGlnGluAla
895 TTCTACGGCAAATGGCGCAACAGCATCGATGTGGCGGTCAAAACGCTGCGCGAAGGCACCATGTCCACGGCCTGCTTTCCTTCAGGAGGCC
AlaIleMetLysLysPheArgHisAsnArgLeuValAlaLeuTyrAlaValCysSerGlnGluGluProIleTyrIleValGlnGluTyr
985 GCGATTATGAAGAAGTTCCGACACAACCGCCTGGTGGCCCTCTATGCCGTTTGCTCGCAGGAGGAGCCAATTTACATCGTGCAGGAGTAC
MetSerLysGlySerLeuLeuAspPheLeuArgGluGlyAspGlyArgTyrLeuHisPheGluAspLeuIleTyrIleAlaThrGlnVal
1075 ATGTCCAAGGGCAGTCTGCTGGACTTCTTGCGCGAGGGCGATGGCCGCTACTTGCACTTCGAAGATCTCATCTACATAGCCACCCAGGTG
AlaSerGlyMetGluTyrLeuGluSerLysGlnLeuIleHisArgAspLeuAlaAlaArgAsnValLeuIleGlyGluAsnAsnValAla
1165 GCCAGCGGTATGGAGTATCTAGAGTCCAAGCAGCTCATCCATCGCGATCTGGCGGCCCGTAATGTGCTGATCGGAGAGAATAATGTGGCG
LysIleCysAspPheGlyLeuAlaArgValIleAlaAspAspGluTyrCysProLysGlnGlySerArgPheProValLysTrpThrAla
1255 AAGATTTGTGATTTTGGACTGGCCCGTGTCATCGCGGATGACGAGTACTGCCCCAAGCAGGGATCCCGGTTTCCGGTCAAGTGGACGGCG
ProGluAlaIleIleTyrGlyLysPheSerIleLysSerAspValTrpSerTyrGlyIleLeuLeuMetGluLeuPheThrTyrGlyGln
1345 CCCGAGGCGATCATATACGGCAAGTTCTCGATCAAGTCGGACGTGTGGTCCTATGGCATTCTGCTGATGGAGCTATTCACGTACGGACAA
ValProTyrProGlyMetHisSerArgGluValIleGluAsnIleGluArgGlyPheArgMetProLysProThrAsnHisTyrPhePro
1435 GTGCCCTATCCGGGCATGCATAGTCGGGAGGTGATTGAGAACATCGAGCGCGGTTTCCGCATGCCGAAGCCAACGAATCACTACTTCCCG
AspAsnIleTyrGlnLeuLeuLeuGlnCysTrpAspAlaValProGluLysArgProThrPheGluPheLeuAsnHisTyrPheGluSer
1525 GACAACATTTATCAGCTGCTGCTCCAGTGCTGGGATGCTGTGCCCGAGAAGCGGCCGACATTCGAGTTCTTAAACCACTACTTCGAGTCC
PheSerValThrSerGluValProTyrArgGluValGlnAspOC
1615 TTCTCGGTCACGTCGGAGGTGCCGTATCGAGAGGTGCAACACTAAACAGAGTCCAGC

Figure 2-7. Comparison of the sequences of chicken pp60$^{c\text{-}src}$ and the predicted protein product of the *Drosophila c-src* locus. The deduced amino acid sequence of a cDNA derived from *Drosophila c-src* is compared with the amino acid sequence of chicken pp60$^{c\text{-}src}$ as described by Takeya and Hanafusa (1983). Identities are indicated by (:). Sequences that are conserved in tyrosine-specific protein kinases are indicated by (*) below the chicken sequence (Hunter and Cooper, 1985).

FIGURE 2-7


(Dr) MGNKCCSKRQDQELALAYPTGGYKKSDYTFGQTHINSSGGGNMGGVLGQKHNNGGSLDSF
(Ch) MGSSK-SKPKDPSQ--------RRRSLEPPDSTHHGGFPASQTPNKTAAPDTHRTPSRSE
61 YTPDPNHRGPLKIGGKGGVDIIRPRTTPTGVPGVVLKRVVVSLYDYKSRDESDLSFMKGI
52 GTVATE---PKLFGGFNTSDTVTSPQRAGALAGGV--TTFVALYDYESRTETDLSFKKGE
121 RMEVIDDTESDWWRVVNLTTRQEGLIPLNFVAEERSVNSEDWFFENVLRKEADKLLLAEE
107 RLQIVNNTEGDWWLAHSLTTGQTGYIPSNYVAPSDSIQAEEWYFGKITRRESERLLLNPE
181 NPRGTFLVRPSEHNPNGYSLSVKDWEDGRGYHVKHYRIKPLDNGGYYIATNQTFPSLQAL
167 NPRGTFLVRESETTKGAYCLSVSDFDNAKGLNVKHYKIRKLDSGGFYITSRTQFSSLQQL
241 VMAYSKNALGLCHILSRPCPKPQPQMWDLGPELRDKYEIPRSEIQLLRKLGRGNFGEVFY
227 VAYYSKHADGLCHRLTNVCPTSKPQTQGL---AKDAWEIPRESLRLEVKLGQGCFGEVWM
301 GKWRNSIDVAVKTLREGTMSTAAFLQEAAIMKKFRHNRLVALYAVCSQEEPIYIVQEYMS
284 GTWNGTTRVAIKTLKPGNMSPEAFLQEAQVMKKLRHEKLVQLYAVVS-EEPIYIVTEYMS
361 KGSLLDFLREGDGRYLHFEDLIYIATQVASGMEYLESKQLIHRDLAARNVLIGENNVAKI
343 KGSLLDFLKGEMGKYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKV
421 CDFGLARVIADDEYCPKQGSRFPVKWTAPEAIIYGKFSIKSDVWSYGILLMELFTYGQVP
403 ADFGLARLIEDNEYTARQGAKFPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVP
481 YPGMHSREVIENIERGFRMPKPTNHYFPDNIYQLLLQCWDAVPEKRPTFEFLNHYFESFS
463 YPGMVNREVLDQVERGYRMPCPPEC--PESLHDLMCQCWRRDPEERPTFEYLQAFLEDYF
541 VTSEVPYREVQD
521 TSTEPQYQPGENL

the first 10 amino acids of the protein (see Chapter 1). The conserved sequences include the glycine at position 2 that is the site of myristate addition. There is no amino acid that clearly corresponds to serine-17, the major site of serine phosphorylation of chicken pp60$^{c\text{-}src}$.

The greatest homology between the two proteins lies within the carboxy-terminal 47 kd of the proteins. In this region, the sequences can be aligned such that 243 of 425 amino acids are identical. A total of 60 amino acid residues in this region are conserved among many known tyrosine-specific kinases (Hunter and Cooper, 1985). Fifty-six of these residues are conserved in the *Drosophila* protein. Included among these are tyrosine-434, which corresponds to the major site of tyrosine phosphorylation in pp60$^{v\text{-}src}$, and lysine-312, which corresponds to the lysine that marks the ATP binding site of all protein kinases. In addition, tyrosine-547 appears to correspond to tyrosine-527 of chicken pp60$^{c\text{-}src}$. This is the major site of tyrosine phosphorylation and an important site of regulation of chicken pp60$^{c\text{-}src}$.

2.5 Discussion

The experiments presented in this chapter demonstrated that *Drosophila* cells contain a protein(s) that possesses tyrosine-specific protein kinase activity and is antigenically related to pp60$^{v\text{-}src}$. In addition, three loci were molecularly cloned that

are each capable of hybridizing to *v-src* sequences. The sequence reported above demonstrates clearly that one of these loci, residing at map position 64B, is capable of encoding a protein that is very similar to chicken pp60$^{c\text{-}src}$. The other two loci have also been at least partially sequenced by others and each is capable of encoding a protein that possesses all of the hallmarks of a tyrosine-specific protein kinase (Hoffman *et al.*, 1983; J. Jackson, personal communication). The locus residing at 64B is more closely related to chicken *c-src* than either of the other two loci and has therefore been chosen for further study and named *Drosophila c-src*. However, it should be noted that *c-src* is a member of a larger gene family that includes the *yes*, *fgr*, *syn* and *slk* genes (Kitamura *et al.*, 1982; Nishizawa *et al.*, 1984; Semba *et al.*, 1986; Kawakami *et al.*, 1986). The protein product of *Drosophila c-src* is equally related to the products of each of these genes. Furthermore, the gene products of these vertebrate genes are more closely related to each other (approximately 70-80% identity) than any of them is to the product of the 64B locus (approximately 40% identity). The 64B locus could equally well be named for any of these genes, though the name *Drosophila c-src* will be used as a convenience. The relationships between these genes does suggest the possibility that *Drosophila* does not possess separate homologs of each of the vertebrate genes and that a single ancestral gene has diversified into the various vertebrate genes since the divergence of Chordates and Arthropods.

CHAPTER THREE

CHARACTERIZATION OF RNA PRODUCED BY DROSOPHILA C-SRC

3.1 Introduction

An understanding of the number, structure and localization of the RNA products of the *Drosophila c-src* locus is essential to uncovering the role of this gene in the growth and development of the fruit fly. This is true for two reasons. In the preceding chapter, the primary sequence of a cDNA derived from the *Drosophila c-src* locus was described. However, no evidence was presented that addressed the issue of whether this cDNA, and the protein it encodes, is representative of all of the RNAs produced by the locus. Secondly, knowledge of the localization of the mRNA produced by the locus may suggest a physiological role for the gene and a phenotype for mutants of the gene.

3.2 The RNAs produced by Drosophila c-src

To determine the number, size and developmental regulation of *c-src* transcripts, Northern blots of polyadenylated RNA were prepared from whole *Drosophila* at various stages of development (Simon *et al.*, 1985). The blots were probed with a nick-translated DNA fragment of 700 bp that includes the carboxy-terminus of the protein predicted by the cDNA sequence (Probe A in Fig. 3-1). The results are shown in Figure 3-2. Three *c-src* transcripts were detected. Each is regulated in an independent fashion during development. A 3.5 kb transcript was detected during the initial 6

Figure 3-1. Map of the *Drosophila c-src* gene. The direction of transcription is from left to right. The positions of the initiation and termination codons, as determined by the sequence shown in Figure 2-6, is indicated by the AUG and TAA, respectively. The fragments designated A and B were used as probes for Northern blot and *in situ* hybridization. The map units are kilobasepairs. (A=*Apa*I; B=*Bam*HI; E=*Eco*RI; H=*Hind*3; P=*Pst*I; S=*Sal*I)

FIGURE 3-1

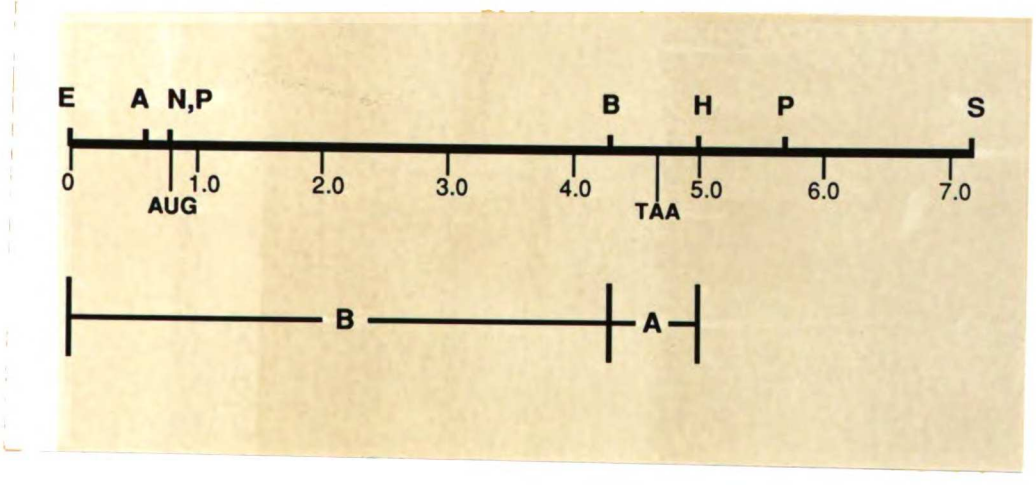
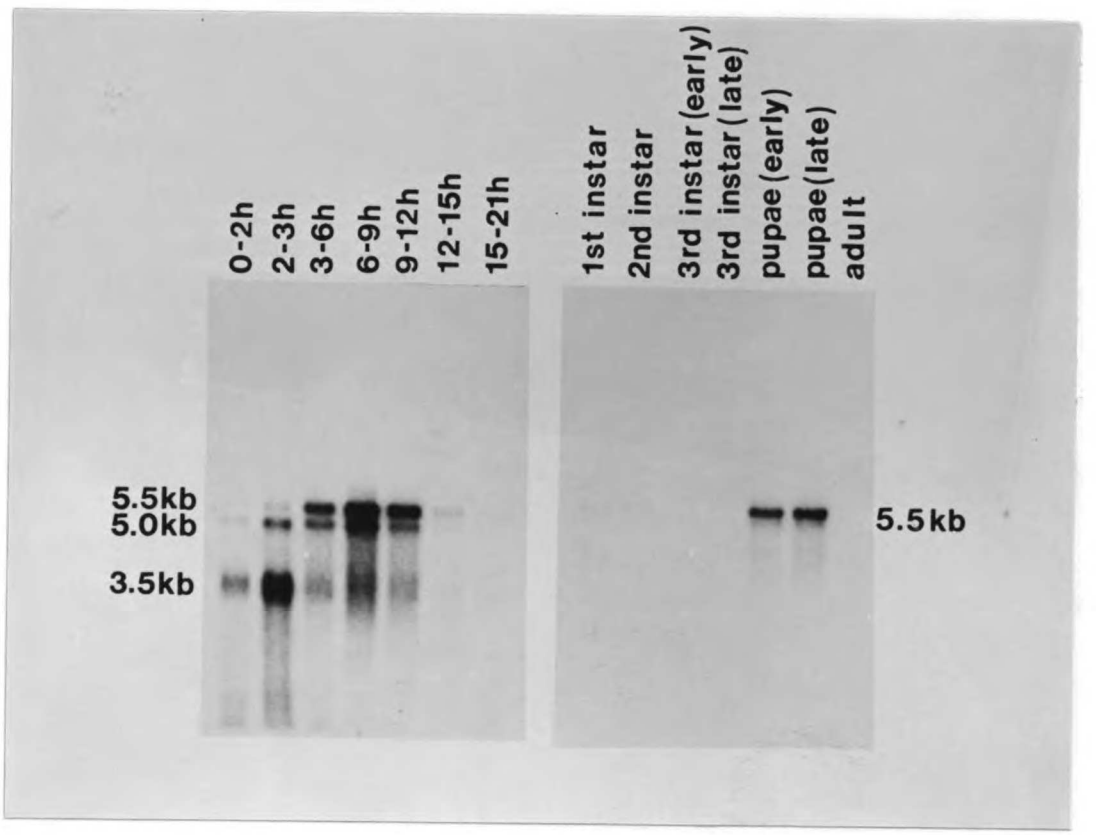


Figure 3-2. Developmental Expression of *Drosophila c-src*. Polyadenylated RNA (2 μg per lane) was prepared from whole animals at various stages of development, fractionated in formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized with Probe A (see Figure 3-1). The time points are given as hours after egg-laying for the embryonic period. Later timepoints are: first instar, 24-48 hr; second instar, 48-72 hr; early third instar, 72-96 hr; late third instar, 96-120 hr; early pupae, 120-168 hr; late pupae 168-216 hr. RNA sizes were estimated from stained yeast ribosomal RNAs.

FIGURE 3-2


0-2h
2-3h
3-6h
6-9h
9-12h
12-15h
15-21h
1st instar
2nd instar
3rd instar (early)
3rd instar (late)
pupae (early)
pupae (late)
adult
5.5kb
5.0kb
3.5kb
5.5kb

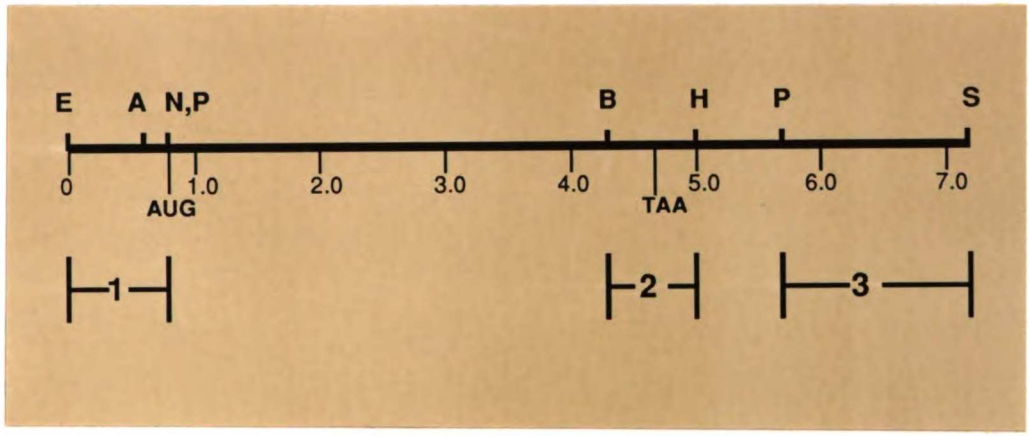
hours of embryonic development. A 5.0 kb transcript was also present during early development and persisted until approximately 12 hours after egg deposition. A 5.5 kb transcript accumulated from the onset of gastrulation until late in embryogenesis at 15 hours and was again apparent during the pupal period. After longer exposure of the blot, low levels of transcripts could also be detected during both the adult (3.5 and 5.0 transcripts) and larval (5.5 kb transcript) periods (data not shown).

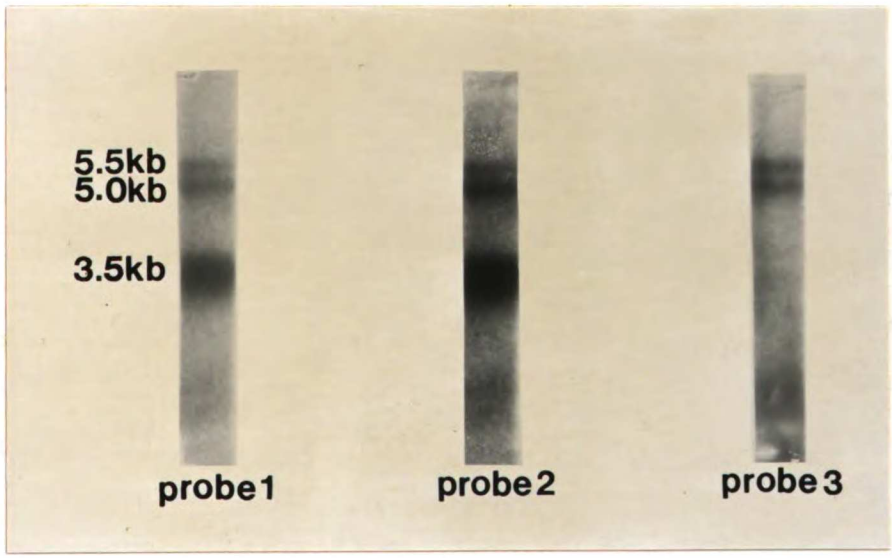
In order to determine the nature of the differences between the three RNA species, a number of hybridization probes were derived from the region in which the coding seqences defined by the cDNA lie. The results of probing RNA from 0 to 7 hour old embryos with these probes are shown in Figure 3-3. The results suggest that the major difference between the 3.5 kb transcript and the 5.0 and 5.5 kb transcripts is due to differential use of a polyadenylation site. Using probes that lie within or immediately upstream of the the protein coding region defined by the cDNA sequence, all three transcripts were detected. In contrast, probes from the sequences downstream of the coding region only detected the larger transcripts. These results also suggest that the sequenced cDNA is a representative of the 3.5 kb transcript class, since the cDNA ends near the *HindIII* site and is terminated by a run of approximately 30 adenosines (data not shown).

These experiments imply that all three transcripts are derived from *c-src*. However, one cannot conclude that all three

Figure 3-3. Topography of *c-src* RNAs. The upper panel is a map of the *c-src* region. The direction of transcription is from left to right. The map units are kilobasepairs. The positions of the initiation and termination codons, as determined by the sequence shown in Figure 2-6, is indicated by the AUG and TAA, respectively. The fragments designated 1, 2, and 3 were used as probes for Northern blot analysis of RNA from 0-7 hour old embryos (lower panel). Each lane contained 5 μg of polyadenylated RNA. (A=*Apa*I; B=*Bam*HI; E=*Eco*RI; H=*Hind*3; P=*Pst*I; S=*Sal*I)

FIGURE 3-3


E
A
N,P
B
H
P
S
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
AUG
TAA
1
2
3


5.5kb
5.0kb
3.5kb
probe1
probe2
probe3

transcripts encode identical proteins. Two lines of evidence do support the notion that only a single *c-src* protein is produced. First, sequence analysis of a number of cDNAs, including 3 clones that must be derived from one of the larger transcripts, has failed to provide evidence for a second protein product. This conclusion must be partially discounted because only 80% of the coding region of each clone has been examined. Second, analysis of proteins produced by the locus has failed to indicate the existence of a second form of the protein (see Chapter 4). However, both of these results are inconclusive and the possibility remains that several different *c-src* proteins are produced.

3.3 Localization of c-src RNA

The localization of *c-src* transcripts in developing embryos, larvae and pupae was examined by *in situ* hybridization of RNA in frozen sections (Simon *et al.*, 1985). The probe used in these experiments was a plasmid containing a 4.3 kb fragment of the *c-src* locus (Probe B in Fig. 3-1). This probe contains no repetitive sequences and hybridizes to all three *c-src* transcripts (see Fig. 3-3). To ensure that any hybridization detected was due to specific binding of *c-src* transcripts, parallel experiments were performed in which the parental plasmid, pEMBL9, was used as a probe. Slides that were hybridized with the control plasmid exhibited no increase in signal over the embryo sections relative

to blank areas of the slide.

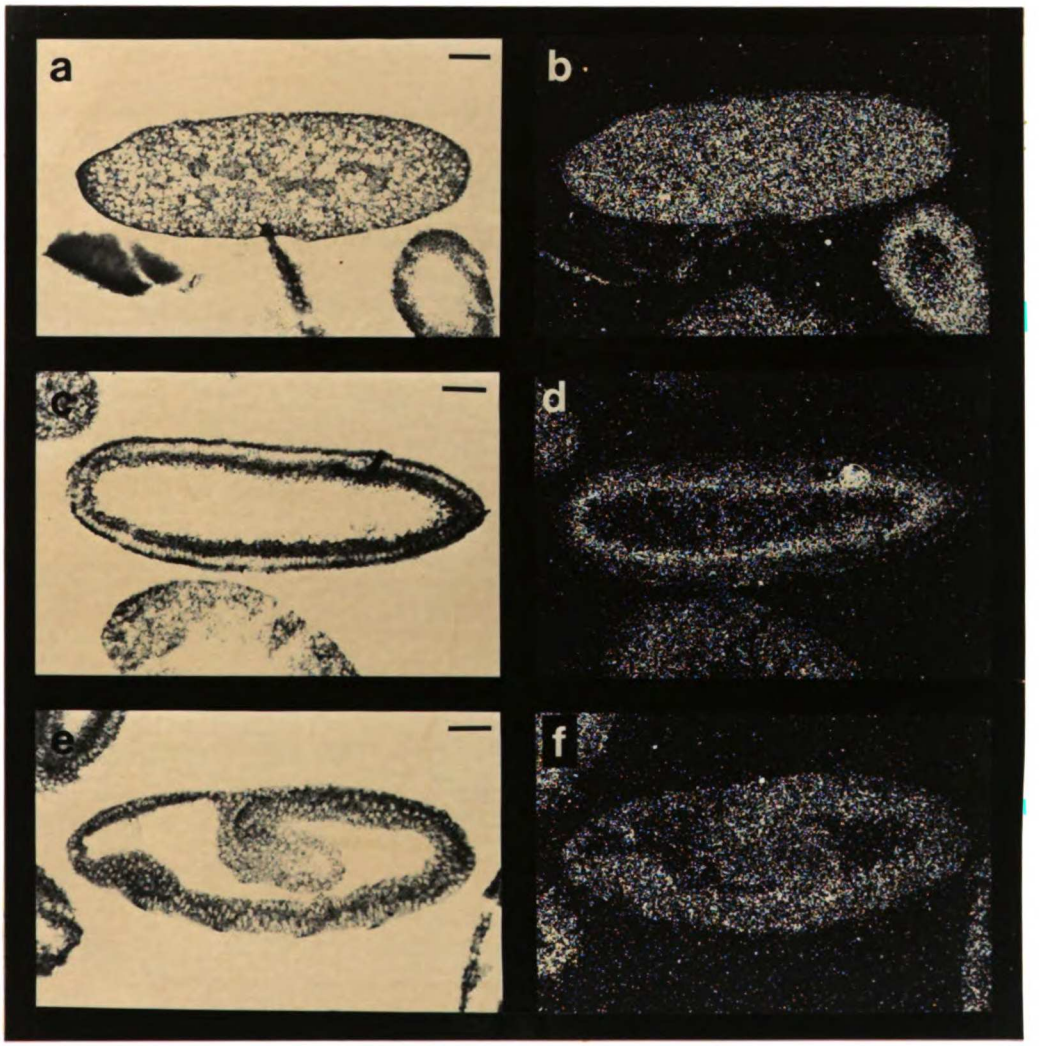
3.3.1 Embryos

During the first 2.5 hours of embryogenesis at 25°C, the nuclei undergo 13 synchronous divisions in a syncytial preblastoderm and are then enclosed by cell membranes to form the cellular blastoderm (for a review of embryonic development, see Fullilove *et al.*, 1978). *c-src* transcripts were found to be evenly distributed throughout the preblastoderm (Fig. 3-4 A and B). This RNA presumably represents maternal message, since little RNA synthesis occurs during the preblastoderm period (Zalokar, 1976). Cellular blastoderm stage embryos showed accumulation of *c-src* RNA primarily in the cytoplasm of the newly formed cells (Fig. 3-4 C and D). The yolk region was virtually devoid of grains.

Immediately after the cellular blastoderm is formed, gastrulation begins. A primitive mesoderm is formed by invagination of cells along the longitudinal ventral midline (the ventral furrow). Rudiments of the anterior and posterior midgut are formed by invaginations at either end of the ventral furrow. The ventral mesodermal and ectodermal cells (the germ band) then extend around the posterior pole and anteriorly along the dorsal surface of the embryo. This process (germ band extension) involves both cell migration and cell division and is complete at approximately 6 hours. Throughout the period of germ band extension, *c-src* mRNA

Figure 3-4. Localization of *c-src* transcripts in 0-6 hr embryos. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography. Orientation of the embryos are anterior to the left. (A) is a section of a pre-blastoderm embryo. *c-src* RNA is distributed uniformly throughout the embryo (B). (C) is a slightly oblique section of an embryo just prior to cellular blastoderm formation (2.5 hr). Grains are distributed in the cytoplasm of all of the newly forming blastoderm cells (D). Only low levels of *c-src* RNA can be detected in the central yolk mass. (E) is a sagittal section of a 5.5 hr embryo, the germ band of which is almost completely extended. *c-src* RNA is present in all of the cells but not in the yolk (F). The horizontal bars correspond to 50 μm.

FIGURE 3-4



was distributed uniformly in all the cells of the embryo (Fig. 3-4 E and F).

After approximately 8.5 hours of embryonic development, the extended germ band begins to return to a position along the ventral midline by migration around the posterior pole (germ band retraction). At this time, the first signs of regional expression of *c-src* became apparent. While low levels of *c-src* RNA were detected throughout the embryo, the newly forming visceral mesoderm showed considerably elevated levels of *c-src* transcripts (Fig. 3-5 A,B,C and D). This was most clearly seen in cross section (Fig. 3-6). The grains accumulated over cells that will form the smooth muscle surrounding the gut. The final mitotic divisions of these cells occur at approximately 8 hours after the start of embryonic development (Poulson, 1950).

Examination of late embryos revealed another site of *c-src* expression. While total *c-src* expression in late embryos was low (Fig. 3-2), the brain and ventral nerve chord displayed elevated levels of *c-src* transcripts (Fig. 3-5 E and F). The cells of the visceral musculature have completed differentiation at this time and no longer express *c-src*.

3.3.2 Larvae and pupae

Two distinct sets of cells coexist in the larvae of a holometabolous insect such as *Drosophila* (Bodenstein, 1950). Many of

Figure 3-5. Localization of *c-src* transcripts in 9-18 hr embryos. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography. Orientation of the embryos are anterior to the left. (A) is a lateral sagittal section of a 9.5-10 hr embryo. Abundant *c-src* expression can be detected in a group of cells that lie ventral to the midgut and dorsal to the developing nerve chord (B). (C) is a frontal section of an embryo of approximately the same age as in (A). The identity of the site of hybridization in (A-D) is more easily discerned in cross section (Figure 3-6). (E) is a lateral sagittal section of an 18 hr embryo. *c-src* RNA accumulation is apparent in the brain (br) and ventral nerve chord (vc). The horizontal bars correspond to 50 μm.

FIGURE 3-5

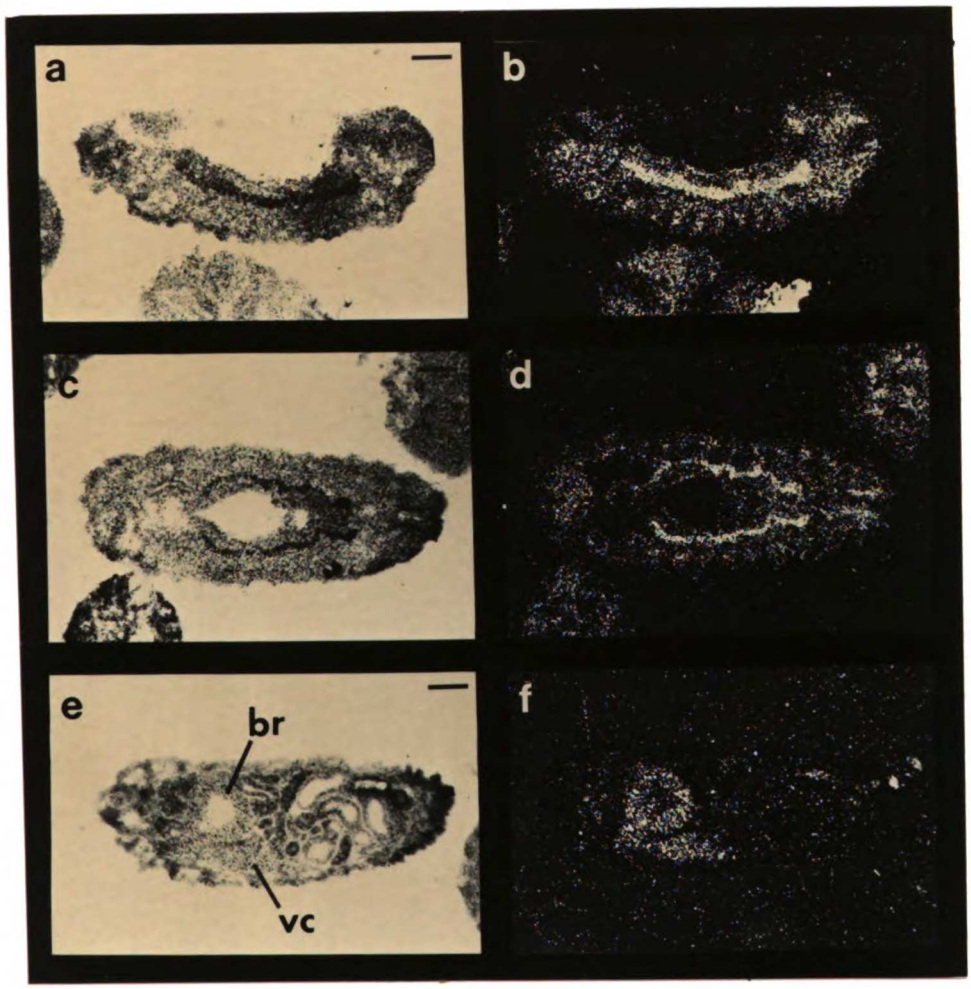
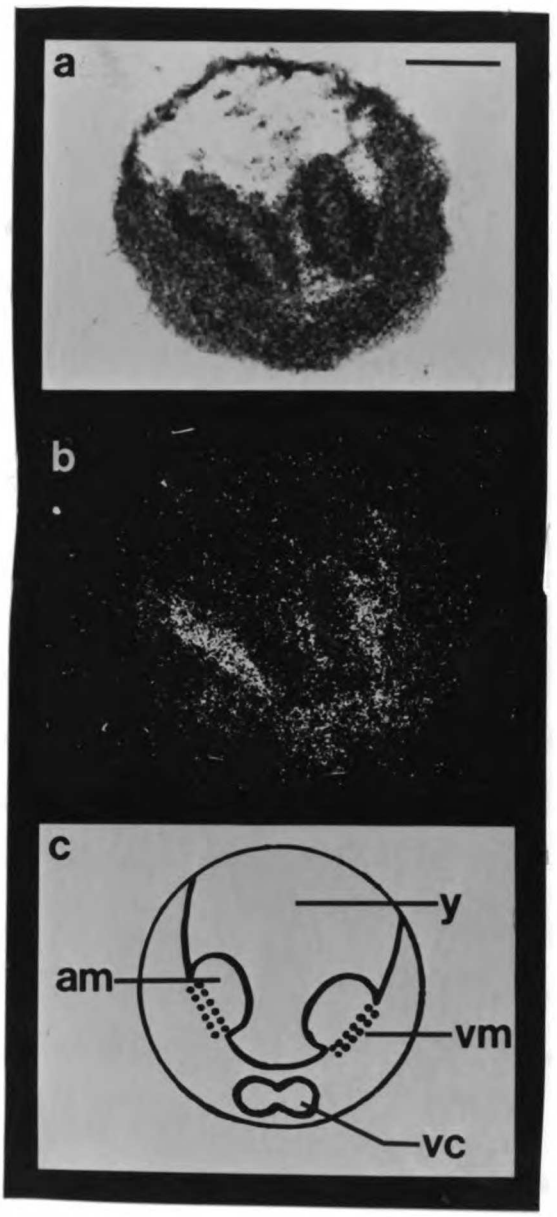


Figure 3-6. Localization of *c-src* transcripts in 10-11 hr embryos. (A) and (B) are corresponding bright- and dark-field micrographs of a slightly oblique cross-section of a 10-11 hr embryo. (C) is a schematic cross-section of an 11 hr embryo (adapted from Fullilove *et al.*, 1978). Abundant *c-src* expression is detected in the visceral mesoderm that will form the smooth muscle of the gut. Lower levels of *c-src* RNA are apparent in the region of the ventral nerve chord. The horizontal bar indicates a length of 50 μm. Abbreviations are: am, anterior midgut; vc, ventral nerve chord; vm, visceral mesoderm; y, yolk.

FIGURE 3-6



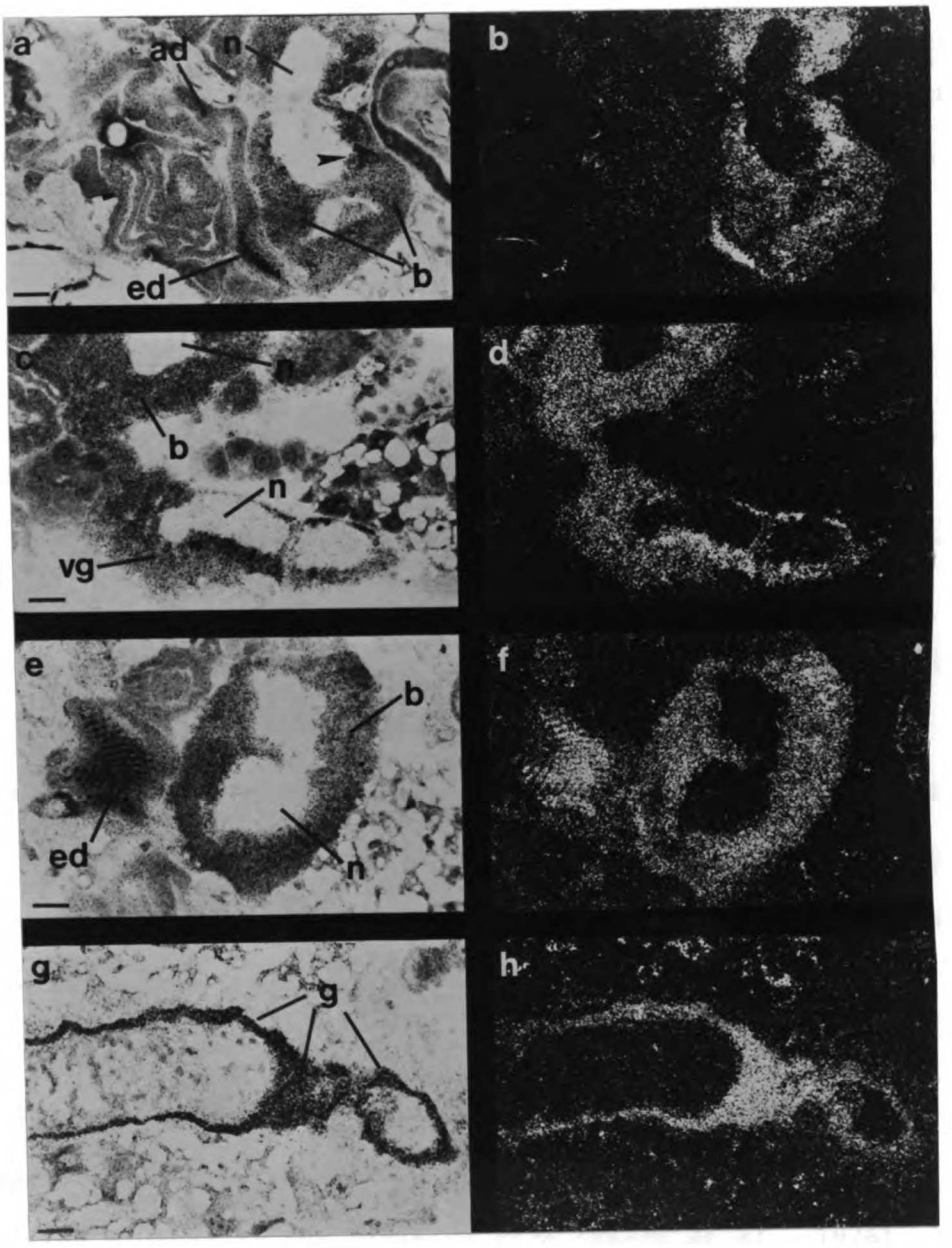
the cells that perform functional roles in the larva itself become polytene and grow by cell enlargement. These cells will histolyze during the pupal period and will not contribute to the adult form. Other cells remain diploid and will form the adult (or imago) during the pupal period. Some of the diploid cells form discrete groups, called imaginal discs, that divide throughout the larval period. Others exist as small pockets of diploid cells among the polytene cells and do not commence cell division until the onset of the pupal period. The nervous system of the animal is exceptional in that both new imaginal cells and some larval cells form the nervous system (Kankel *et al.*, 1980).

Expression of *c-src* RNA was examined in climbing stage third instar larvae (5.5 days old) and in early pupae (6 to 24 hours after puparium formation). Attempts to examine later pupal time points were unsuccessful because of nonspecific binding of probe to the newly forming adult cuticle. Abundant expression of *c-src* RNA was detected in the brain and ventral ganglia, in the eye-antennal imaginal disc, and in the cells that surround the pupal gut (see below, Fig. 3-7). Very low levels of *c-src* were also detected in the gonads and in all imaginal discs (data not shown). No *c-src* RNA was apparent in other tissues.

Expression of *c-src* RNA was abundant in brain and nerve chord at both of the stages that were examined (Fig. 3-7 A to F). Although all regions of the nervous system displayed abundant amounts of *c-src* RNA, the signal was not distributed uniformly

Figure 3-7. Localization of *c-src* transcripts in larvae and pupae. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography. (A) is a view of a frontal section of a climbing stage third instar larva. Grains accumulate over the entire brain and the eye region of the eye-antennal imaginal disc (B). The arrow indicates a region of the brain that is the site of especially abundant *c-src* RNA accumulation. Only low levels of *c-src* RNA can be detected in the the head and antennal portions of the eye-antennal discs. (C) is a view of a sagittal section of an early pupa. *c-src* expression is apparent in both the brain and ventral ganglia (D). (E) is a section of an early pupa. *c-src* RNA can be detected in both the brain and eye-antennal disc of this pupa (F). Note that the regular array of the photoreceptor clusters (ommatidia) is apparent in the accumulation of silver grains. (G) is a view of a sagittal section of an early pupa. Hybridization can be detected along the walls of the gut (H). The signal is especially clear in the center of the field where the surface of the gut wall has been exposed. The horizontal bar in each of the bright field images corresponds to 50 μm. Abbreviations are as follows: ad, antennal portion of the eye-antennal imaginal disc; b, brain; ed, eye portion of the eye-antennal imaginal disc; g, gut; n, neuropil; vg, ventral ganglia.

FIGURE 3-7



throughout the nervous system. One region of the brain, located in the dorsal and medial portion of each of the brain hemispheres, showed exceptionally intense hybridization (Fig. 3-7 A and B). The specific function of this region of the brain is not known.

The eye-antennal imaginal discs are groups of cells that form the adult compound eye and most of the head capsule. The region of the disc that forms the eye was the site of the most abundant *c-src* RNA expression in both third instar larvae and early pupae (Fig. 3-7 A,B,E and F). During this period, the cells in this region of the disc undergo their final mitotic divisions and commence differentiation into the mature retina (Ready *et al.*, 1976). This process occurs in an extremely organized manner. A wave of mitosis and differentiation sweeps across the monolayer of the disc from posterior to anterior. This wave is marked by a deep furrow (the morphogenetic furrow) that moves across the disc surface. As the furrow passes, the nuclei of the cells are forced to the basal surface of the disc. The nuclei of the photoreceptor cells then rise in a defined sequence and their corresponding cells differentiate to form each photoreceptor (ommatidial) cluster. As a consequence of this ordered process, the temporal sequence of ommatidial development is displayed across the disc. Each developing ommatidium is approximately two hours older than the neighboring cluster on the anterior side (Ready *et al.*, 1976). This spatial display of development provided a unique opportunity for observing the timing of *c-src* expression during

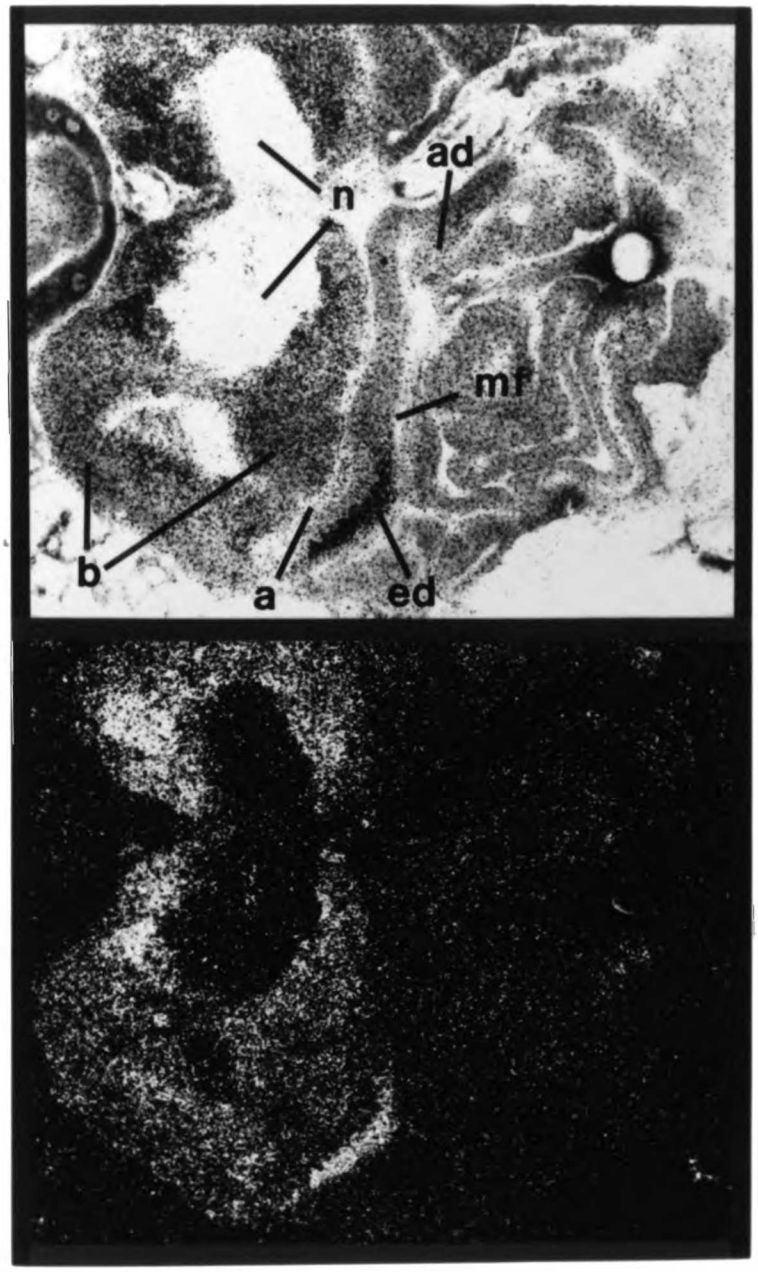
differentiation.

The timing of *c-src* expression in the eye-antennal disc of a third instar larva can be seen in Figure 3-8. The approximate position of the morphogenetic furrow is indicated. This position can be inferred from the position of the axons that extend from photoreceptor cells behind the furrow. Expression can be seen in the cell nuclei and cell bodies that have risen to the apical surface of the disc behind the furrow. Only low levels of *c-src* RNA were detected in cells that lie anterior to the furrow and in the regions of the disc that form the antennae and head cuticle (Fig. 3-7 A and B). This result strongly suggests that *c-src* is predominantly expressed in the photoreceptor cells. Unfortunately, the preservation of morphology in frozen sections is inadequate for determining the exact temporal relationship between *c-src* expression and the earliest markers of neuronal differentiation of the photoreceptors due to uncertainty about the exact position of the morphogenetic furrow. This determination will probably require antibody staining of eye-antennal disc whole mounts. The data does allow the conclusion that the onset of *c-src* RNA accumulation occurs in the photoreceptor cells at approximately the time of terminal differentiation.

During the early pupal period, cells that surround the gut also expressed high levels of *c-src* RNA (Fig. 3-7 G and H). Examination of larval sections showed that no corresponding accumulation of transcripts in any cells in the vicinity of the

Figure 3-8. The timing of *c-src* expression in the eye-antennal imaginal disc. (A) and (B) are corresponding bright and dark-field photomicrographs of a sagittal section of a third instar larva. Anterior is to the left. The approximate position of the morphogenetic furrow is indicated by the arrow. The position of the furrow was inferred from the position of the photoreceptor axons. *c-src* RNA accumulation is apparent in the photoreceptor cells that rise to the apical surface of the disc behind the furrow. Much less *c-src* RNA is expressed by the cells in front of the furrow. Abbreviations are as follows: a, axons; ad, antennal portion of eye-antennal imaginal disc; b, brain; ed, eye portion of eye-antennal imaginal disc; mf, morphogenetic furrow; n, neuropil.

FIGURE 3-8



gut. Although the exact identity and fate of these pupal cells is unknown, their position suggests that they may be the pupal analogs of the visceral muscle cells that express *c-src* during the embryonic period.

3.4 Discussion

The RNA analysis described in this chapter has provided evidence that the *Drosophila c-src* gene is transcribed in a complex pattern during the development of the fly. Three RNAs are produced. The expression of each of these transcripts is temporally regulated in a distinctive manner during the life cycle of the fly. *In situ* analysis showed that *c-src* RNA is expressed in a tissue-specific manner during much of the *Drosophila* life cycle. After the early embryonic period, expression of *c-src* is largely restricted to the smooth muscles of the gut, the developing compound eyes, the brain and the ventral ganglia.

The discovery of multiple *c-src* transcripts raises a number of important issues that have not as yet been analyzed satisfactorily. The most important of these are whether all three of the *c-src* transcripts produce the same protein and whether all three RNAs are transcribed from a single promoter. Since the structures of the RNAs have not been extensively analyzed, these questions remain unanswered. The present data merely suggest that a major portion of the difference between the 3.5 kb RNA and the

5.0 and 5.5 kb RNAs can be explained by differential usage of polyadenylation signals and a resulting increase in the length of the 3′ untranslated sequences.

The ability of the *v-src* gene of Rous sarcoma virus to stimulate the growth of cells suggests that the closely related *c-src* gene might also serve as a signal for mitosis. *In situ* analysis of *Drosophila c-src* does not support this hypothesis. The discrepancy is most evident for the embryonic smooth muscle cells of the gut. These cells complete their final divisions at 6-8 hours after the beginning of embryogenesis and form functional muscles by the end of embryonic development at 22 hours (Poulson, 1950). Abundant *c-src* expression was detected from 9 to 14 hours after fertilization and thus correlates with the differentiation rather than with the proliferation of these cells. A similar lack of correlation between *c-src* expression and cell proliferation was seen in the developing eye. Intense *c-src* expression was observed in photoreceptor cells during differentiation rather than during proliferation. It was not possible to assess the correlation of proliferation, differentiation and *c-src* expression in the brain because differentiated and proliferating cells are intermingled there throughout most of *Drosophila* development (White and Kankel, 1978; Kankel *et al.*, 1980).

The pattern of expression of *Drosophila c-src* is remarkably similar to that of chicken *c-src*. The expression of pp60$^{c\text{-}src}$ is elevated in several neural tissues of the chicken, including brain

and retina (Cotton and Brugge, 1938; Levy *et al.*, 1984). Furthermore, *in situ* anlysis of the developing retina and cerebellum of chickens by immunocytochemistry suggests that pp60$^{c\text{-}src}$ specifically accumulates in nonproliferating, differentiating neural cells. The similarity of the expression patterns in flies and chickens strongly suggests that the function of *c-src* has been conserved between insects and vertebrates. The nature of this function is still unknown, but the data suggests that *c-src* performs a function that is important for the differentiation of smooth muscle and neural cells.

CHAPTER FOUR

CHARACTERIZATION OF PROTEIN PRODUCED BY DROSOPHILA C-SRC

4.1 Introduction

The preceding chapters have provided insight into the possible enzymatic activity and localization of the protein product of *Drosophila c-src*. However, DNA sequence homology to other tyrosine kinases does not really prove that a gene encodes a tyrosine kinase and RNA localization does not necessarily correlate completely with protein localization. Direct examination of the protein(s) produced by *c-src* is therefore essential for the understanding of *c-src* function. The approach chosen was to raise antibodies that specifically recognize *c-src* protein. These specific antibodies were then employed to characterize *c-src* protein activity and structure and to localize *c-src* protein in developing flies.

4.2 Overproduction of c-src protein in a Drosophila cell line

In order to assay the ability of the antisera to specifically recognize *c-src* protein, a system for inducible expression of *c-src* protein in a *Drosophila* cell culture line, Schneider Line 2 (SL2), was developed. The *c-src* coding region was first placed downstream of the heat-shock inducible hsp70 promoter. This hs-src cassette was then placed into a modified version of the pcopneo vector that encodes resistance to the antibiotic G-418 in *Drosophila* cells (Rio and Rubin, 1985). This construct was then modified to delete some of the DNA between the hsp70 promoter and

the *c-src* initiation site. These manipulations created the pcopneo-hssrc plasmid (see Fig. 4-1). This plasmid was then transfected into SL2 cells. The G-418 resistant cells, called SL2-SRC cells, were grown and analyzed as a mass population without single cell cloning.

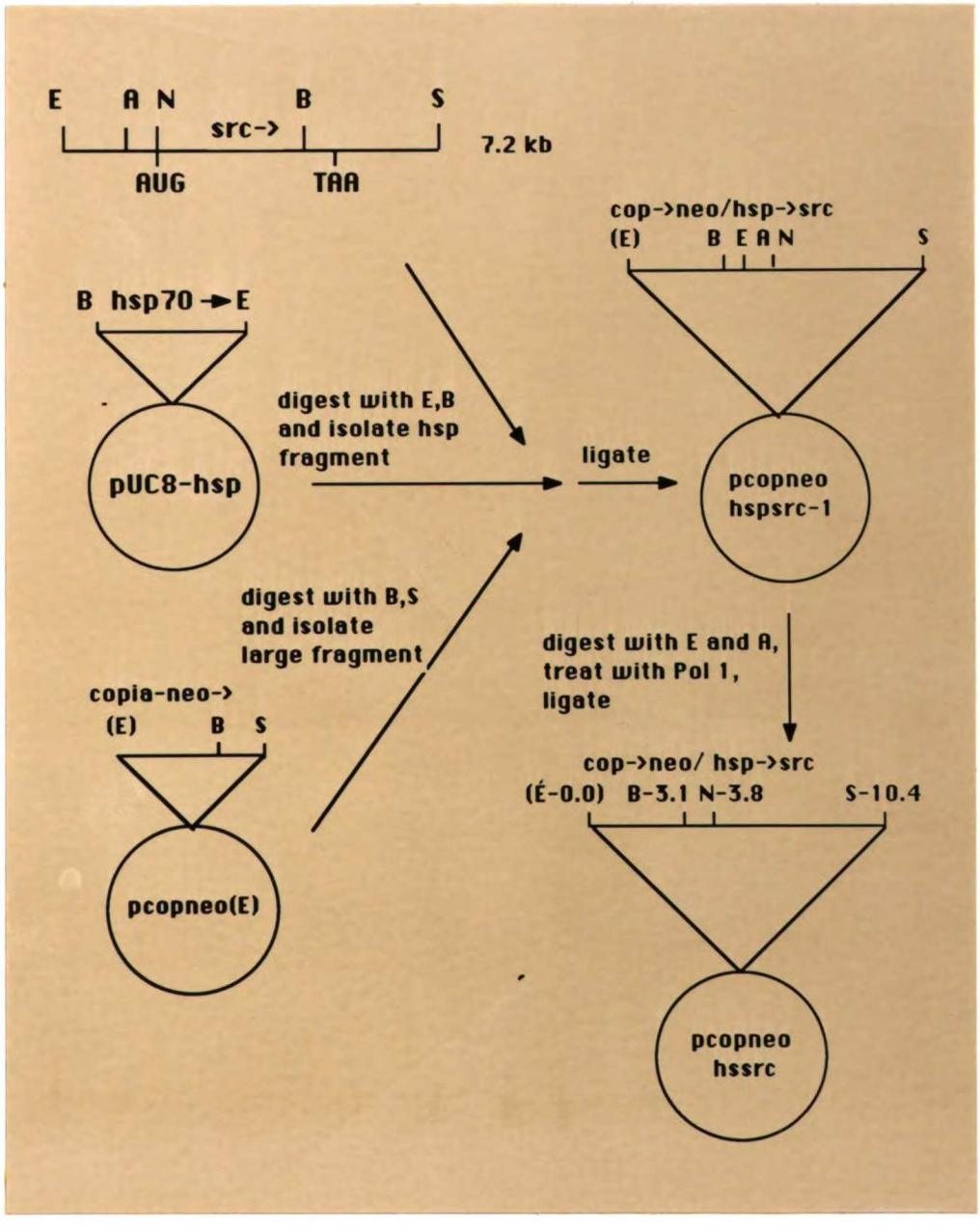
4.3 The production of antisera

Antisera that specifically recognize *c-src* protein were produced by immunizing rabbits with synthetic peptides whose sequences were predicted by the *c-src* cDNA sequence described in Chapter 2. Three peptides were chosen. Two of the peptides were derived from within the predicted kinase domain of the protein (positions 485-495 and 501-511), while one represented the carboxy-terminal 13 amino acids of the predicted protein. The first two peptides were coupled to the carrier Keyhole Limpet Hemacyanin (KLH) before immunization. Tuberculin PPD was used as the carrier for the carboxy-terminal peptide. Antisera were collected and assayed for ability to recognize *c-src* protein.

Western blots of proteins isolated from nonheat-shocked and heat-shocked SL2 and SL2-SRC cells were prepared and probed with each of the anti-peptide antisera at a 1:250 dilution. The antisera raised against the two internal peptides were unable to recognize any proteins that were specifically induced in the SL2 cells by heat-shock (data not shown). The antisera to the carboxy

Figure 4-1. Construction of a *c-src* expression vector. The plasmid pcopneo-hssrc was constructed in a two step process. First, a 7.2 kilobasepair *Eco*RI/*Sal*I fragment from λ64B1-10 (see Figure 5-7)that contains the *c-src* coding region, a 500 basepair *Bam*HI/*Eco*RI fragment that contains the hsp70 promoter (from H. Stellar), and a *Bam*HI/*Sal*I fragment of the pcopneo(E) vector (the pcopneo vector of Rio And Rubin (1985) modified to eliminate the sole *Eco*RI site) were ligated to form pcopneo-hspsrc-1. This plasmid was then digested with *Eco*RI and *Apa*I, treated with Pol I in the presence of all four nucleotides, and then ligated to form pcopneo-hssrc. The numbers shown in the final diagram of pcopneo-hssrc are distances in kilobasepairs from the *Eco*RI site of pcopneo. The arrows indicate the direction of transcription of the copia-driven *neo* gene and the hsp-driven *c-src* gene. (A=*Apa*I; B=*Bam*HI; E=*Eco*RI; N=*Nco*I; S=*Sal*I).

FIGURE 4-1


E A N B S
src->
7.2 kb
AUG
TAA
cop->neo/hsp->src
(E) B E A N S
B hsp70 -> E
pUC8-hsp
digest with E,B
and isolate hsp
fragment
ligate
pcopneo
hspsrc-1
digest with B,S
and isolate
large fragment
digest with E and A,
treat with Pol 1,
ligate
copia-neo->
(E) B S
pcopneo(E)
cop->neo/ hsp->src
(E-0.0) B-3.1 N-3.8 S-10.4
pcopneo
hssrc

terminal peptide were able to recognize a 62 kilodalton protein that was specifically induced in the SL2 cells after heat-shock (Fig. 4-2). Four lines of evidence support the identification of the 62 kd protein as the protein product of *c-src*: 1) the protein is induced by heat-shock only in the cells that have received the pcopneo-hssrc construction, 2) the protein is the size predicted for the *c-src* protein by the sequence of the *c-src* cDNA, 3) the protein is recognized by antisera directed against a peptide predicted to be included within the *c-src* protein, and 4) this interaction can be blocked by the addition of excess peptide. One of the carboxy-terminal antisera, serum 11, was particularly strong in its reaction with pp62$^{c\text{-}src}$ and was used for all further characterization.

4.4 Enzymatic activity of the c-src protein

The availability of antisera that react with pp62$^{c\text{-}src}$ and of cells that overproduce pp62$^{c\text{-}src}$ allowed a direct evaluation of the ability of the protein to phosphorylate tyrosine residues. Extracts prepared from SL2 and SL2-SRC cells before and after heat-shock treatment were immunoprecipitated with serum 11. These immunoprecipitates were then assayed for the ability to phosphorylate either endogenous proteins or a random copolymer of glutamate, alanine, and tyrosine. The results are shown in Figure 4-3. The extent of phosphorylation of the polymer correlated well

Figure 4-2. Western blotting analysis of expression of pp62$^{c\text{-}src}$ in Schneider Line 2 cells. Immunoblotting of cell extracts was performed as described in Appendix 1 using an antiserum that recognizes the carboxy-terminal 13 amino acids of pp62$^{c\text{-}src}$. The lanes are: 1) normal Schneider Line 2 (SL2) cells; 2) normal SL2 cells that had been heat-shocked at 37^{o} for 1 hr and allowed to recover at 24^{o} for two hours; 3) SL2 cells that had been transfected with pcopneo-hssrc (SL2-SRC cells); 4) SL2-SRC cells heat shocked for 1 hr at 37^{o} and allowed to recover for 2 hr. The position of molecular weight markers is shown to the left (M_r x 10^3).

FIGURE 4-2

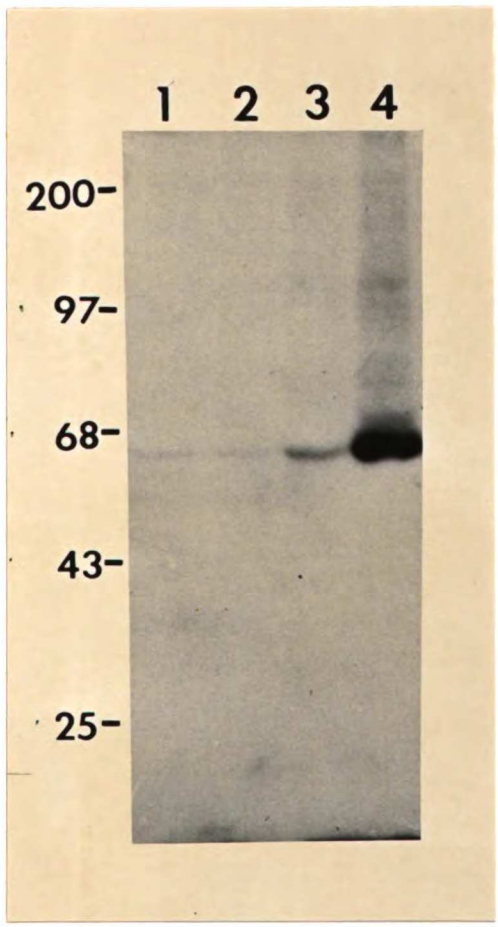
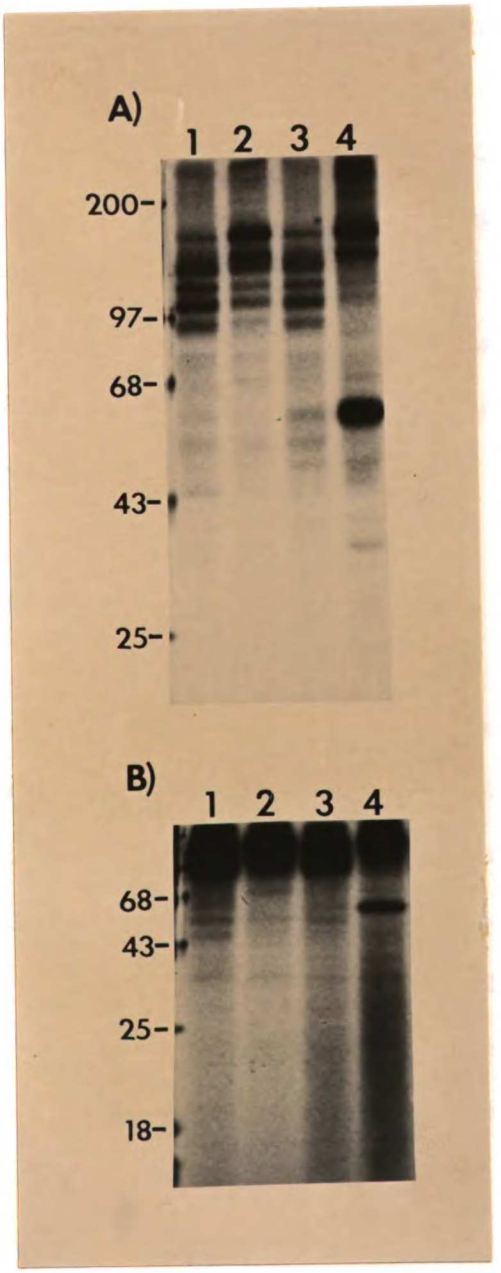


Figure 4-3. *Drosophila c-src* encodes a tyrosine-specific protein kinase. Immunoprecipitates of *Drosophila* cells were reacted with either [γ^{32}P]-ATP (upper panel) or [γ^{32}P]-ATP and a synthetic polymer of glutamate, alanine, and tyrosine (lower panel) and then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The antiserum used recognizes the carboxy-terminal 13 amino acids of pp62$^{c\text{-}src}$. The lanes are: 1) normal Schneider Line 2 (SL2) cells; 2) normal SL2 cells that had been heat-shocked at 37^{o} for 1 hr and allowed to recover at 24^{o} for two hours; 3) SL2 cells that had been transfected with pcopneo-hssrc (SL2-SRC cells); 4) SL2-SRC cells heat shocked for 1 hr at 37^{o} and allowed to recover for 2 hr. The position of molecular weight markers is shown to the left (M_r x 10^3).

FIGURE 4-3



with the amount of pp62$^{c\text{-}src}$ present in the immunoprecipitates. In addition to the labelling of the polymer, a 62 kd protein was phosphorylated when [γ^{32}P]-ATP was added to the immunoprecipitate of the heat-shocked SL2-SRC cells. This labelling occured both in the absence and presence of the polymer and was due to the autophosphorylation of a tyrosine residue of pp62$^{c\text{-}src}$ (Fig. 4-4 and data not shown). These results strongly suggest that pp62$^{c\text{-}src}$ is directly capable of phosphorylating tyrosine residues of proteins. However, the remote possibility that some protein that binds to pp62$^{c\text{-}src}$ is responsible for the activity cannot be completely discounted.

4.5 Modifications of the c-src protein

The chicken pp60$^{c\text{-}src}$ protein is modified both by phosphorylation and myristylation (discussed in Chapter 1). In order to determine whether this was also true for the *Drosophila* protein, SL2 and SL2-SRC cells were labelled in parallel with [^{35}S]-methionine, [$^{32}P_i$], and [^{3}H]-myristate and then immunoprecipitated with serum 11. The results of the experiments are shown in Figure 4-5. Serum 11 was able to detect proteins from the [^{35}S]-labelled SL2-SRC extracts but not from the [^{35}S]-labelled SL2-SRC extracts. While only a single 62 kilodalton band was detected from the nonheat-shocked cells, a doublet consisting of 62 and 64 kd bands was detected from the heat-shocked cells. This was a surprising

Figure 4-4. Phosphoamino acid analysis of pp62$^{c\text{-}src}$. [^{32}P]-labelled pp60$^{c\text{-}src}$ was analyzed for phosphoamino acid composition. The result of analysis of *in vitro* labelled pp62$^{c\text{-}src}$ (see Figure 4-3, lane 4) is shown in the left panel. The result of analysis of *in vivo* labelled pp62$^{c\text{-}src}$ (see Figure 4-5, lane 5) is shown in the right panel. In both cases, phosphorylation is predominantly of tyrosine residues. Marker phosphoamino acids were visualized by ninhydrin staining.

FIGURE 4-4

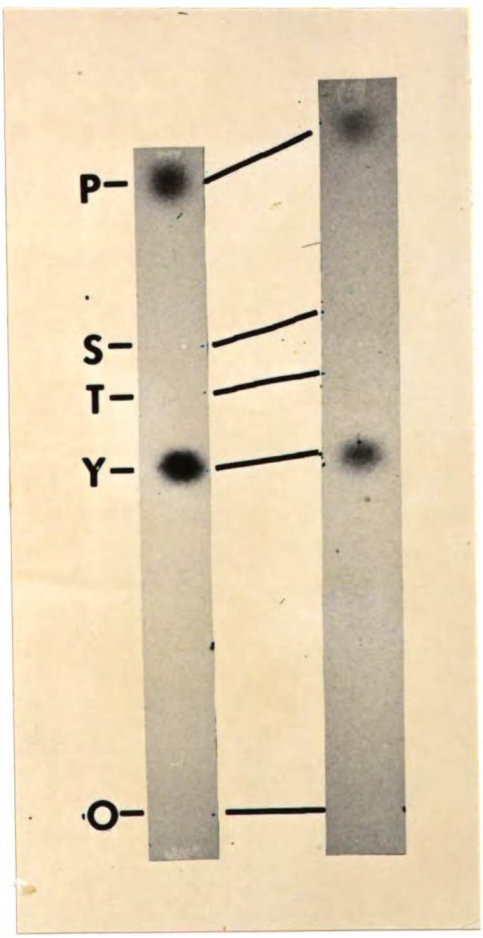
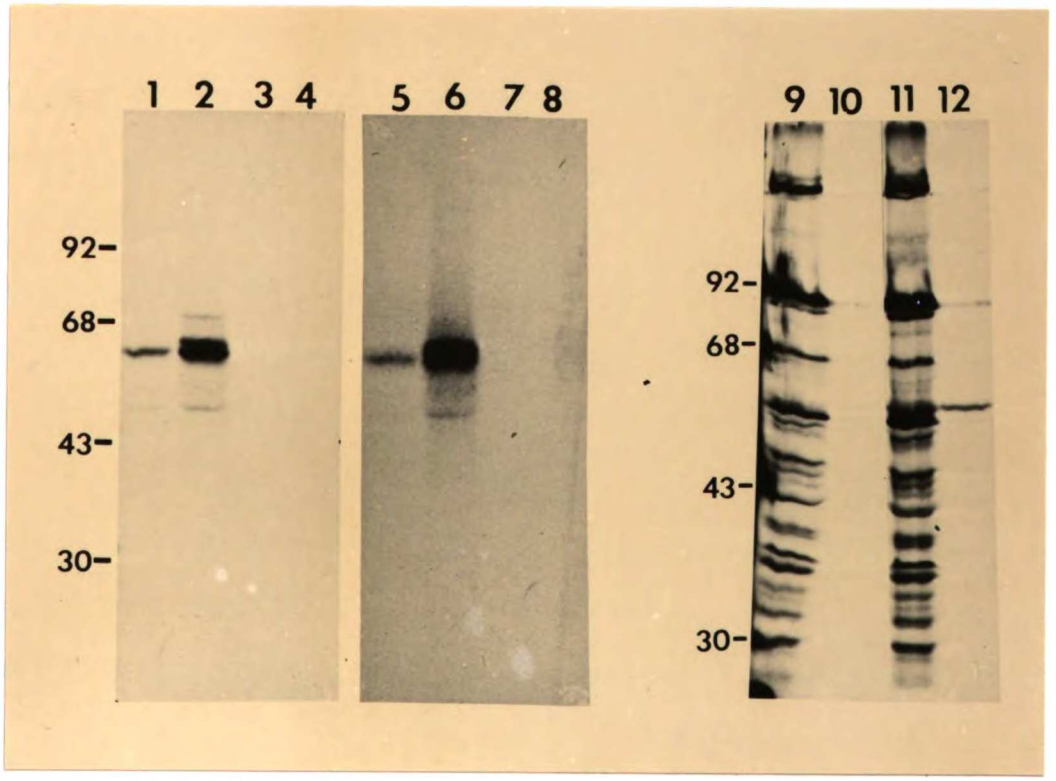


Figure 4-5. Modification of *c-src* protein in SL2-SRC cells. SL2 and SL2-SRC cells were labelled with either [^{35}S]-methionine (lanes 1-4), [^{32}P]-orthophosphate (lanes 5-8), or [^{3}H]-myristic acid (lanes 9-12) and immunoprecipitated using an antiserum that recognizes the carboxy-terminal 13 amino acids of pp62$^{c\text{-}src}$. The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The lanes are: 1) SL2-SRC cells without heat shock; 2) SL2-SRC cells after heat shock; 3) SL2 cells without heat shock; 4) SL2 cells after heat shock; 5) SL2-SRC cells without heat shock; 6) SL2-SRC cells after heat shock; 7) SL2 cells without heat shock; 8) SL2 cells after heat shock; 9) unprecipitated extact from SL2 cells; 10) immunoprecipitated extract from SL2 cells; 11) unprecipitated extract from SL2-SRC cells; 12) immunoprecipitated extract from SL2-SRC cells. The position of molecular weight markers is shown to the left of each panel (M_r x 10^3).

FIGURE 4-5



result since Western blotting had detected only a single pp62$^{c\text{-}src}$ band from these same cells. Partial protease digestion of the proteins in each of the band was conducted to verify that the upper band was actually a form of *c-src* protein (Cleveland and Kirschner, 1977). The V8 digestion patterns of the two bands were very similar (data not shown). This supports the notion that both of these proteins are derived from *c-src*. The nature of the difference between the two forms is unknown. However, the fact that the second form was not detected during Western analysis, during which antigens are generally thought to be largely denatured, might suggest that the difference is a modification within the region of the carboxy-terminal peptide.

The same pattern of proteins was detected after labelling of the cells with [$^{32}P_i$]. Phosphoamino acid analysis of the pp62$^{c\text{-}src}$ showed that only tyrosine residues were phosphorylated (Fig. 4-4). The actual site(s) of phosphorylation has not been determined. The absence of serine phosphorylation distinguishes pp62$^{c\text{-}src}$ from the chicken *c-src* protein. This difference may be explained by the lack of an obvious analog in the *Drosophila* protein to the major site of serine phosphorylation (serine-17) in the chicken protein, pp60$^{c\text{-}src}$ (Collett *et al.*, 1979a; Cross and Hanafusa., 1983). The same pattern of bands was not detected after labelling with myristate. This was due to severely diminished incorporation of labelled myristate into any cellular proteins during heat-shock (data not shown). Therefore, SL2-SRC cells were

labelled overnight at 25° and immunoprecipitated with serum 11. Figure 4-5,C shows the total crude extracts of the cells and the immunoprecipitation of an equivalent amount of extract. pp62$^{c\text{-}src}$ was labelled with myristate. The position of myristate addition was not definitively determined, but is presumed to be the same as that of pp60$^{v\text{-}src}$, glycine-2 (Buss and Sefton, 1985; Schultz *et al.*, 1985). The conservation that was observed between the first ten amino acids of the chicken and *Drosophila* proteins (Chapter 2) may therefore be an indication of the sequences that are required for myristate addition.

4.6 Localization of the c-src protein

The RNA localization experiments described in Chapter 3 provided information about the possible locations within the developing fly where *c-src* might function. However, the conclusions that could be drawn from those experiments were limited by technical problems with hybridizing sections prepared from timepoints later than puparia and by uncertainty that the distribution of protein completely mimicks that of the RNA. The development of antisera that recognize pp62$^{c\text{-}src}$ provided the means to address these issues by directly examining the distribution of protein.

Frozen sections of flies at several stages of development were collected and stained with peptide affinity purified antibodies

from serum 11. The antibodies that were retained in the sections were then detected by staining with gold-coupled secondary antibodies followed by silver deposition on the gold particles (see Appendix I). This technique allowed very sensitive detection of pp62$^{c\text{-}src}$ antigen. However, background staining of many tissues was observed. The ability of the addition of excess peptide to block the staining was used to distinguish between staining of pp62$^{c\text{-}src}$ and background non-specific binding to sections. The problem of non-specific background was particularly severe when sections of early embryos were stained. Therefore, no data from the early embryonic period is presented.

The earliest specific staining of sections was detected in 10 hour old embryos. Staining was observed in the smooth muscle cells that surround the gut (Fig. 4-6). The staining of these cells persisted until approximately 14 hours after egg deposition. The next site of staining detected was in the brains and ventral ganglia of embryos approximately 14 hours after egg deposition (Fig. 4-7). The brain and ventral ganglia are each structured with all of the cell bodies situated around the periphery of a central neuropil that consists of a mass of axonic projections from the neuronal cell bodies (Kankel *et al.*, 1980). The staining was detected almost exclusively in the neuropil region. This staining of the neuropil was apparent throughout the remainder of embryonic development. The abundance of *c-src* protein in both the smooth muscle cells and neurons correlates very well with the observed

Figure 4-6. Localization of *c-src* protein in 10 hr embryos. *c-src* protein accumulation was detected by staining frozen sections (10 μm) with an antiserum that recognizes the carboxy-terminal 13 amino acids of pp62$^{c\text{-}src}$ as described in Appendix 1. The location of *c-src* protein is indicated by the presence of dark silver grains. Bright-field photomicrographs were taken before (right) and after (left) staining with Giemsa stain. The sections are oriented with anterior to the right. (A) and (B) are views of a frontal section of a 10 hr embryo. Staining of the visceral mesederm (vm) is apparent. (C) and (D) are views of a similar section except that the antiserum was first incubated with 1 μg/ml of the immunizing peptide. No staining is apparent. The horizontal bars correspond to 50 μm.

FIGURE 4-6

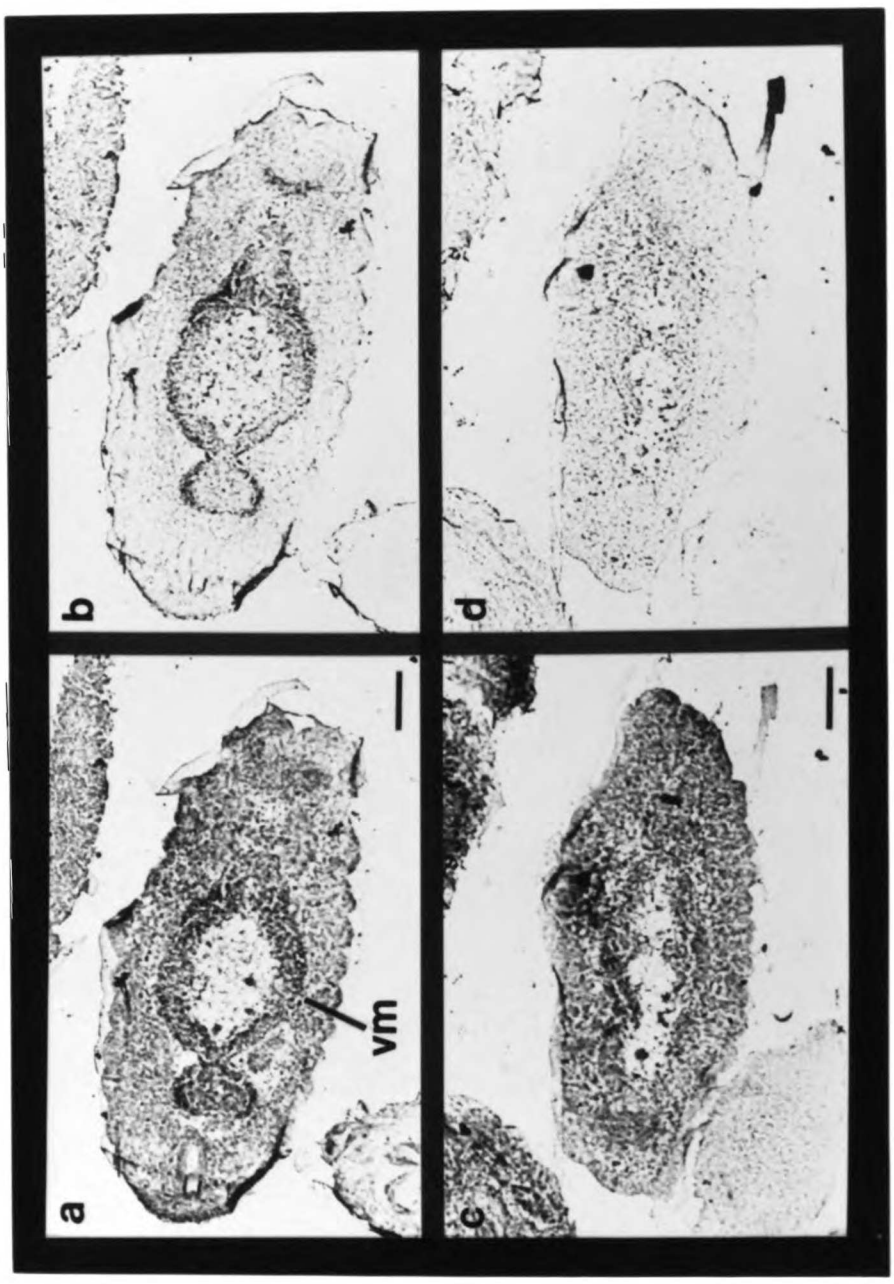
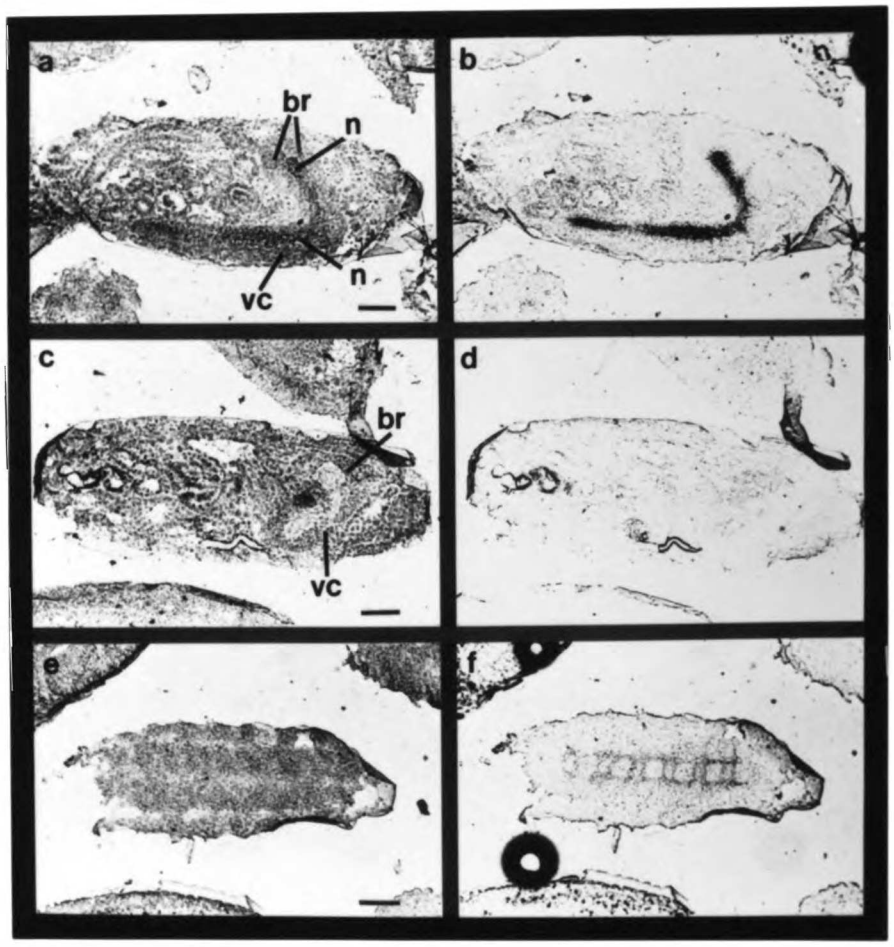


Figure 4-7. Localization of *c-src* protein in 16 hr embryos. *c-src* protein accumulation was detected by staining frozen sections (10 μm) with an antiserum that recognizes the carboxy-terminal 13 amino acids of $pp62^{c\text{-}src}$ as described in Appendix 1. The location of *c-src* protein is indicated by the presence of dark silver grains. Bright-field photomicrographs were taken before (right) and after (left) staining with Giemsa stain. The sections are oriented with anterior to the right. (A) and (B) are views of a sagittal section of a 16 hr old embryo. *c-src* protein accumulation is apparent in the neuropils (n) of the brain (br) and ventral nerve chord (vc). (C) and (D) are views of a similar section except that the antiserum was first incubated with 1 μg/ml of the immunizing peptide. No staining is apparent. (E) and (F) are views of a frontal section through the ventral nerve chord. *c-src* protein accumulation is apparent in the axon bundles that connect the ventral ganglia. The horizontal bars correspond to 50 μm.

FIGURE 4-7



abundance of RNA in these cells during this period.

The next stage to be examined was climbing stage third instar larvae. The neuropils of the brain and ventral ganglia continued to be stained as in the late embryonic period (Fig. 4-8). However, a new site of staining was also noted. The axons that project from the developing photoreceptor cells of the eye-antennal imaginal disc to the brain stained heavily. This result agrees very well with the observed abundance of *c-src* RNA in cells of the eye-antennal discs. Furthermore, it confirms the identification of the photoreceptors as the particular cell-type of the eye-antennal disc that express the RNA.

The pattern of staining was very similar during the puparial period except that very weak staining was also detected in cells that surround the gut (Fig. 4-8 and data not shown). These presumably are the same cells that surrounded the gut and expressed *c-src* RNA. This gut staining did not persist into the later pupal stages. The staining of the neuropils of the brain did persist into later pupal periods, but declined sharply in intensity in phaerate adults and was almost undetectable in adults (Fig. 4-9).

4.7 Discussion

This chapter has described the development of an antiserum that specifically recognizes the products of the *c-src* locus and its use to study their activity, modifications, and localization.

Figure 4-8. Localization of *c-src* protein in larvae and puparia. *c-src* protein accumulation was detected by staining frozen sections (10 μm) with an antiserum that recognizes the carboxy-terminal 13 amino acids of $pp62^{c\text{-}src}$ as described in Appendix 1. The location of *c-src* protein is indicated by the presence of dark silver grains. Bright-field photomicrographs were taken before (right) and after (left) staining of the sections with Giemsa stain. The sections are oriented with anterior to the top. (A) and (B) are views of a frontal section of a late third instar larva. Intense staining is apparent in the axons of the optic nerve and the neuropile of the brain. Staining can also be observed in the cell bodies of the the photoreceptor, which are near the apical surface of the eye disc. The arrow in (A) indicates a site of non-specific binding of the secondary antibodies. (C) and (D) show the results of staining a similar section as that in (A) and (B) except that prior to staining the primary antibody was incubated with 1 μg per ml of the immunizing peptide. Note that staining is abolished by this treatment. (E) and (F) are views of a frontal section of a puparium stage animal. The pattern of staining is similar to that of (A) and (B). Abbreviations are as follows: b, bodies of brain cells; bn, neuropil of the brain; ed, eye portion of the eye-antennal imaginal disc; on, optic nerve. The horizontal bars correspond to 50 μm.

FIGURE 4-8

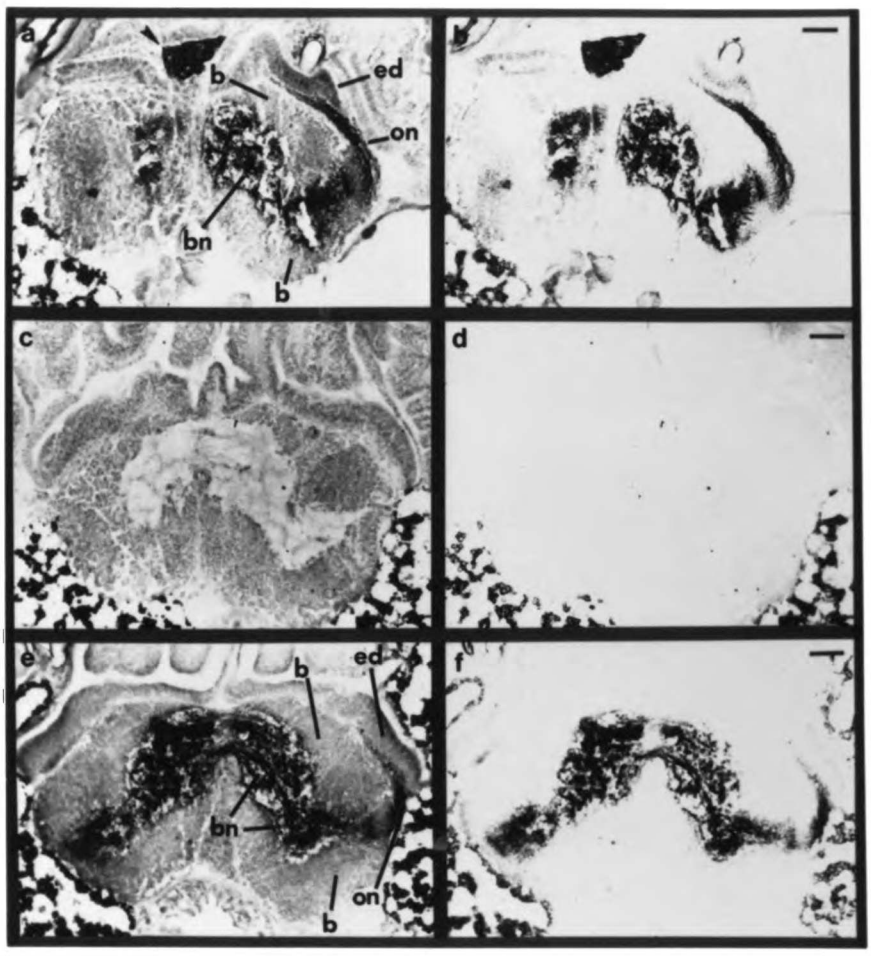
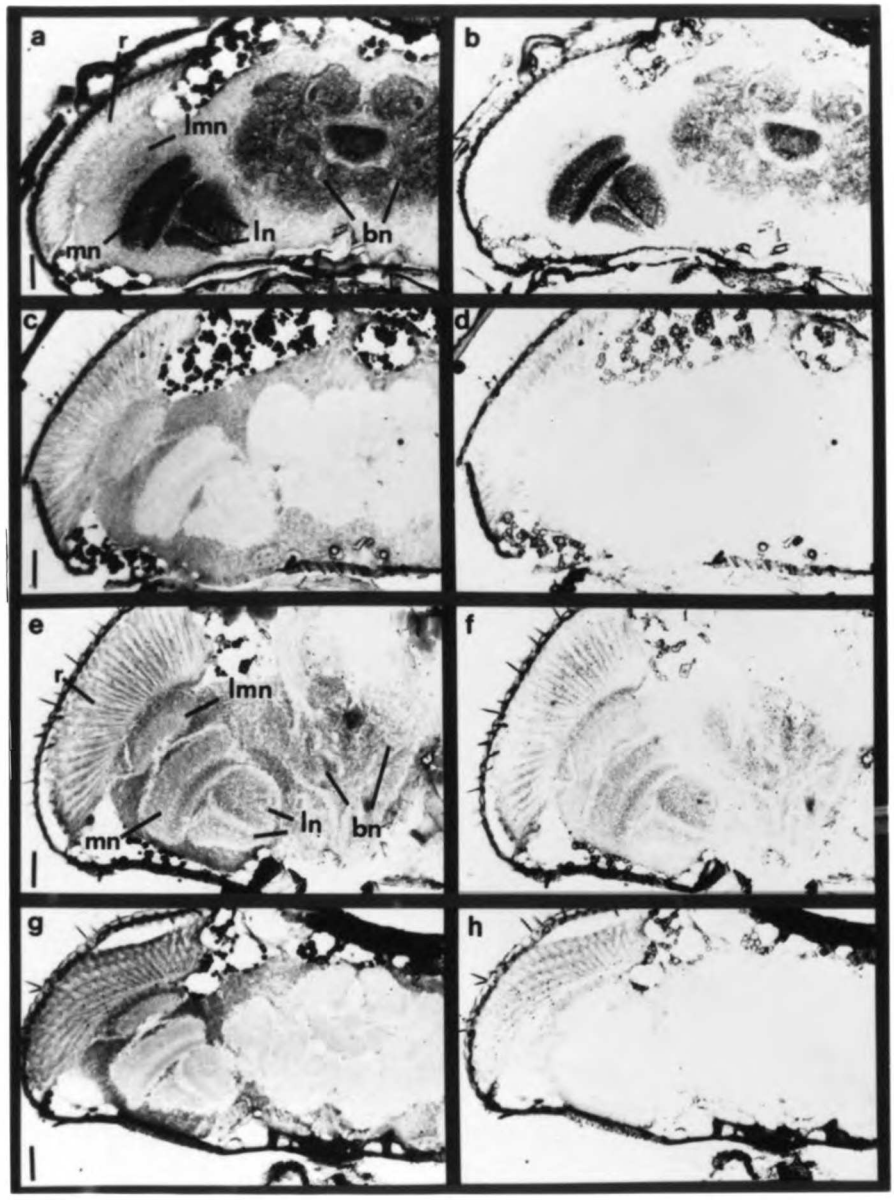


Figure 4-9. Localization of *c-src* protein in phaerate adults and adults. *c-src* protein accumulation was detected by staining frozen sections (10 μm) with an antiserum that recognizes the carboxy-terminal 13 amino acids of pp62$^{c\text{-}src}$ as described in Appendix 1. The location of *c-src* protein is indicated by the presence of dark silver grains. Bright-field photomicrographs were taken before (right) and after (left) staining of the sections with Giemsa stain. The sections are oriented with anterior to the top. (A) and (B) are views of a section across the head of a phaerate adult. Staining is apparent in the neuropils of the brain, lobula, and medulla. (C) and (D) show the results of staining a similar section as that in (A) and (B) except that prior to staining the primary antibody was incubated with 1 μg per ml of the immunizing peptide. Note that staining is abolished by this treatment. (E) and and (F) are views of a section across the head of a 1 day old adult. The pattern of staining is similar to that observed in (A) and (B) except that the intensity of staining is markedly reduced. (G) and (H) show the results of staining a similar section as that in (E) and (F) except that prior to staining the primary antibody was incubated with 1 μg per ml of the immunizing peptide. Note that staining is abolished by this treatment. The vertical bars in the images correspond to 50 μm. The abbreviations used are: bn, brain neuropile; lmn, laminar neuropile; ln, lobular neuropile; mn, medullar neuropile; r, retina.

FIGURE 4-9



When produced from cells grown at 25°, the product of the locus is a myristylated phosphoprotein possessing an apparent molecular weight of 62 kilodaltons, pp62$^{c\text{-}src}$. This protein was shown to be capable *in vitro* of phosphorylating tyrosine residues of exogenously added protein substrates. When produced from heat-shocked cells, two products of the locus are produced. One of the products is a 62 kd protein that is so far indistinguishable from pp62$^{c\text{-}src}$. The second product is a 64 kilodalton protein that is very similar to pp62$^{c\text{-}src}$. The cause of the shift in mobility is unknown. The mix of *c-src* proteins produced after heat-shock is also capable of phosphorylating the tyrosine residues of exogenous substrates and in addition is capable of autophosphorylation on tyrosine. The sequence analysis presented in Chapter 2 strongly predicted that the products of this locus would be tyrosine-specific protein kinases. These results verify that prediction.

The antibody staining experiments have provided information about the location of *c-src* protein within cells. The staining of neuronal cells clearly indicates that the bulk of *c-src* protein resides in processes that extend from the main cell body. The present data do not allow any conclusions to be drawn about the mechanism by which *c-src* protein is directed to axons. The intense staining of *c-src* protein in axons may either be the result of active transport or may merely be the reflection of the high membrane content of these structures. While no direct proof that the *Drosophila c-src* protein is membrane-associated has been

presented, the homology of pp62$^{c\text{-}src}$ to chicken pp60$^{c\text{-}src}$ and the myristylation of pp62$^{c\text{-}src}$ both suggest that this is likely to be the case. Immunoelectron microscopy will be required to ascertain exactly where *c-src* protein resides in axons.

The antibody staining experiments presented in this chapter both confirm and extend the analysis of *c-src* RNA localization presented in Chapter 3. The RNA analysis suggested that *c-src* might play a role in the differentiation of neurons and smooth muscle cells. However, it was not possible to rigorously exclude the possibility that *c-src* protein persisted in these cells after differentiation. Therefore, the possibility remained that the function of *c-src* was actually in the mature cells. The observation that the amount of *c-src* protein in the photoreceptor axons and the neuropil declines during development until it is barely detectable in adults strongly suggests that *c-src* is not involved in mature neuronal function. The details of neuronal and smooth muscle expression of *c-src* protein suggest instead that the gene is involved in the differentiation of these cell types.

CHAPTER FIVE

GENETICS OF C-SRC AND THE 64B REGION

5.1 Introduction

The main goal of the work described in this thesis was to establish an experimental system in which the function of *c-src* could be studied by genetic means. At present, inferences about the role of *c-src* must be drawn either from studies of dominant alleles of the gene or from correlative studies of where the products of the gene are either located or activated. These studies have their utilities, but suffer from a crucial flaw. There is no way in which these studies can ever prove that *c-src* is involved in any particular cellular process. The effects of dominant alleles of a gene can never indicate with certainty the role of the unmutated gene because of the possibility that the dominant allelles have acquired capabilities that are qualitatively different from those of the normal gene. Furthermore, presence or activation of a protein cannot indicate the nature of the role of the protein in that cell. One possible way to understand the role of *c-src* would be to study the effects of a complete lack of *c-src* function. By observing which processes are affected by the deficiency, one could begin to understand what the action of *c-src* might be.

One possible method for obtaining such a lack of *c-src* function is to mutate the chromosomal copies of the gene so that no functional protein can be produced. The goal of the work described in this chapter was to isolate and characterize such mutations.

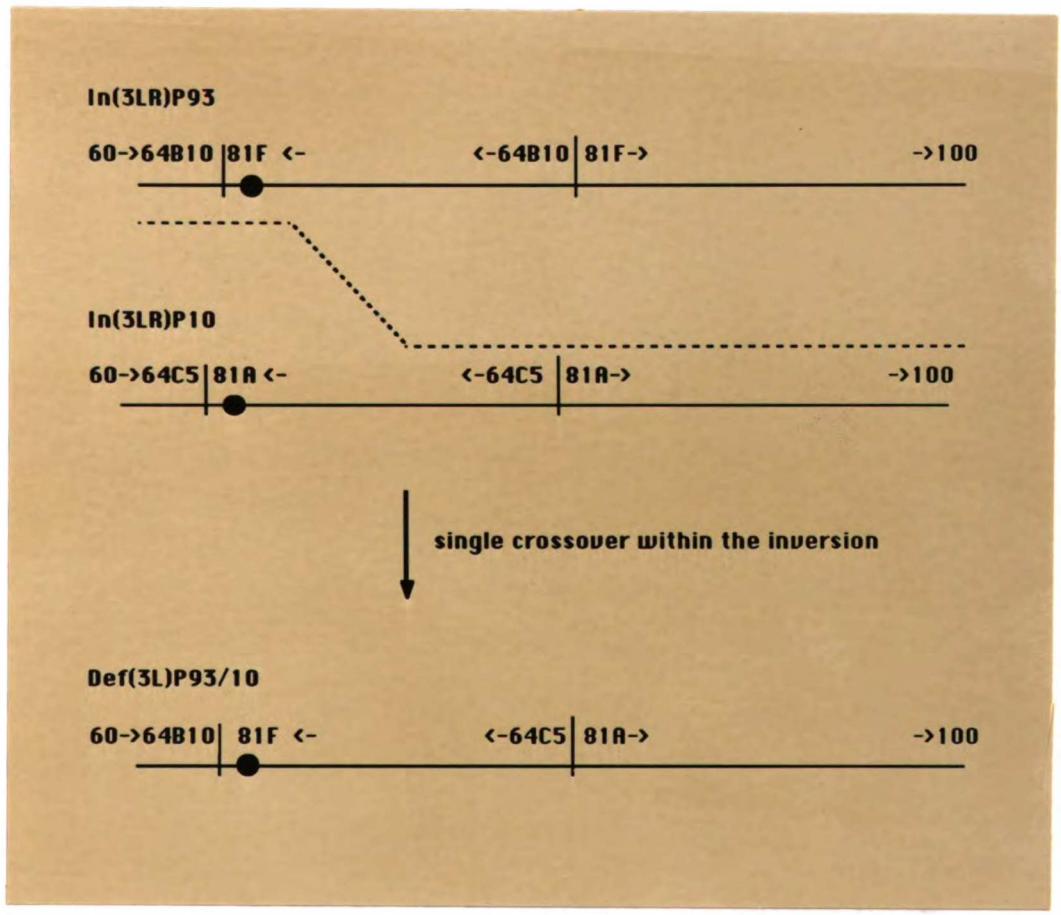
The choice of experimental organism was dictated by several factors. Since there was no reliable way to predict *a priori* what, if any, phenotype might result from mutations in *c-src*, genetic techniques were required that permitted the isolation of mutants on the basis of either the chromosomal location or DNA sequence of the gene. The ideal organisms for such an approach would be prokaryotes or yeast. However, hybridization studies had previously indicated that sequences related to *c-src* could only be detected in metazoan organisms (Shilo and Weinberg, 1981). Therefore, the most genetically tractable of the metazoans, *Drosophila melanogaster* was chosen.

5.2 Isolation of mutations

The mutagenesis scheme was designed to identify and isolate flies heterozygous for mutations in the region surrounding *c-src*. In order to accomplish this it was necessary to first create a chromosome with a deletion that spanned *c-src*. This was accomplished using a pair of chromosomes each of which carries a pericentric inversion with one endpoint in the 64BC region and the other in the 81 region (provided by L. Craymer). The two chromosomes were placed in a single female fly and allowed to recombine. A single crossover within the inversions generated a chromosome, Def(3L)P10/93, that is deficient in the 64BC region and carries a duplication of the 81 region (Fig. 5-1). This chromosome is

Figure 5-1. Construction of Def(3L)P93/10. Female flies bearing the chromosomes In(3LR)P93 and In(3LR)P10 were generated by crossing males carrying In(3LR)P93 to females carrying In(3LR)P10. A single crossover event within the inversions in the germ cells of these females generated Def(3L)P93/10. Such a chromosome was recovered by crossing the females bearing the two inversions to males bearing the balancer chromosome TM3. The deficiency chromosome was then recovered and balanced over TM3.

FIGURE 5-1


In(3LR)P93
60->64B10 81F <-
<-64B10 81F->
->100
In(3LR)P10
60->64C5 81A <-
<-64C5 81A->
->100
single crossover within the inversion
Def(3L)P93/10
60->64B10 81F <-
<-64C5 81A->
->100

homozygous lethal due to the deficiency and was maintained as a balanced stock. That Def(3L)P10/93 is actually deficient for the *c-src* locus was shown by using restriction endonuclease polymorphisms (Fig. 5-2). The results clearly show that the deficiency chromosome contributed no *c-src* band when a Southern blot of DNA from various flies was probed with a *c-src* probe.

Flies containing Def(3L)P10/93 were then used to isolate mutations that map within the the 64BC region. The detailed schemes are shown in Figure 5-3 and Figure 5-4. The schemes essentially consisted of testing the F_1 male progeny of mutagenized male flies by individually crossing them with females that were heterozygous for Def(3L)P10/93. In this way, individual mutagenized third chromosomes were tested for their ability to be complemented by Def(3L)P10/93. If a chromosome contained either a lethal or phenotypic mutation in the region deleted in the deficiency chromosome, then this mutation would be expressed as either inviability or phenotypic change when heterozygous with Def(3L)P10/93. Chromosomes that contained such mutations were maintained in balanced stocks as described in Figure 5-3 and Figure 5-4. Due to the nature of the screening process, only lethal mutations and those with very dramatic phenotypic changes were isolated. Many minor phenotypic changes may have been missed.

Aproximately 10,000 X-irradiated and 7,000 EMS-treated chromosomes were tested in this fashion. 40 X-ray and 37 EMS alleles were isolated. Flies containing many of these mutant

Figure 5-2. Chromosomes Def(3L)P93/10, x^{37}, and e^{13} are deficient for the

c-src gene. DNA was prepared from flies, digested with *Eco*RI, run on a 0.8% agarose gel, blotted onto nitrocellulose, probed with a *c-src* probe (Figure 3-1, probe B), and autoradiographed. The fly stock from which the DNA was prepared for each lanes are:

A) Adh^{fn4}, pr, cn ; $Src^{7.0}$

B) Adh^{fn4}, pr, cn // + ; $Src^{7.0}$ // Def(3LR)P93/10

C) + ; Def (3LR)P93/10 // DcxF

D) Adh^{fn4}, pr, cn // + ; $Src^{7.0}$ // x^{37}

E) + ; x^{37} // DcxF

F) Adh^{fn4}, pr, cn // + ; $Src^{7.0}$ // e^{13}

G) + ; e^{13} // DcxF

The positons of stained molecular weight markers are indicated on the left (in kilobasepairs).

FIGURE 5-2

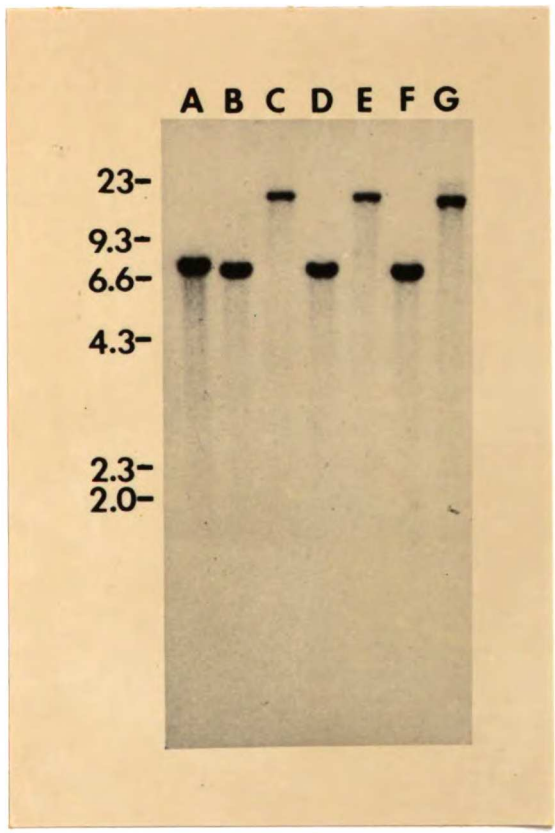


Figure 5-3. Mutagenesis of the 64B region (I). This mutation screen was used to isolate mutant chromosomes x^2 and x^5.

FIGURE 5-3

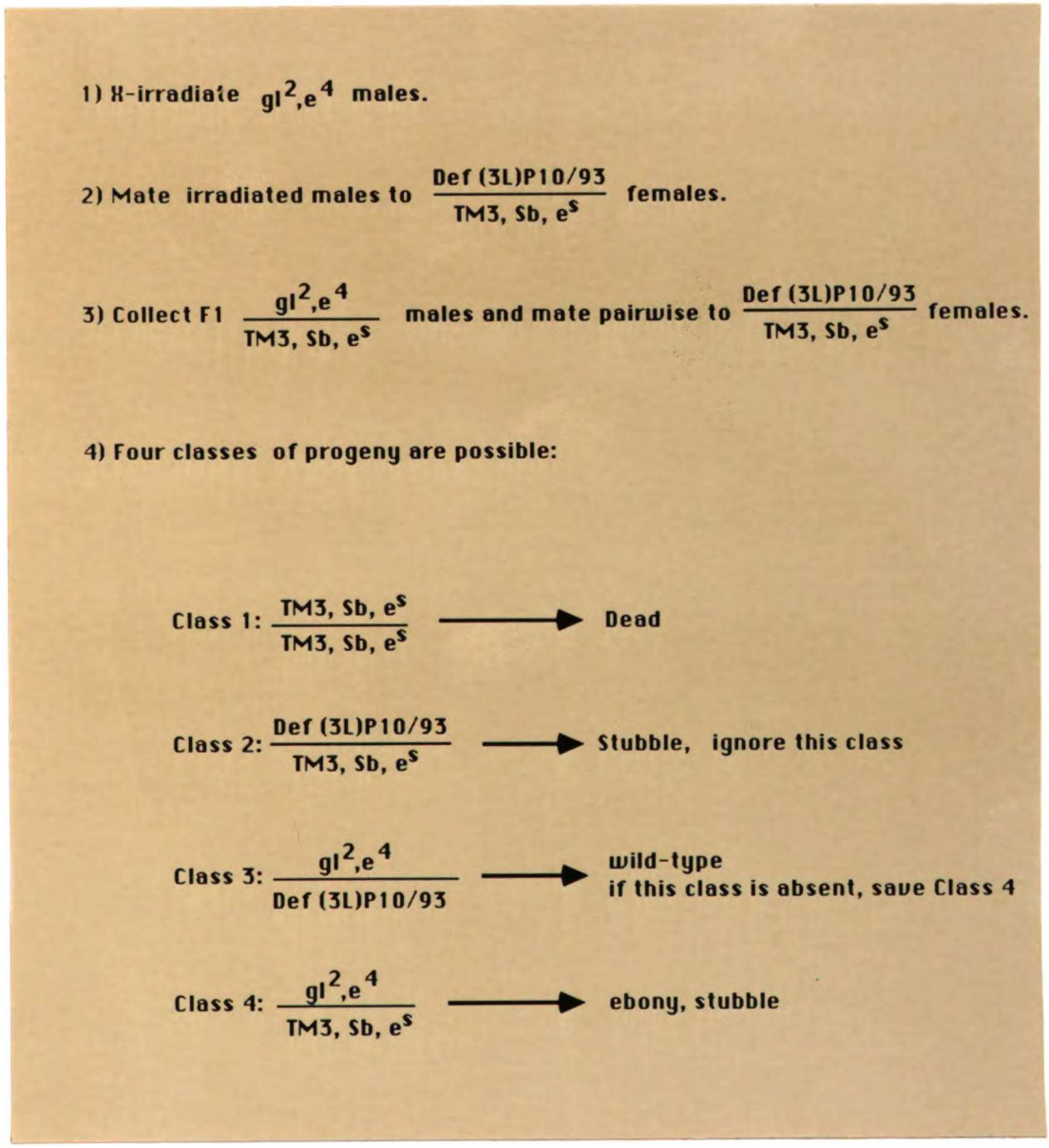
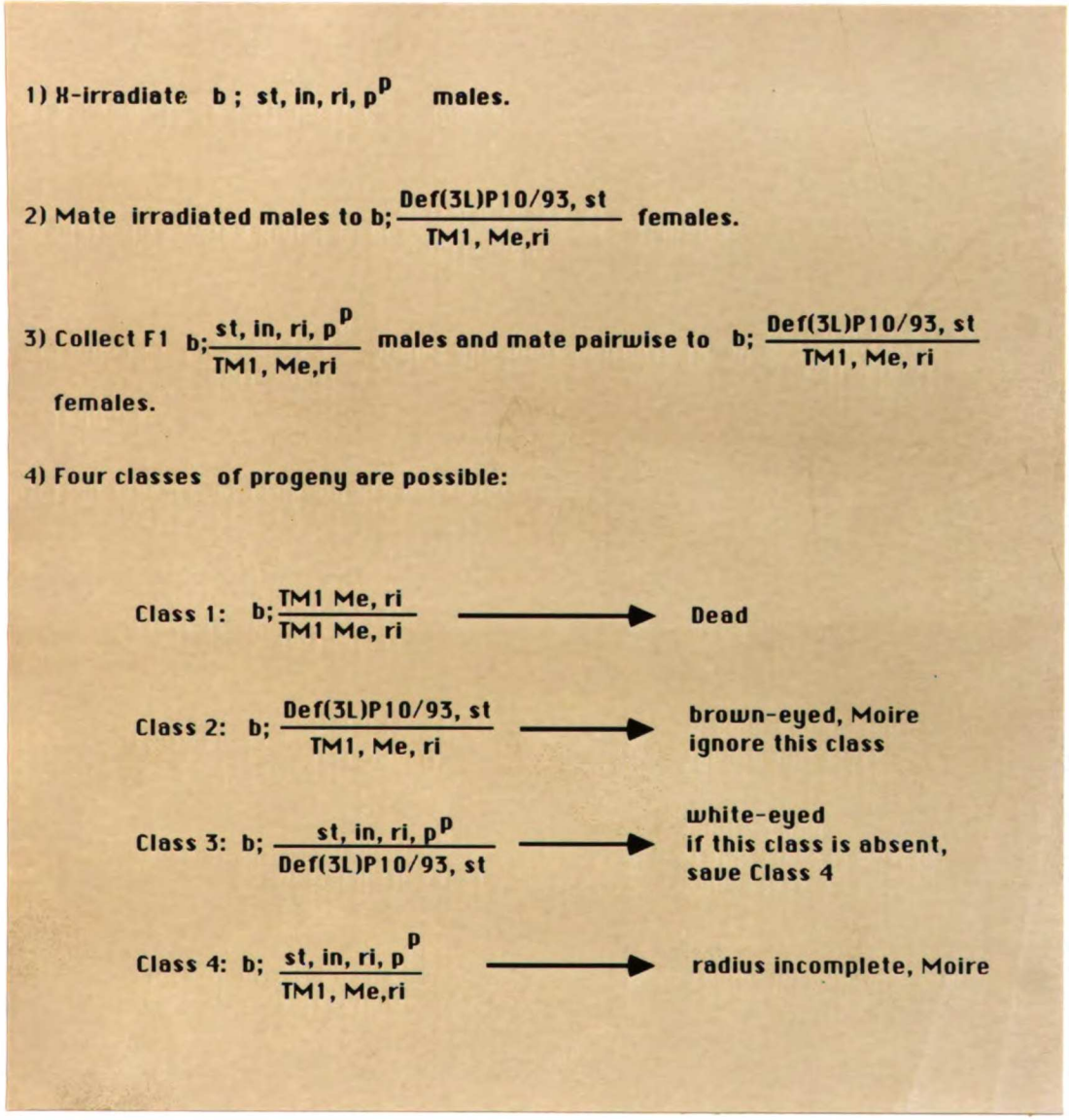

1) X-irradiate gl2,e4 males.
2) Mate irradiated males to Def (3L)P10/93 / TM3, Sb, es females.
3) Collect F1 gl2,e4 / TM3, Sb, es males and mate pairwise to Def (3L)P10/93 / TM3, Sb, es females.
4) Four classes of progeny are possible:
Class 1: TM3, Sb, es / TM3, Sb, es → Dead
Class 2: Def (3L)P10/93 / TM3, Sb, es → Stubble, ignore this class
Class 3: gl2,e4 / Def (3L)P10/93 → wild-type
if this class is absent, save Class 4
Class 4: gl2,e4 / TM3, Sb, es → ebony, stubble

Figure 5-4. Mutagenesis of the 64B region (II). This mutation screen was used to identify mutant chromosomes x^{23}, x^{27}, x^{29}, x^{37}, x^{38}, x^{39}, and x^{40}. A similar screen was used to identify mutant chromosomes e^{4}, e^{11}, e^{13}, e^{15}, e^{26}, and e^{37} except that the male flies were treated with ethyl methyl sulfonate rather than with X-irradiation.

FIGURE 5-4


1) X-irradiate b ; st, in, ri, p^p males.
2) Mate irradiated males to b; Def(3L)P10/93, st / TM1, Me,ri females.
3) Collect F1 b; st, in, ri, p^p / TM1, Me,ri males and mate pairwise to b; Def(3L)P10/93, st / TM1, Me, ri females.
4) Four classes of progeny are possible:
Class 1: b; TM1 Me, ri / TM1 Me, ri → Dead
Class 2: b; Def(3L)P10/93, st / TM1, Me, ri → brown-eyed, Moire ignore this class
Class 3: b; st, in, ri, p^p / Def(3L)P10/93, st → white-eyed if this class is absent, save Class 4
Class 4: b; st, in, ri, p^p / TM1, Me,ri → radius incomplete, Moire

chromosomes were then mated to each other in order to group the mutations into complementation groups. In the course of these crosses, it became clear that two of the mutations that had been isolated, x^{37} and e^{13}, were unable to complement mutations that were members of different complementation groups. This suggested that these chromosomes might contain deletions that removed several genes. These chromosomes were therefore tested to ascertain whether the *c-src* locus had been deleted. The results of such a test are shown in Figure 5-2. In both cases the coding region of *c-src* is absent. Cytological analysis revealed that the x^{37} chromosome is deficient for the 64A and 64B regions (data not shown), while the e^{13} chromosome contains a complicated rearrangement that could not be deciphered (data not shown). The isolation of the two additional deficiency chromosomes allowed the analysis of the other mutant chromosomes to be narrowed. Only those that cannot be complemented by any of Def(3L)P10/93, x^{37}, and e^{13} are potential *c-src* mutations.

Each of the mutations that had been isolated was first tested for its ability to be complemented by the x^{37} chromosome. 9 X-ray and 5 EMS induced alleles were unable to be complemented by the *c-src* deficient x^{37} chromosome. These mutant chromosomes were then tested for their ability to complement each other. The results of the complementation crosses are illustrated in Figure 5-5. Three simple recessive lethal complementation groups (Groups I, II, and III) were discovered. These groups consist of alleles that are

each unable to complement each other but are fully capable of complementing any member of another group. An additional complementation group (Group IV) consisting of 5 members was also discovered which demonstrates a complex pattern of interactions (Fig. 5-6). All members of this group are fully capable of complementing members of groups I, II, and III. The group is united by the inability to complement the e^{37} mutation. However, different alleles produce different phenotypes when placed in trans from e^{37}. For instance, x^{33} and e^{11} are both lethal when placed in the same fly as e^{37}, while x^{40} and x^{39} each produce roughened eyes and outstretched wings in combination with e^{37}. Furthermore, x^{33} and e^{11} can be fully complemented by x^{39}. These interactions suggest that these mutations may define a complex locus with multiple functions.

The mutant chromosomes that had failed to be complemented by the x^{37} chromosome were then tested for their ability to be complemented by the e^{13} chromosome. The lethality of groups I, II, and III cannot be rescued by the e^{13} chromosome. The observed pattern for group IV was more complicated. e^{37}, x^{39}, and x^{40} cannot be rescued by the e^{13} chromosome. e^{11} can be completely rescued. The lethality of x^{33} can be rescued, but some of the x^{33}/e^{13} progeny have roughened eyes. These results eliminate the e^{11} and x^{33} mutations from being potential *c-src* mutations.

Figure 5-5. Complementation groups in the vicinity of *c-src*. Three lethal complementation groups that map to the immediate vicinity of the *c-src* gene were identified using the mutagenesis schemes described in Figures 5-3 and 5-4.

FIGURE 5-5

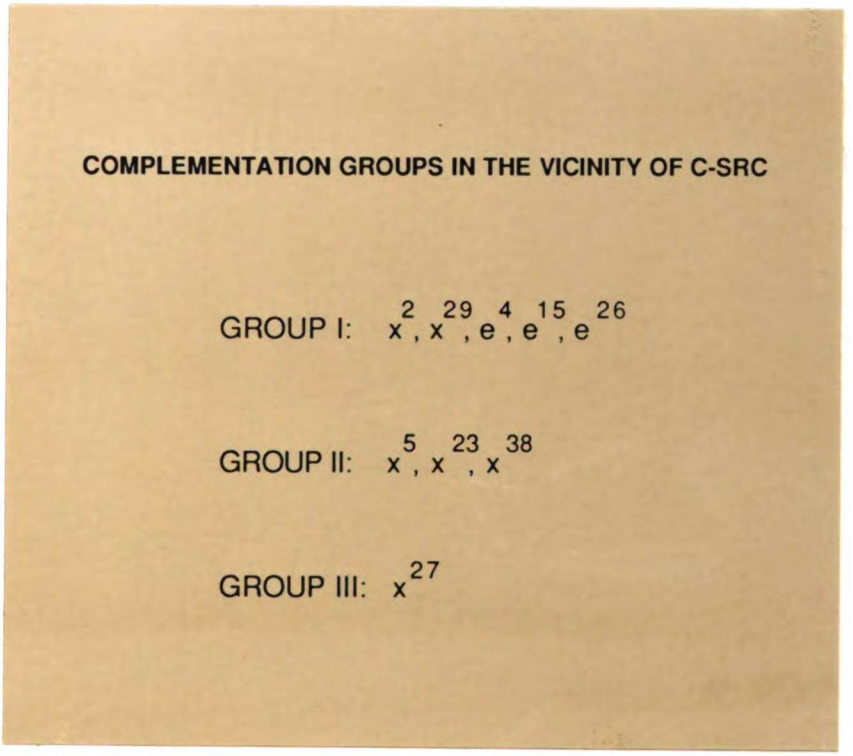
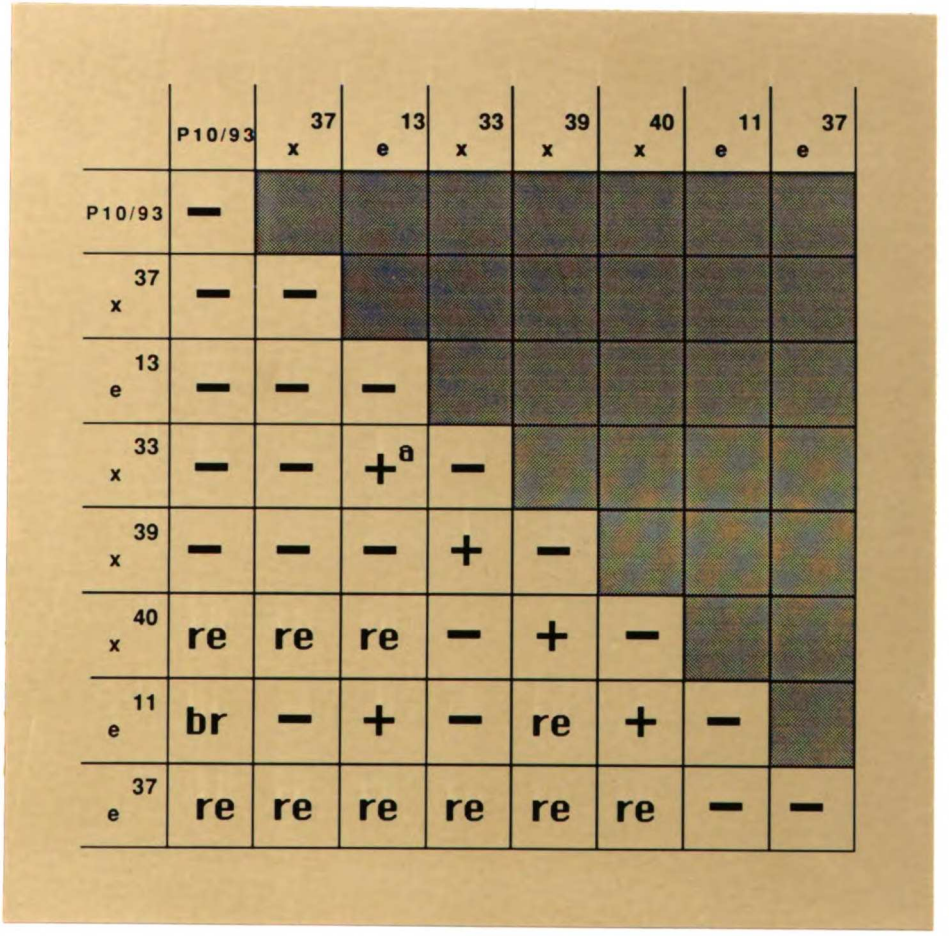

COMPLEMENTATION GROUPS IN THE VICINITY OF C-SRC
GROUP I: x^{2}, x^{29}, e^{4}, e^{15}, e^{26}
GROUP II: x^{5}, x^{23}, x^{38}
GROUP III: x^{27}

Figure 5-6. Complementation analysis of Group IV. Mutant chromosomes x^{33}, x^{39}, x^{40}, e^{11}, and e^{37} were assayed for their ability to complement each other and the *c-src*-deficient chromosomes Def(3L)P10/93, x^{37}, and e^{13}. The group demonstrates a complex set of interactions that suggests the presence of multiple overlapping functions. (-) represents a failure to recover any progeny possessing the two indicated chromosomes. (+) indicates that progeny containing the indicated chromosomes were wild-type. (re) indicates that the progeny with the indicated chromosomes were viable but possessed roughened eyes. ($+^a$) indicates that 90% of the progeny containing the indicated chromosomes were wild-type, while 10% possessed roughened eyes.

FIGURE 5-6


P10/93
x 37
e 13
x 33
x 39
x 40
e 11
e 37
P10/93 −
x 37 − −
e 13 − − −
x 33 − − +a −
x 39 − − − + −
x 40 re re re − + −
e 11 br − + − re + −
e 37 re re re re re re − −

5.3 Testing for c-src function

The next step in the isolation of *c-src* mutations was to determine which, if any, of these mutations lie within the *c-src* locus. The first attempt was to analyze the *c-src* locus of each of the mutant chromosomes by Southern blotting in order to determine whether any mutagen-induced rearrangements could be detected. In order to accomplish this it was first necessary to map the extent of the locus.

The protein experiments described in Chapter 4 had suggested that the polyadenylation sites of the *c-src* must lie upstream of the *Sal*I site that was used to construct the pcopneo-hssrc vector. This defined the 3′ end of the transcription unit. In order to locate the 5′ end of the gene, a *c-src* cDNA fragment that contained approximately 900 bp of leader sequence upstream of the initiator methionine was used to probe a genomic library of Drosophila DNA cloned in bacteriophage λ (Maniatis *et al.*, 1978). The library was simultaneously screened with a probe that represented the first kilobasepair that lies immediately upstream of the initiator methionine in the genomic DNA. If the sequences from which the mRNA leader is derived lie immediately upstream of the coding region, then these two probes should contain the same sequences and hybridize with the same recombinant phage. The two probes did not detect the same phage. All of the plaques that hybridized with the genomic probe also hybridized with the cDNA probe. However, the

cDNA probe also detected a number of phage that did not hybridize to the genomic probe. This implied that the 5′ end of the cDNA spanned an intron of probably greater than 10 kb. In order to determine the actual distance and to isolate the intervening DNA, the plaques that hybridized to either of the two probes were isolated and analyzed. From these phage, it was possible to isolate and map the entire region that was spanned by the cDNA. The map of this region is shown in Figure 5-7. Northern blots were conducted using probes from the upstream region in order to locate the upstream exon (Fig. 5-8). The upstream region of homology to the *c-src* cDNA hybridizes to all three of the *c-src* RNAs, while those slightly upstream detect a new message that does not appear to be related to *c-src*. This RNA is presumably derived from the next transcription unit along the chromosome. However, it must be noted that this only limits the position of the 5′ end of the *c-src* transcription unit if *c-src* does not overlap the next gene. The possibility remains that the locus actually contains another intron and extends further 5′ than the phage shown in Figure 5-8. An example of overlapping transcription units has been described in flies (Henikoff *et al.*, 1986). Definitive proof that the *c-src* transcription unit does not extend further upstream will require more detailed analysis of the promoter region.

DNA from each of the bacteriophage λ clones shown in Figure 5-7 was then used to probe Southern blots of genomic DNA isolated from flies heterozygous for each mutant chromosome and a third

Figure 5-7. The *c-src* region. Bacteriophage λ clones were isolated on the basis of their ability to hybridize with *c-src* cDNA clones. The λ clones were then mapped using a number of restiction endonucleases. The clones define a chromosomal region of approximately 49 kilobasepairs. The map of this region (E=*Eco*RI, S=*Sal*I) is indicated. The positions of the *c-src* initiation and termination codons are indicated by (AUG) and (TAA), respectively. The map units are kilobasepairs.

FIGURE 5-7

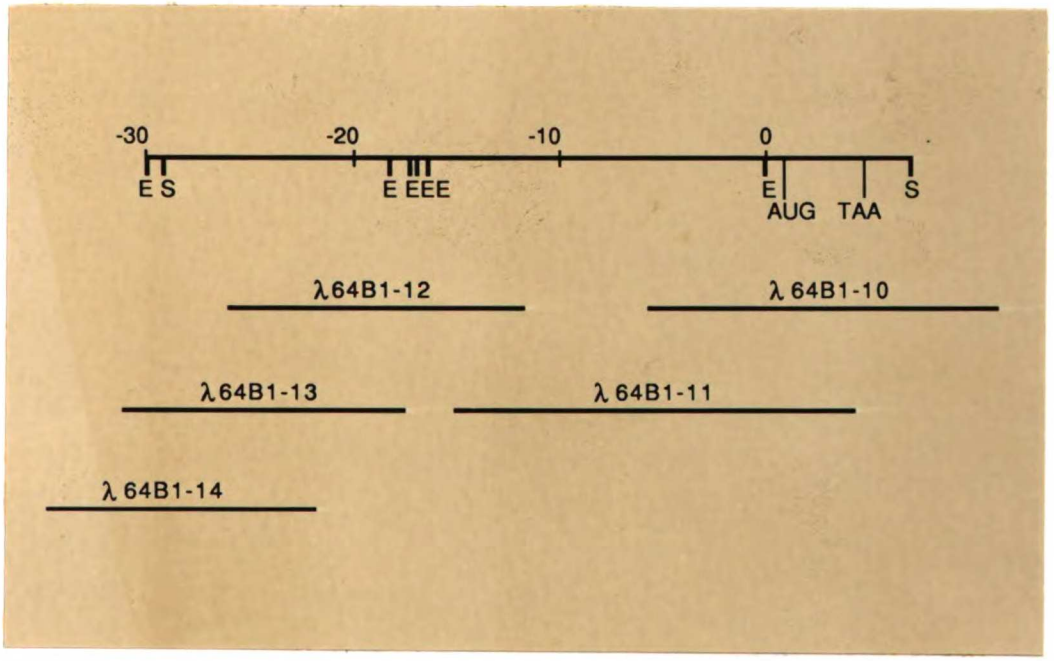
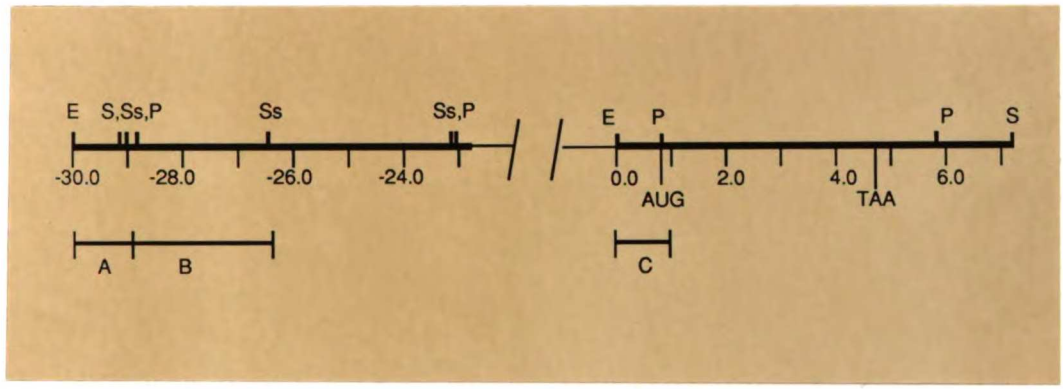
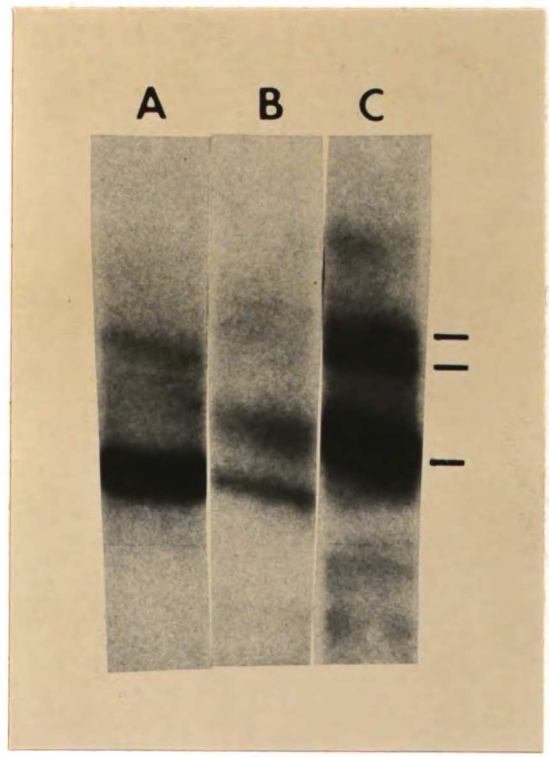

-30
-20
-10
0
E S
E EEE
E
AUG
TAA
S
λ64B1-12
λ 64B1-10
λ64B1-13
λ 64B1-11
λ 64B1-14

Figure 5-8. The possible extent of the *c-src* transcription unit. The upper panel is a map of the *c-src* region of the third chromosome. The map units are kilobasepairs. The indicated restriction enzyme sites are: E=*Eco*RI, P=*Pst*I, S=*Sal*I, and Ss=*Sst*I. The positions of the *c-src* initiation and termination codons are indicated by (AUG) and (TAA), respectively. The probes used in the lower panel are indicated below the map. The lower panel is the results of probing Northern blots (0.9% formaldehyde-agarose gels) containing 2 μg of polyadenylated RNA from 0-7 hour old embyros with the indicated probes. The position of the three previously defined *c-src* RNAs is indicated on the left (see Figure 3-2). Probe A detects an RNA species that does not appear to be derived from *c-src*.

FIGURE 5-8





chromosome balancer (either TM3 or DcxF). No chromosomal abnormalities were observed (data not shown). Since many, if not most, mutations are not associated with rearrangements that could be detected on Southern blots, the experiments described above do not absolutely eliminate any of the mutants from being *c-src* mutations.

A second approach to identifying *c-src* mutations is to directly assay the coding capacity of the *c-src* alleles present on the mutant chromosomes. The first step was to molecularly clone the *c-src* DNA from a number of the mutant chromosomes. This was facilitated by the observation that while the *c-src* coding regions of the mutated chromosomes are contained in a single *Eco*RI fragments of 15 kb, the *c-src* coding region of another fly stock (Adh^{fn4}, pr, cn; $Src^{7.0kb}$) is contained in a 7.0 kb *Eco*RI fragment. Flies containing the mutated chromosome were crossed to flies containing the polymorphic chromosome in order to generate flies in which the only 15 kb fragment that contained *c-src* was derived from the mutated third chromosome. DNA was then prepared from these flies, digested with *Eco*RI, and cloned into the bacteriophage λ vector EMBL 4 (Frischauf *et al.*, 1983). This vector requires an insert size of greater than 9 kb. Therefore, only the alleles from the mutated chromosomes were cloned. The bacteriophage containing *c-src* were isolated by hybridization with a *c-src* probe and used for further characterization. The alleles analyzed were from the x^2, e^4, x^5, x^{38}, x^{27}, e^{37}, and x^{39} chromosomes.

The cloned *c-src* alleles were examined in two ways. First,

the DNA sequence of much of the coding region of each of the alleles was determined. The coding region of each allele was cloned into the m13mp9 sequencing vector. A series of oligonucleotide primers were then synthesized and used as primers for sequencing reactions. The primers chosen were spaced at approximately 250 bp intervals along the gene. This strategy permitted the rapid determination of 80% of the coding sequence of each *c-src* allele. No differences in sequence were detected that would change the amino acid sequence of the *c-src* protein (data not shown). The only differences that were detected were polymorphisms that correlated with the genetic background from which the allele was derived.

The ability of the cloned *c-src* alleles to encode active pp62$^{c\text{-}src}$ was also examined. The *c-src* expression vector, pcopneo-hssrc, was reconsructed for each of the cloned alleles. These constructions were then individually transfected into Schneider L2 cells. The cells containing each construct were then heat-shocked and assayed for *c-src* tyrosine kinase activity as desribed in Chapter 4. The results are shown in Figure 5-9. All of the alleles were capable of encoding pp62$^{c\text{-}src}$ molecules that possess tyrosine kinase activity.

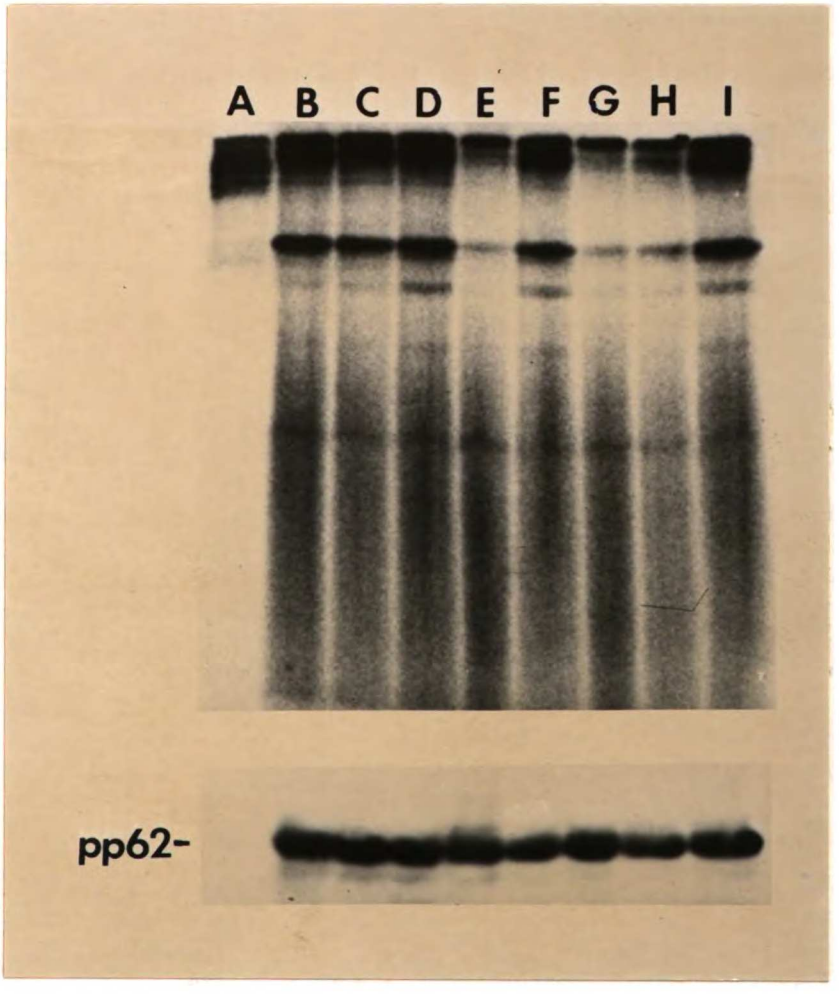
5.4 Discussion

This chapter has described the identification of a number of

Figure 5-9. The *c-src* genes cloned from mutant chromosomes encode pp62$^{c\text{-}src}$ proteins that possess tyrosine-specific protein kinase activity. The *c-src* loci of mutant chromosomes were cloned into the *c-src* expression vector pcopneo-hssrc and assayed for their ability to encode proteins possessing the ability to phosphorylate synthetic polymers of glutamate, alanine, and tyrosine. Immunoprecipitates of *Drosophila* cells that had been stably transfected with the expression vectors and heat-shocked were reacted with [γ^{32}P]-ATP and a synthetic polymer of glutamate, alanine, and tyrosine (upper panel) and then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The antiserum used recognizes the carboxy-terminal 13 amino acids of pp62$^{c\text{-}src}$. The lower panel shows the results of probing a Western blot of these same extracts with the antiserum. The position of pp62$^{c\text{-}src}$ is indicated on the left. The lanes are: A) normal Schneider Line 2 (SL2) cells; (B) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the Canton S wild-type chromosome; C) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the x^2 mutant chromosome; D) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the e^4 mutant chromosome; E) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the x^5 mutant chromosome; F) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the x^{27} mutant chromosome; G) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the e^{37} mutant

chromosome; H) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the x^{38} mutant chromosome; I) SL2 cells transfected with the pcopneo-hssrc plasmid containing the *c-src* from the x^{39} mutant chromosome. Each of the tested alleles was capable of encoding an active tyrosine-specific kinase.

FIGURE 5-9



genetic complementation groups that map to a small region of the *Drosophila* genome that contains the *c-src* locus. Several strategies were employed in order to determine whether any of the genetic loci identified by the different complementation groups correspond to the *c-src* locus. The various mutant chromosomes were examined both for DNA rearrangements that might have been induced by mutagenesis and for any changes in the protein coding ability of their *c-src* alleles. No rearrangements or alterations were discovered. Thus, none of the identified complementation groups can be assigned to *c-src*.

Unfortunately, none of the strategies used to identify *c-src* mutations can eliminate the possibility that one of the complementation groups does correspond to *c-src*. For instance, a point mutation in a promoter sequence would not have been detected by any of the experiments described above. Therefore, the status of the search for *c-src* mutations remains unclear. Three possibilities exist. First, one of the identified complementation groups may correspond to *c-src*. Determining whether this is correct will require the development of a test that permits the conclusion that a group is not *c-src* (see Chapter 6 for a discussion of how this might be accomplished). Second, an absence of *c-src* function might have either no phenotype or an extremely subtle phenotype. The mutagenesis scheme that was undertaken only allows the isolation of recessive mutations that are lethal or cause extremely obvious phenotypic defects. The possibility that *c-src* causes subtle

phenotypic abnormalities that were missed is impossible to disprove except by the isolation of *c-src* mutations. The only argument against this is that the *c-src* gene is highly conserved during evolution and thus must be important. There is, however, no compelling reason to believe that the lack of an evolutionarily important function must lead to a phenotype that is obvious to the human eye. Finally, mutations of *c-src* may not have been generated during the mutagenesis. Certainly, the available data does not strongly support the conclusion that the 64BC region of the chromosome has been saturated for complementation groups. Although as many as 40 mutations have been recovered from a single complementation group uncovered by Def(3L)P10/93 (data not shown), Group III is represented by only one member. However, the large size of the *c-src* locus suggests that the gene would be a rather sizable target for X-ray induced chromosomal rearrangements.

CHAPTER SIX

FUTURE DIRECTIONS

The goal of the work described in this thesis was to establish a system for studying the effects of a complete lack of *c-src* function. It was hoped that an examination of the consequences of such a deficiency would indicate the nature of the processes in which *c-src* plays a role. The approach was to attempt to eliminate *c-src* function by genetically mutating the normal chromosomal copies of *c-src*. *Drosophila melanogaster* was chosen as the experimental animal for two reasons. First, there was reason to believe that fruit flies might possess a *c-src* gene (Shilo and Weinberg, 1981). Second, the genetics of flies are facile enough that there was at least a chance that *c-src* mutations could be isolated.

Since the *c-src* locus was first discovered and has been exclusively studied in vertebrate organisms, it was first necessary to demonstrate that the Drosophila genome contains a *c-src* locus. The locus was isolated by reduced stringency hybridization and mapped to polytene chromosome positon 64B. Sequence analysis of a cDNA derived from the locus clearly showed that at least one protein produced by the locus is remarkably homologous to that produced by chicken *c-src* and a number of closely related vertebrate genes. The protein encoded by the *Drosophila* gene was then identified and shown to possess the same enzymatic activity, tyrosine-specific protein kinase activity, that the vertebrate *c-src* proteins possess.

Study of the expression of *Drosophila c-src* provided

additional evidence that the 64B locus is the true homolog of the vertebrate *c-src* genes. Evidence has been gathering from studies of chicken and rat *c-src* protein production and modification that neuronal cells may be an important site of *c-src* action (discussed in Chapter 1). *In situ* detection of *Drosophila c-src* RNA and protein showed that, after a short period of ubiquitous expression during early embryogenesis, the major sites of *c-src* expression were in neuronal and smooth muscle cells. This correlates very well with the vertebrate data and strongly suggests that the *Drosophila* gene performs a very similar function to that performed by vertebrate *c-src*. The nature of this putative *c-src* function is unclear, but the timing of expression in *Drosophila* suggests that a role in the differentiation, but not mature function, of neurons and smooth muscle cells is likely.

The identification and chromosomal mapping of the *Drosophila c-src* locus provided the information necessary to begin a search for flies bearing recessive mutations in the *c-src* gene. The isolation scheme was a screen for mutagen-treated chromosomes that contain recessive mutations that could not be complemented by chromosomes bearing deletions of the *c-src* locus. A number of such mutants were obtained and mapped into 4 complementation groups. The next crucial issue was which, if any, of these groups represented the *c-src* locus. The mutations were tested for *c-src* defects in three ways. All of the mutant chromosomes were screened by Southern blotting for mutagen-induced chromosomal rearrangements

that might eliminate *c-src* function. The *c-src* alleles of a selected set of the mutant chromosomes were molecularly cloned and partially sequenced. Finally, these same cloned alleles were expressed in a cultured cell line and the produced *c-src* proteins were assayed for tyrosine-specific protein kinase activity. All of these tests failed to reveal any abnormalities. Therefore, it was impossible to conclude that any of the isolated complementation groups correspond to *c-src*.

Unfortunately, none of the strategies used to identify *c-src* mutations could eliminate the possibility that one of the complementation groups does correspond to *c-src*. For instance, a point mutation in a promoter sequence would not have been detected by any of the experiments described above. Therefore, the possibility remains that *c-src* mutations have been isolated, but not identified. To remedy this situation and prove decisively whether *c-src* is represented by one of the complementation groups will require testing the mutations in a way that allows a negative result to be meaningful. One possible approach to accomplishing this goal would be to place a competent copy of *c-src* into the *Drosophila* genome using P-element mediated transformation (Rubin and Spradling, 1982) and test whether this introduced gene can rescue any of the complementation groups. The certainty that a negative result means that the complementation group in question is not *c-src* would then be equivalent to the certainty that the introduced *c-src* gene is fully competent.

This approach would be straightforward except for the size of the *c-src* locus. The mapped transcription unit comprises an area of over 30 kb. This is approaching the maximum size that can be inserted into P element vectors. Since the promoter region has not been characterized, the locus could be considerably larger. Thus, an absolute priority for future work is a detailed mapping of the extent of the sequences that are required for normal transcription of the three *c-src* RNA species. Once a P-element containing *c-src* has been placed in the genome, it will be necessary to establish with complete certainty that *c-src* RNA is being expressed from the introduced copy. This will fortunately be a relatively easy task. The polymorphic *c-src* allele, $Src^{7.0kb}$, produces different sized RNA species than those of wild-type alleles (data not shown). The introduced *c-src* allele can therefore be genetically crossed into the polymorphic background and directly tested for its ability to produce wild-type *c-src* RNA.

If none of the mutations can be rescued by the introduced *c-src* gene, then either insufficient numbers of mutations were collected to find all of the complementation groups in the region uncovered by the deficiency chromosomes or *c-src* mutations do not have a phenotype that could be scored in this screen. The first possibility could be addressed by conducting a new round of mutagenesis on a much larger scale than that described in Chapter 5. The second explanation would require a change in strategy. One alternative would be to make an educated guess as to what the

mutant phenotype might be and to design an appropriate mutation screening procedure. For example, one might try a screen that tests for impaired neural function. A second approach would be to isolate more deletions in the region around *c-src*. This could perhaps be most easily accomplished by demanding the reversion of dominant genetic markers that map close to *c-src*. Several P insertions in the region that carry appropriate markers for this purpose have been reported (Levis *et al.*, 1985; Spradling and Rubin, 1983). One might hope to isolate deletions with endpoints in *c-src*. By placing into the same fly chromosomes containing two such deletions that extend in opposite directions from *c-src*, a complete lack of *c-src* activity could be created.

Each of the tasks for the future that have been discussed are both difficult and time-consuming. Therefore, a reasonable question arises as to whether this approach to understanding *c-src* function warrants such continued effort. This author believes that it does. The simple fact remains that the kinds of experiments that are being conducted on the vertebrate *c-src* genes cannot easily prove what *c-src* does. Without some method for observing the effects of a lack of *c-src* activity, understanding the role of *c-src* in normal growth and development may prove immensely difficult.

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APPENDIX ONE

MATERIALS AND METHODS

Analysis of DNA

The basic techniques employed in the analysis of *Drosophila*, bacteriophage λ, bacteriophage M13, and plasmid DNA have been described by Maniatis *et al.* (1982). Only modifications of procedures described in that text and procedures not included in that text are described below.

Hybridization conditions

Two sets of hybridization conditions were employed for hybridization to DNA immobilized on nitrocellulose filters. Hybridization experiments in which *v-src* sequences were used to screen bacteriophage λ libraries of *Drosophila* DNA or in which cloned *Drosophila* sequences were used to probe Southern blots of *v-src* DNA were conducted under conditions of reduced stringency. These conditions were prehybridization for 12 hours at 42° in 35% (vol/vol) formamide/ 750 mM NaCl/ 75 mM sodium citrate/ 65 mM KH_2PO_4/ 5mM EDTA/ 0.1% polyvinyl pyrrolidone/ 0.1% Ficoll/ 0.1% bovine serum albumin/ 500 μg per ml denatured salmon sperm DNA, followed by hybridization for 36 hours to [^{32}P]-labelled probe in the same solution. The filters were then washed for 6 hours at 55° with several changes of 300 mM NaCl/ 30 mM sodium citrate/ 0.1% sodium dodecyl sulfate (SDS). All other hybridization to nitrocellulose filters were conducted under conditions of normal

stringency. These conditions were prehybridization for 2 hours at 42° in 50% (vol/vol) formamide/ 750 mM NaCl/ 75 mM sodium citrate/ 0.1% polyvinyl pyrrolidone/ 0.1% Ficoll/ 0.1% bovine serum albumin/ 200 μg per ml denatured salmon sperm DNA, followed by hybridization to labelled probe for 24 hours. The filters were then washed for 6 hours at 42° in 15 mM NaCl/ 1.5 mM sodium citrate/ 0.1% SDS/ 0.1% sodium pyrophosphate.

Preparation of DNA from adult flies

Large scale preparations of *Drosophila* DNA were performed by the following method. Approximately 100 adult flies were disrupted by douncing in 2 mls of a 4:1 mixture of 100 mM NaCl/ 200 mM sucrose/ 100 mM EDTA/ 30 mM Tris (*p*H 8) and 250 mM EDTA/ 2.5% SDS/ 500 mM Tris (*p*H 9). The solution was then heated to 65° for 30 minutes. Potassium acetate was then added to a final concentration of 1 M. The solution was then placed on ice for 1 hour and then centrifuged at 10,000 rpm in a Beckman JS13 rotor. The supernatant was then removed to a new tube and extracted twice with a 1:1 mixture of phenol and chloroform. The remaining supernatant was then precipitated with 2 volumes of ethanol. The DNA was collected by centrifugation, dissolved in 1 ml of 1mM EDTA/ 10 mM Tris (*p*H 8), and reprecipitated by the addition of sodium acetate to 250 mM followed by the addition of 2 mls of ethanol. The precipitate was again collected by centrifugation, dissolved in 100 μl of 1 mM

EDTA/ 10 mM Tris (*p*H 8), and stored at -20°. Approximately 3 μl of this solution was used for each lane of Southern blots.

Preparation of genomic libraries of fly DNA

Genomic libraries of *Drosophila* DNA were prepared using the bacteriophage λ vector EMBL4 (Promega Biotech). One μg of EMBL4 DNA that had been digested with the restriction endonucleases *Eco*RI and *Bam*HI, extracted with phenol, precipitated by the addition of ethanol, and dissolved in 1 mM EDTA/ 10 mM Tris (*p*H 8) was ligated to 0.1 μg of *Eco*RI-digested *Drosophila* DNA in a final volume of 10 μl using T4 DNA Ligase. Three μl of the ligation mix was then packaged using a Gigapack kit (Stratagene Inc.). Typically this resulted in the production of a library of 5 x 10^4 independent phage. The plating and screening of these phage was then performed as described by Maniatis *et al.* (1982).

In situ hybridization to polytene chromosomes

The salivary glands of wandering third instar larvae were dissected and squashed as described by Gall and Pardue (1971) onto slides that had been pretreated as described by Brahic and Haase (1978). The squashes were then heat-treated and prepared for hybridization as described by Bonner and Pardue (1976). The slides were hybridized in 300 mM NaCl/ 30 mM sodium citrate/ 30 mM sodium

phosphate (pH 7)/ 40% formamide/ 10% dextran sulfate/ 300 μg per ml denatured salmon sperm DNA/ 1 x 10^7 cpm of probe DNA labelled by nick-translation to a specific activity of 1 x 10^7 cpm with tritiated nucleotides. Hybridization was for 18 hours at 42° under siliconized coverslips using 20 μl per slide. The slides were then washed, autoradiographed and stained as described by Gall and Pardue (1971).

Sequence Analysis

Sequence determination was performed by the method of Sanger *et al.* (1977). M13 subclones for sequencing of cDNA clones were obtained either by subcloning defined resriction fragments into the M13 mp8/9 vectors (Messing and Vieira, 1982) or by the random deletion method of Hong (1982). The genomic clones derived from mutant chromosomes were sequenced using a series of specific primers that were derived from the cDNA sequence and were synthesized by the UCSF Biomolecular Resource Center.

Analysis of RNA

Isolation and Analysis of RNA

Polyadenylated RNA was prepared and analyzed by Northern

blotting exactly as was described by Poole *et al.* (1985) except that the blots were hybridized under the normal stringency conditions that were described above for the hybridization of DNA bound to nitrocellulose filters.

In situ hybridization of RNA in sectioned Drosophila

In situ hybridizations to sectioned (8μ) embryos, larvae, and pupae were performed by a modification of the technique of Hafen *et al.* (1983) exactly as described by Kornberg *et al.* (1985). The probes were prepared by nick-translation in the presence of tritiated nucleotides and possessed specific activities of approximately 8 x 10^7 cpm. Autoradiography was at 4^o for 1 month for embryonic sections and for two months for larval and pupal sections.

Analysis of Protein

Production of antisera

Peptides that represent portions of the *Drosophila c-src* protein were synthesized by either Sequamat Inc. or Penisula Laboratories. The peptides were coupled to either Keyhole Limpet Hemacyanin (Sigma) or Tuberculin PPD (Statens Serum Institute) by

mixing an equal volume of 1 mg/ml carrier solution in PBS (PBS is 25 mM sodium phosphate (*p*H 7.5)/ 150 mM NaCl) with 1 mg/ml peptide in PBS. Glutaraldehyde was then added to this solution to a final concentration of 0.05%. The coupling was allowed to proceed overnight at room temperature with vigorous stirring. The proteins were then precipitated by the addition of 4 volumes of acetone/ 0.01% concentrated HCl. After 1 hour at -70°, the precipitate was collected by centrifugation and resuspended in PBS in a volume of 1 ml per mg of carrier protein used.

This carrier-peptide mix was then used to raise antisera in rabbits. Each rabbit was first immunized by subcutaneous injection of an emulsion of 200 μl of the carrier-peptide suspension, 500 μl of Freund's Complete Adjuvant (Sigma), and 300 μl of water. The rabbits were subsequently boosted at two week intervals by subcutaneous injection of an emulsion of 100 μl of carrier-peptide suspension, 500 μl of Freund's Incomplete Adjuvant (Sigma) and 400 μl of water. The rabbits were bled from an ear vein after approximately 3 months of boosting and 7 days after the most recent boost. The blood was allowed to clot for 1 hour at room temperature and then for 12 hours at 4°. The blood cells were then removed from the serum by centrifugation in a clinical centrifuge. The serum supernatant was supplemented with thimerosol to 0.01%, aliqouted, and stored at -70°. The rounds of bleeding and boosting continued for several months.

Western blotting and kinase assays

Combined kinase assays and Western blots were performed as follows. All steps prior to the addition of Sample Buffer were performed at 0° unless otherwise noted. 1 x 10^7 cells were plated onto a 100 mm tissue culture flask and allowed to settle overnight. For experiments that required heat-shocking of cells, the cells were placed at 37° for 1 hour and then allowed to recover at room temperature for 2 hours. The cells were then washed and lysed in 200 µl of Kinase Lysis Buffer (9.4 mM KH_2PO_4/ 23.9 mM Na_2HPO_4/ 200 mM KCl/ 1% NP-40). The sample was then clarified by two successive rounds of centrifugation for 2 minutes in a microfuge. The supernatant was then removed to a new tube. Fifty µl of the supernatant was removed for Western analysis and added to a tube containing 25 µl of 30% glycerol/ 6% SDS/ 375 mM Tris (*p*H 6.8)/ 15% 2-mercaptoethanol. This sample was stored at -20° until it could be analyzed. 100 µl of the remaining supernatant was used to test for kinase activity. This 100 µl of supernatant was added to a tube containing 800 µl of Kinase Lysis Buffer and 100 µl of 2 M KCl. Five µl of antiserum was then added to the tube. After 45 minutes, 30 µl of a 50% slurry of Protein A-Sepharose beads (Pharmacia) was added and the tube was incubated for an additional 30 minutes with constant inversion. The beads were then spun to the bottom of the tube by centrifugation for 15 seconds in a microfuge and washed twice with Kinase Lysis Buffer. This was

followed by a wash with Kinase Assay Buffer (22.7 mM KH_2PO_4/ 10.6 mM Na_2HPO_4/ 100 mM KCl/ 10 mM $MgCl_2$/ 0.1% NP-40). The beads were then resuspended in 225 μl of Kinase Assay Buffer. 100 μl of the suspension was added to a tube containing 10 μcuries of [γ^{32}P]-ATP and 25 μg of a synthetic peptide polymer containing a 6:3:1 ratio of glutamate, alanine, and tyrosine (Sigma) and incubated at 20° for 20 minutes with occasional agitation. The reaction was terminated by the addition of 50 μl of 30% glycerol/ 6% SDS/ 375 mM Tris (*p*H 6.8)/ 15% 2-mercaptoethanol. This sample was stored at -20° until it could be analyzed. An additional 100 μl of the bead suspension was then added to a tube containing 10 μcuries of [γ^{32}P]-ATP and incubated at 20° for 20 minutes with occasional agitation. The beads were then washed two times with Kinase Lysis Buffer and resuspended in 75 μl of 10% glycerol/ 2% SDS/ 125 mM Tris (*p*H 6.8)/ 5% 2-mercaptoethanol. This sample was also stored at -20° until it could be analysed.

The kinase assay samples were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) followed by autoradiography. The autophosphorylation assays were analyzed on 9% gels, while the peptide phosphorylation assays were analyzed on 14% gels.

The Western blotting samples were chromatographed on a 9% SDS-polyacrylamide gel. The gel was then soaked in 20% methanol/ 192 mM glycine/ 20 mM Tris-base for 20 minutes and then electro-blotted onto nitrocellulose overnight at 20 volts using a Biorad Electroblotting Apparatus. The nitrocellulose sheet was then

incubated for 30 minutes in 2% milk powder in TBS (TBS is 25 mM Tris (*p*H 8.1)/ 150 mM NaCl). This was followed by a 120 minute incubation in TBS/ 5% calf serum/ 0.5% antiserum. The blot was then washed for 15 minutes twice with TBS/ 5% calf serum/ 0.5% Tween-20 and once with TBS/ 5% calf serum. The blot was then incubated for 60 minutes in TBS/ 5% calf serum/ 5 x 10^5 cpm of iodinated donkey anti-rabbit antibodies ($F(ab)_2$ fragment from Amersham). Following this incubation, the blot was washed for 30 minutes five times with TBS/ 5% calf serum/ 0.5% Tween-20. The blot was then placed on film for autoradiography.

Metabolic labelling of cells

Drosophila tissue culture cells were labelled overnight with [^{3}H]-myristate by the addition of 1 mCi of [^{3}H]-myristate in 10 μl of dimethylsulfoxide to 1 x 10^7 cells in a volume of 2 mls of Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum. In order to label cells with [^{35}S]-methionine or [^{32}P]-orthophosphate, 5 x 10^7 cells were placed in a 15 ml conical tube and centrifuged at 1000 rpm in a Beckman clinical centrifuge. The cells were resuspended in 10 mls of Tris-buffered saline (UCSF Tissue Culture Facility). The cells were washed in this manner three times. For methionine labelling, cells were then resuspended in 1 ml of 90% Schneider's Drosophila medium (minus methionine and yeastolate)/ 10% dialyzed fetal bovine serum (dialyzed against 10

mM Hepes *p*H 7)/ 500 μCi of [^{35}S]-methionine. For phosphate labelling, cells were then resuspended in 1 ml of 90% Schneider's *Drosophila* medium (minus sodium phosphate and potassium phosphate)/ 10% dialyzed fetal bovine serum/ 1 mCi of [^{32}P]-orthophosphate. Cells were either labelled for either 3 hours at room temperature or labelled for 1 hour at 37^{o} followed by 2 hours at room temperature.

Immunoprecipitation of labelled extracts

All steps in the preparation and immunoprecipitation of extracts were performed at 0^{o}. Labelled cells were placed in 10 mls of Tris-buffered saline and pelleted at low speed. The supernatants were removed and discarded. The cells were then lysed in 1 ml of Lysis Buffer (25 mM Tris *p*H 8.0/ 150 mM NaCl/ 0.5% NP-40/ 0.5% sodium deoxycholate/ 0.2% SDS). The DNA in each sample was sheared by repeated passage through a 26 gauge syringe.

Five μl of antiserum was added to each extract. After 45 minutes, 30 μl of a 50% slurry of Protein A-Sepharose beads (Pharmacia) was added. After an additional 30 minute incubation, the beads were collected by centrifugation for 15 seconds in a microfuge. The beads were resuspended and washed 4 times with Lysis Buffer. The beads were then resuspended in 75 μl of 10% glycerol/ 2% SDS/ 125 mM Tris (*p*H 6.8)/ 5% 2-mercaptoethanol. The samples were then denatured by boiling and analyzed by electro-

phoresis through 9% SDS-polyacrylamide gels and autoradiography. Gels containing [^{3}H]- or [^{35}S]-labelled proteins were flourographed using PPO/DMSO (New England Nuclear) before autoradiography.

Phosphoamino acid analysis

One-dimensional phosphoamino acid analysis was performed as described by Hunter and Sefton (1980) except that following hydrolysis and subsequent removal of the hydrochloric acid by lyophilization, the phosphoamino acids were purified by ion exchange as described by Cooper and Hunter (1981). The phosphamino acids were analyzed by electrophoresis on cellulose thin layer plates at *p*H 3.5 for 60 minutes at 600 volts. The plates were then dried and autoradiographed for 1-10 days prior to marker detection by ninhydrin staining.

Two dimensional analysis of total cellular phosphoamino acids was performed exactly as described by Snyder *et al.* (1983).

Antibody staining of sectioned Drosophila

Antibody staining of sectioned *Drosophila* larvae, pupae, and adults was performed using a modification of the Immunogold technique recommended by Boehringer Mannheim Inc. Frozen sections (10 μm) were prepared on gelatin coated slides. After 30-60 minutes of drying at room temperature, the sections were fixed in

PBS (25 mM sodium phosphate *p*H 7.5/ 150 mM NaCl) containing 2% paraformaldehyde for 30 minutes. The slides were then washed in PBS for 5 minutes twice and then placed overnight in PBSBTG (PBS/ 0.5% bovine serum albumin/ 0.1% Triton X-100/ 5% normal goat serum. The slides were then incubated for 30 minutes in PBSBTG containing 2.5% affinity-purified serum raised against the carboxy-terminal region of pp62$^{c\text{-}src}$. The slides were then washed 3 times for 15 minutes in PBSBT (PBS/ 0.5% bovine serum albumin/ 0.1% Triton X-100). The slides were then incubated for 30 minutes with PBSBTG containing 1.25% Immunogold goat anti-rabbit antiserum (Boehringer Mannheim, 5 nm particles). The slides were then washed 4 times for 15 minutes in PBS/ 0.5% bovine serum albumin, twice for 5 minutes in PBS. The slides were then fixed in 2% glutaraldehyde for 15 minutes. Subsequently, the slides were washed twice in PBS, twice in water and then developed using an INTENSE silver developing kit (Boehringer Mannheim). The development was exactly as described by the manufacturer except that the slides underwent 3 rounds of 5 minutes each of development. After fixation, the slides were stained with Giemsa stain and examined.

Affinity purification of antibodies

Affinity purification of antibodies raised against synthetic peptides was performed using columns of CNBr-activated Sepharose (Pharmacia) to which peptide had been coupled. The coupling was

performed by taking 1 ml equivalent of beads, swelling these beads in 1 mM HCl for 15 minutes, washing the beads with 100 mM $NaHCO_3$/ 500 mM NaCl and adding 2 mls of 100 mM $NaHCO_3$/ 500 mM NaCl/ 2 mg per ml peptide. The coupling reaction was for 2 hours at room temperature. After blocking reactive groups with 1.0 M Ethanolamine (*p*H 8) for 2 hours at room temperature, the beads were washed with PBS, then with 100 mM citrate buffer (*p*H 2.5), and then with PBS again.

Five mls of serum was then incubated with 1 ml of packed coupled beads by repeatedly allowing the serum to run through a column containing the beads. The column was then washed with 10 mls of PBS. The proteins that bound to the column were then eluted with 100 mM citrate buffer (*p*H 2.5). One ml fractions were collected and immediately neutralized with 1 M NaOH. The fractions containing proteins were pooled, dialyzed overnight against PBS, supplemented with thimerosol to 0.01%, and stored at 4°. The OD_{280} of the resultant antibody solution was 0.46.

Drosophila Culture

Maintenance of stocks

Fly stocks were maintained at either room temperature or 25° on standard cornmeal-yeast medium. The genetic markers and

balancer chromosomes are as described by Lindsley and Grell (1972).

Mutagenesis of flies

Mutagenesis was either by X-irradiation with 4000 rads from a 250 KeV source or by overnight feeding of ethyl methane sulphonate (25 mM EMS in 10% sucrose). Adult male flies were mutagenized in groups of approximately 100 and then mated to 200 virgin females. After three days of mating, the males were removed and discarded. The females were then transferred daily to a new yeasted bottle until they ceased to lay eggs. The appropriate male progeny of these females were then individually mated in small vials to 2-3 females. The progeny of these mating were examined after three weeks for appropriate markers that would indicate the presence of a mutation in the 64B region of the chromosome. Male progeny bearing potentially mutant chromosomes were then recovered and the chromosome balanced by mating with females carrying appropriate balancer chromosomes.

Complementation Analysis

Complementaion analysis was performed by mating 3 males with 5 females in a small vial. Failure to complement was indicated by the absence of progeny that do not carry a balancer chromosome after the emergence of at least 40 adults. Successful complemen-

tation was indicated by the presence of at least five progeny not carrying either of the balancer chromosomes.

Drosophila Tissue Culture

Drosophila tissue culture cells were grown in flasks at 24° in either Schneider's *Drosophila* medium (Gibco)/ 10% fetal bovine serum for Schneider Line 2 cells and Echalier's medium (Echalier and Ohanassian, 1970) for K_c cells. Cells were maintained at densities of from 1-5 x 10^6 cells per ml. Transfection of Schneider Line 2 cells was performed exactly as described by Rio and Rubin (1985). Selection for transfectants was by supplementation of the medium with 1 mg per ml G-418.